

Novel *in vivo* biosensors for monitoring of  
mammalian cell cultures

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# Declaration

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Forscht, wo ihr was zum Forschen findet. Das Unerforschbare laßt ungeründet.

— *Erich Kästner, Das fliegende Klassenzimmer*

It is only by means of the sciences of life that the quality of life can be radically changed.

— *Aldous Huxley, Foreword to Brave New World*

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# Abstract

Mammalian cell cultures are used for production of biopharmaceuticals, *e.g.* monoclonal antibodies. Only mammalian hybridoma cells contain the pathways for antibody production, but due to their multicellular origin the cells have complex nutrient requirements. Cell growth and antibody production are limited by supply of essential nutrients such as glutamine and accumulation of toxic waste products such as lactate. Many attempts have been made at tackling these challenges, *e.g.* by optimising growth media to keep metabolite concentrations at optimal levels. These approaches have been hampered by our ability to monitor relevant cell culture parameters such as metabolite concentration dynamics in real time.

The aim of this study is to develop a solution to this problem using a synthetic biology approach. Whole-cell bacterial biosensors for important culture parameters, glutamine, leucine, alanine and lactate, were designed, built and characterised. The biosensors were designed from natural metabolite-sensing systems, specifically the *Escherichia coli* *Ntr* regulon, *Lrp* regulon and *lldPRD* operon and the *Bacillus subtilis* GlnK-GlnL system. Characterisation of the biosensors in defined medium using known lactate concentrations was followed by validation in mammalian cell culture media and using cell culture samples.

A lactate sensor based on the *lldPRD* operon showed a reliable lactate-response during initial characterisation and was chosen to determine lactate concentrations in cell culture samples in parallel with lactate analysis using a bioprofiler. Generally, the lactate concentrations from the two methods showed a good match. Data points where the results differed showed that there are some sources of error in the usage of the biosensor that could be addressed in future.

The results of this study also highlight the many challenges of applying synthetic biology constructs to complex industrial contexts. The biosensors presented in this study are more generally applicable in any experimental context that requires sensing of metabolites.

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# Publications

## Research papers:

Engineering a biopart library using parts from the natural and complex *E. coli* Lrp operon (*in preparation*).

Novel lactate biosensor for bioprocessing (*in preparation*).

## Review papers:

Goers, L., Freemont, P. and Polizzi, K. M. (2014), 'Co-culture systems and technologies: taking synthetic biology to the next level', *Journal of the Royal Society Interface* 11(96), pp. 1-13.

## Book chapters:

Goers, L., Kylilis, N., Tomazou, M., Yan Wen, K., Freemont, P. and Polizzi, K. (2013), *Engineering microbial biosensors*, Vol. Volume 40, Academic Press, chapter 5, pp. 119-156.

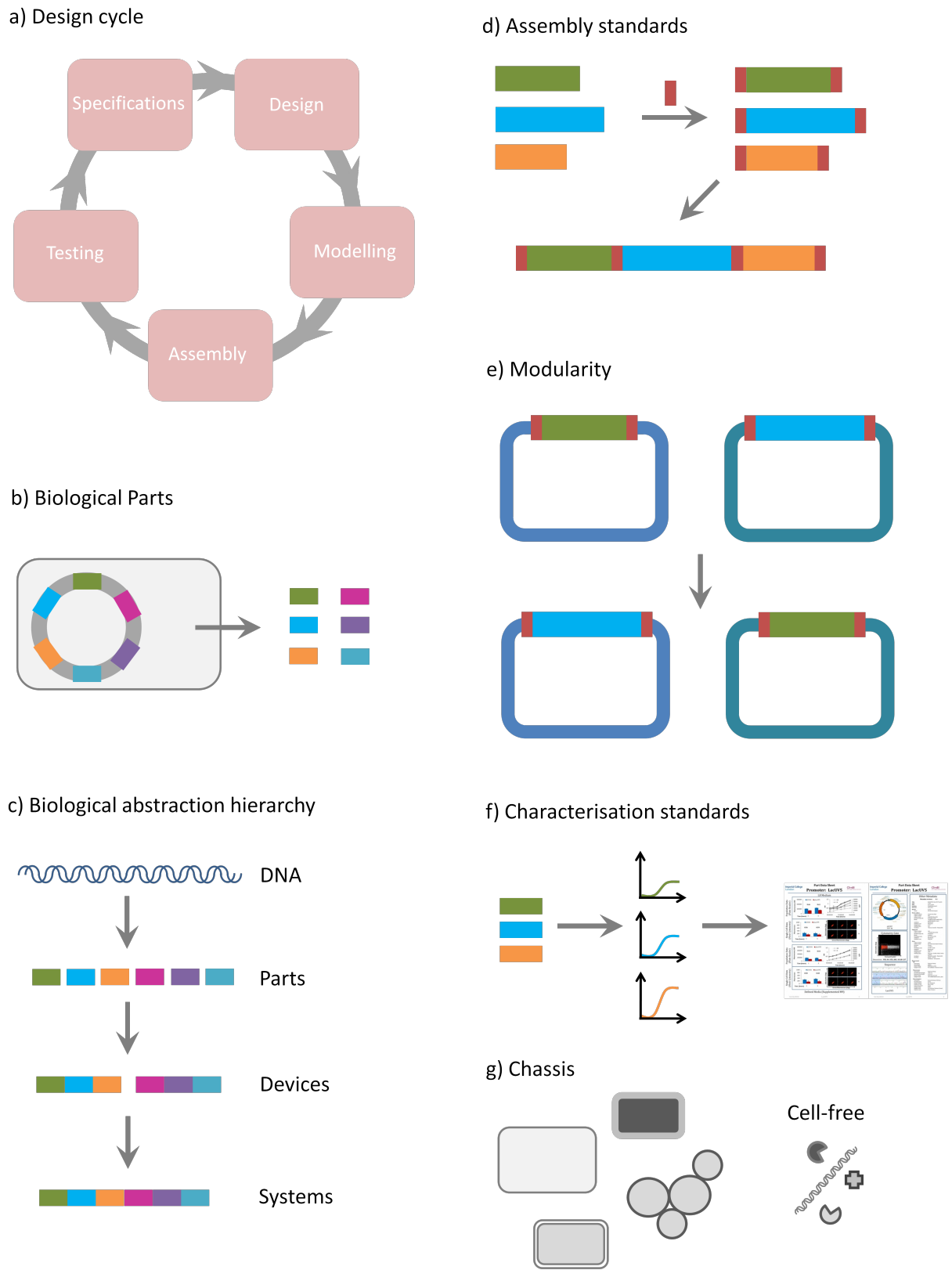
# 1 Introduction

## 1.1 Synthetic Biology

Synthetic biology is a new applications-driven field with the aim to apply engineering principles to biology. The idea is that biological molecules are parts, which come together to form devices and these in turn can form complex *de novo* systems with predictable behaviour (Weber and Fussenegger, 2011). Using engineering principles, such as modularity, characterisation and standardisation at every level of a biological abstraction hierarchy, far more complex and predictable systems than ever before can be engineered according to specifications. By synthetically reconstructing a pathway, we can gain greater understanding of the underlying processes (Kampf and Weber, 2010). To help the rapid assembly and testing of so-called biological “parts”, high throughput DNA assembly and part characterisation platforms and standards are being developed (Canton et al., 2008; Shetty et al., 2008). This fast-developing field has been reviewed numerous times (Canton et al., 2008; Freemont and Kitney, 2012; Kitney and Freemont, 2012; Church et al., 2014). Successful outcomes of this field so far include biological logic gates (Miyamoto et al., 2012), pattern-generators (Basu et al., 2005) and biosensors (Gu et al., 2010; Wang, Barahona and Buck, 2013).

### 1.1.1 Principles of synthetic biology - Parts, assembly and characterisation

Synthetic biology is based on a number of foundational principles of systematic design (Figure 1.1). Projects that aim to design and build complex biological systems should follow the design cycle, which outlines the steps in the process (Figure 1.1a). Biological parts are biological objects that perform a biological function and may be engineered to meet specified design or performance requirements (Canton et al., 2008) (Figure 1.1b). They are usually engineered DNA sequences based on natural ones (Canton et al., 2008). For instance, the biological object may be a promoter, performing the biological function of gene expression control. This promoter may be designed and engineered to switch on gene expression in response to the presence of red light, which represents the performance requirement. Biological parts are



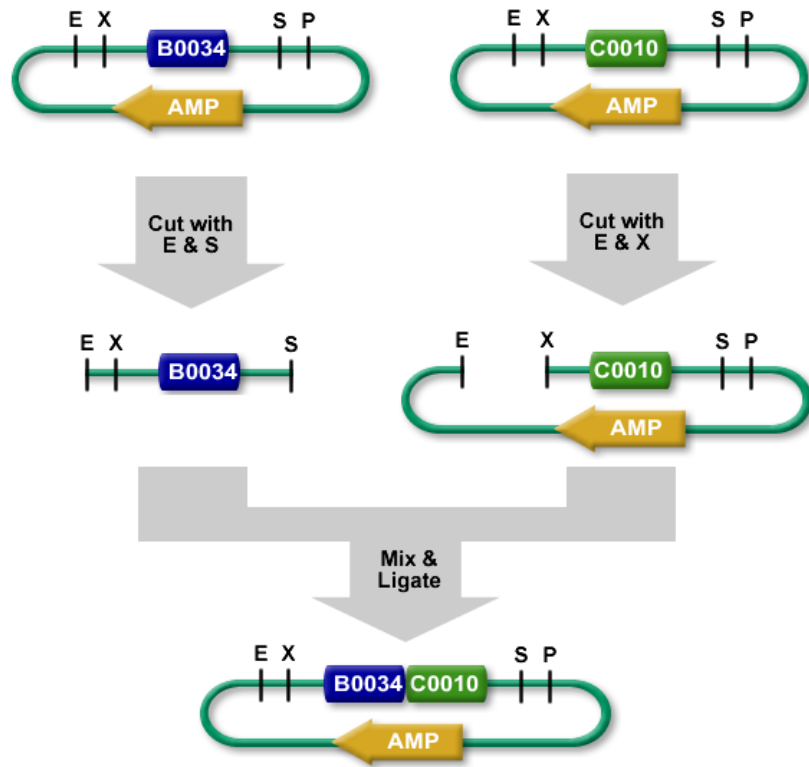
**Figure 1.1:** Principles of synthetic biology - Systematic design. a) The design cycle. b) Biological parts. c) Biological abstraction hierarchy. d) Assembly standards. e) Modularity. f) Characterisation standards. g) Chassis choice. Further detail in the main text.

collected in libraries or registries such as the Registry of Standard Biological Parts [<http://partsregistry.org>], which today already holds thousands of parts.

Engineering biology is a less daunting task when we use a biological abstraction hierarchy (Figure 1.1c). The idea here is that bioengineers can concentrate on working at a particular level of the hierarchy, *e.g.* devices, and treat the lower levels as “black boxes”, where the exact mechanisms of action do not need to be known. Building devices and systems from parts is supported by assembly standards and varied assembly methods (Figure 1.1d). After a genetic design for the desired circuit has been drawn up, the circuit needs to be assembled physically. There are numerous DNA assembly methods, but assembly remains one of the limiting steps in synthetic biology. Common assembly methods include restriction enzyme-based methods (for example BioBricks, Figure 1.2), PCR-based methods (for example the Gibson DNA Assembly method) or gene synthesis by commercial companies (Ellis et al., 2011; Goers et al., 2013). Another helpful concept in systems design is modularity (Figure 1.1e). This means that modules could be exchanged for different part of the same type, without having to significantly change the others. Assembly standards can help enforce this.

Once the construct is assembled, the next step in the design cycle is characterisation (Figure 1.1f). This involves experimental testing to understand the behaviour of the part. The exact method of characterisation depends on the parts to be characterised. One of the developments in synthetic biology is the establishment of standardised characterisation procedures (Canton et al., 2008; Kelly et al., 2009). Results of characterisation can be recorded in standardised data sheets (Canton et al., 2008). Ratiometric analysis allows comparison of data across institutions (Kelly et al., 2009). Biological systems need a chassis, meaning an organism in which they can be implemented (Canton et al., 2008). Chassis choice opens up many questions for implementation and needs to be carefully considered (Figure 1.1g). Model organisms such as *Escherichia coli*, *Bacillus subtilis* or *Saccharomyces cerevisiae* have been commonly used in science and industry and are therefore well established with molecular biology tools available. There are also cell-free systems available, which have the advantage of reduced biological complexity (Chappell et al., 2013).

Synthetic biology is still a young field and there are many challenges still to be overcome. Systems often suffer from cross-talk and context dependency. We have limited knowledge and understanding of many biological parts and systems. Biological systems will not work in all contexts and over arbitrarily long times. During characterisation it needs to be determined whether the system fulfils the performance requirements. It is useful to define conditions under which a system fails during characterisation (Canton et al., 2008).



**Figure 1.2:** BioBrick standard assembly. Figure taken from [[http://parts.igem.org/Assembly:Standard\\_assembly](http://parts.igem.org/Assembly:Standard_assembly)].

## 1.2 Biosensors

Biosensors represent an increasingly important field (Belkin, 2003; French and Gwenin, 2012; Goers et al., 2013; Park et al., 2013). The term biosensor has been given a number of definitions. Generally, it refers to any, at least partially biological, entity that be used to monitor a parameter of interest or target molecule (Goers et al., 2013). Biosensors come in many forms, *e.g.* purified proteins attached to electrodes or incorporated into membranes. Some biosensors are fully biological systems, such as whole cells, others have a biological detection unit and information is passed on to and processed by a chemical or electrical component. Biosensors predate synthetic biology but many have been created using synthetic biology principles (Checa et al., 2012). Numerous examples of biosensors have been created *e.g.* (Aleksic et al., 2007; Voigt, 2012; Siedler et al., 2013). Biosensors form the basis of most larger synthetic biology circuits (Bacchus and Fussenegger, 2013). Logic gates, such as AND or NOT, and pathways start with a point of input, which is detected by a biosensor. Biosensors are therefore needed to build such systems.

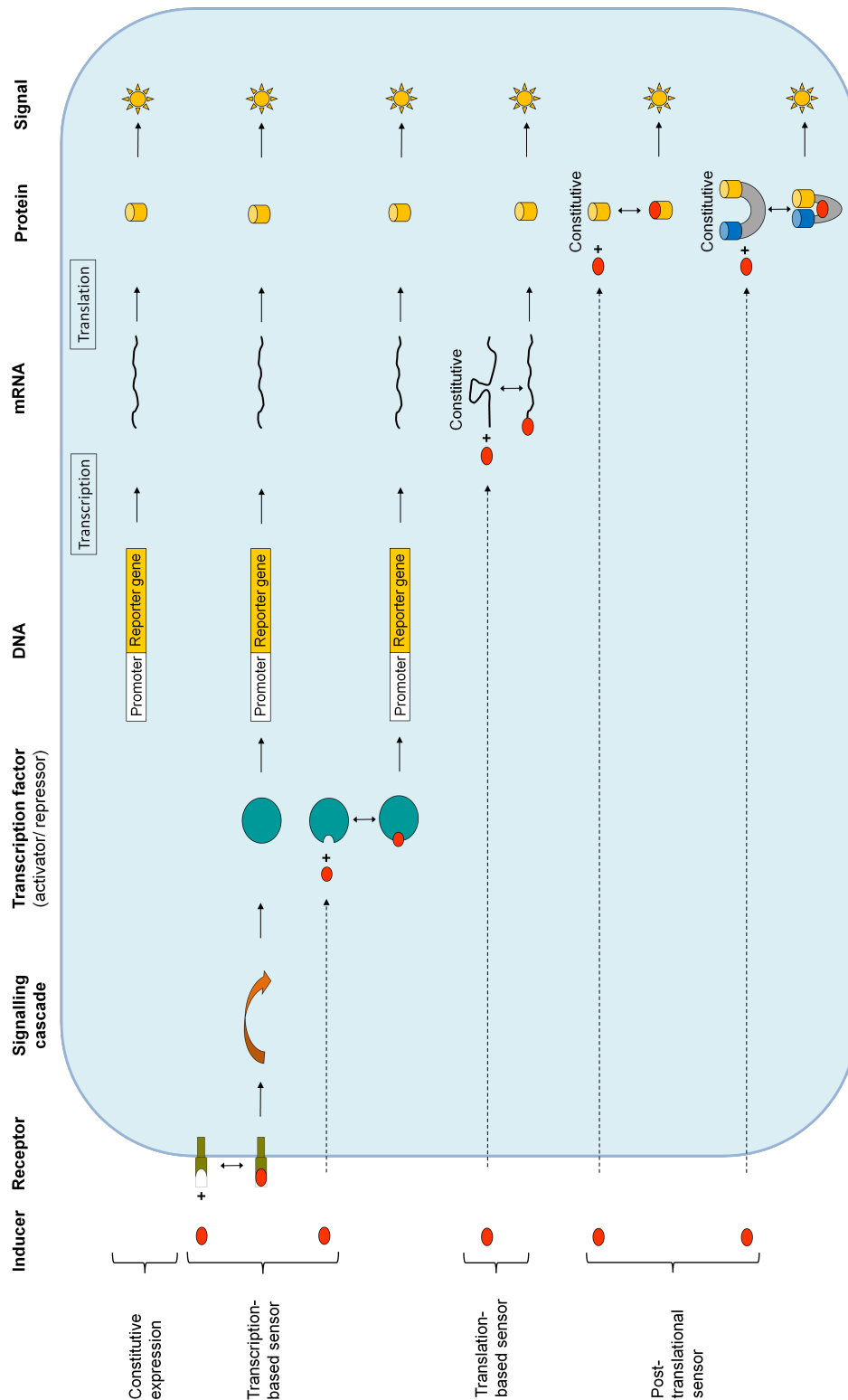
Being based on biological elements can bring advantages and disadvantages (Belkin, 2003; French and Gwenin, 2012; Goers et al., 2013). The advantages include high

sensitivity and specificity, due to the underlying enzymatic or other biomolecular mechanisms, and cost-effectiveness. The disadvantages are that biological elements may not be as resistant to extremes of temperature and pH as other kinds of sensors, and current limitations in our ability to engineer biological elements.

### 1.2.1 Whole-cell biosensors

Cells naturally monitor their external environment and respond to what they detect. The underlying mechanisms tend to involve three processes: detection, transduction and response (Checa et al., 2012; Miyamoto et al., 2012). Whole-cell biosensors are genetically engineered cells that express a detection mechanism for the compound of interest, which is linked to an output, often in the form of a detectable reporter protein (Muranaka, 2009). Whole-cell biosensors tend to make use of natural sensing and signalling pathways of cells, specifically the pathways of protein synthesis (Goers et al., 2013). Sensor mechanisms can be transcriptional, translational or post-translational (Marchisio and Rudolf, 2011) (Figure 1.3; Table 1.1). Depending on the underlying detection mechanism, the target compound could be extracellular or intracellular. Some “traditional” biosensors do not detect any specific metabolite and instead monitor general cell growth and well-being. These are used as environmental biosensors to detect general toxicity or “nutrient bioavailability” (Belkin, 2003; Darwent et al., 2003; Goers et al., 2013). Over 100 different genetically encoded biosensors have been developed for diverse targets, including ions, molecules and enzymes (Palmer et al., 2011). Whole-cell biosensors have been previously reviewed (Daunert et al., 2000; van der Meer and Belkin, 2010; Marchisio and Rudolf, 2011; Goers et al., 2013).

The mechanism of the biosensor defines the possible downstream effects (Goers et al., 2013). Transcriptional or translational biosensors can translate the target molecule input into transcription or translation, both of which ultimately lead to protein expression. FRET (Förster resonance energy transfer) sensors provide an output that can be measured, but which does not easily translate into a biological output such as protein expression.



**Figure 1.3:** Mechanisms of different types of whole-cell biosensors, including transcription-based, translation-based and post-translational. In all biosensors, detection of a target molecule leads to an observable signal of some kind, but different biological mechanisms connect the two. More detail on the different kinds of biosensors can be found in the main text and in Table 1.1. Figure and legend adapted with permission from Goers et al. (2013).



Whole-cell biosensors have advantages and disadvantages in their use over other biosensors such as purified proteins (van der Meer and Belkin, 2010). Many of the advantages relate to synthetic biology in general. There is potential for large size of biosensor cell populations, rapid growth rate, low cost, improved stability and easy maintenance (Belkin, 2003; Park et al., 2013). These factors mean that whole-cell biosensors lend themselves for being used in remote areas where complex chemical equipment and storage may be scarce. Once made, the biosensors can be made easily by culturing, and do not require ongoing purification of proteins (Muranaka, 2009). A whole-cell biosensor allows for the sensing input to be complex, *e.g.* through logic gates and by combining several sensors. This allows for complex information processing (Muranaka, 2009). Limitations of working with whole-cell biosensors are as follows. The availability of appropriate natural sensors is limited, and creating new ones might require elaborate protein engineering or directed evolution (Muranaka, 2009). Limited information (*e.g.* structural, regulatory) may be available about natural systems, thus making a synthetic biology approach difficult. Cellular metabolism can interfere with sensing mechanisms (cross-talk). Only a few bacterial biosensors have been commercialised so far, due to problems with legislation concerning GM organisms and technical hurdles, such as shelf life and scalability (van der Meer and Belkin, 2010). Whole-cell biosensors have generally been used to detect toxicity and bioavailability of contaminants in water and soils (?). A well known example of an environmental whole-cell biosensor that is moving towards commercial application is the arsenic biosensor based on the 2006 Edinburgh iGEM project [<http://www.arsenicbiosensor.org/>].

### 1.2.1.1 Reporters for biosensors

Any biosensor needs to link detection of a target to an observable output signal (Figure 1.3; Table 1.1). Common types of signal include fluorescence, bioluminescence and colour change. But there are also many other kinds of outputs. For instance, general cell well-being or electrical potential. The most suitable choice will depend on the context in which the biosensor is to be used. Reporter genes and their uses for synthetic biology biosensors have been previously reviewed (French et al., 2011). Some common biosensor outputs will be described below. This summary of reporters for biosensors is based on that given in (Goers et al., 2013).

Fluorescence is a commonly used biosensor output. GFP and other fluorescent proteins are widely used in molecular biology. These proteins emit fluorescence at a certain wavelength when exposed to another wavelength of light. They offer

**Table 1.1:** Characteristics of different classes of biosensors. Table adapted with permission from Goers et al. (2013).

	<b>Transcription-based</b>	<b>Translation-based</b>	<b>Posttranslational</b>
<b>Response time</b>	Slow (minutes to hours), though there are exceptions (Purnick and Weiss, 2009)	Intermediate (minutes)	Fast (seconds)
<b>Target location</b>	Intra- or extracellular (receptor)	Intracellular	Intra- or extracellular (depends on protein localisation)
<b>Form</b>	Inducible promoters, cell-surface receptors, signalling proteins	RNA switches	Immobilised enzymes, multidomain fusion proteins, surface-displayed proteins
<b>Metabolic burden on the cell</b>	Reporter protein only expressed in the presence of inducer. Transcription factor could be constitutively expressed	Need to make constitutive mRNA	Need to express constitutive protein
<b>Ease of construction</b>	Can be easy to construct using existing plasmids	Engineering of DNA is straightforward these days	Can be difficult to construct, as may require protein engineering
<b>Specificity</b>	As the recognition of the target is protein-based, it can be very specific. The final output may not be a direct measure of the target molecule due to downstream steps in protein synthesis	Target recognition can be very specific with well-designed aptamer. The final output is a more direct measure of the target molecule than for transcriptionbased sensors	As the recognition of the target is protein-based, it can be very specific. The final output is a more direct measure of the target molecule than for translationbased sensors
<b>Output</b>	Can be linked to gene expression.	Can be linked to protein synthesis. Other outputs also possible.	Cannot be linked to gene expression. In case of FRET, fluorescence output only
<b>Examples</b>	Arsenic sensor (Aleksic et al., 2007); Amino acid sensor (Mustafi et al., 2011)	Theophylline sensor (Desai and Gallivan, 2004); Doxorubicin and kanamycin sensors (Ferguson et al., 2013)	Glutamine and glucose FRET sensors (Behjousiar et al., 2012); Laconic lactate FRET sensor (San Martín et al., 2013)

an output that can be detected with great specificity and sensitivity. Several fluorescent proteins can be used in the same experiment to allow monitoring of several parameters in parallel and they allow very intuitive visualisation. The use of more than one fluorescent protein is fundamental to FRET-based biosensors. Being derived from natural proteins, they can be produced by cells and their genes can be incorporated into gene circuits as reporter genes. This makes them very suitable outputs for transcription- and translation-based biosensors. Many molecular biology techniques exist for measuring fluorescence with cells being grown in various formats.

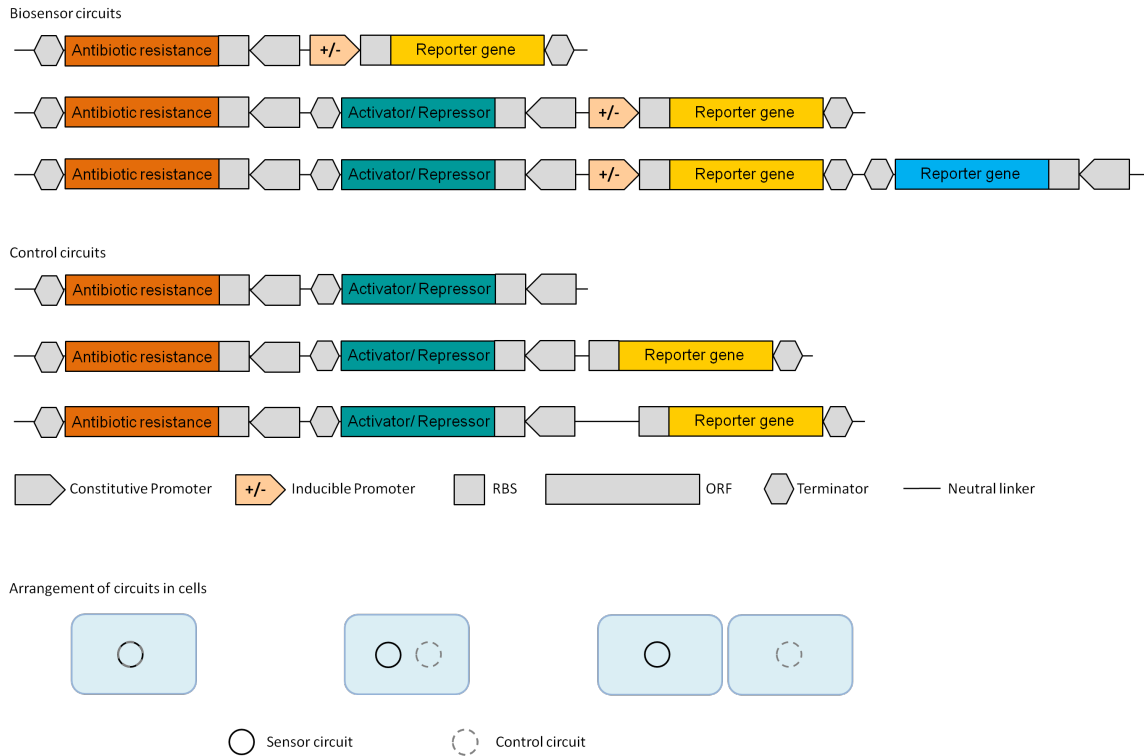
An alternative biosensor output to fluorescence is bioluminescence. Proteins such as firefly luciferase are similar to fluorescent proteins in their use and advantages for the engineering of biosensors, although the mechanism by which a detectable output is produced differs. Here, the protein itself does not emit light, but it acts as an enzyme that catalyses a reaction that leads to light emission. This means that the reaction substrate needs to be provided during the experiment, although in some cases, the genetic construct can be modified as well to allow the cells to biosynthesise the substrate (French et al., 2011).

Some biosensors use colour change as their output. For any sensor that is intended to be used in the field (*e.g.* in a field test kit or in a hospital at point of care), an output that can be detected without the use of expensive equipment is desirable, making a colour change more suitable for such contexts than fluorescence or bioluminescence. This is a more varied category than the earlier mentioned and includes a number of very different systems. Most of such systems require the addition of a substrate that is chemically transformed into a related colour-producing compound. A commonly used reporter that causes a colour change is the lacZ (b-galactosidase) gene and the chromogenic compound 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Xgal). The 2010 Imperial College London iGEM team used the compound catechol and the enzyme catechol 2,3-dioxygenase (C23O) to produce a yellow colour [[http://2010.igem.org/Team:Imperial\\_College\\_London](http://2010.igem.org/Team:Imperial_College_London)]. The 2009 Cambridge iGEM team made a set of colour generators using pigments [<http://2009.igem.org/Team:Cambridge>]. The 2006 Edinburgh iGEM team made an arsenic sensor with an output that resulted in a pH change, which could be visualised using a coloured pH indicator [[http://2006.igem.org/wiki/index.php/University\\_of\\_Edinburgh\\_2006](http://2006.igem.org/wiki/index.php/University_of_Edinburgh_2006)].

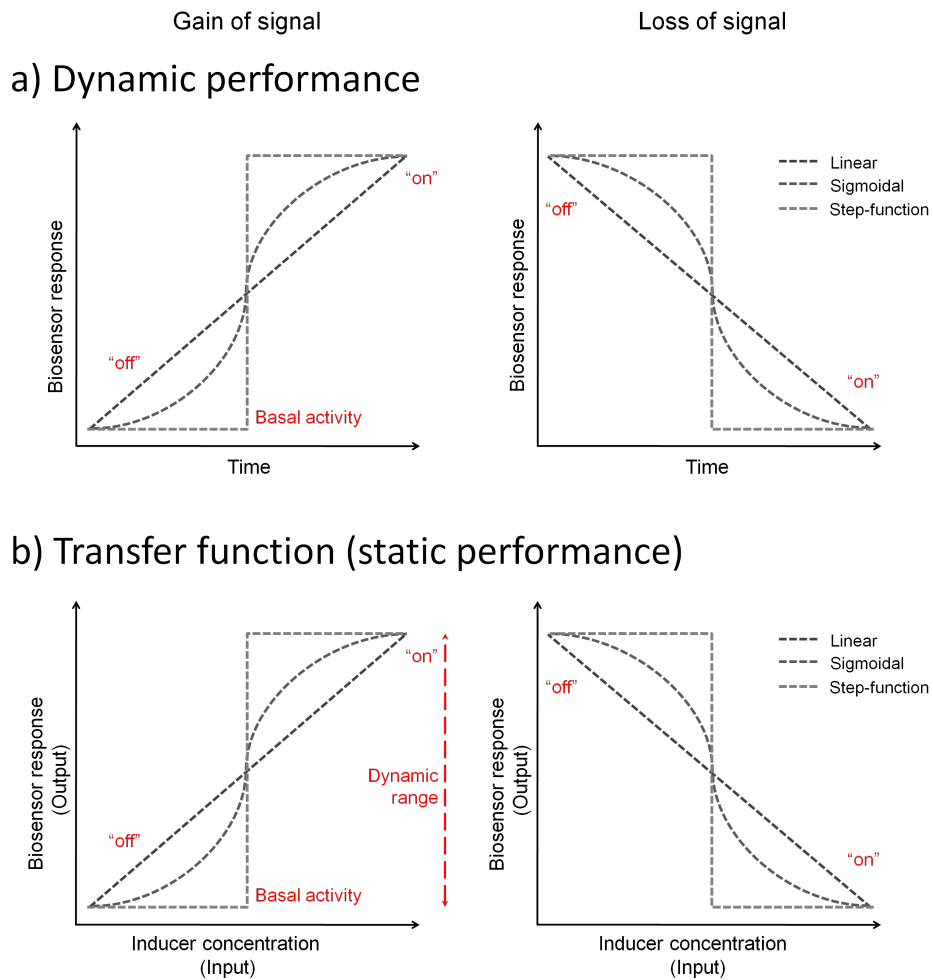
### **1.2.1.2 Engineering whole-cell transcriptional biosensors**

The process of engineering whole-cell biosensors was extensively outlined by Goers et al. (2013). When using synthetic biology principles, then the methods here will be similar to the process outlined in Figure 1.1. When planning the design

and assembly of a biosensor construct, there are certain general principles, even though each construct will have specific context-dependent elements (Figure 1.4). Appropriate control constructs need to be characterised alongside the biosensor constructs. The methods of characterisation will depend on the sensor design. Output characteristics of biosensors can be varied and, depending on system requirements, there may be several useful kinds of dynamics (Figure 1.5).



**Figure 1.4:** Typical elements of a transcriptional biosensor and control cell gene circuits and possible arrangements of biosensor and control circuits in the cells. Biosensor constructs can contain different basic elements. The relevant transcription factor could be overexpressed (green). An additional reporter protein can be constitutively expressed for ratiometric analysis (blue). Control circuits lack the responsive element that acts as the biosensor, but could contain all other parts that are in the biosensor construct. Biosensor and control constructs could be placed in the same cell or in different cells. In the case of ratiometric analysis they can be located on the same plasmid. Figure and legend adapted with permission from Goers et al. (2013).



**Figure 1.5:** Idealised possible biosensor response characteristics. a) Dynamic performance. This shows possible behaviours of biosensors over time after addition of inducer. b) Static performance. This shows possible behaviours of biosensors over concentrations of inducer. Various parameters can be gained from such curves that can be used to describe the behaviour of biosensors in comparison with other sensors. Figure and legend adapted with permission from Goers et al. (2013).

## 1.3 Mammalian cell bioprocessing

### 1.3.1 Biopharmaceuticals

Biopharmaceuticals have become increasingly important in recent years (Reichert et al., 2005; van Berkel et al., 2009; Kyriakopoulos and Kontoravdi, 2013). Biopharmaceuticals include protein compounds such as hormones, growth factors, therapeutic enzymes, vaccines and therapeutic monoclonal antibodies (mAbs) and related products, of which the latter are currently the predominant and fastest-growing group (Coco-Martin and Harmsen, 2008; Beck et al., 2010; Kyriakopoulos and Kontoravdi, 2013). Clinical successes and approvals of therapeutics are numerous (Coco-Martin and Harmsen, 2008; Beck et al., 2010; Weiner et al., 2010). MABs are currently used largely for treating cancer, inflammatory and orphan diseases (Beck et al., 2010). Examples of currently used mAb cancer therapeutics include Trastuzumab (Herceptin) for breast cancer, and Rituximab (Mabthera) for non-Hodgkin lymphoma. Biopharmaceuticals such as mAbs are very complex molecules and can be used to very specifically treat human conditions (Kyriakopoulos, 2014). Past and recent successes promise continued developments in this field with many new mAbs expected to be developed in coming years (Coco-Martin and Harmsen, 2008). To expand the applicability and affordability of these therapeutics, improvements in efficiency, effectiveness and specificity are sought at all stages of the development, production and use of mAbs (Beck et al., 2010). MAB-based cancer therapeutics are very costly. Process developments accounts for a large fraction of the cost of bringing a drug to the market (Harms et al., 2002). Limiting factors in mAb production include the timescale of the required cell cultures and the yield of drug protein from these cell cultures (Kyriakopoulos, 2014). Reducing production and processing costs by improving the methods used is therefore an important aspect of improving affordability of mAbs and other drugs (Beck et al., 2010; Zhou et al., 2011).

### 1.3.2 Bioprocessing

The aforementioned biopharmaceutical compounds are produced by large cultures of mammalian cells in the field known as bioprocessing (Hu and Aunins, 1997). Commonly used cell types in bioprocessing include CHO (Chinese hamster ovary), mouse or human hybridoma and myeloma cells, of which mostly hybridoma cells, and increasingly CHO cells, are used for the production of mAbs. Due to their

multicellular origin mammalian cells are difficult to culture and have complex nutrient requirements. For a lot of other industrial bioprocesses, the organisms of choice are unicellular microbial cells, *e.g.* bacteria and fungi, which are more easily cultured. Bacteria have many advantages over mammalian cells in bioprocessing, including ease of manipulation, rapid growth rates and simpler nutrient requirements. Before mammalian cell culture techniques matured, non-mammalian cells were also more commonly used for biopharmaceuticals. However, only mammalian cells contain the post-translational modification pathways of glycosylation that are essential for the proper structure and function of mAbs (del Val et al., 2010) and over the last few years, there have been many more approved therapeutic proteins produced by mammalian cells than by microbial or yeast expression systems (Coco-Martin and Harmsen, 2008). While expression systems in *Pichia pastoris* are being considered (Vogl et al., 2013), today, mammalian cells are the only kind of cells that can produce mAbs and be efficiently grown in lab or industrial settings.

### **1.3.3 Metabolites are limiting factors in bioprocessing**

All aspects of hybridoma (and generally mammalian) cell culturing have been subject to study and attempted improvements (Hu and Aunins, 1997). As in any cell culture, hybridoma cell cultures are limited by nutrient availability and metabolic waste accumulation (Figure 1.6a). In many different kinds of cells these waste products limit cell growth and mAb production (Glacken et al., 1986; Ozturk et al., 1992; Kurano et al., 1990). Limiting factors in hybridoma cell cultures have been reviewed by Newland et al. (1990).

The major limiting nutrients for hybridoma cultures are glutamine and glucose, but there are also vitamins, salts and serum components. The major metabolic waste products are ammonia and lactate (Hu and Aunins, 1997). Ideally, for maximum product yield, waste products need to be kept to a minimal level, whereas nutrients need to be kept at optimal levels, which does not automatically imply maximal levels (see Fig. 1.6b). However, all these factors are very cell line-dependent.

For pharmaceutical purposes a very large amount of mAbs needs to be produced using bioreactors, where the mammalian cells grow at high proliferation rates (Newland et al., 1990). Initial process development is usually done in small volumes using flasks. These processes are then upscaled to the volumes of industrial bioreactors. This is a complex process requiring much optimisation as cells behave differently in large bioreactors compared to flasks.





In the bioreactor cells are initially subjected to very high glutamine and glucose concentrations; much higher than those needed for growth and mAb production. This results in high uptake rates of nutrients and subsequent high rates of waste accumulation leading to inhibitory levels of waste products and metabolic byproducts such as ammonia and lactate (Figure 1.6c) (Newland et al., 1990; Paredes et al., 1999). These byproducts reduce cell viability and product yields as well as introducing unwanted variability into cell culture bioprocesses (Young, 2013). Cell lines based on tumour cells (such as myeloma and hybridoma cells) especially produce a large amount of lactate (Newland et al., 1990). Mammalian cells and tumour cell lines in particular are “wasteful” with metabolites, as they do not have a regulated “metabolic policy” like healthy cells would do in a natural context (Bonarius et al., 1996; Young, 2013). There is therefore a need for controlling cellular nutrient intake and metabolism to optimise cell growth and production for industrial purposes. Some ways in which this has been done will be outlined in the following section.

### **1.3.3.1 Glutamine and ammonia**

Glutamine and ammonia are metabolically linked in mammalian bioprocessing (Figure 1.6c). This complex problem will be discussed here in detail to illustrate limiting factors in mammalian bioprocessing. Glutamine in particular needs to be present at optimal levels and is an example for a limiting factor in bioprocessing that has extensively been subject to study and attempted improvement. Amino acid metabolism plays an important role in mammalian cell metabolism (Sheikh et al., 2005; Selvarasu, Karimi, Ghim and Lee, 2010). However, many aspects of amino acid metabolism are still unknown (Kontoravdi, Wong, Lam, Lee, Yap, Pistikopoulos and Mantalaris, 2007). The amino acid glutamine is involved in several physiological processes within the cell as an energy source and precursor for nucleotides, lipids, all non-essential amino acids and hence proteins (Newland et al., 1990; Jeong and Wang, 1995; Europa et al., 2000). In the blood and many tissues glutamine occurs at the highest concentration of all amino acids (Newland et al., 1990). It has long been recognised that glutamine has many functions. Hans Krebs noted that although “most amino acids have multiple functions, glutamine appears to be the most versatile” (Krebs, 1980; Wilmore and Rombeau, 2001), and glutamine catabolism can occur through eight different metabolic routes (Newland et al., 1990; Häggström, 1991; Vriezen and van Dijken, 1998). For these reasons, mammalian cell cultures require glutamine to be supplied in the culture medium (Eagle, 1956).

The high proliferation rates as occur in bioprocessing result in rapid glutamine depletion (Jenkins et al., 1992). Cell cultures that lack sufficient glutamine will undergo cell death (Jeong and Wang, 1995). Glutamine has been identified as one of the most important limiting factors for cell growth and proliferation and hence mAb production (Jeong and Wang, 1995). Provision of increased glutamine in the culture medium cannot solve this problem, not only as the cells produce ammonia as a byproduct of glutamine metabolism, but also as glutamine has a short half life and decomposes extracellularly to produce toxic ammonia (Newland et al., 1990). Most of the ammonia secreted by CHO cells comes from glutamine metabolism from the reaction catalysed by glutaminase (Street et al., 1993). Different cell types display different tolerances to ammonia, *e.g.* myeloma cells (one of the constituents of hybridoma cells) are very sensitive to ammonia (Newland et al., 1990), while other cell types, HDF and Vero, show no sensitivity to ammonia (Butler and Christie, 1994). Ammonia production from cellular metabolism is more significant than from decomposition in the medium, especially in the later stages of a culture (at high cell density), but decomposition still has an effect worth noting (Newland et al., 1990). Both the ammonium ion ( $\text{NH}_4^+$ ) and ammonia ( $\text{NH}_3$ ) tend to be present in the culture medium, although the former predominates at a culture pH of about 7 (Newland et al., 1990). Ammonia and ammonium can also have different effects (Schneider et al., 1996). Glutamine consumption also varies with presence of other metabolites, *e.g.* at low glucose concentrations, glutamine consumption is regulated by glucose and vice versa (Newland et al., 1990). As glutamine requirements are cell population-dependent (O’Callaghan and James, 2008), there is no “one-size-fits-all” solution for providing glutamine to mammalian cell cultures. Specific mAb productivity (*i.e.* productivity per cell) is less sensitive than cell growth to the effects of ammonia (Newland et al., 1990). However, volumetric mAb productivity (productivity per volume) can be strongly affected, not only due a lower number of viable cells, but also as ammonia may change mAb glycosylation patterns (Andersen and Goochee, 1995; Wong et al., 2005).

It has long been known that cells release specific amino acids into the culture medium during cell growth, specifically aspartate (Burleigh et al., 2011) asparagine and alanine (Butler, 1987; Miller et al., 1988; Newland et al., 1990; Bonarius et al., 1996; Vriezen et al., 1997; Paredes et al., 1999; Europa et al., 2000; Sheikh et al., 2005; Selvarasu et al., 2009; Selvarasu, Kim, Karimi and Lee, 2010; Selvarasu, Karimi, Ghim and Lee, 2010; Burleigh et al., 2011; Young, 2013). It is thought that secretion is preceded by an intracellular build-up of ammonia (Newland et al., 1990), and hence acts as a way to deal with the excess toxic waste product, with alanine being a major endproduct of glutamine metabolism (Street et al., 1993). It

was shown that alanine replaced ammonia as the major byproduct of glutamine-metabolism at ammonia concentrations above 5 mM, although the exact pathways involved are not yet fully elucidated (Butler, 1987; Miller et al., 1988; Newland et al., 1990; Sheikh et al., 2005). This is one way in which ammonia reduces the metabolic efficiency of cells, by forcing excretion of valuable metabolites (Schneider et al., 1996). Alternatively, it has been suggested that ammonia accumulation simply causes inefficient “overflow” metabolism (Burleigh et al., 2011). A study using NMR with labelled nitrogen showed evidence that in cell culture metabolism glutamine forms ammonia and that ammonia subsequently forms metabolites such as alanine (Street et al., 1993).

The glutamine challenge in mammalian cell culture and proposed solutions have been discussed several times, *e.g.* by Newland et al. (1990); Schneider et al. (1996) and Genzel et al. (2005). It consists of two main aspects: (1) strong need for glutamine source in the culture medium for cell growth and hence mAb production and (2) toxicity of the glutamine byproduct ammonia, both from cellular metabolism and glutamine decomposition in the medium. It has been argued that if the accumulation of metabolites, such as ammonia, could be reduced, then a higher cell density could be attained (Europa et al., 2000; Hu and Himes, 1989). So far there has not been a complete solution to the glutamine-problem, which may partially be because the metabolic pathways of glutamine and ammonia are still not fully elucidated in mammalian cell culture (Newland et al., 1990; Young, 2013). Mammalian cells have complex and variable behaviour and their metabolism and its regulation is often not completely understood (Newland et al., 1990; Europa et al., 2000; Sheikh et al., 2005; Selvarasu et al., 2009). There is also the problem that a lot of what is known about hybridoma cell metabolism is from studying mouse and human cell and genome data rather than human cell metabolism directly (Sheikh et al., 2005; Selvarasu, Karimi, Ghim and Lee, 2010). This type of data may not give an accurate picture, as hybridoma cells cannot simply be equated with either human or mouse cells. These unique kinds of cells act differently to the cells from which they are derived. There are a number of focussed studies of mammalian cell metabolism (Fernandez et al., 1988), but a complete, experimentally validated, overall picture of hybridoma cells is missing. Some studies have been conducted to obtain experimental ‘omics’ data on these cells (Yee et al., 2008; Meleady, 2007). Approaches include genetic engineering (Selvarasu, Kim, Karimi and Lee, 2010) and systems biology approaches (O’Callaghan and James, 2008; Kondragunta et al., 2012; Young, 2013). Studies are further complicated by the fact that consumption and fate of metabolites are all interdependent, *e.g.* glutamine metabolism is interdependent on glucose metabolism and possibly serum concentration (Figure 1.6) (Newland et al., 1990; Vriezen et al.,

1997). There is therefore a need to study the cells that are used industrially in more detail to understand their behaviour and optimise their utilisation.

There are a variety of ways in which scientists have tackled the glutamine and ammonia problem, which are summarised below. There are several main principles that have been used: decreasing glutamine need of the cells or preventing ammonia accumulation (either by preventing ammonia being formed in the first place or removing it).

(1) Use of alternative expression systems. Cell types other than mammalian cells can have simpler nutrient requirements, but would have to be genetically engineered to be able to produce mAbs. For example, bacteria can usually live on simple nitrogen sources, such as ammonia and produce glutamine. Attempts have been made to use the Baculovirus system (Deparis et al., 2003), bacteria (Carter et al., 1992; Spiess et al., 2013) or plants (Mullard, 2011). Alternatively, there are certain kinds of mammalian cells that do not show ammonia sensitivity, such as HDF and Vero cells (Butler and Christie, 1994) or mammalian cells that have an intrinsic ability to produce glutamine. CHO cells (Tjio and Puck, 1958) are the most commonly used cells in the production of recombinant biopharmaceuticals and are ever more commonly used to produce mAbs (Schlatter et al., 2005; Jayapal et al., 2007). They naturally contain a glutamine synthetase enzyme, and can thus produce glutamine. However, they still commonly need growth medium supplemented with glutamine or nitrogen sources like glutamate, as they cannot produce enough to cover their needs (Altamirano et al., 2000).

(2) Genetic engineering of mammalian cells. One way to overcome this problem has been the genetic engineering of the mammalian cell line NS0 to insert a gene for a glutamine synthetase enzyme. The presence of the enzyme acts as a selectable marker in glutamine-free medium. This is commonly used as an expression system called GS System<sup>TM</sup> ([\[www.1onza.com\]](http://www.1onza.com), glutamine synthetase gene expression system). Hybridoma cells have also been engineered to lower accumulation of ammonia and lactate (Paredes et al., 1999). Some hybridoma cells have been successfully engineered to contain a glutamine synthetase enzyme, which made them glutamine-independent but also reduced growth rate (Bell et al., 1995). There have been recent attempts to combine metabolic engineering of mammalian cells with fed-batch feeding strategies (see below) to prevent waste product accumulation (Young, 2013).

(3) Decreasing or eliminating glutamine in the medium. It is possible to some extent to change mammalian cell metabolism by adaptation. The exact growth conditions (medium and bioreactor) influence mammalian cell metabolism and can be modified

to overcome the glutamine problem (Vriezen and van Dijken, 1998). Adapting cells to very low glutamine concentrations works for some, but not all, mammalian cells (Bell et al., 1995). Glutamine can also be provided to mammalian cell cultures in a different, non-ammoniogenic form. Provision of glutamine can be through dipeptides such as Ala-Gln or Gly-Gln, which are much more stable than molecular glutamine, and hence decrease ammonia accumulation by non-enzymatic decomposition [[www.sigmaaldrich.com](http://www.sigmaaldrich.com)] (Butler and Christie, 1994; Christie and Butler, 1994b). Certain media components can be changed or replaced to change glutamine requirements. In some cases, glutamine can be replaced by high concentrations of pyruvate (Genzel et al., 2005). This approach allowed MDCK, BHK21 and CHO-K1 cells to grow in glutamine-free medium with no need for an adaptation step. However, all these cell lines contain some endogenous glutamine synthetase activity, so it is unlikely that this approach will work with hybridoma cells. Low glucose lowers glutamine uptake (Vriezen and van Dijken, 1998), which shows interdependence of different metabolic pathways (Figure 1.6c). Glutamine and glucose have been replaced with galactose and glutamate in CHO cell medium (Altamirano et al., 2000, 2001, 2004). Replacement of glutamine with glutamate was attempted as early as 1956 by Eagle (Eagle, 1956; Newland et al., 1990; Hassell and Butler, 1990; McDermott and Butler, 1993; Butler and Christie, 1994; Christie and Butler, 1994a). Decreasing glutamine concentration or increasing glucose concentration may reduce glutamine-consumption and hence ammonia production (Jeong and Wang, 1995). But the glutamine-replacement tends to only work with cell types that contain glutamine synthetase (Newland et al., 1990). When oxygen uptake rate is unconstrained then cells take up less glutamine (to a similar level as other amino acids) (Vriezen and van Dijken, 1998; Sheikh et al., 2005). This strategy is also used to adapt cells to various other conditions, *e.g.* serum-free media (Europa et al., 2000) and lower glucose (Newland et al., 1990) or pyruvate, but not all cell lines can be adapted to everything (Genzel et al., 2005).

(4) Removal of ammonia from culture medium. Removal of ammonia is not straightforward because of its high solubility and ion exchange characteristics (Newland et al., 1990). While there have been promising approaches, none of them fully solve the problem. Approaches have included addition of ammonia degrading enzyme to culture media (Newland et al., 1990), dialysis bags (Iio et al., 1985), *in situ* removal of ammonium and lactate through electrical means for hybridoma cultures (Chang et al., 1995) and *in situ* removal of ammonia and fed-batch addition of glutamine and glucose (Park et al., 2000). Perfusion culture to remove ammonia improved cell growth and mAb production and keeps ammonia concentration under an inhibitory level (Matsumura and Nayve, 1995).

A very interesting approach involved co-culture with rat liver cells (hepatocytes) to remove ammonia from the culture medium of CHO cells (Choi et al., 2000). Some of these approaches have also been used in the attempt to make artificial livers. In some microbial industrial processes, *e.g.* amino acid production, microbial contaminants that grow on by-products can actually increase the yield and purity of the product (Hermann, 2003)

(5) Fed-batch culturing mode. A common and well known strategy to reduce ammonia toxicity is the semi-continuous fed-batch culturing mode (Glacken et al., 1986; Ljunggren and Häggström, 1990; Xie and Wang, 1994; Ljunggren and Häggström, 1994; Linz et al., 1997; Nadeau et al., 2000; Hermann, 2003). This method reduces glutamine and glucose concentration in culture medium (Europa et al., 2000), reducing accumulation of toxic byproducts. Fed batch is also useful as a research tool, as it allows the conditions in the culture medium to be changed during the course of a run, for instance to elicit a metabolic change in the mammalian cells (Europa et al., 2000). Addition of nutrients can be according to the results of on-line medium analysis (Lee et al., 2003) or a pre-determined algorithm. Most industrial bioreactors now use the fed-batch mode instead of the previously used batch-mode in which the cell culture is provided with all the nutrients needed for the entire run at the beginning. There is great demand for automatisation in the bioprocessing industry, already quite successful in microbial production (Hermann, 2003). Generally, fed-batch is a very successful approach, however it requires extensive on-line monitoring of cultures or alternatively knowledge about culture dynamics that can feed into a model.

Automatic sensing and monitoring and adding (interface with computer) has been discussed in bacterial bioprocessing (Hermann, 2003). For mammalian cell bioprocessing this is not quite possible yet, as the monitoring alone is challenging enough.

Overall, in order to maximise product yield from mammalian cell cultures, it is important to provide an ideal growth environment for the cells at all times. Since the environment is constantly changing during the course of a cell culture, ways to monitor various culture parameters on-line are needed. By monitoring culture parameters in detail and in real time, we can gather information needed to optimise culture conditions and upscale processes. In short, bioprocess control demands bioprocess monitoring.

### **1.3.3.2 Glucose and lactate**

Glucose is the main source of carbon in mammalian cell cultures. Lactate (also known as lactic acid) is a byproduct of cellular glucose metabolism. High levels of lactate can decrease medium pH below the optimal range (Newland et al., 1990). This is generally not a great problem in short term pH-controlled cultures, however it does become problematic in longer cultures, which are what the field of bioprocessing is working towards (Newland et al., 1990).

At later culture stages, some cells can use lactate as a carbon source when glucose has been depleted (Newland et al., 1990). Such processes can introduce unwanted variability into processes. Attempted solutions to this overlap to some extent with the attempted solutions to the glutamine problem (see above). For example, replacement of medium components has also been used to address lactate accumulation, as glucose has been replaced with fructose, galactose, or mannose (Glacken et al., 1986; Barngrover et al., 1985; Newland et al., 1990). Similarly for metabolic rewiring of mammalian cells (Young, 2013). By keeping glucose at a low level, mammalian cell metabolism was altered to a state with decreased lactate production (Europa et al., 2000). siRNA has been used to knock down genes in lactate metabolism to reduce lactate production by mammalian cells and increase productivity (Zhou et al., 2011).

### **1.3.4 Synthetic biology in mammalian bioprocessing**

While synthetic biology has already been applied to microbial bioprocessing, there have so far only been limited applications in mammalian bioprocessing (Rollié et al., 2012). One aspect of synthetic biology as relevant to mammalian cell bioprocessing is the genetic engineering of mammalian cells. In recent years there have been rapid advances in designing and implementing synthetic circuits and more complex systems in mammalian cells, largely led by the Fussenegger group at the ETH (Weber et al., 2007; Weber, 2009; Weber et al., 2009; Weber and Fussenegger, 2011; Ausländer and Fussenegger, 2013; Bacchus and Fussenegger, 2013), including artificial cell-to-cell communication (Wang et al., 2008), gene switches (Ausländer and Fussenegger, 2013) and many other systems. More work needs to be done to advance synthetic biology in mammalian cells and overcome current limitations. One of the limitations is random integration into the genome of transgene elements in mammalian cells. Systems to overcome this include the Cre/Lox recombinase and FRT/FLP recombinase (Weber and Fussenegger, 2002). Weber (2009) suggested that the synthetic biology “BioBrick” registry be extended to include “CytoBricks”,

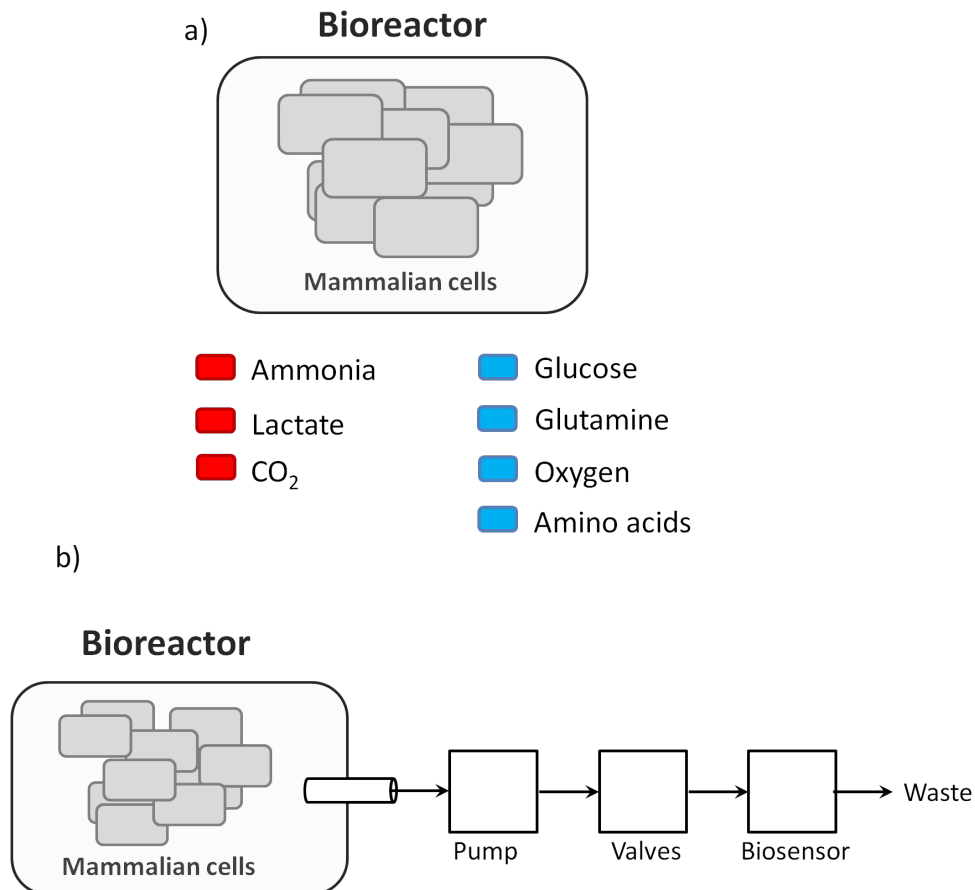
well-characterised mammalian cell lines containing transgenes. Some examples of mammalian cell engineering were mentioned above in tackling limitations in bioprocessing. One example of mammalian synthetic biology used in bioprocessing is the development of intracellular FRET biosensors for glutamine and glucose for mammalian cells (Behjousiar et al., 2012). These are intended to be used during early stage process optimisation.

## 1.4 Monitoring of metabolites in bioprocessing

As discussed previously, bioprocess monitoring is of major importance in bioprocessing. In mAb production by hybridoma cells, control of key nutrients is essential to cell viability and production and control is only possible with some level of monitoring (Mulchandani and Bassi, 1995). Whilst optimising a process, the aim is often to monitor a large number of parameters to learn as much as possible about the cells, which are useful for scale-up (Harms et al., 2002). As there are many potential parameters of interest, there are a number of methods for (mammalian) bioprocess monitoring. Parameters of interest include oxygen and carbon dioxide levels, pH, cell mass, cell viability and the concentrations of ions and metabolites, including those already discussed (Figure 1.7a). The various methods for bioprocess monitoring have been previously discussed (Mulchandani and Bassi, 1995; Harms et al., 2002; French and Gwenin, 2012). There are stringent requirements for sensors that are to be used in bioprocessing, including sterility, temperature and pH tolerance, life-time and detection range, which limits the number of sensor methods that reach a useful level of applicability (Harms et al., 2002).

Sensors are usually based on electronic, chemical or enzymatic mechanisms. Purified proteins attached to electrodes or incorporated into membranes are often used as biosensors in bioreactors (Inaba et al., 2003; Kwan et al., 2004). Numerous examples of such biosensors have been created *e.g.* for glutamine (Cattaneo, Male and Luong, 1992; Cattaneo, Luong and Mercille, 1992; White et al., 1994; Mulchandani and Bassi, 1996), glutamate (White et al., 1994; Mulchandani and Bassi, 1996) or lactate (White et al., 1994; Rohm et al., 1996; Male et al., 1997). Certain commercially available systems combine a number of such sensors for monitoring multiple parameters in parallel and some of these allow on-line measurements: Yellow Springs Instruments [[www.ysi.com](http://www.ysi.com)], Nova Bioprofile-Analyzers [<http://www.novabiomedical.com/>], *e.g.* (Derfus et al., 2010), or Finesse [<http://finesse.com/>] analysers. For instance, equipment from the Nova bioprofile range use ion-selective electrode potentiometry, amperometry, and enzymatic reaction-dependent





**Figure 1.7:** Bioprocess monitoring. a) Parameters of interest that are commonly monitored. Cellular waste products (red) and compounds that are needed by the cells (blue). b) Possible monitoring method using flow injection analysis. Small amounts of culture medium are diverted from the main reactor and channelled towards sensors for monitoring and analysis. See more detail in the main text. Diagram adapted from Mulchandani and Bassi (1995).

biosensors to monitor electrolytes and metabolites in samples (Derfus et al., 2010). Some analysers use the acquired data to control the feed of a fed-batch culture (Wong et al., 2005; Genzel et al., 2005). Amino acids can be measured using HPLC methods. However, even though the technology is constantly improving, many of these methods have a number of disadvantages in their use, as they tend to be expensive, offline, work-intensive, have limited detection sensitivity (Table 1.2) and require large sample volumes and sample processing. Automatic monitoring and feed control (using an interface with a computer) is already being studied in bacterial bioprocessing (Hermann, 2003). For mammalian cell bioprocessing this is not quite possible yet, as the monitoring alone presents a great challenge.

The use of whole-cell microbial biosensors in bioprocessing has been discussed (Mulchandani and Bassi, 1995; Bracewell and Polizzi, 2014). Generally, the

**Table 1.2:** Detection ranges of existing monitoring processes for cell culture metabolites.

Metabolite	Detection range	Reference
Glucose	0.2 - 15.0 g/L	Nova Biomedical
Lactate	0.2 - 5.0 g/L	Nova Biomedical
Glutamine	0.2 - 6.0 mmol/L	Nova Biomedical
Glutamate	0.2 - 6.0 mmol/L	Nova Biomedical
Ammonium	0.2 - 25.0 mmol/L	Nova Biomedical

advantages and limitations of whole-cell biosensors that were discussed above, apply especially in a complex environment such as this. There are potential problems with contamination of mammalian cultures with microbial cells and the sensors may show decreased specificity in the complex culture medium. However, the microbial cells can also be maintained for a long time using cheap growth medium (Mulchandani and Bassi, 1995). Smart reactor set-up can be used to vastly lower the chances of contamination. Biosensor cells could be immobilised using gel entrapment (Mulchandani and Bassi, 1995). Hybridoma and biosensor cells can also be kept apart by making use of flow injection analysis (FIA) or similar concepts (Mulchandani and Bassi, 1995; Male et al., 1997; Mayer et al., 1999; Harms et al., 2002) (Figure 1.7b). In this system, a small sample is taken from the main reactor medium and injected into a carrier stream of buffer solution that continuously flows past a detector (Mulchandani and Bassi, 1995). Because the sensors do not come in contact with the main culture in this scenario, the requirements for the sensors can be less stringent and do not require sterility (Mulchandani and Bassi, 1995). The environment of the sensors could be tailored to the requirements of the sensor cells. FIA analysis systems using enzymatic assays have for example been developed for on-line monitoring of glutamine and glutamate (Mayer et al., 1999).

There are certain metabolite parameters that are of particular interest in bioprocessing, specifically those discussed above. And while many different kinds of sensors have been developed for many different kinds of metabolites, there are still gaps in what we can monitor in bioprocessing, especially in terms of basic metabolites. Biosensors for bioprocessing that can report on availability of carbon sources such as glucose and glutamate would be useful (Yeomans et al., 1999). Real time visualisation of metabolites would be useful (Fehr et al., 2004). As far as the author knows, no whole-cell sensors have been made yet for many important bioprocessing parameters, and there is a need for specific amino acid biosensors in the field (Kwan et al., 2004). Whole-cell biosensors for metabolites would allow monitoring of more complex processes, *e.g.* glutamine-need of mammalian cells by measuring fundamental metabolites and cellular biosensors would allow integration

of several monitored signals. Cells behave differently in large bioreactors than flasks. It is the up-scaling step of bioprocess development that is in particular need for novel sensors.

The suggestion here is that a synthetic biology approach could be used to engineer whole-cell microbial biosensors for mammalian cell culture to be used in process monitoring and optimisation.

## **1.5 Natural bacterial sensing systems relevant to bioprocessing**

A literature search to find natural bacterial sensing mechanisms for metabolites relevant to bioprocessing was carried out. The results are shown in Table 1.3. The aim was to find suitable natural systems that could be used to engineer biosensors used in bioprocessing. There already exist various biosensors for many amino acid and metabolites, but many of them have an output that can be measured, but which does not easily translate into a biological transcription output, *e.g.* FRET sensors (Marvin and Hellinga, 1998; Dattelbaum and Lakowicz, 2001; De Lorimier et al., 2002; Tolosa et al., 2003; Fehr et al., 2004), largely summarised by Dwyer and Hellinga (2004).

**Table 1.3:** Natural sensing mechanisms (or pre-existing biosensors) for interesting bioprocessing variables.

Target	Transcriptional	Translational	Post-translational
<b>Glutamine</b>	GlnK/GlnL ( <i>Bacillus</i> ) (Satomura et al., 2005; Ye et al., 2009)	glnA RNA motif (Ames and Breaker, 2011)	glutamine permease operon glnHPQ ( <i>E. coli</i> )
	GlnL/GlnG ( <i>C. glutamicum</i> ) (Rehm, 2010; Ye et al., 2009)	Downstream-peptide motif (Ames and Breaker, 2011)	glutamine FRET sensor (Behjousiar et al., 2012)
	PII/NRI ntrC ( <i>E. coli</i> (Ninfa and Atkinson, 2000))		glutaminase/glutamate dehydrogenase colourimetric assay
	GS/TnrA ( <i>Bacillus</i> ) (Satomura et al., 2005)		
<b>Glucose</b>	<i>Arabidopsis</i> (Li et al., 2006)	-	glucose oxidase
	rcsF (phosphorelay glucose and zinc sensor)		
	Crr (glucose-specific enzyme IIA component of PTS)		
<b>Ammonia</b>	soybean GS15 (cytosolic glutamine synthetase gene promoter) (Tercé-laforge et al., 1999)	-	-
	GlnK		
	$\sigma$ 54 transcription factor (Charbit, 1996)		
<b>Lactate</b>	lldPRD operon (Aguilera et al., 2008)	-	lactate oxidase lactate dehydrogenase lldR FRET sensor (San Martín et al., 2013)
<b>Alanine</b>	Lrp (leucine-responsive regulatory protein)	-	-
<b>Arginine</b>	artPIQMJ; argT	-	
<b>Asparagine</b>	ans operon ( <i>Bacillus</i> ; <i>Rhizobium etli</i> ) (Ortuño-Olea and Durán-Vargas, 2000)	-	-
<b>Aspartate</b>	Taz chemoreceptor (Jin and Inouye, 1993)	-	gltI (Periplasmic glutamate-aspartate binding protein)
<b>Cysteine</b>	cysH operon	-	-

Continued on next page

Table 1.3 – continued from previous page

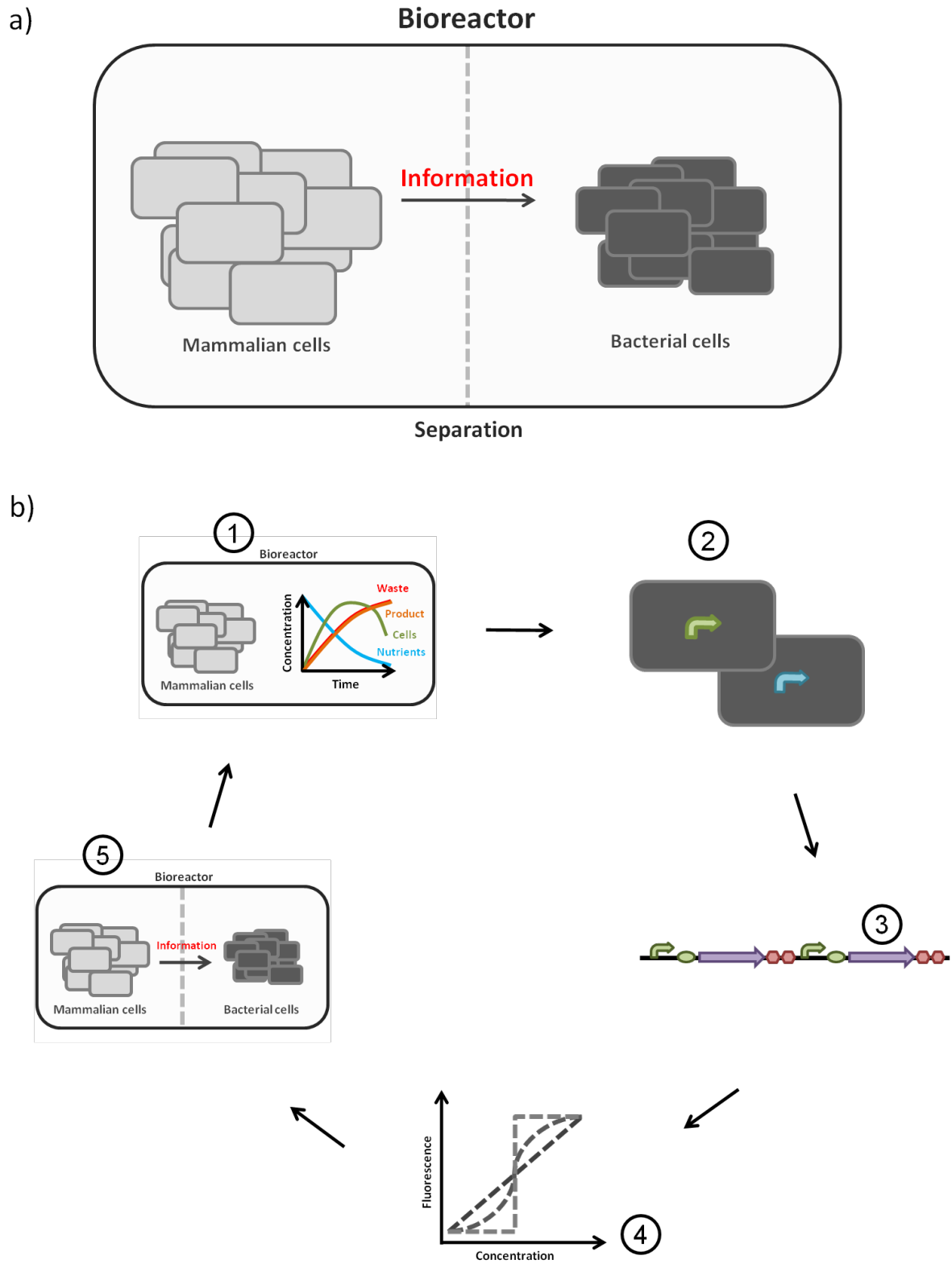
Target	Transcriptional	Translational	Post-translational
<b>Glutamate</b>	Check (Ye et al., 2009)	-	gltI (Periplasmic glutamate-aspartate binding protein) Glutamate FRET sensor (Palmer et al., 2011)
<b>Glycine</b>	-	glycine riboswitch (Mandal et al., 2004)	ProX (Glycine betaine/L-proline transport system binding protein)
<b>Histidine</b>	histidine attenuator (Vitreschak et al., 2004)	HisPMQJ (histidine transporter)	-
<b>Isoleucine</b>	Threonine-isoleucine-dependent attenuator (Vitreschak et al., 2004)	-	-
<b>Leucine</b>	Lrp (Leucine-responsive regulatory protein) (Wang et al., 1994)  Leucine attenuator (Vitreschak et al., 2004)	-	-
<b>Lysine</b>	-	Lysine riboswitch (Vitreschak et al., 2004)	-
<b>Methionine</b>	Met repressor, MetJ, recognises SAM  Lrp ( <i>C. glutamicum</i> ) (Mustafi et al., 2011)	-	-
<b>Phenyl-alanine</b>	TyrR operon (Pittard et al., 2005)	-	-
<b>Proline</b>	-	-	ProX (Glycine betaine/L-proline transport system binding protein)
<b>Serine</b>	-	-	-
<b>Threonine</b>	Threonine-isoleucine-dependent attenuator (Vitreschak et al., 2004)	-	-
<b>Tryptophan</b>	Trp operon  TyrR regulator (Pittard et al., 2005)	-	Trp FRET sensor (Kaper et al., 2007)
<b>Tyrosine</b>	TyrR regulator (Pittard et al., 2005)	-	-
<b>Valine</b>	-	-	-

## 1.6 Aim of this project and thesis overview

The aim of this work was to tackle some of the challenges in bioprocessing using a new synthetic biology approach. Bacterial whole-cell biosensors for metabolites have been engineered using the principles of synthetic biology. The model context chosen for these sensors were target metabolites relevant in bioprocessing (Figure 1.8a). This should lead to a “platform” involving biosensing, and interactions between bacterial and mammalian cells that could be used in other contexts with minimal modifications. Many of the principles that have previously been used in trying to tackle the limitations of bioprocessing fed into the approach. Useful characteristics of bacterial cells, such as fast production, robustness, flexibility, ability to be stably transformed with genes, were used. Sensors for amino acids and other metabolites will find applications in many fields (Mustafi et al., 2011). There have been studies placing metabolite sensors directly inside mammalian cells (Behjousiar et al., 2012). This can provide useful intracellular information, but also pose a metabolic burden to the cells (Harms et al., 2002). The sensors proposed here would give extracellular information and could be transferred to other systems and cell types more easily. There already exist various biosensors for many amino acid and metabolites, but many of them have an output that can be measured, but which does not easily translate into a biological transcription output, *e.g.* FRET sensors (Marvin and Hellinga, 1998; Dattelbaum and Lakowicz, 2001; De Lorimier et al., 2002; Tolosa et al., 2003; Fehr et al., 2004), largely summarised in (Dwyer and Hellinga, 2004). Here the aim is to design transcriptional or translational sensors that can be linked up to a biological output. This would allow the sensors to possibly be linked up to various possible reactions from the cells.

The principles of systematic design were followed in this project. Initially, specifications for the biosensors were drawn up (Figure 1.8b(1)). This involved mammalian cell culture experiments that defined the environment in which these sensors were to be used. These can be found in chapter 3 (Hybridoma cell culture characteristics).

Natural sensor mechanisms were researched and biosensors designed (Figure 1.8b(2)). Biosensor design fed into assembly (Figure 1.8b(3)). Biobrick format was used to make use of existing parts and so that the devices would be compatible with other constructs in the synthetic biology community. Several biosensors were designed in this study. Details can be found in chapters 5 (*E. coli lldPRD* lactate-responsive operon), 6 (*E. coli* Lrp operon) and 7 (*E. coli* Ntr regulon) and 8 (*Bacillus subtilis* GlnK-GlnL system). The next step was biosensor characterisation (Figure 1.8b(4)). The sensors were tested with an easily detectable output (*e.g.* presence of green



**Figure 1.8:** a) Bacterial whole cell biosensors for bioprocessing. b) Approach for engineering bacterial whole cell biosensors for bioprocessing. Design cycle specific to thus project. 1) Specifications. 2) Design. 3) Assembly. 4) Testing. 5) Implementation.

fluorescent protein (GFP)) and real time sensing with limited time delay (but allowing for *e.g.* transcription) (Belkin, 2003) under standard conditions. Details on characterisation protocol development can be found in chapter 4 (Development of a biosensor characterisation protocol). Details can be found in chapters 5 (*E. coli lldPRD* lactate-responsive operon), 6 (*E. coli* Lrp operon) and 7 (*E. coli* Ntr regulon) and 8 (*Bacillus subtilis* GlnK-GlnL system).

To be implemented in a mammalian cell culture context, the biosensor cells needed to function in complex medium (mammalian cell growth medium) in bioreactor conditions (Figure 1.8b(5)). These experiments can be seen in chapter 9 (Biosensors in Bioprocessing).



## **2 Materials and methods**

### **2.1 Hybridoma cell culture**

#### **Cell line**

The murine hybridoma cell line ATCC-CRL1606, producing a mAb (HFN7.1) against human fibronectin (Schoen et al., 1982), was obtained from the American Type Culture Collection and was subcultured as recommended.

#### **Cell culture at different glutamine concentrations**

Cells were cultured in 500 ml Erlenmeyer flasks (Corning, UK) at 37°C in an atmosphere containing 5% CO<sub>2</sub> on an orbital shaking platform rotating at 125 rpm. The basal growth medium was 100 ml glutamine-free DMEM (Sigma, D6546) supplemented with 10% (v/v) calf bovine serum (ATCC, 30-2030) and either 0 mM Gln, 2 mM Gln, 4 mM Gln, 6 mM Gln, 8 mM Gln or 10 mM Gln (Sigma, G3126). Cells had previously been grown in DMEM (Gibco, Invitrogen, 52100-039) containing 4 mM Gln. Glutamine was sterilised by filtration.

#### **Cell culture for sample collection**

Cells were cultured in 1L Erlenmeyer flasks at 37°C in an atmosphere containing 5% CO<sub>2</sub> on an orbital shaking platform rotating at 125 rpm. The basal growth medium was 200 ml dulbecco's modified eagle's medium (DMEM) (Gibco, Invitrogen, 52100-039), supplemented with 10% (v/v) calf bovine serum (ATCC, 30-2030).

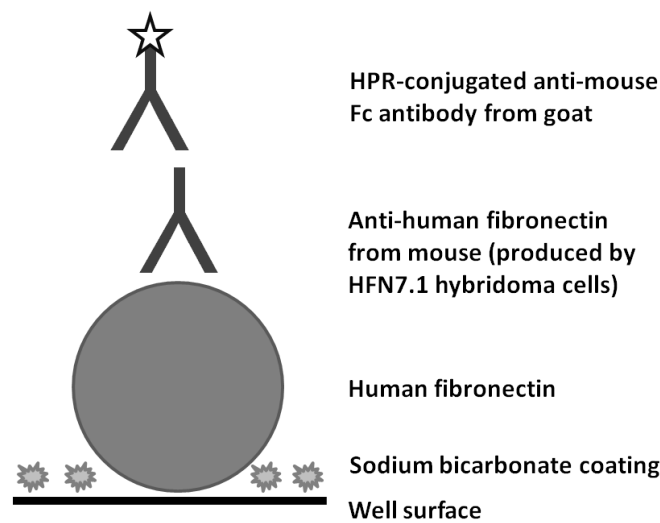
#### **Cell enumeration**

Viable cell concentrations were determined using the Trypan blue exclusion method (Patterson Jr, 1979). Small volumes of cell culture were samples and diluted with water as appropriate. Samples were then mixed with an equal volume of trypan blue. Using a light microscope, cells were determined to be alive (transparent) or

dead (blue) and counted. Integral viable cell concentration (IVC) was calculated as the integral of the viable cell concentration over time.

### ELISA for antibody detection

Antibody analysis of culture supernatant samples was performed using an optimised protocol based on that described in (Kontoravdi, 2007). Optimisation of this assay used a number of different 96-well plates: medisorb (Nunc, 467320)\*, multisorb (Nunc, 467340) and microtitre (sterilin, 611F96). Two brands of human fibronectin were tested: (BD, 354008)\* and (Sigma-Aldrich, F0895). Two anti-human fibronectin mAbs were tested for use as the standard: mouse monoclonal anti-human fibronectin (HFN7.1, abcam, ab80923)\* and mouse monoclonal anti-human fibronectin (Sigma-Aldrich, F0791). Two HPR-conjugated anti-mouse Fc immunoglobins were tested for use as the secondary antibody: (Sigma-Aldrich, A0168) and (Dako, P0447)\*. Reagents marked with a star were used in the ELISA analysis of culture supernatant samples.



**Figure 2.1:** ELISA to specifically detect anti-human fibronectin antibody produced by HFN7.1 hybridoma cells.

### Metabolite analysis

Samples of culture supernatant were analysed using a Bioprofile 400 Analyzer (Nova Biomedical) according to the manufacturer's guidelines. Cells were removed by centrifugation to obtain supernatant samples. No further sample processing was done. The Bioprofile Analyzer was used to measure ammonia, glucose, glutamine and lactate concentrations.

## 2.2 CHO cell culture

### Cell line

A CHO-S cell line containing a fluorescent fusion protein integrated into the genome was obtained from Antony Constantinou (CSynBI, Imperial College London).

### Cell culture conditions

Cells were cultured in a 250 ml Erlenmeyer flask (Corning, UK) at 37°C in an atmosphere containing 5% CO<sub>2</sub> on an orbital shaking platform rotating at 125 rpm. The basal growth medium was 100 ml CD CHO medium (Invitrogen, UK), supplemented with 8 mM glutamine (Invitrogen, UK) and HT supplement (11067-030, Gibco).

## 2.3 Assembly of bacterial biosensor constructs

### Bacterial strains

Bacterial strains used in this study are summarised in Table 2.1.

**Table 2.1:** Bacterial strains used in this study

Bacterial strain	Source	Genotype
<i>Escherichia coli</i> DH5 $\alpha$ ( $\alpha$ -Select)	Bioline	F- deoR endA1 recA1 relA1 gyrA96 hsdR17(rk-, mk+) supE44 thi-1 phoA $\Delta$ (lacZYA argF) U169 $\Phi$ 80lacZ $\Delta$ M15 $\lambda$ -
<i>Bacillus subtilis</i> subtilis 168	Bacillus Genetic Stock Center (BGSC)	trpC2

### Assembly of DNA constructs

The BioBrick assembly method was used in the assembly of all DNA constructs. Lactate sensor constructs were assembled by undergraduate students Sharmilah

Veteryan and Katarzyna Roguska. Glutamine transporter constructs were assembled by undergraduate student Kai Jiang. Nitrogen metabolism constructs were assembled by undergraduate student Jonathan Chan. Plasmid pSB1A2 as used for all constructs.

## Promoter sequences

The promoter sequences used in the biosensors in this study are summarised in Table 2.2.

**Table 2.2:** Promoters used in this study.

Primer	Sequence
<i>E. coli NtrC-responsive promoters</i>	
argTp	TTTTGC AACCGCGATC AAATCCTCGA CATTTTGTTT CTGCCATTCA ATCGAAACGC TGCATTCAA CCGCTATACC TGCTATCTTC AACTTCAGGA CAATAATGCA ACGTCTTATT AACATATTTA ACGTTGAATG TTA CTGTTGT CGTCAAGATG GCATAAGACC TGCATGAAAG AGCCT
astCp2	TTGTTAATGA TGTCAACGAT GCGCAAAAA ATGCCCGCTT TTGGTGCGCG CTGCGTCAGA ATGGCGCAGT AATTTCCAGT AAATTTGCTC AATGATCACA ATTCCAGAAT TATATTTTAC TTCTGCATAA CATTGCGTTT TTTATCTTT TTATTCACCC ACATCGCAA CGTATCACT TTATATGCAC TTTAAATGCA TATGGTTTGG TTATAACTCC TTGATTTCCA GTTAGCCTCC GCCGTTTATG CACTTTTATC ACTGGCTGGC ACGAACCTG CAATCTACAT TTA
glnAp1	GTCCCTTTGT GATCGCTTTC ACGGAGCATA AAAAGGGTTA TCCAAAGGTC ATTGCACCAA CATGGTGCTT AATGTTTCCA TTGAAGCACT ATATTGGTGC AACATTACACA TCGTGGTGCA GCCCTTTTGC ACGATGGTGC GCATGATAAC GCCTTTTAGG GGCAATT
glnAp2	ATTGCACCAA CATGGTGCTT AATGTTTCCA TTGAAGCACT ATATTGGTGC AACATTACACA TCGTGGTGCA GCCCTTTTGC ACGATGGTGC GCATGATAAC GCCTTTTAGG GGCAATTTAA AAGTTGGCAC AGATTTGCTT TTATCTTTTT TA
glnHp2	ATCCATCGCT GATGGTGCA GACTTTAGTA CCCGATAAAA GCGGCTTCCT GACAGGAGGC CGTTTTGTTT TGCAGCCCAC CTCAACGCAC TTATTTAGTG CATCCATCTG CTATCTCCAG CTGATTAAGT AAATTTTTTG TATCCACATC ATCACACAAT CGTTACATAA AGATTGTTTT TTCATCAGGT TTTACGCTAA ATAATCACTG TGTTGAGTGC ACAATTTTAG CGCACCAGAT TGGTGCCCCA GAATGGTGCA TCTTCAGGT ATTGCCCTAT AAATCGTGCA TCACGTTTTT GCCGATCTC GAAAAATCAA GGAGTTGCAA AACTGGCACG ATTTTTTCAT ATATGTGAAT G
glnKp	AGCGCA ATATTTTCATC GTTGGTGCAA AAATGTAACG CACTGTGCAC TGTCATAGTG CGTTTTTCATT TTCAAACCTC TTAACCTCCT GCTCTCTTTC TCGTTTTTCA TTTCTGGCAC ACCGCTTGCA ATACCTTCTT C
glnLp	TTGTCAGCTA TCTGTAGCCC ATCTCTGCAT GGGCTTTTTT CTCCGTCAAT TCTCTGATGC TTCGCGCTTT TTATCCGTAA AAAGCTATAA TGCATAAAA TGGTGCAACC

Continued on next page

Table 2.2 – continued from previous page

Primer	Sequence
nac	CGG GCGCATATT AATGGCAGTG CATGGTGATG TCAAAGGGTG AAATGCATTT TTTTGTGTTGT TTGCTGTCAA TTTCAACAAA GTGGTGAGCT TTCAATCTTA TTGGGTAATG AACCATCGTG GTGCATACCC TCCTTTTATA GGGCAGGGGA ACGCGACAGC TGATTAAGG AGCAAATGAC GAATCTATCA TAATCTCTTA TTTGACAGTT GGTTAGCTTG TACATCAACA CAAAATAAA ACTGGCAAGC ATCTTGCAAT CTGGTTGTA
<i>E. coli</i> LRP-responsive promoters	
fimBp1	GAAGTTTAA GTCGTAGTGA CCAAAGCTAT ATTTACCAAC GAATGTAGAT GAAAAAATCA TCTCCTGCGT TCCCCCATAT CTCTAGGATA AAAAGGAATG TAACAATCTC ATGGCGTAAG CTGACGAATC AGCAGGAATA ATCGCTAGGG ACCTAAGAAT TAGCATGATA ATAGCCACTA AGAAATTAAT GCGCTCCATG AAATAGCCAT TTTGTGGCAA ATGGAGTTGA CTAATAATGT CATATGTGAG ACGGCTAGTT GAACGAATAT TAAATTTTGC TGAATTTTTT ATGTTGATTT TACTTGTTAC AGAACATATC ACATGATATA TAGATAAGAT TAGTTGCATT AATGATGAGG GTTATTATTA GATTCGTATC CGATTGATAA ATATATAAAG GTACATAGCA TGCAAGAGCA TGGCGTTTGT ATGGCAACGT TATTATAATT AACAGTTGCT AC
fimBp2	GCACCTTTATACCTGTT ATACCAGATC AAAAATCACG CAATCCATAC AA- CAAAACCA GATTTGCAAT TCGTGTCAACA AAATATGTCG ATCTTTTTTCT AAGAGGAAGA TGCCATGTGA AGCCAGACGA ACACCTGCGG TGGTCTTCAA AAACTAAAGA TCTTAGTTTA ACTATTTGTT TTATAAATAA TTTATTAAGA GTCTAAACAA GGGGAGCTTT GCAAGCTAAC TCAGTGAGCT TGGTAAAAT CAGTGTTTAC CCGCCATCAG GCTGAGCATA ATTCTCATCA TGAAATATGT TTCTGGTTT GTGGCTTGTA ACTGGTCACT TCTGAAGTCG ATCTGGAGAG GCTTGTGAT GTTGGTGTGTT TCAGGATGAT GTTTCACTTA GTTTGTTTGC CGTATCGCCC GGCGAATGGC TGTGATTGAG GAAGGTTTAA GTCGTAGTGA CCAAAGCTAT ATTTACCAAC GAATGTAGAT GAAAAAATCA TCTCCTGCGT TCCCCCATAT CTCTAGGATA AAAAGGAATG TAACAATCTC ATGGCGTAAG CTGACGAATC AGCAGGAATA ATCGCTAGGG ACCTAAGAAT TAGCATGATA ATAGCCACTA AGAAATTAAT GCGCTCCATG AAATAGCCAT TTTGTGGCAA ATGGAGTTGA CTAATAATGT CATATGTGAG ACGGCTAGTT GAACGAATAT TAAATTTTGC TGAATTTTTT ATGTTGATTT TACTTGTTAC AGAACATATC
gltBp	TTAAGGCAGT ATAAAATGCT GGTTTTGTGTCG TCAGTTCAAG GCAGGATAAG GGTTAACACA CCTTATGAC AGTCAGGAAT TGACTGTTTC TCTAACGACT TCCCTTTTAG CCTTAAAGAT AAAATCCATT TTAATTTTCAAG TCATTTAATA AAGAATTTTG CGCTAAAGCA CATTTCTGTA CCAATAAGCT TGCCATTTGA CCTGTATCAG CTTTCCCGAT AAGTTGGAAA
ilvIHp1	GAATG TCTGGTTTAT TCTGCATTTT TTATTGAATG TAGAATTTTA TTCTGAATGT GTGGGCTCTC TATTTTAGGA TTAATTAATA AAATAGAGAA ATTGCTGTAA GTTGTGGGAT TCAGCCGATT TATTATCAAT TTAATCCTCT GTAATGGAGG ATTTTATCGT TTCTTTTTCAC CTTTCCCTCT GTTTATCTT ATTACCCCGT GTTTATGTCT CTGGCTGCCA ATTGCTTAAG CAAGATCGGA CGGTTAATGT GTTTTA
leuLp	AACCACCGCA GCACAATTAG CTAATTTTAC GGATGCAGAA CTCACGCTGG CGGGACGTTT TTATTGCGTC AGGGTTGACA TCCGTTTTTG TATCCAGTAA CTCTAAAAGC ATATCGCATT CATCTGGAGC TGATTTAATG ACTCACATCG TTGCTTTTAT CGGTCTACTA CTAATAAACG CATCTTCTTT GCGCGGTAGA CGAGTGAGCG GCATCCAGCA TTAAGCCAGC ACGCAGTCAA AAAAAAACC CGCGCCATTG CGCGGGTTTT TTTATGCCCG AAGCGAGGCG CTCTAAAAGA GACAAGGACC CAAACC

Continued on next page

Table 2.2 – continued from previous page	
Primer	Sequence
livJp	TGAGAGTCCGGGGTTTTTGTTTT      TTGGGCCTCT      GTAATAATCA ATTTCCCCTC CGGCAAAACG CCAATCCCCA CGCAGATTGT TAATAAACTG TCAAAATAGC TATTCCAATA TCATAAAAAT CGGGTATGTT TTAGCA
<i>E. coli lldR-responsive promoter</i>	
lldp	CTTTACCAG ACATCTCCCC CCACAAGAAT TGGCCCTACC AATTCTTCGC TTATCTGACC TCTGGTTCAC AATTCCCAA TTAAAACCTCA CATCAATGTT GCCAATACAT AACATTTAGT TAACCATTCA TTGTCATTAT CCCTACACAA CACAATTGGC AGTGCCACTT TTACACAACG TGTGACAAGG AGATGAGCAA CAGACTCATT ACACGATGTG CGTGGACTCC
<i>B. subtilis GlnK/GlnL-responsive promoter</i>	
PM8J2-706	TGCTTATTTT CAAAATGTTT TTGTCGTATT TTGTATGATT CTGTAGTCTC CATTATACAG TAATTAT

## Polymerase chain reaction

Polymerase chain reaction (PCR) to obtain biosensor promoters was carried out using a variety of conditions, using the Pfu Turbo kit (Promega, UK) and Phusion High-Fidelity DNA Polymerase (Thermo Scientific, UK). Detail of PCR reaction conditions is given in Table 2.3. PCR primers (Invitrogen) used are summarised in Table 2.4. For *E. coli* Ntr-responsive promoters, PCR with blunt primers was followed by secondary PCR with primers containing the Biobrick prefix and suffix. For the *E. coli* promoters, *E. coli* genomic DNA from JM109 or DH5 $\alpha$  was used as a template. *E. coli* DH5 $\alpha$  genomic DNA was obtained by boiling cells in water at 98°C for 10 min. 1-2  $\mu$ l of genomic DNA template were used in PCR reactions. Either Pfu polymerase (Stratagene) or Phusion high-fidelity polymerase (FisherScientific) were used. Promoter sequences were mostly taken from [ecocyc.org]. For the *B. subtilis* PM8J2-706 promoter, two overlapping oligonucleotides, together representing the whole promoter sequence were used in primary PCR, followed by secondary PCR with primers containing the Biobrick prefix and suffix.

**Table 2.3:** PCR conditions used in this study

Amplified fragment	Polymerase	Annealing temperature (°C)	Product length (bp)
<i>E. coli NtrC-responsive promoters</i>			
argTp	Pfu	45	181
astCp2	Phusion	62	293
glnAp1	Phusion	70	167
glnAp2	Phusion	64	152
glnHp2	Pfu	45	361
glnKp	Phusion	63	136
glnLp	Phusion	60	90
nac	Phusion	70	272
argTp-Biobrick	Pfu	50	240
astCp2-Biobrick	Pfu	50	352
glnAp1-Biobrick	Pfu	50	226
glnAp2-Biobrick	Pfu	59	211
glnHp2-Biobrick	Pfu	50	420
glnKp-Biobrick	Pfu	50	195
glnLp-Biobrick	Pfu	50	149
nac-Biobrick	Pfu	50	331
<i>Bacillus GlnK/GlnL-responsive promoter</i>			
PM8J2-706	Pfu	61	67
PM8J2-706-Biobrick	Pfu	50	126
<i>E. coli Lrp-responsive promoters</i>			
Clp	/	/	345
fimBp1	Phusion	52	462
fimBp2	Phusion	58	747
gltBp	Phusion	54	230
ilvIH	Phusion	56	261
leuLp	Phusion	63	317
livJp	Phusion	53	139
oppAp	Phusion	50	642
fimBp1-Biobrick	Phusion	52	521
fimBp2-Biobrick	Phusion	58	806
gltBp-Biobrick	Phusion	54	289
ilvIH-Biobrick	Phusion	56	320
leuLp-Biobrick	Phusion	63	376
livJp-Biobrick	Phusion	53	198
oppAp-Biobrick	Phusion	50	701

**Table 2.4:** Primers used in this study. (Briobrick extensions are underlined, overlapping sequenced are bold.)

Primer	Sequence (5'-3')
<i>E. coli NtrC-responsive promoters</i>	
argTp-F	TTTTGCAACCGCGATCAAATCC
argTp-R	AGGCTCTTTCATGCACATCTTG
astCp2-F	TTGTTAATGATGTCAACGATG
astCp2-R	TAAATGTAGATTGCAGGGTTC
glnAp1-F	GTCCCTTTGTGATCGCTTTCAC
glnAp1-R	AATTGCCCTAAAAGGCGTTATC
glnAp2-F	ATTGCACCAACATGGTGC
glnAp2-R	TAAAAAAGATAAAGCGAAATCTGTGCC
glnHp2-F	ATCCATCGCTAACTTTAGTAC
glnHp2-R	CATTCACATATATGAAAAAATCGTG
glnKp-F	AGCGCAATATTTTCATCGTTG
glnKp-R	GAAGAAGGTATTGCAAGCGG
glnLp-F	TTGTCAGCTATCTGTAGCCC
glnLp-R	GGTTGCACCATTTTAGTGC
nac-F	CGGGGCGCATATTAATGGCAG
nac-R	TACAACCAGATTGCAAGATGCTTG
<i>E. coli NtrC-responsive promoters - Biobrick</i>	
argTp-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTTTTGCAACCGCGATCAAATC
argTp-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTAAGGCTCTTTCATGCACATCTTG
astCp2-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTTGTTAATGATGTCAACGATG
astCp2-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTATAAATGTAGATTGCAGGGTTC
glnAp1-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTCCCTTTGTGATCGCTTTC
glnAp1-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTAAATTGCCCTAAAAGGCGTTATC
glnAp2-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGATTGCACCAACATGGTGC
glnAp2-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTATAAAAAAAGATAAAGCGAAATCTGTGCC
glnHp2-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGATCCATCGCTAACTTTAGTAC
glnHp2-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTACATTCACATATATGAAAAAATCG
glnKp-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGAGCGCAATATTTTCATCGTTG
glnKp-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTAGAAGAAGGTATTGCAAGCGG
glnLp-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTTGTCAGCTATCTGTAGCCC
glnLp-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTAGGTTGCACCATTTTAGTGC
nac-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGCGGGGCGCATATTAATGGCAG
nac-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTATAACAACCAGATTGCAAGATGC
<i>Bacillus GlnK/GlnL-responsive promoter</i>	
PM8J2-706-F	TGCTTATTTTCAAAAATGTTTTTGTGCGTATTTTGTATGATTCTGTAGTCTC
PM8J2-706-R	ATAATTACTGTATAATGGAGACTACAGAATCATACAAAATACGACAAAAAC
<i>Bacillus GlnK/GlnL-responsive promoter - Biobrick</i>	
PM8J2-706-BB-F	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTGCTTATTTTCAAAAATGTTTTTGT
PM8J2-706-BB-R	GTTTCTTCCTGCAGCGGCCGCTACTAGTATAAATTACTGTATAATGGAGAC

Continued on next page



Table 2.4 – continued from previous page	
Primer	Sequence (5'-3')
<i>E. coli Lrp-responsive promoters</i>	
Clp-blunt-F	TTTTTAACTTTAAATTCAT
Clp-blunt-R	AGAAATATAACCAGACC
fimBp1-blunt-F	GAAGGTTTAAGTCGTAGTG
fimBp1-blunt-R	GTAGCAACTGTTAATTATAAAC
fimBp2-blunt-F	GCACCTTTATACCTGTTATACC
fimBp2-blunt-R	GATATGTTCTGTAAACAAGTAAAATC
gltBp-blunt-F	TTAAGGCAGTATAAAATGCTG
gltBp-blunt-R	TTTCCAACCTTATCGGG
ilvIHp1-blunt-F	GAATGCTCTGGTTTATTCTGC
ilvIHp1-blunt-R	TAAAACACATTAACCGTCCG
leuLp-blunt-F	AACCACCGCAGCACAATTAG
leuLp-blunt-R	TGGTTTGGGTCCTTGTCTC
livJp-blunt-F	TGAGAGTCCGGGG
livJp-blunt-R	TGCTAAAACATACCCG
oppAp-blunt-F	ATTCCTTTCAAATGAAAC
oppAp-blunt-R	GGTTACTTTATCGTTACG
<i>E. coli Lrp-responsive promoters - Biobrick</i>	
fimBp1-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGGAAGGTTTAAGTCGTAGTG</u>
fimBp1-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTAGCAACTGTTAATTATAAAC</u>
fimBp2-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGGCACCTTTATACCTGTTATACC</u>
fimBp2-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTAGATATGTTCTGTAAACAAGTAAAATC</u>
gltBp-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTTAAGGCAGTATAAAATGCTG</u>
gltBp-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTATTTCCAACCTTATCGGG</u>
ilvIHp1-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGGAATGCTCTGGTTTATTCTGC</u>
ilvIHp1-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTATAAAAACACATTAACCGTCCG</u>
leuLp-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGAACCACCGCAGCACAATTAG</u>
leuLp-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTATGGTTTGGGTCCTTGTCTC</u>
livJp-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTGAGAGTCCGGGG</u>
livJp-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTATGCTAAAACATACCCG</u>
oppAp-BB-F	<u>GTTTCTTCGAATTCGCGGCCGCTTCTAGAGATTCTTTCAAATGAAAC</u>
oppAp-BB-R	<u>GTTTCTTCCTGCAGCGGCCGCTACTAGTAGGTTACTTTATCGTTACG</u>
<i>B. subtilis colony PCR primers</i>	
AmyE-short-F1	ATGTTTGCAAAACGATTCAAAAC
AmyE-short-R2	TCAATGGGGAAGAGAACCG
AmyE-near-F	GAGTATTCCAACTGGACACATGG
AmyE-near-R	CTGGAAAAGAAAAGAGGCGTACTG
Bac-colPCR-Cmr-F	GCAGTTTCTACACATATATTCGCAAG
Bac-colPCR-GFP-R	ATTCCAATTTGTGTCCAAGAATG

## **Gel electrophoresis**

PCR products were analysed by gel electrophoresis using agarose gels of appropriate concentration (1-2.5% w/v in TAE) with addition of GelRed (Cambridge Bioscience) or SYBR safe (Invitrogen) according to the manufacturer's protocol. The DNA HyperLadder V (Bioline) or HyperLadder I (Bioline) was used as a standard. Resolved DNA fragments were visualised using a UV transilluminator (Biorad).

## **Gel purification**

Resolved DNA fragments were extracted from agarose gels by isolation of gel slice and purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol.

## **PCR purification**

PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol.

## **Restriction enzyme digests**

Restriction enzyme digests were typically performed in 50  $\mu$ l total volume using the appropriate combination of BioBrick enzymes, *EcoRI*, *SpeI*, *XbaI* and *PstI* (Promega or NEB), according to the manufacturer's guidelines.

## **Ligation**

Ligation reactions were performed using T4 DNA ligase (Promega, UK) in 10  $\mu$ l according to the manufacturer's guidelines.

## **Chemically-competent cells and transformation**

Chemically-competent *E. coli* DH5 $\alpha$  cells were either purchased (C2987H, NEB 5-alpha Competent *E. coli* (High Efficiency); NEB) and transformed according to the manufacturer's protocol or made from non-competent *E. coli* DH5 $\alpha$  cells and transformed according to a protocol by the Blaser group (<http://www.med.nyu.edu/>

medicine/labs/blaserlab/v1-protocols.html; NYU, NY, USA). Typically, 100  $\mu$ l of transformation mixture were plated on LB agar plates with suitable antibiotics.

### **Agar plates and antibiotic concentrations**

For *E. coli* ampicillin was used where appropriate at the recommended concentrations (usually 100  $\mu$ g/mL). For *B. subtilis* used CAM (5  $\mu$ g/ml) according to (Harwood and Cutting, 1990).

### ***E. coli* colony PCR**

*E. coli* colonies were picked and boiled at 98°C for 5 min in 100  $\mu$ l of water of which 17.25  $\mu$ l as template in 25  $\mu$ l colony PCR reactions using GoTaq polymerase (Promega). The BioBrick Vf2 and Vr primers were mostly used for colony PCR.

### **Plasmid purification**

Miniprep isolation of plasmids from *E. coli* cells was done using peqGOLD Plasmid Miniprep Kit I (peqlab, Erlangen, Germany) according to the manufacturer's protocols. Midiprep isolation of plasmids *E. coli* cells was done using QIAGEN Plasmid Midi Kit according to the manufacturer's protocols.

### ***B. subtilis* transformation**

*B. subtilis* transformation was done according to the protocol used by the 2008 Imperial College iGEM team [[http://2008.igem.org/Team:Imperial\\_College/Transformation](http://2008.igem.org/Team:Imperial_College/Transformation)] with some modifications. Approximately 2  $\mu$ g of midiprep plasmid DNA were used in the transformation. Cells were then incubated with the DNA for 2 h at 37°C. No Tbase and glucose solution were added to cells. 100  $\mu$ l of the cell mix were plated onto LB agar plates. Plates were incubated for two days at 37°C and colonies were then selected for testing.

### **Amylase test**

Correct integration of constructs into the AmyE site was checked using an amylase test based on the protocol used by the 2008 Cambridge iGEM team [[http://2008.igem.org/Team:Cambridge/Bacillus\\_subtilis\\_transformation](http://2008.igem.org/Team:Cambridge/Bacillus_subtilis_transformation)]. Starch for LB agar

starch plates was purchased from Sigma-Aldrich (S9765-100G). Negative control was untransformed *B. subtilis* cells and positive control was *B. subtilis* cells with an insert in the AmyE locus kindly provided by Gary Dixon. 10 ml of Gram's iodine stain (Sigma-Aldrich, HT902-8FOZ) were added to plates and the plates were then incubated for 5 min, after which the stain solution is decanted.

### ***B. subtilis* colony PCR**

Large *B. subtilis* colonies were picked and boiled at 98°C for 5 min in 100  $\mu$ l of water. Samples were centrifuged at high speed for 2 min and 2  $\mu$ l used as template in 25  $\mu$ l colony PCR reactions. PCR conditions are summarised in Table 2.5.

**Table 2.5:** *B. subtilis* colony PCR conditions used in this study

Primers	Polymerase	Annealing temperature (°C)	Product length with insert (bp)	Product length without insert (bp)
AmyE-short-F1; AmyE-short-R2	Phusion	67	3451	1527
AmyE-near-F; AmyE-near-R	Phusion	69	2158	234
Bac-colPCR-Cmr-F; Bac-colPCR-GFP-R	Phusion	65	1014	0

### **DNA sequencing**

DNA constructs were checked for correctness using various commercial services, including Eurofins MWG operon, GATC and Biosource DNA sequencing service.

### **Glycerol stocks of bacterial cells**

For both *E. coli* and *B. subtilis* glycerol stocks were made by mixing equal volumes of bacterial overnight cultures and 50% (v/v) glycerol solution. Stocks were stored in cryotubes at -80°C.

## 2.4 Characterisation of bacterial biosensor constructs

Over the course of this study, a range of characterisation experiments were carried out that informed changes to the protocol. Details on protocol development can be found in results chapter 4.

### M9 minimal medium and plates

The recipe used for M9 minimal medium was taken from [http://openwetware.org/wiki/Endy:M9\\_medium/minimal](http://openwetware.org/wiki/Endy:M9_medium/minimal).  $\text{NH}_4\text{Cl}$  has used as a nitrogen source. Depending on the experiment, glycerol or glucose was used as a carbon source. For M9 agar plates, 14 g/L agar were added. Single colonies of *E. coli* cells containing promoter characterisation or the control construct were streaked onto the plate in a grid-formation, incubated over night at 37°C and analysed qualitatively the next day using a blue light transilluminator.

### MM minimal medium

The *B. subtilis* glutamine sensor was tested in MM medium made as by Yoshida et al. (2000).

### BMG plate reader

Fluorescence and O.D. measurements for biosensor characterisation and growth curves were performed using a BMG Omega Polarstar fluorometer in a 96-well plate format (Costar, 3370) at a temperature of 37°C. The sampling time and gain were varied according to the experiment. Fluorescence excitation filter 485 nm and emission filter 510 nm was used. Data was analysed and visualised using Matlab and Microsoft Excel.

For the detailed characterisation of individual NtrC promoters, colonies of cells containing a characterisation construct and control cells were used to inoculate LB cultures (with appropriate antibiotic) in 14 ml culture tubes, which were incubated for several hours at 37°C.  $\sim 40 \mu\text{l}$  of these cultures were used to inoculate 5 ml overnight cultures of DMEM and M9, both supplemented with 18.7 mM  $\text{NH}_4\text{Cl}$  and appropriate antibiotic. The following morning, cultures were diluted with fresh

media and grown up further during the day. In the evening the OD600 of each culture was measured and cultures were diluted with fresh media to an appropriate starting OD. After  $\sim 1$  h, cultures were induced by adding appropriate volumes of a 200 mM glutamine stock to achieve concentrations of 0, 2.5, 5, 7.5 and 10 mM glutamine. Three replicates of 100  $\mu\text{l}$  of each culture were then transferred to a 96-well plate and measured for several hours.

## Robotic characterisation

This experimental protocol and subsequent data analysis was adapted from one developed and used by Chris Hirst (Hirst, 2014) (Figure 2.2). Experiments were carried out using a Aviso-GmbH Theonyx robotic platform linked to a plate reader (Synergy HT, Biotek) and a shaking incubator (Ventura 2000, Mikura). 1-5 ml LB cultures of the *E. coli* biosensors and control cells were grown during the day and used to inoculate 5 ml overnight cultures in M9, DMEM or LB medium (or other appropriate media). On the next morning, cultures were transferred to 96-well plate format. Using the Theonyx platform the cells then undergo dilution, outgrowth, in some cases a second dilution and assaying with OD and fluorescence measurements every 10 min. Cells were induced by addition of 25  $\mu\text{l}$  of appropriate inducer concentration in appropriate solvent to the 100  $\mu\text{l}$  of cell culture in the well, forming 125  $\mu\text{l}$  of liquid in each well-post-induction. Shaking of cell cultures during these experiments was usually at 700 rpm. Fluorescence excitation filter 485 nm and emission filter 528 nm was used with a bandpass of 20. Appropriate fluorescence sensitivities were chosen in the plate reader settings for characterisation of different biosensors. During data analysis, OD outliers were excluded and medium blank controls and control cell fluorescence subtracted from sample data. Data analysis was done using Microsoft Excel. Plotting was done using Microsoft Excel and Mathematica. Raw fluorescence data was processed with an R-based spline-fitting algorithm written by Catherine Ainsworth (unpublished). Growth curves show sample absorbance values at OD600 with the average absorbance for media subtracted. A trendline with the following equation was fitted to a graph showing fluorescence vs corrected OD600 of the control cells:

$$FL_{control} = OD_{control} \times m + c \quad (2.1)$$

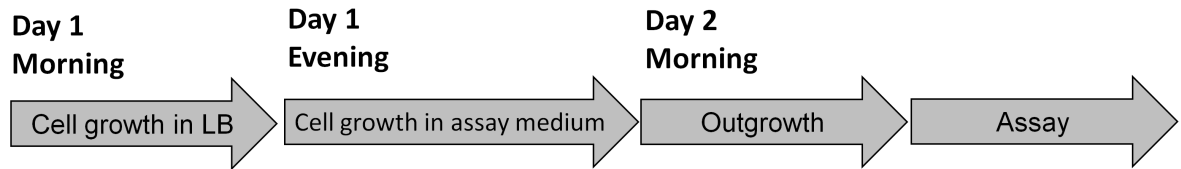
The equation of this trendline was used to subtract cell autofluorescence from biosensor cell fluorescence as follows:

$$FL_{i,corrected} = FL_i - ((OD_i \times m) + c) \quad (2.2)$$

Fluorescence production (GFP synthesis rate) was calculated as follows:

$$\text{GFP synthesis rate} = \frac{FL_i - FL_{i-1}}{(OD_i - OD_{i-1})/2} \quad (2.3)$$

In order to calculate lactate concentrations in cell culture samples, GFP synthesis rate values from the standard lactate concentrations from an appropriate time point of the time course were chosen to make a standard curve. GFP synthesis rate values from the cell culture samples were used to interpolate the lactate concentrations using the GraphPad Prism software.



**Figure 2.2:** Workflow for promoter characterisation using Theonix robotic platform.

## Flow cytometry

Cells were grown up overnight. Cells were measured using a FACScan flow cytometer (Becton Dickinson) with a 96-Well Automated Micro-Sampler (Cytex). Data was acquired using CellQuest software (Becton Dickinson) with wells being sampled on high flow rate for 20 seconds. Data was analysed using CyFlogic or FlowJo software.

## 2.5 *E. coli* and *B. subtilis* growth curves

### Using DMEM in flasks

Single colonies of *E. coli* *DH5α* and *B. subtilis* 1012 were used to inoculate 10 ml of LB broth (Miller, 1.10285.0500) and grown overnight (37°C, 225 rpm). Overnight cultures were diluted 200-fold in 200 ml of DMEM (Gibco, Invitrogen) supplemented

with 10% (v/v) calf bovine serum (ATCC, 30-2030) and grown at 37°C with shaking at 225 rpm. Duplicate O.D. measurements were obtained using a Biomate 5 spectrophotometer (ThermoSpectronic).



## **3 Hybridoma cell culture characteristics**

### **3.1 Introduction**

In order to design bacterial biosensors for mammalian cell bioprocessing, experiments first needed to be carried out to study hybridoma cell culture more closely and derive performance specifications for potential biosensors.

#### **3.1.1 Hybridoma cell line**

The mammalian cells used here were of the murine hybridoma cell line ATCC-CRL1606 producing an industrially-relevant mAb (HFN7.1) against human fibronectin. These cells were chosen for a number of reasons that make them very suitable for this project: they are an established model cell line in bioprocessing (Kontoravdi, Asprey, Pistikopoulos and Mantalaris, 2007; Selvarasu, Kim, Karimi and Lee, 2010), they require glutamine in the culture medium and an assay has been developed to detect the mAb produced by these cells (Kontoravdi, 2007). These cells do not contain sufficient glutamine synthetase activity to sustain growth in glutamine-free medium. It has been suggested that glutamine synthetase activity is over time irreversibly repressed in many hybridoma cell lines that are grown at high glutamine concentrations, as glutamine has been shown to suppress glutamine synthetase (Juurlink, 1987; Newland et al., 1990). These cells grow in suspension as opposed to being adherent cells. They therefore require mixing, which should prevent heterogeneity in cultures and simplify conditions during experiments.

#### **3.1.2 Metabolite concentrations in bioprocessing**

Specifications for the biosensors concern the target compound, detection threshold, accuracy, signal output and host chassis. An appropriate detection range is required. Typical concentration ranges of metabolites in mammalian cell cultures were researched (Table 3.1).

**Table 3.1: Concentrations of metabolites in mammalian cell culture (over the course of ~100-150h).** Values were taken from the literature from experiments using the same or a similar cell type and similar conditions. Common growth medium concentrations were also taken into account as for a lot of compounds, the greatest concentration will be at time zero, and hence equal to the concentration of the growth medium. Concentration ranges depend on how long the cells are being cultured and growth conditions as well as culturing mode (Male et al., 1997).

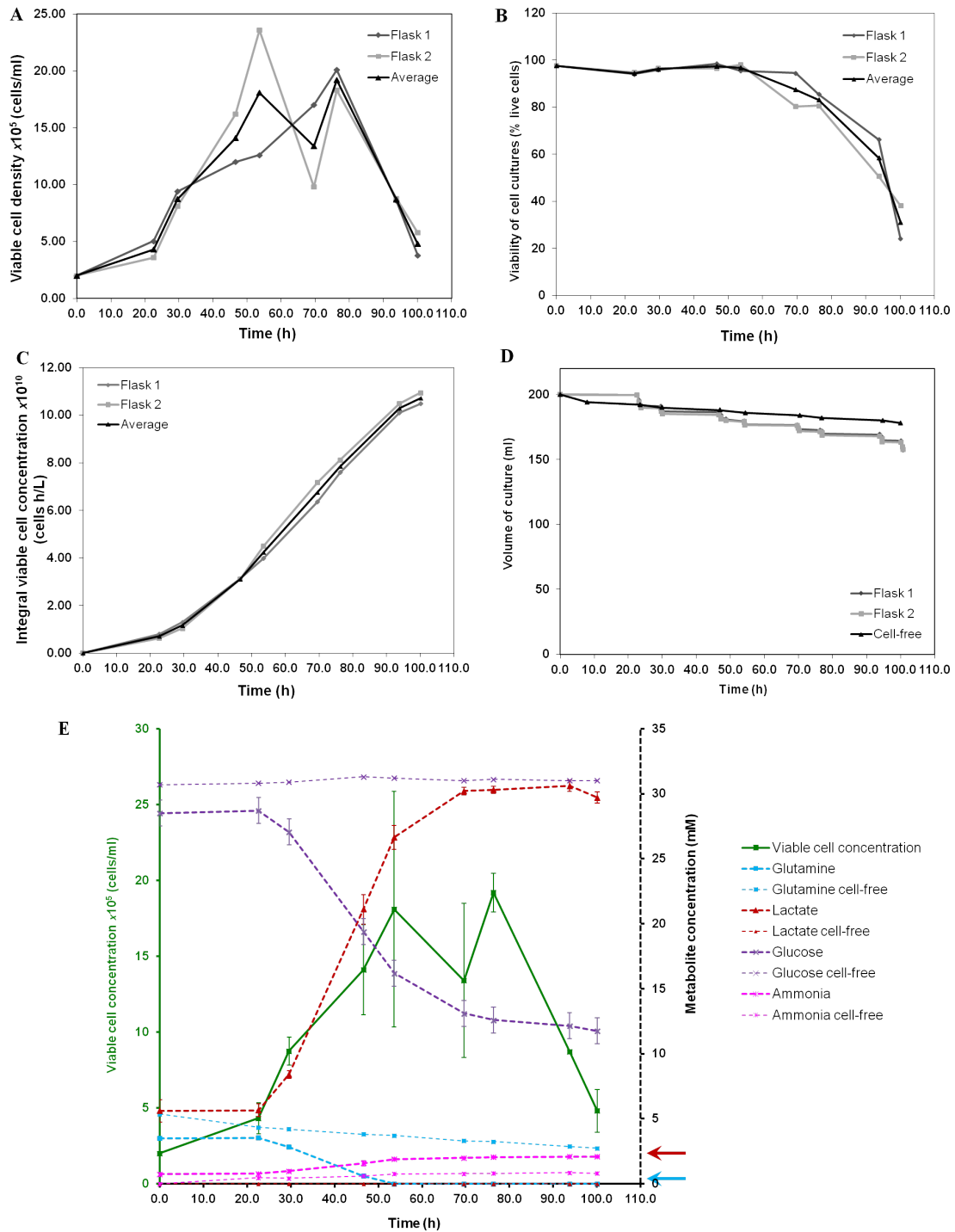
Metabolite	Concentration range	Reference
Glucose	0 – 25 mM	Kontoravdi, Asprey, Pistikopoulos and Mantalaris (2007); Selvarasu et al. (2009); Invitrogen
Ammonia	0 – 6 mM (variable)	Kontoravdi, Asprey, Pistikopoulos and Mantalaris (2007); Selvarasu et al. (2009)
Lactate	0 – 80 mM	Male et al. (1997); Selvarasu et al. (2009)
Serum (components)	N/A	
mAb	0 – 2.5 g L <sup>-1</sup>	Kontoravdi, Asprey, Pistikopoulos and Mantalaris (2007); Selvarasu et al. (2009)
Cell	0 – 5x10 <sup>6</sup> cells ml <sup>-1</sup>	Selvarasu et al. (2009)
<b>Amino Acids</b>		
Alanine	0 – 0.6 mM (up to ~6 mM if using ala-gln dipeptide)	Christie and Butler (1994a); Selvarasu et al. (2009)
Arginine	0 – 1.0 mM	Selvarasu et al. (2009); Invitrogen
Asparagine	0 – 0.3 mM	Selvarasu et al. (2009)
Aspartate	0 – 0.4 mM	Selvarasu et al. (2009)
Cysteine	0 – 0.2 mM	Invitrogen
Glutamate	0 – 0.7 mM	Selvarasu et al. (2009)
Glutamine	0 – 5 mM	Kontoravdi, Asprey, Pistikopoulos and Mantalaris (2007); Selvarasu et al. (2009); this work
Glycine	0 – 0.5 mM (up to ~6 mM if using gly-gln dipeptide)	Christie and Butler (1994a); Selvarasu et al. (2009); Invitrogen
Histidine	0 – 0.5 mM	Selvarasu et al. (2009); Invitrogen
Isoleucine	0 – 1.2 mM	Selvarasu et al. (2009); Invitrogen
Leucine	0 – 1.2 mM	Selvarasu et al. (2009); Invitrogen
Lysine	0 – 1.2 mM	Selvarasu et al. (2009); Invitrogen
Methionine	0 – 0.5 mM	Selvarasu et al. (2009); Invitrogen
Phenylalanine	0 – 0.4 mM	Invitrogen
Proline	/	
Serine	0 – 0.4 mM	Selvarasu et al. (2009); Invitrogen
Threonine	0 – 1.2 mM	Selvarasu et al. (2009); Invitrogen
Tryptophan	0 – 0.08 mM	Invitrogen
Tyrosine	0 – 0.7 mM	Selvarasu et al. (2009); Invitrogen
Valine	0 – 0.5 mM	Invitrogen

## 3.2 Experimental Results

### 3.2.1 Characteristics of hybridoma cell growth

To explore the growth characteristics of CRL1606 hybridoma cells a batch over-growth experiment was carried out (Figure 3.1). Figure 3.1(A) shows the viable cell density. The cells show typical growth characteristics with an initial lag phase ( $\sim 0.0-20.0$  h), followed by exponential growth phase ( $\sim 20.0-55.0$  h), stationary phase ( $\sim 55.0-75.0$  h) and death phase ( $\sim 75.0-100.0$  h). There is great variance in cell counts during late exponential and stationary phase. Fig. 3.1(B) shows the percentage viability of the cells. Viability is near 100% at the beginning of the batch culture. The beginning of the stationary phase marks the point at which cell division rate and viability decrease due to depletion of nutrients and accumulation of waste products. Figure 3.1(C) shows the integral viable cell concentration (IVC). This represents the number of viable cells contributing to the expression of cell activity during the accumulated time of cell growth. In short, the IVC is representative of the antibody-producing power of the cells. As expected the IVC increases with time and cell density, but increases more slowly towards the end of the culture run. Sample taking from cultures affects culture volume but this was kept to a minimum (Figure 3.1(D)).

Extracellular culture samples were analysed for metabolite concentrations (Figure 3.1(E)). This shows that the onset of stationary phase correlates with the depletion of glutamine rather than glucose. Comparing culture flasks with a cell-free culture shows that, as expected, glutamine concentration decreases to some extent even in the absence of cells and some ammonia accumulates. Glucose however stays at a constant level in the absence of cells and no lactate is detected. Over the course of the experiment, glutamine concentration in the cell-free flask approximately halved, giving glutamine a half life of approximately 100 hours under these conditions. Table 3.2 shows the concentration ranges of metabolites found in this experiment. All metabolites and cell concentrations shown in Table 3.2 fall into the ranges from Table 3.1. Only glucose shows a slightly higher concentration at the beginning of the culture, which could be due to glucose from the added serum.



**Figure 3.1:** Batch overgrowth of CRL1606 hybridoma cells in DMEM. **(A)** Viable cell density (cells/ml). **(B)** Percentage viability. **(C)** Integral viable cell concentration (cells h/L). **(D)** Volume changes of cultures due to sample taking. **(E)** Culture supernatant metabolites analysis using a Nova BioProfile Analyzer. Arrows indicate lower concentration detection limits. Data are based on two replicate culture flasks. Error bars are standard deviation of samples from these two flasks.

**Table 3.2:** Metabolite concentration ranges in mammalian cell culture experiment shown in Figure 3.1.

<b>Metabolite</b>	<b>Concentration range</b>
Cell	$2.00 \times 10^5$ - $1.92 \times 10^6$ cells/ml
Glutamine	<0.2 - 3.49 mM
Lactate	5.6 - 29.7 mM
Glucose	11.75 - 28.7 mM
Ammonia	0.73 - 2.095 mM

### 3.2.2 CRL1606 hybridoma glutamine requirement

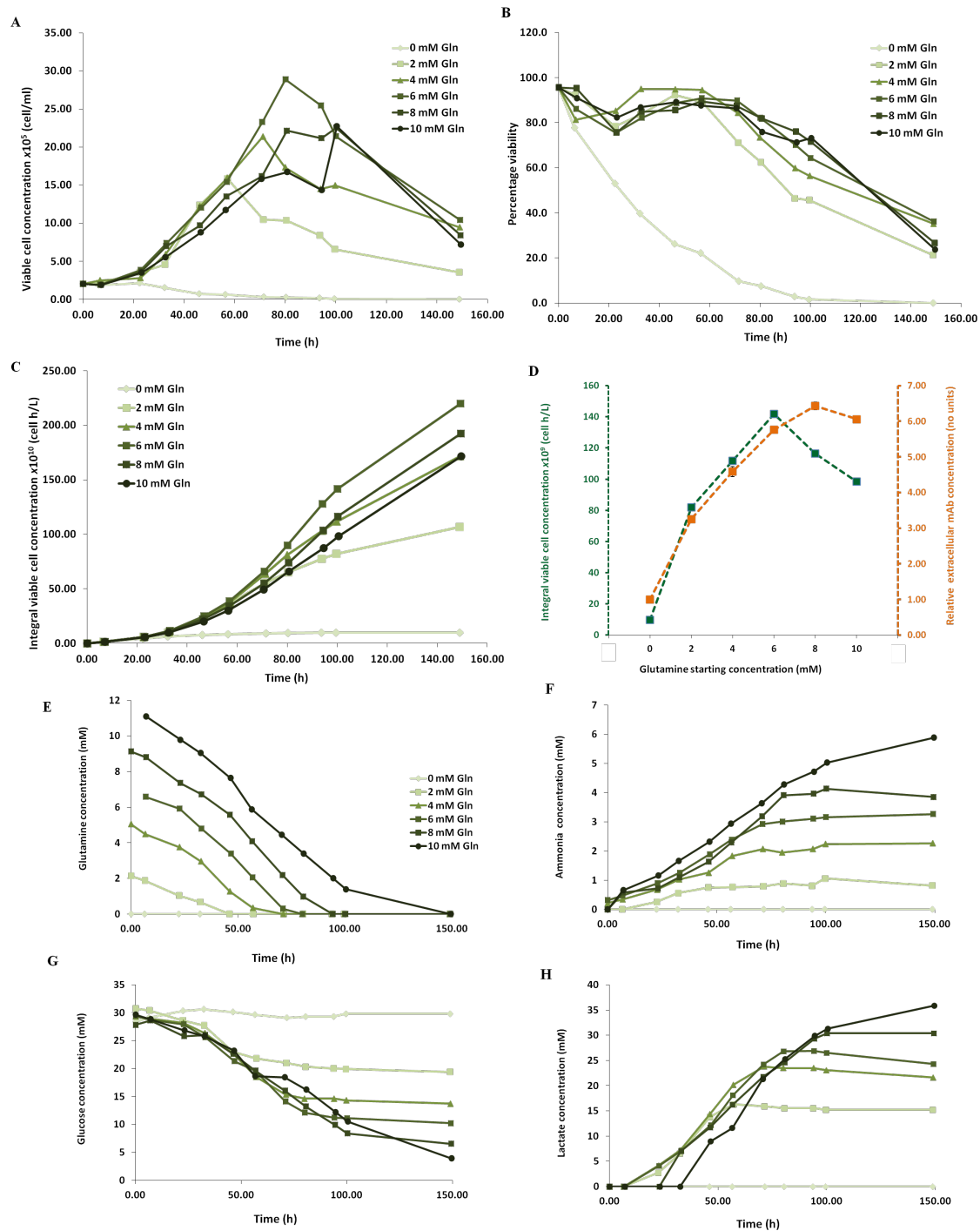
An experiment was carried out to study cell culture characteristics when changing the concentration of essential metabolites. The aim was to help identify interesting targets for biosensing, important limiting factors for culturing these cells. Cells were cultured with different starting concentrations of glutamine. Glutamine concentrations used ranged from 0 mM to 10 mM. Typical concentrations in commonly used mammalian growth media range from 2 mM to 8 mM. Cell growth was monitored (Figure 3.2(A)). Cells supplemented with 0 mM glutamine were unable to divide and underwent cell death, reaching a cell density of essentially zero after  $\sim 90.0$  h. As all the other cultures that were supplemented with varying concentrations of glutamine were able to grow and divide, this shows the strong need for glutamine for these hybridoma cells. The highest cell density of all cultures was reached by the 6 mM glutamine culture after  $\sim 80.0$  h with a cell density of  $\sim 3.00 \times 10^6$  cells/ml. The cultures with lower glutamine concentrations, 2 mM and 4 mM, reached lower maximum cell densities and entered stationary phase and death phase earlier, after  $\sim 55.0$  h and  $\sim 70.0$  h, respectively. None of the cultures apart from possibly 8 mM Gln and 10 mM glutamine showed a pronounced stationary phase, but seemed to go from exponential phase straight to death phase.

During the early lag phase ( $\sim 0.0$ - $10.0$  h) cells at 8 mM glutamine showed the highest viability (Figure 3.2(B)). During the exponential phase ( $\sim 20.0$  -  $65.0$  h) cells at 4 mM glutamine showed the highest viability. Only the cells at 0 mM glutamine never showed any increase in viability. The 6 mM glutamine culture displayed the highest IVC of all the cultures at all times, *i.e.* higher than both higher and lower glutamine concentrations (Figure 3.2(C)). These results confirmed that these cells require glutamine, but that at high glutamine concentrations ( $>6$  mM) growth was inhibited.

Culture supernatant samples were also analysed for the mAb product using an ELISA method. Figure 3.2(D)) shows samples taken after 100 h of cell culture (*i.e.* the second to last time point on Figure 3.2(A)-(C)). Relative mAb concentration reaches an optimum at 8 mM glutamine, which was a higher glutamine concentration than the 6 mM glutamine optimum for integral viable cell concentration. The data showed that the CRL1606 hybridoma cells need glutamine to be provided at optimum concentration for growth and mAb production. mAb production is often greatest during stationary phase, which does seem most pronounced at 8 mM glutamine.

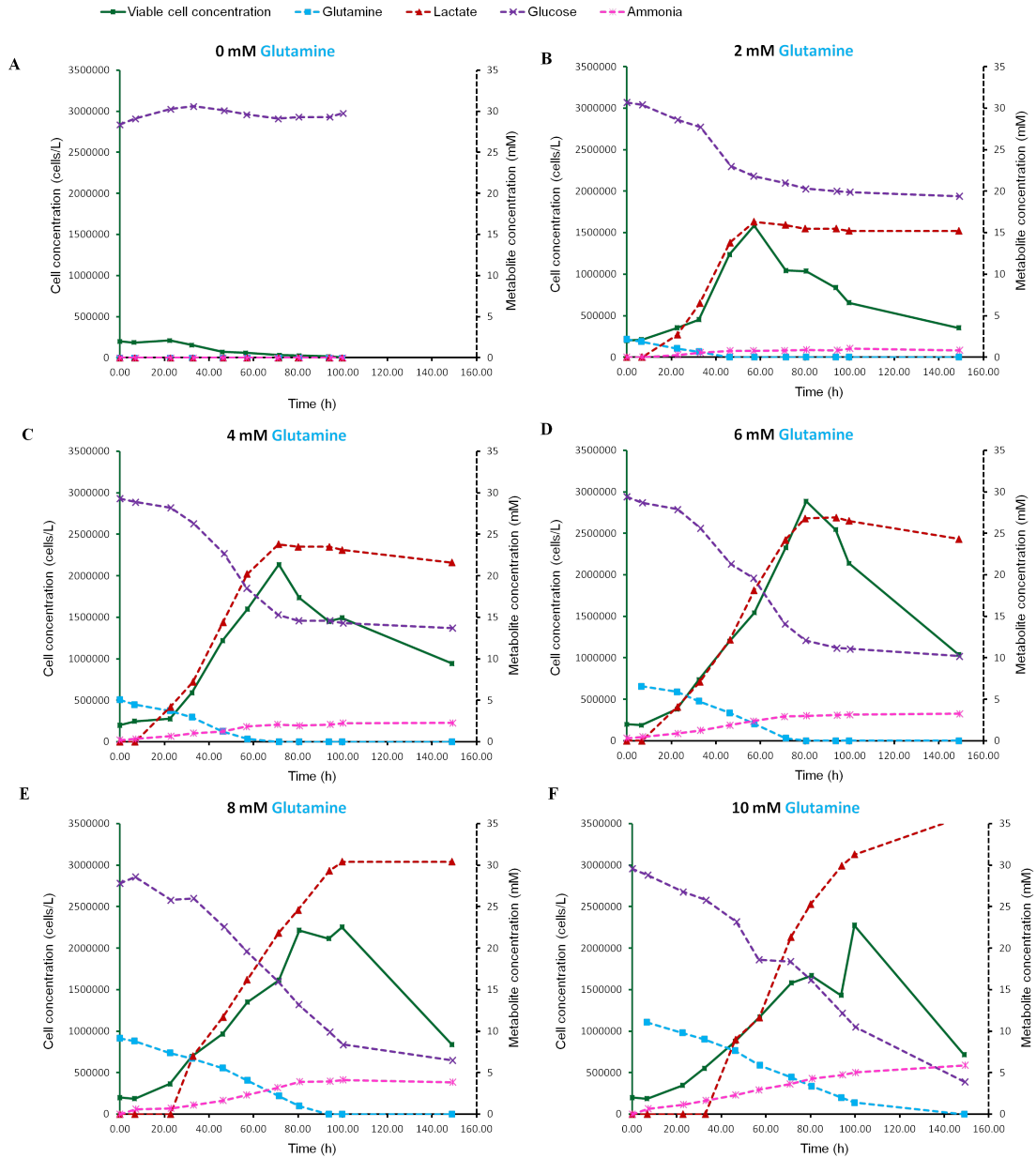
Extracellular culture supernatant samples were collected at each time point for

metabolite analysis. Results are shown in Figure 3.2(E)-(H). The nutrients glutamine and glutamate, as well as the metabolic waste products ammonia and lactate were measured. As expected, the nutrient concentrations decrease as they are being used up by the cells. Metabolic waste products increase in concentration, as cells secrete them. The differences in cell growth at different glutamine concentrations are reflected in the rate at which metabolites are produced or used up. To clarify how changes in metabolite concentrations and cell growth relate to each other, time courses for each flask are shown in Figure 3.3. These results again that cell growth is largely correlated with glutamine depletion and ammonia accumulation. Glutamine is thus confirmed as a relevant cell culture parameter for these cells.



**Figure 3.2:** CRL1606 hybridoma cells batch overgrowth in DMEM growth medium supplemented with different concentrations of glutamine. **(A)** Viable cell concentration. **(B)** Percentage viability. **(C)** Integral viable cell concentration. **(D)** Optimum glutamine starting concentration in terms of cell growth and mAb production after  $\sim 100$ h of cell culture. Error bars of mAb concentration show standard deviation of three replicate measurements in the same experiment. Relative mAb concentration was measured using an ELISA. **(E)-(H)** Extracellular metabolite concentrations. **(E)** Glutamine. **(F)** Ammonia. **(G)** Glucose. **(H)** Lactate. A single culture flask per glutamine concentration was used.





**Figure 3.3:** Metabolites and cell growth during CRL1606 hybridoma cells batch overgrowth in DMEM growth medium supplemented with different concentrations of glutamine shown by glutamine concentrations. (A) 0 mM glutamine. (B) 2 mM glutamine. (C) 4 mM glutamine. (D) 6 mM glutamine. (E) 8 mM glutamine. (F) 10 mM glutamine. Missing data points were caused by values being larger or smaller than the detection limit. A single culture flask per glutamine concentration was used.

### 3.3 Summary of results

Hybridoma cell cultures were carried out to learn more about cell growth and metabolite dynamics in this potential biosensor context. Initially, a hybridoma batch culture was carried out. Cell growth and metabolite concentrations were found to fall within ranges expected from the literature. Hybridoma cultures at different glutamine concentrations were then carried out to further investigate limiting factors in cell culture. It was found that glutamine is an important limiting metabolite for cell growth and mAb production in the conditions used here.

While antibody production by hybridoma cells was tested, the glycosylation patterns of the mAb product were not analysed here. It is possible that changing the glutamine concentration changes these patterns as glycosylation is very sensitive to culture conditions (van Berkel et al., 2009; Wong et al., 2010).

These cultures refine specifications for potential metabolite biosensors used in bioprocessing. Table 3.1 and the results in this section indicate required detection ranges for metabolites. Although it not be necessary for sensors to cover the whole range in each case, as long as they can detect certain critical threshold levels.

Based on the results herein, the literature overview in the introduction and the availability of natural sensing systems (see Introduction) it was decided that a small number of sensing systems should be selected to be tested for their suitability as biosensors in bioprocessing. The selected systems were the lactate-responsive *E. coli* LIPRD system, leucine/alanine-responsive *E. coli* Lrp system, glutamine-responsive *E. coli* Ntr system, and the glutamine-responsive *B. subtilis* GlnK/GlnL system. Glutamine is an extremely important cell culture nutrient. The results in this chapter show that the depletion of glutamine correlates with the onset of stationary and death phase for the hybridoma cells. Lactate is a common waste product in cell cultures, as was discussed in the introduction. It accumulated to high concentrations in the hybridoma cultures shown in this chapter. Alanine is a common cellular metabolism byproduct in cell cultures that is indicative of ammonia stress in cells.

Experiments towards engineering biosensors from these systems can be seen in subsequent chapters.

## 4 Development of a biosensor characterisation protocol

### 4.1 Introduction

After exploring the parameters of mammalian cell culture and choosing bacterial sensing systems to test as biosensors, the next step was to develop a suitable protocol for characterising these systems. The crucial promoter elements from the selected bacterial sensing systems were incorporated into testing constructs and tested.

General parts characterisation and biosensor engineering was previously discussed in the introduction. Several landmark synthetic biology parts characterisation papers discuss standardisation of genetic constructs and characterisation protocols (Canton et al., 2008; Kelly et al., 2009). A lot of successful biosensors in the field respond to compounds that are largely orthogonal to intracellular metabolism, *i.e.* they are not produced within the cells and come from the external environment. However, some of the target compounds in this work are metabolites that can be produced within bacterial cells and are found in many common growth media. Such factors needed to be considered in the characterisation protocol used here. There are some existing examples of biosensors with amino acid targets, such as an intracellular sensor for methionine and other branched amino acids (Mustafi et al., 2011).

This chapter outlines the characterisation protocol development using example data from several of the promoters studied in this work. The systems used here include promoters of the lactate-responsive *E. coli* LIPRD system (Chapter 5), leucine/alanine-responsive *E. coli* Lrp system (Chapter 6), and the glutamine-responsive *E. coli* Ntr system (Chapter 7). More details on the background, assembly and characterisation results on these promoters can be found in these subsequent chapters. Data shown in this chapter relates to experiments that informed the characterisation set-up.

## 4.2 Choice of experimental growth conditions and equipment

When working in liquid medium, the important parameters to measure for biosensors were cell growth and fluorescence, to estimate promoter activity. It was therefore decided to carry out these experiments using a plate reader and 96-well plates to allow high-throughput characterisation.

### 4.2.1 Growth media

The Ntr promoters were used to set up a protocol for quantitative characterisation experiments of the promoter constructs. An important question was what kind of growth medium should be used during characterisation experiments. There are many different growth media to choose from, the most common distinction being rich or complete media on one hand and minimal media on the other. The glutamine-responsive promoters would need to be studied in a glutamine-free medium which could be supplemented with known concentrations of glutamine. The mammalian growth medium DMEM represents the context in which the biosensors would eventually be used. However, DMEM is a complex medium which normally contains glutamine, leucine and alanine. Fortunately, glutamine-free varieties are available. LB is the most commonly used rich growth medium for supporting growth of bacterial species such as *E. coli* and *B. subtilis* in molecular biology. LB can display batch-to-batch variation, although due to the rich availability of nutrients this rarely affects bacterial growth. However, as it contains a source of amino acids in the form of mixed peptides, it is not a suitable medium to use for testing of dynamics of the glutamine- or leucine/alanine-responsive systems. It could, however, be used for the lactate-responsive system. LB is commonly used in synthetic biology, *e.g.* Gardner et al. (2000); Stricker et al. (2008). A common defined minimal growth medium for characterising bioparts is M9 medium, which is free of amino acids. Carbon and nitrogen sources used can be chosen as needed. M9 has been used in synthetic biology, *e.g.* Basu et al. (2005), as well as promoter characterisation in *E. coli* (Zaslaver et al., 2009). It was therefore decided that promoter characterisation would be carried out in DMEM and M9 where possible.

For mammalian cell culture, including the CRL 1606 hybridoma cells growth experiments reported in the previous chapter, DMEM is often supplemented with phenol red as a pH indicator. To test if phenol red would interfere with fluorescence measurements, experiments were carried out in DMEM with phenol red, DMEM

without phenol red and M9. Results showed that phenol red interferes with green fluorescence detection (Figure 4.1(A)). It was therefore decided that DMEM without phenol red supplement, and M9 medium would be used for subsequent promoter characterisation.

## 4.2.2 Effect of target metabolites on measurements

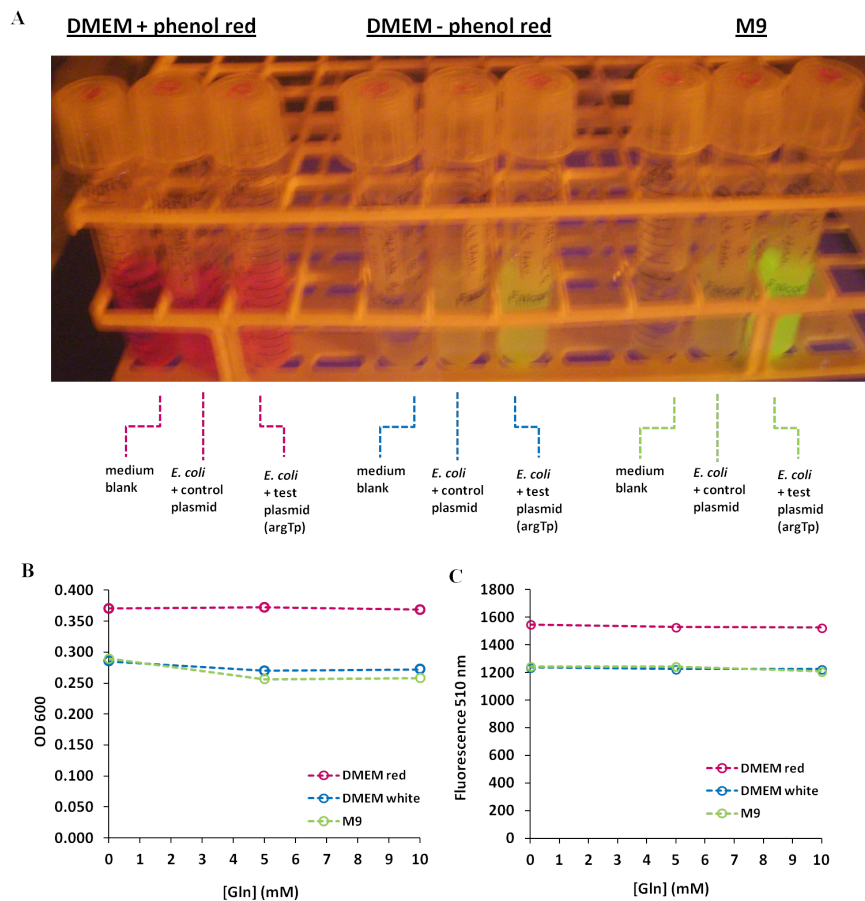
In a characterisation experiment of a glutamine-responsive promoter, cells expressing the characterisation construct would be grown in media containing varying concentrations of glutamine and cell growth (600 nm wavelength) and green fluorescence (485 nm wavelength) would be measured. The effect of glutamine concentration in cell-free media on absorbance measurements at 600 nm and fluorescence measurements at 485 nm was tested (Figure 4.1(B) and (C)). It was found that glutamine concentration has no effect on these measurements, and therefore there would be no need for a medium blank for each glutamine concentration in subsequent characterisation experiments.

A similar test was carried out for lactate (Figure 4.2). It was found that both OD600 and fluorescence at 510 nm decreased a small amount with increasing lactate concentration. Lactate has been suggested to form higher-order structures at higher concentrations in the presence of divalent ions (Cariati et al., 1977), which could cause this trend. This effect could lead to slight underestimates of the effects of lactate on the *lldPp* promoter.

## 4.2.3 Cell growth dynamics

Growth behaviour in the available liquid media was studied. For this, biosensor cells containing lactate-sensing constructs were used.

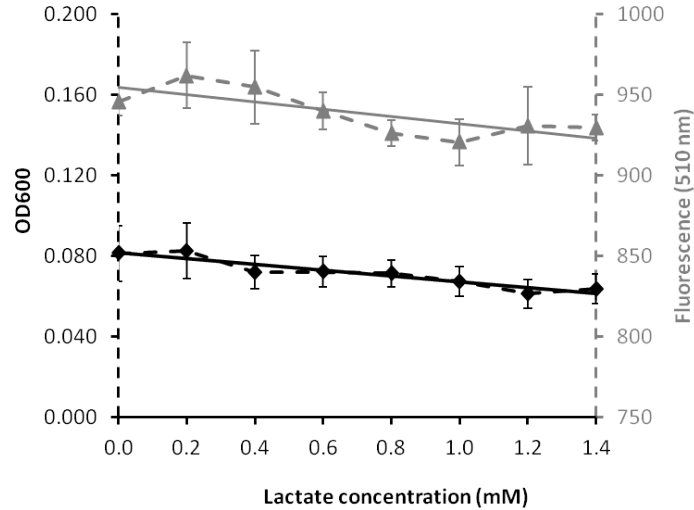
The growth of the lactate sensor cells was studied in relevant growth media for biosensor characterisation, LB, M9 and DMEM (Figure 4.3). The cells grew well in LB medium (Figure 4.3(A)). *lldPp+lldR+lldP* grew at a lower rate than the others, suggesting that overexpression of the permease inhibits the cells. In M9 minimal medium, cells grew at a lower rate than in LB and *lldPp+lldR+lldP* did not grow at all (Figure 4.3(B)). The added metabolic burden of permease overexpression or the effects from the presence of additional transporters seem to prohibit these cells from growing in this minimal medium. This is also seen in DMEM medium. It was therefore decided to not pursue work with the *lldPp+lldR+lldP* construct (see Chapter: *E. coli lldPRD* lactate-responsive operon). The DMEM used here did



**Figure 4.1: (A) Suitability of different glutamine-free growth media for quantitative characterisation of glutamine-responsive promoters.**

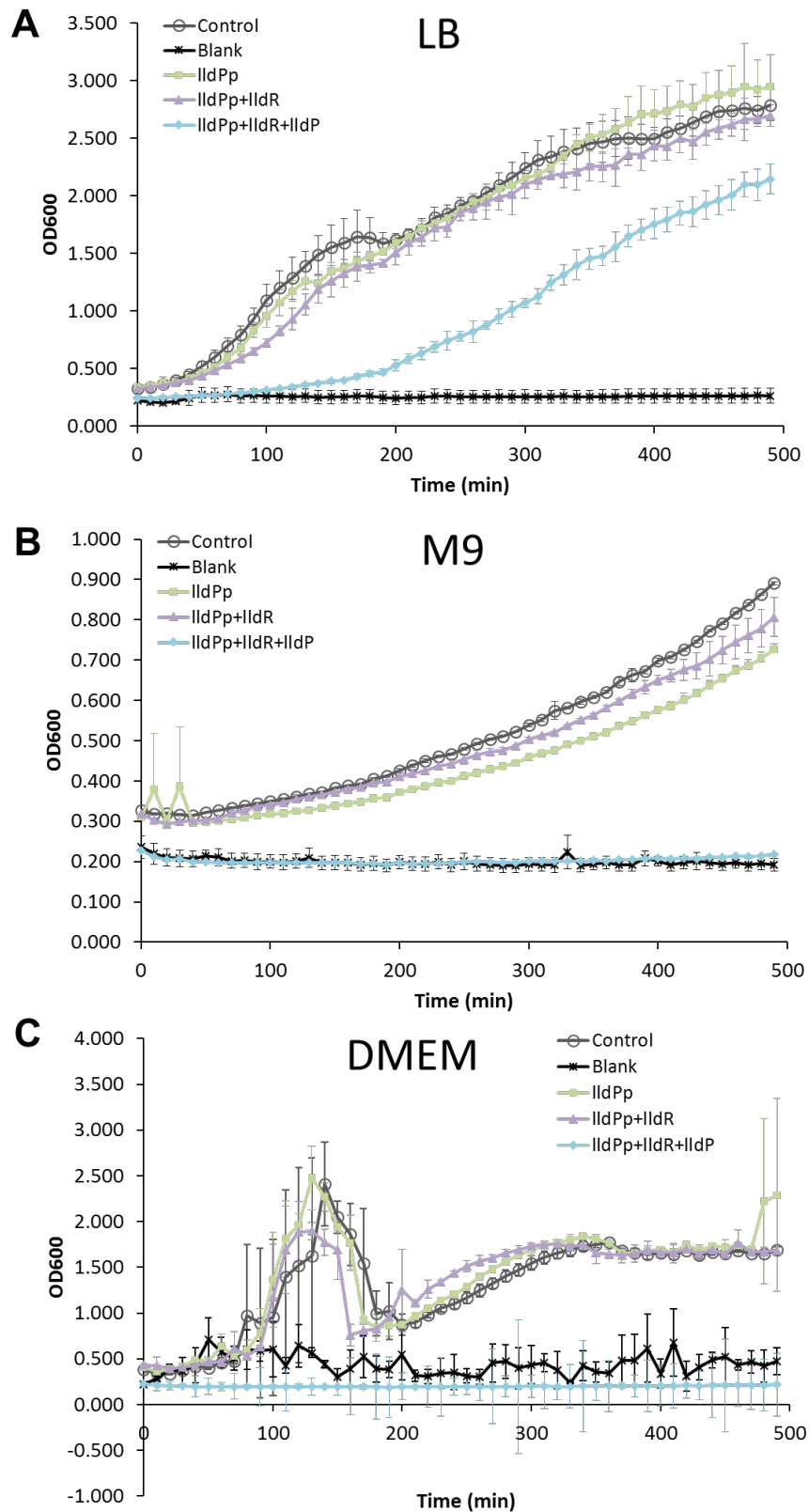
The media tested were DMEM with or without a supplement of phenol red and M9 medium. The three culture tubes for each medium hold a medium blank or *E. coli* cells with control or test plasmids. (B) Absorbance at 600 nm of different concentrations of glutamine in cell-free growth media. (C) Fluorescence at 485 nm of different concentrations of glutamine in cell-free growth media.

not contain added serum or glutamine, instead using  $\text{NH}_4\text{Cl}$  as a nitrogen source. The *Blank* trace showed noise in the OD signal (Figure 4.3(C)). The peak at time 150 min in the traces for *lldPp*, *lldPp+lldR* and *Control* is thought to result from precipitation of medium components. DMEM is known to contain components that can precipitate out of solution, *e.g.* sulphur and calcium. The medium is also optimised to be used under high  $\text{CO}_2$  (*i.e.* acidic) conditions, which were not used here. Basic conditions can promote precipitation of certain ions. Notably this precipitation does not occur in the *Blank*, nor does it occur in *lldPp+lldR+lldP*, where cells are not growing. The precipitation thus seems to be promoted by the bacterial cells during the exponential growth phase. Following this peak, the trace seems to rejoin the bacterial growth curve, suggesting that precipitated material returned into solution as bacterial cells consumed ions or changed the pH. When *E.*



**Figure 4.2:** OD600 and fluorescence (510 nm) of lactate in cell-free M9 medium.

*coli* or *B. subtilis* cells were grown in DMEM with added serum, the OD followed a smooth curve (Chapter 9). However, that experiment used larger culture volumes in flasks and measurements used cuvettes. Larger volumes and longer times between time points may have masked the trends seen here. The growth rates in M9 and DMEM in particular are relatively low. This in itself is not a problem. Ideally the growth rates should not be too slow as that is an indication that the cells are not growing healthily and so will not react as expected. But the growth rate should also not be too fast for two reasons: if cell division is faster than maturation of the relevant protein (here GFP) then the fluorescence increase may not be visible due to too much dilution and if cells grow too fast then the exponential growth phase is over too quickly to obtain sufficient data for characterisation.



**Figure 4.3: Growth of *E. coli* cells expressing lactate sensor constructs in LB, M9 and DMEM growth media.** OD600 was measured using BMG omega polarstar fluorometer in a 96-well plate. (A) LB. (B) M9. (C) DMEM. Error bars represent standard deviations of three biological replicate wells.



#### 4.2.3.1 Growth medium precipitation

An experiment was carried out to test if any of the culture additives could be causing the precipitation and what additives might prevent it (data not shown). It was shown that in the absence of cells,  $\text{NH}_4\text{Cl}$  does not cause precipitation. Addition of acid to some extent prevents precipitation and base causes precipitation. The manufacturer recommends addition of HEPES if using DMEM in a non- $\text{CO}_2$ -enriched atmosphere to prevent precipitation. However, addition of HEPES increased the levels of precipitation. While precipitation is a problem for OD measurements during promoter characterisation, it may not necessarily interfere with biosensor function. As GFP synthesis rate is calculated using fluorescence and OD600 measurements, interference in either of these measurements disrupts the data analysis. DMEM was therefore found to be largely unsuited to be used as the main growth medium in biosensor characterisation.

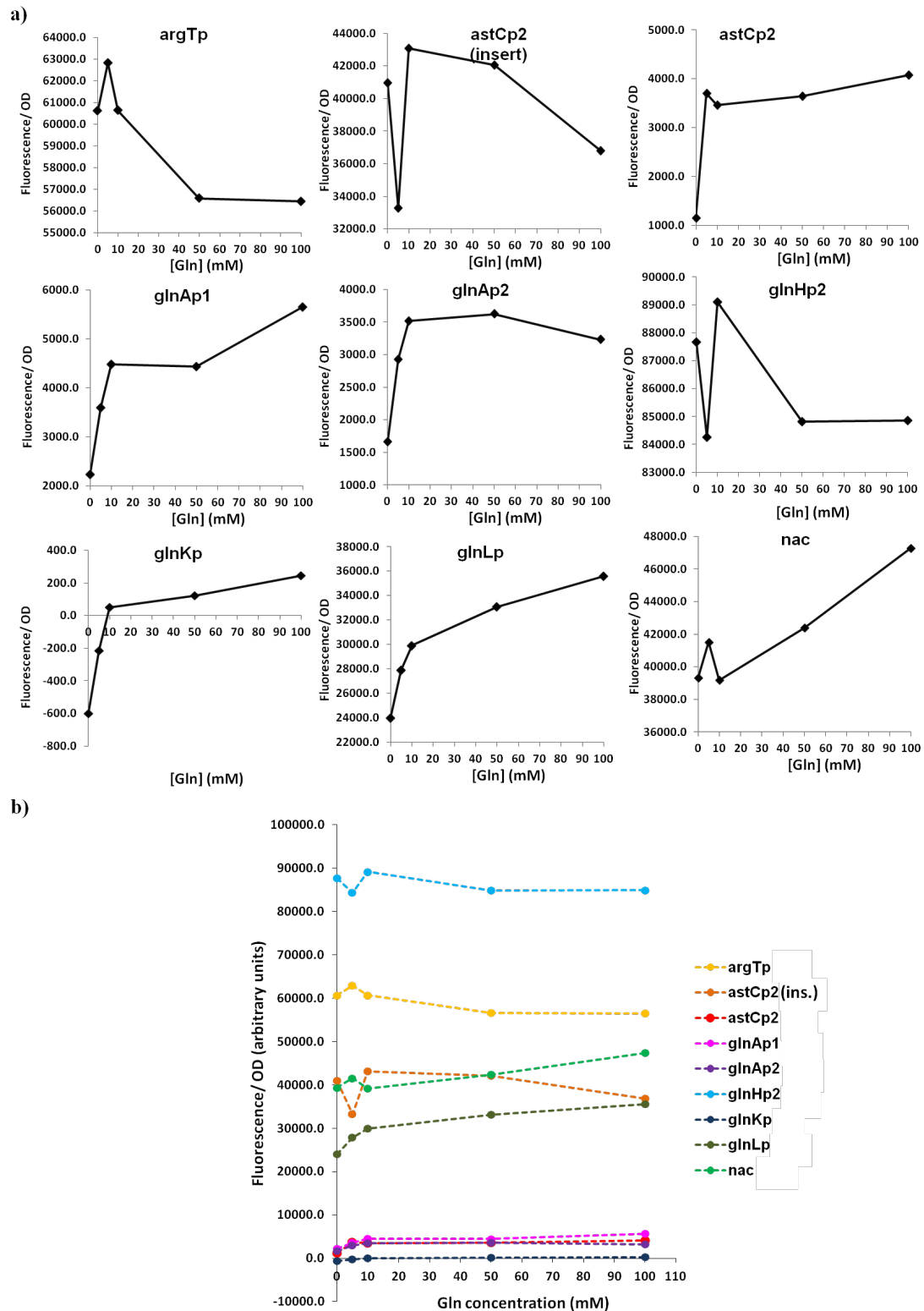
## 4.2.4 Preliminary characterisation

An end-point characterisation experiment of the Ntr promoters over a glutamine concentration range of 0 - 100 mM was carried out (see Figure 4.4). All promoters show different responses to glutamine. Large changes in output can be seen in the glutamine concentration range 0 - 10 mM (the range relevant for mammalian cell cultures), and smaller changes can be seen in the range 10 - 100 mM. A second experiment was therefore carried out, which explores the glutamine concentration range of 0 - 10 mM more closely (Figure 4.5). This shows that target concentration ranges may need to be optimised. Note that such end-point experiments can be prone to error due to differences in growth rates and growth phases between cells in the different wells that are not being accounted for. The corrected fluorescence for *glnKp* shows negative values in both Figure 4.4 and (Figure 4.5). While absolute fluorescence cannot give negative values, the fluorescence values here have been corrected by subtraction of fluorescence from the control cells. These control cells contain the same construct as the biosensor cells, except lacking the relevant responsive promoter. It is therefore possible for control cells to give higher fluorescence than the sensor cells if sensor cell fluorescence is low, leading to negative values. The next step was to develop a protocol for dynamic time-resolved experiments.

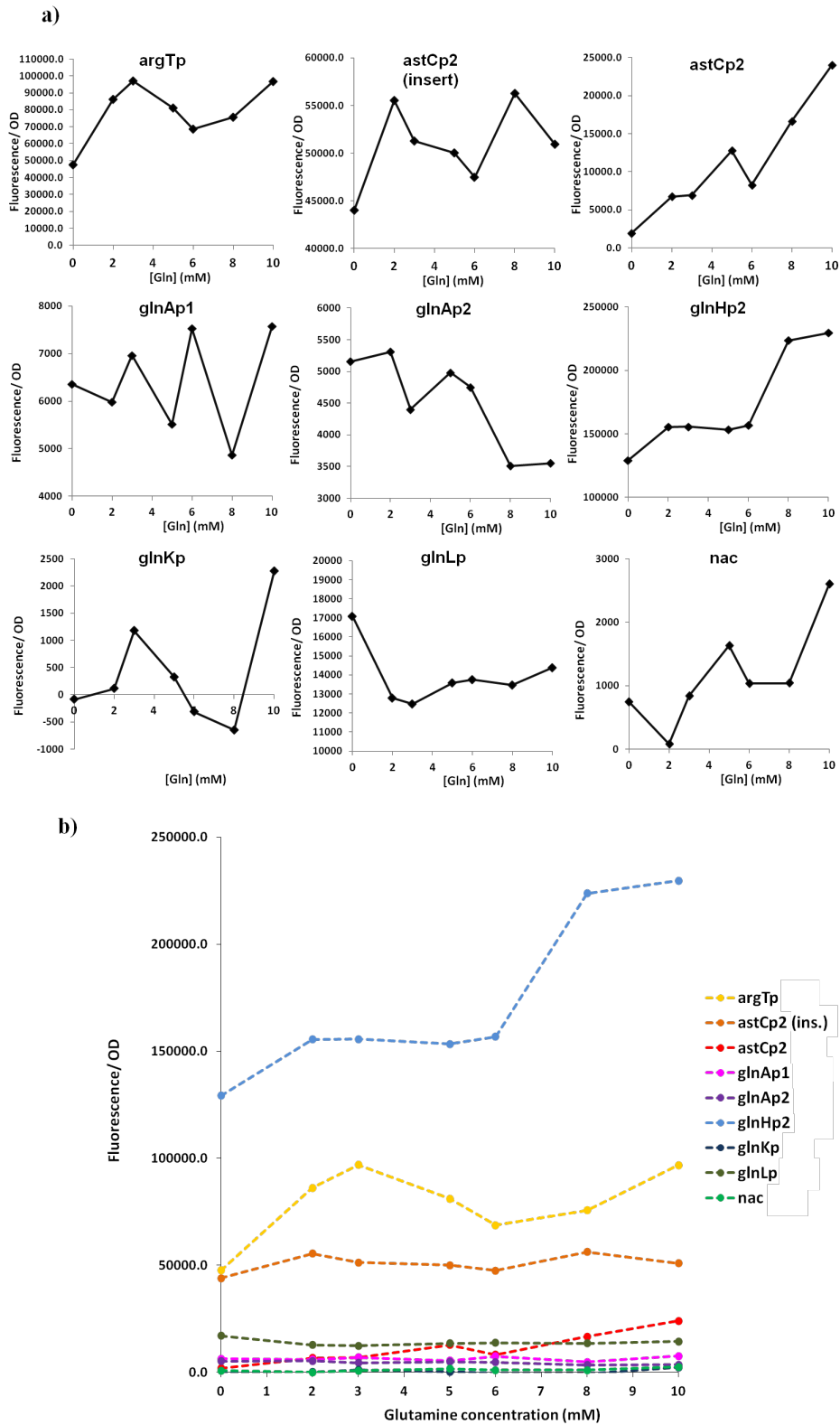
### 4.2.4.1 Equipment choice

More detailed time-resolved characterisation experiments in M9 were then carried out for the Ntr promoters. However, these experiments did not produce usable characterisation data (data not shown). It was observed that the experimental set-up, which involved many time-sensitive manual pipetting steps lead to time delays in induction, settling of cells at the bottom of wells (*i.e.* insufficient mixing) and volume errors and hence inconsistent and noisy data. Replicate cultures on the same plate often showed differences for example in growth rates, which made them difficult to combine during data analysis.

Therefore, it was decided to use a plate reader integrated into a robotic liquid-handling platform and a protocol as developed by Christopher Hirst (Hirst, 2014). This would increase consistency across samples and experimental through-put. Hirst developed an automated characterisation methodology specifically focused on promoters and suitable for both inducible and constitutive promoters. The standardised workflow has been optimised to enable parts characterisation under highly reproducible growth conditions, which should thus produce high quality data.



**Figure 4.4:** Preliminary characterisation of Ntr promoters in M9 medium at glutamine concentrations 1-100 mM. *E. coli* DH $\alpha$  cells containing the various Ntr promoter characterisation constructs were grown in M9 medium on a 96-well plate for 5.5 h at different glutamine concentrations. **a)** individual promoters. **b)** all promoters in comparison. Data are based on single cultures.



**Figure 4.5:** Preliminary characterisation of Ntr promoters in M9 medium at glutamine concentrations 1-10 mM. *E. coli* DH $\alpha$  cells containing the various NtrC promoter characterisation constructs were grown in M9 medium on a 96-well plate for 5.0 h at different glutamine concentrations. **a)** individual promoters. **b)** all promoters in comparison. Data are based on single cultures.

The method is based around the aforementioned robotoc liquid-handling platform with an integrated incubator and plate reader. This allows for reproducible and high-throughput dilution, outgrowth, induction and assaying of biosensor cells. For full detail, see the Materials and Methods section. In short, cells are grown up in cultures overnight. On the day of the characterisation assay, cultures are transferred onto a 96-well plate and diluted with fresh medium. This is followed by an outgrowth phase, induction and regular OD600 and fluorescence measurements. The details, such as durations, can be changed as appropriate. The protocol by Christopher Hirst was adapted for the present biosensors and further developed over time with specific adaptations for each of the biosensors described in this work. Data shown in the later chapters was obtained using different variants of the basic set-up. For instance, the protocol used in this work initially included a dilution of cell cultures, followed by an outgrowth phase and a second dilution before induction, thus following the work flow of Hirst (2014). However, with the very minimal M9 growth medium used here, it was found that only the first dilution of cultures was necessary and leaving out the second dilution vastly improved the biosensor characterisation data that could be collected. This is due to the fact that a lot of the sensors shown in this work produce low levels of fluorescence and so higher cell densities are needed during characterisation in order to distinguish biosensor output from background signal.

### 4.3 Summary of results

The experiments in this chapter showed the development of a general protocol for characterisation of the metabolite-responsive promoters discussed in this work.

Different growth media were considered as the base medium for bacterial growth during biosensor characterisation. It was found that the mammalian growth medium DMEM (as used in Chapter 3) is not a suitable base medium for biosensor characterisation, due to precipitation events, which interfered with OD measurements. A version of the minimal bacterial growth medium M9, containing no amino acids, was found to be a suitable base medium. This is due its defined contents, predictable growth dynamics and lack of interference with fluorescence measurements. However, DMEM would be the base medium of many cell culture samples that could be analysed with the sensors shown in this work. This challenge is addressed in chapter 9 (Biosensors in Bioprocessing).

Preliminary characterisation experiments involved manual loading, dilution and induction in 96-well format. However, the data obtained during these experiments

was found to be unusable due to noise from pipetting errors, time delays and other factors. It was therefore decided to use a robotic characterisation platform.

The protocol that is used in subsequent chapters is based on a robotic platform using 96-well plate format and measurements of absorbance and fluorescence. The protocol is based on one developed by Hirst (2014). Throughout this work, there have been small alterations to the protocol, meaning that datasets shown in the subsequent chapters may use slightly different versions of the basic protocol.

## 5 *E. coli lldPRD* lactate-responsive operon

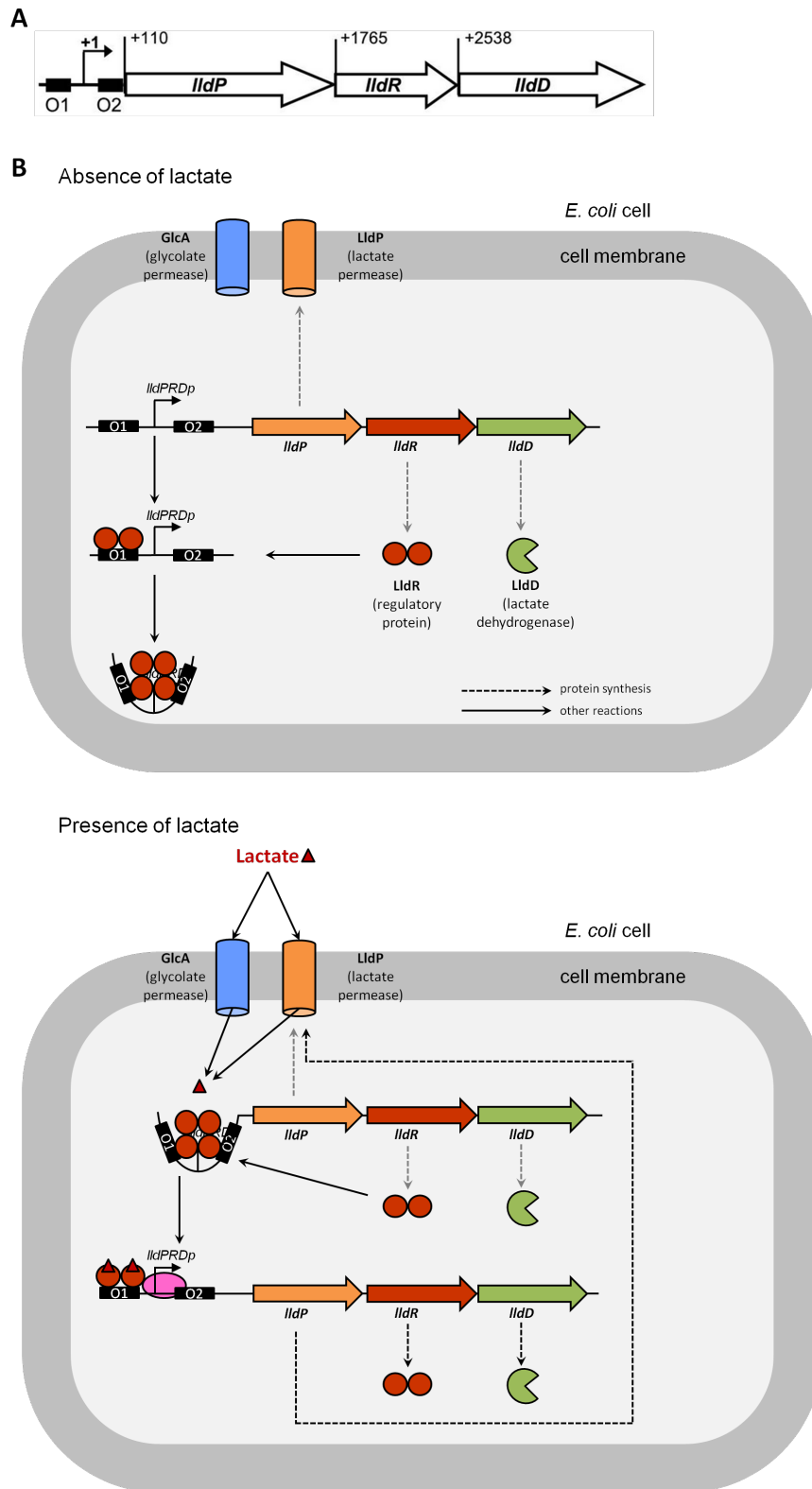
### 5.1 Introduction

#### 5.1.1 Lactate-responsiveness of the *lldPRD* operon

The *lldPRD* operon (previously called *lct*) in *E. coli* is expressed in response to the presence of L-lactate (Núñez et al., 2001; Hua et al., 2007; Aguilera et al., 2008). The operon comprises three genes transcribed from a single promoter (*lldPp*): Lactate permease *lldP* (Núñez et al., 2002), regulatory protein *lldR* and lactate dehydrogenase (Figure 5.1(A)) *lldD* (Dong et al., 1993; Aguilera et al., 2008). D-lactate has also been shown to induce expression from *lldPp*, though to a lesser extent than L-lactate (Núñez et al., 2001). Growth and evolution on medium containing lactate as the carbon source lead to increased transcriptional expression of LldP and LldD, though not LldR (Hua et al., 2007).

LldR was shown to have an N-terminal helix-turn-helix motif that is homologous to that of a number of bacterial transcription factors, including FadR (Dong et al., 1993). It was suggested that LldR functions as a repressor in the absence of lactate (Lynch and Lin, 1996). A comparative study placed LldR in the FadR subfamily of the helix-turn-helix GntR family of bacterial regulators (Rigali et al., 2002; Aguilera et al., 2008). Similar lactate operons exist in other bacterial species. The LldR protein from *Corynebacterium glutamicum* and the operon that it regulates have been extensively characterised (Stansen et al., 2005; Georgi et al., 2008; Gao et al., 2008; Toyoda et al., 2009). Systems have also been found in *Pseudomonas aeruginosa* (Gao et al., 2012) and *Shewanella oneidensis* (Pinchuk et al., 2009).

The structure of the *lldPp* promoter was analysed for transcription factor binding sites and transcription start sites (Lynch and Lin, 1996) and a model for *lldPp* regulation through lactate was proposed. A subsequent extensive study updated this model (Aguilera et al., 2008) (Figure 5.1(B)). In absence of lactate, the LldR transcription factor acts as a repressor of gene expression from this operon. In presence of lactate, LldR activates transcription by aiding the assembly of the transcription machinery.



**Figure 5.1: Lactate response of the *lldPRD* operon in *E. coli*.** (A) Genetic structure of *lldPRD* operon. It consists of three structural genes and an inducible promoter. Figure taken from Aguilera et al. (2008). (B) Gene expression in *lldPRD* operon in absence and presence of lactate. Expression from the *lldPRDp* promoter is thought to be repressed in the absence of lactate and activated in the presence of lactate. Figure is based on information from Dong et al. (1993); Lynch and Lin (1996) and Aguilera et al. (2008).

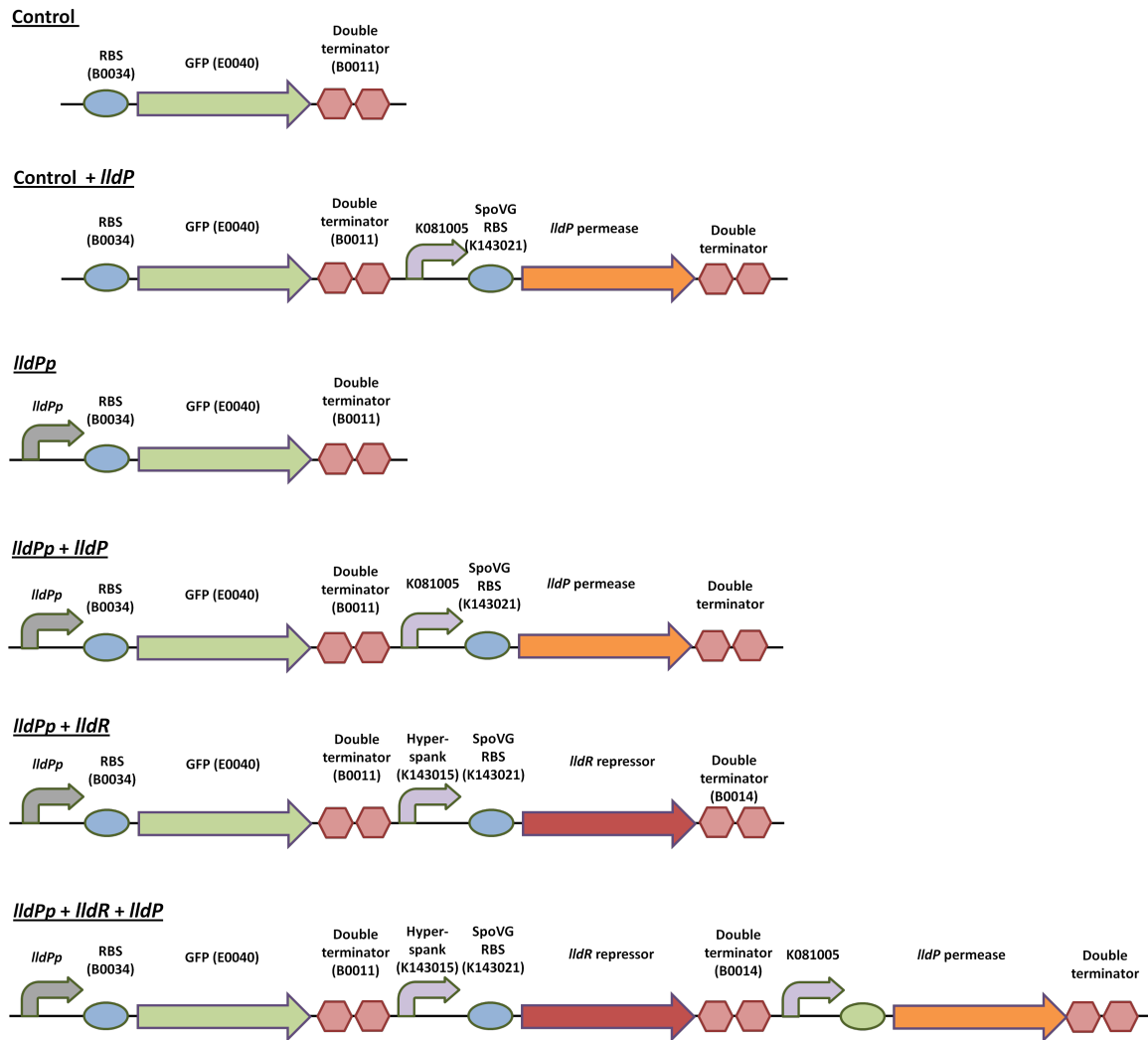


## 5.1.2 Biosensor assembly and preliminary characterisation

It is proposed here that the *lldPp* promoter could be used as part of a whole-cell lactate biosensor. The LldR protein has previously been used as a FRET lactate sensor called Laconic in mammalian cells (San Martín et al., 2013). In that context, the sensor was shown to be quantitatively responsive to lactate in the concentration range from 1  $\mu$ M to 10 mM and was not significantly affected by a large number of other metabolites at the concentrations found in mammalian cells. pH was shown to affect the response of Laconic quantitatively but not qualitatively (San Martín et al., 2013). The effect of pH, however, may be due to the pH sensitivity of the fluorophores rather than the LldR itself.

A number of constructs were made to study the potential of this promoter as a biosensor (Figure 5.2). These constructs were made by undergraduate students Sharmilah Veterayan (Veterayan, 2011) and Katarzyna Roguska (Roguska, 2012). Veterayan (2011) constructed *lldPp* (containing the responsive promoter linked to a GFP gene) and *lldPp+lldR* (additionally overexpressing the transcription factor) and confirmed that they respond to lactate with a linear detection range between 2.5 mM and 0.05 mM. Roguska (2012) constructed variants of *lldPp* and *lldPp+lldR* plasmids containing the *lldP* permease gene (to potentially aid import of lactate and change response time), as well as variants with different plasmid copy numbers (to potentially change the sensitivity range of the sensor). Lactate-response of *lldPp* and *lldPp+lldR* in presence of other carbon sources were also investigated (Roguska, 2012). Confirming previous results (San Martín et al., 2013), it was shown that the sensors were lactate-responsive in the presence of a number of different carbon sources, but that the quantitative fluorescence output differs for these different conditions. This could be due to different carbon sources leading to different levels of intracellular lactate accumulation or due to different carbon sources causing different levels of repression of the *lldPp* promoter. In this work it was found that the *lldPp+lldR+lldP* cells did not show significant growth in M9 or DMEM media and this construct was therefore not characterised further (see Chapter: Development of a biosensor characterisation protocol).

To act as a lactate sensor, the *lldPp* promoter should ideally only change activity in response to lactate concentration and not respond to any other compounds or conditions. In addition to LldR, the *lldPp* promoter is also regulated by the two-component system ArcB-ArcA. ArcB-ArcA reacts to cellular redox state, which is representative of cellular oxygen availability. Expression of the *lldPRD* operon is activated under aerobic conditions and repressed under anaerobic conditions (Iuchi et al., 1994). ArcB is a sensor kinase thought to detect an electron transport



**Figure 5.2:** Lactate biosensor constructs used in this study, made by undergraduate students Sharmilah Veterayan (Veterayan, 2011) and Katarzyna Roguska (Roguska, 2012).

component in reduced form, and is ArcA a response regulator phosphorylated by ArcB during anaerobic growth (Lynch and Lin, 1996). Changes in expression of *lldPRD* can be as much as 30-fold or 90-100-fold in response to respiratory growth conditions, which represents the widest range of responses to ArcA of all operons regulated through this system (Dong et al., 1993; Lynch and Lin, 1996). This may be explained by the fact that the ArcA binding sites in *lldPp* are very similar to the ArcA consensus binding site (Liu and De Wulf, 2004). During transition from anaerobic to aerobic growth conditions, expression from *lldPRD* is rapidly increased ~3-5-fold within 5 minutes (Partridge et al., 2006). The regulation by ArcA could interfere with the use of *lldPp* as a lactate sensor. Using the *lldPp* and *lldPp+lldR* constructs, it was shown that ArcA regulation of *lldPp* could become insignificant in *biosensor 2*, probably because the endogenous ArcB-ArcA expression is too low (Roguska, 2012). Alternatively, presence of nitrate in the

growth medium can partially relieve the anaerobic repression of ArcB-ArcA due to its high redox potential (Iuchi et al., 1994). A possible indirect but small regulatory effect on *lldPRD* by Fnr, a global regulator involved in aerobic metabolism, has also been suggested (Iuchi et al., 1994). Another transcription factor, PdhR, was also suggested to regulate expression of *lldPRD* (Lynch and Lin, 1996), but this was later shown not to be the case (Aguilera et al., 2008).

## 5.2 Results

### 5.2.1 Biosensor characterisation on solid medium

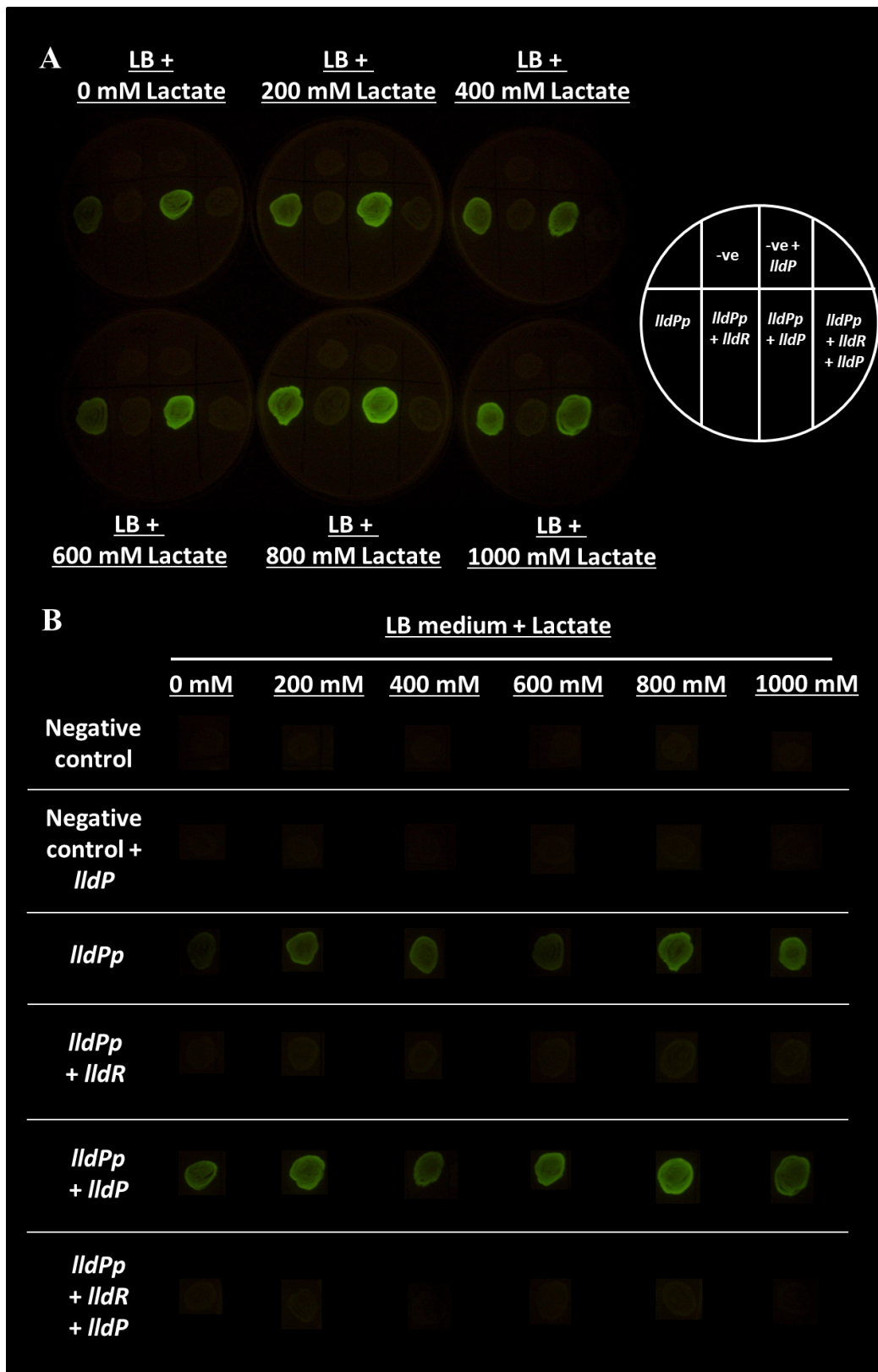
A qualitative biosensor characterisation was carried out. *E. coli* cells expressing the various biosensor constructs were plated on LB agar plates containing varying concentrations of lactate (Figure 5.3). As previously shown (Veterayan, 2011; Roguska, 2012), overexpressing *lldR* leads to lower expression levels of GFP from the *lldPp* promoter. However, fluorescence changes with respect to lactate concentration were not discernible by eye.

### 5.2.2 Biosensor characterisation in liquid medium

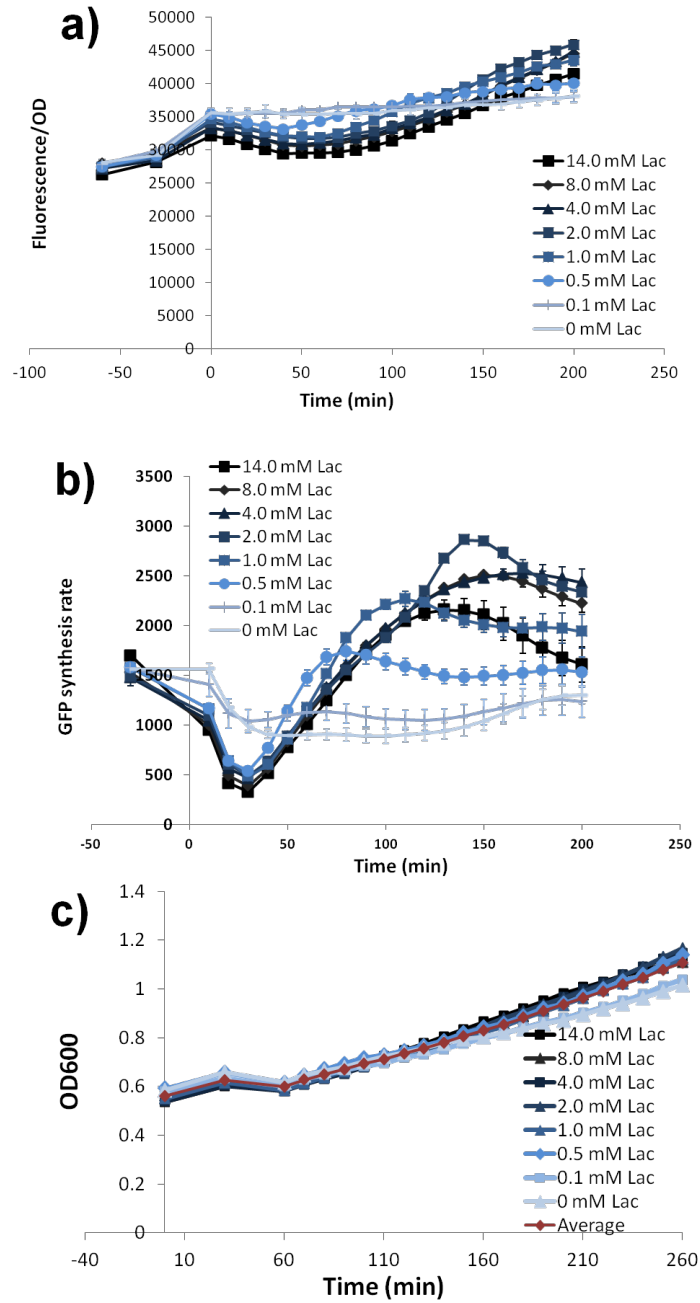
Results shown in this section were always representative of multiple replicate experiments with a minimum two technical replicates of three biological replicates.

The biosensor cells were characterised in M9 medium (containing glycerol as a carbon source) using a robotic plate reader platform. Both *lldPp* sensor cells (Figure 5.4) and *lldPp+lldR* sensor cells (Figure 5.5) were characterised. As mentioned above, *lldPp+lldR+lldP* was also characterised, but these cells showed reduced growth, probably due to additional metabolic burden or toxicity due to the overexpression of the permease.

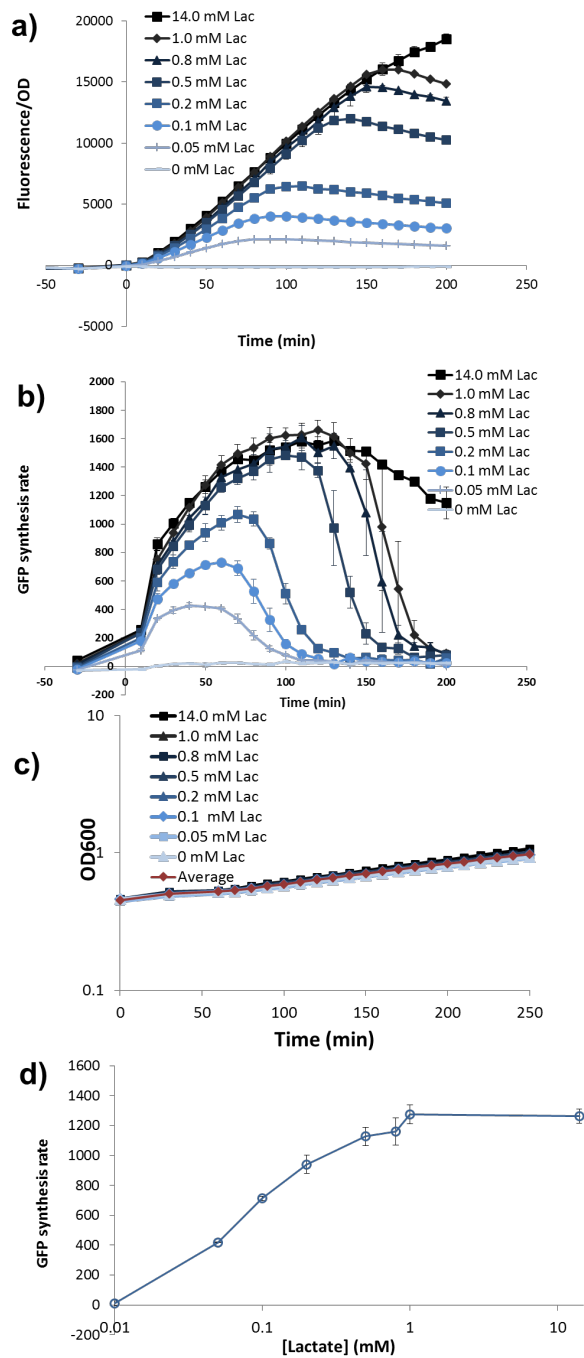
The *lldPp* sensor showed a limited response to lactate and a high baseline fluorescence in absence of lactate (Figure 5.4). The *lldPp+lldR* sensor shows a strong and very defined response to lactate (Figure 5.5). In absence of lactate, sensor cell fluorescence is equal to control cell autofluorescence. Presence of lactate leads to an increase in fluorescence and over time cells arrive at a higher stable fluorescence level, the level of which depends on the lactate concentration.



**Figure 5.3: Lactate sensor characterisation on solid medium.** *E. coli* DH $\alpha$  cells were plated out on plates with LB agar containing different concentrations of lactate and grown for approximately 20 hours. (A) Photo of colonies on plates. (B) Colonies re-ordered for comparison. Plates are shown placed on a UV-illuminator.



**Figure 5.4:** *UdpPp* lactate sensor characterisation. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. Biosensor cells were grown in M9 minimal medium. Error bars represent the standard deviation of three biological replicates.



**Figure 5.5:** *lldPp+lldR* lactate sensor characterisation. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. **d)** Dose-response graph. Biosensor cells were grown in M9 minimal medium. Error bars represent the standard deviation of three biological replicates.

## 5.2.3 Effect of carbon and nitrogen sources on lactate biosensing

### 5.2.3.1 Lactate sensing in presence of glucose

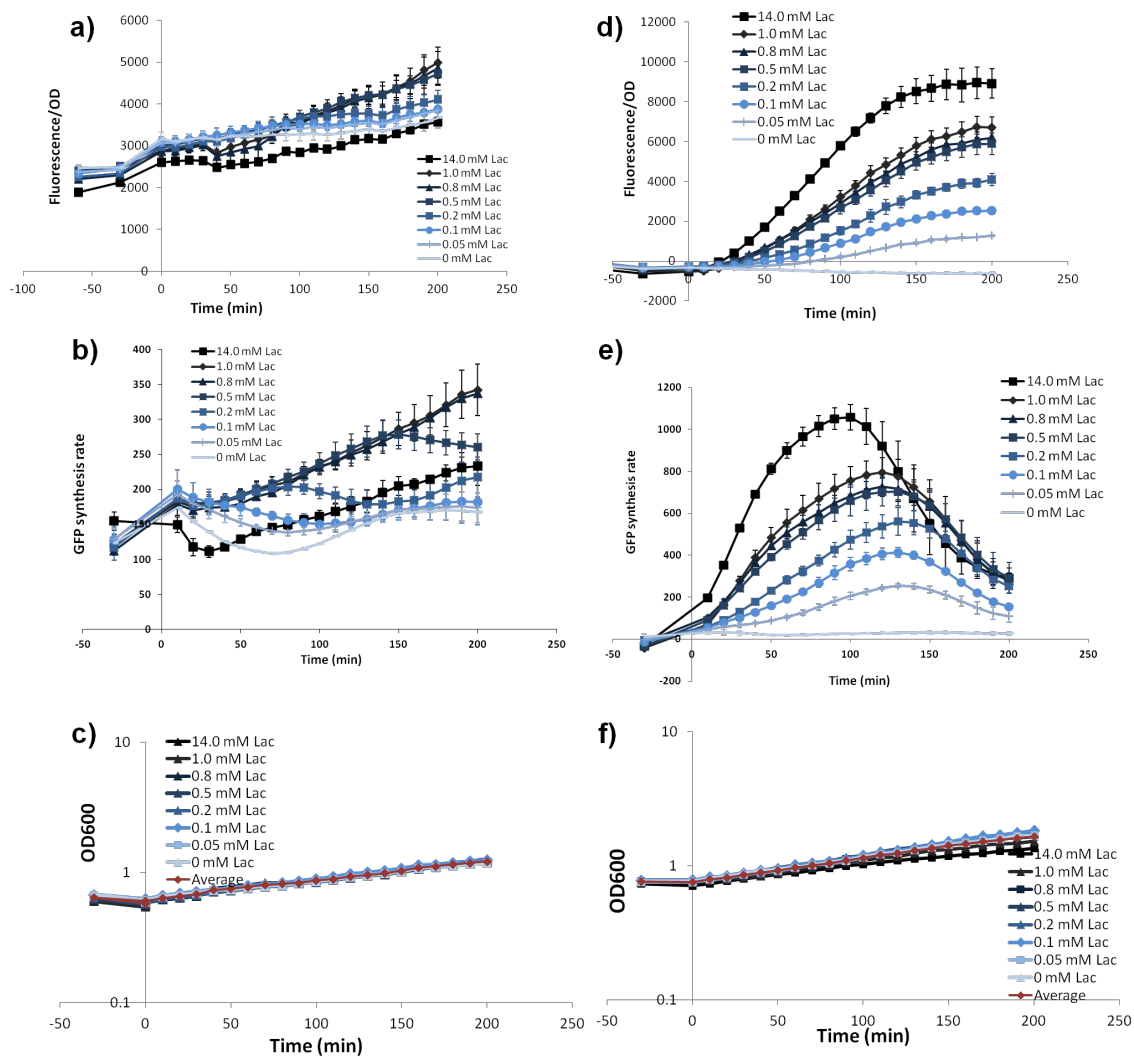
Results from the literature suggest that the *lldPp* promoter is repressed by the presence of glucose. Cells would use glucose as a carbon source in preference to lactate and hence keep expression from the *lldPp* promoter low. This is also similar to other carbon source operons, such as the *lac* operon. It was suggested that the presence of glucose would lower the baseline expression from the *lldPp* promoter in such a way that the *lldPp* sensor cells would show a greater response to lactate. Furthermore, most mammalian cell growth media contain glucose as a carbon source and so it was important to test the sensors in presence of this compound. Both *lldPp* sensor cells and *lldPp+lldR* sensor cells were characterised for their response to lactate in M9 medium containing glucose as a carbon source instead of glycerol (Figure 5.6).

Overall, the results show that the biosensors behave very similarly in the presence of glucose as in the previous experiments in absence of glucose, except that the fluorescence output is lower. *lldPp* still only shows a limited lactate response with a high background baseline and *lldPp+lldR* still shows a very strong lactate response with a low background baseline. Corrected fluorescence values in the negative range are due to calculations of removing background fluorescence from the control cells. This suggests that there is some leaky expression of GFP by the control cells.

### 5.2.3.2 LB medium

Characterisation of the biosensors in LB was attempted but these experiments did not give usable data (data not shown). Cell growth in LB medium is very fast. This means that cells quickly reach stationary phase where biosensor characterisation is difficult as the assumptions of exponential phase no longer hold true. Also, very fast cell division can mask potential GFP accumulation in cells from being detectable. Modifications would have to be made to the protocol to characterise the sensors in this medium.





**Figure 5.6:** Lactate sensor response to lactate in presence of glucose. **a) - c)** show *lldPp* and **d) - f)** show *lldPp+lldR*. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. **d)** Corrected fluorescence. **e)** GFP synthesis rate. **f)** Cell growth. Biosensor cells were grown in M9 minimal medium. Error bars represent the standard deviation of three biological replicates.

## 5.2.4 Other inducers - D-lactate and pyruvate

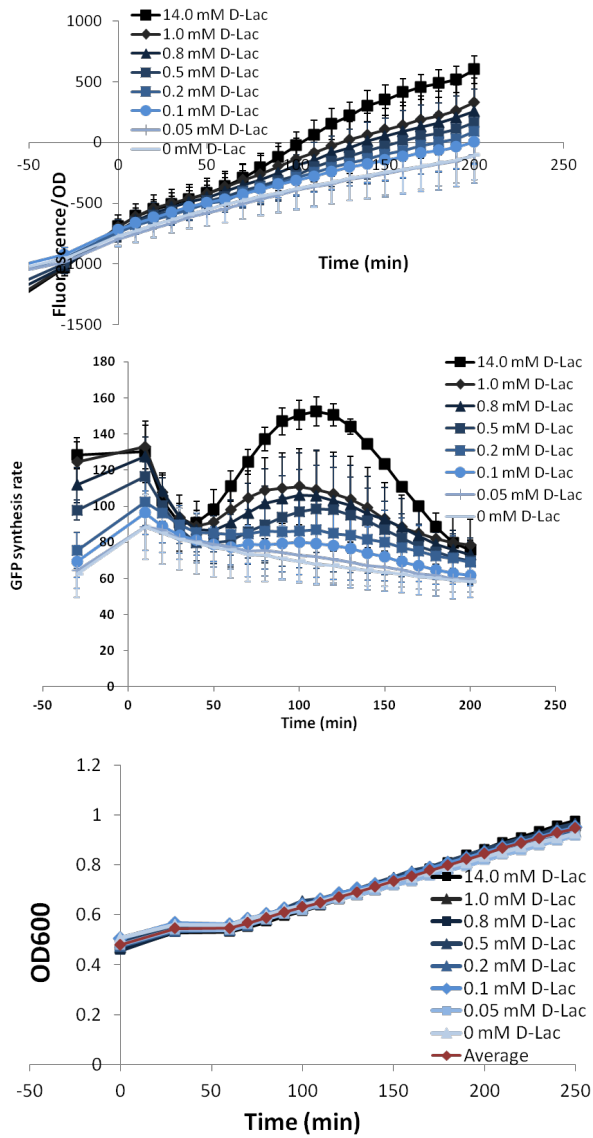
It is possible for biological molecules to interact with more than one target molecule. For a biosensor, this can have both advantages and disadvantages. Responsiveness to several inducers can increase or decrease the possible application range. It reduces specificity, but opens up new sensing targets. It was decided to test the response of the *lldPp+lldR* lactate sensor to D-lactate and pyruvate. Both these molecules are similar to L-lactate in size and structure. Pyruvate is present in many cell culture media and there is potential interest in our Centre for creating a D-lactate sensor.

### 5.2.4.1 D-lactate

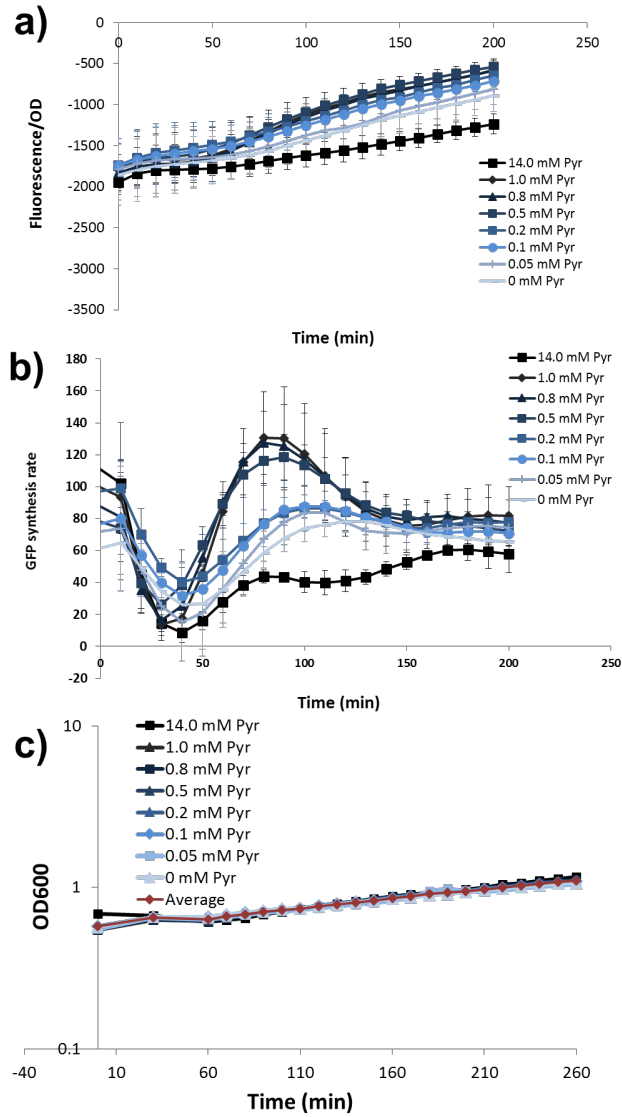
D-lactate has previously been shown to induce expression from *lldPp*, though to a lesser extent than L-lactate (Núñez et al., 2001). Results from characterisation of the response of *lldPp+lldR* to D-lactate confirm these results from the literature (Figure 5.7). As before, corrected fluorescence values in the negative range are due to calculations of removing background fluorescence from the control cells.

### 5.2.4.2 Pyruvate

The *lldPp+lldR* lactate sensor cells were exposed to different concentrations of pyruvate (Figure 5.8). While pyruvate concentrations in the range 0 - 1.0 mM cause a small activation effect, fluorescence at 14 mM pyruvate was always the lowest of all concentrations. As before, corrected fluorescence values in the negative range are due to calculations of removing background fluorescence from the control cells.



**Figure 5.7:** *lldPp+lldR* lactate sensor response to D-lactate. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. Biosensor cells were grown in M9 minimal medium. Error bars represent the standard deviation of three biological replicates.



**Figure 5.8:** *UdpPp+UldR* lactate sensor response to pyruvate. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. Biosensor cells were grown in M9 minimal medium. Error bars represent the standard deviation of three biological replicates.

## 5.3 Summary of results

Lactate sensors based on the *lldPRDp* promoter were characterised for their response to lactate. It was found that a construct containing the promoter linked to a GFP gene, as well as overexpressing the *lldR* regulator (*lldPp+lldR*) showed strong activation by lactate in the range 0-14 mM in liquid M9 medium. A construct containing the responsive promoter, but not overexpressing the *lldR* regulator (*lldPp*) showed a much more limited response to lactate in liquid M9 medium. Characterisation in M9 medium containing glucose or glycerol as a carbon source gave similar results qualitatively, though quantitatively, fluorescence output from the sensor was lower in presence of glucose. Characterisation on solid medium did not show a response that was visible to the eye. Cells with constructs overexpressing the *lldP* permease gene did not grow well enough for characterisation. It therefore seems that overexpression of this gene is damaging to the cells.

The *lldPp+lldR* construct was also characterised for its response to D-lactate and pyruvate. There was a response to D-lactate that was similar to, though less pronounced than, that to L-lactate. This means that protein engineering could potentially be used to create a D-lactate biosensor from this system. While pyruvate concentrations in the range 0 - 1.0 mM cause a small activation effect, fluorescence at 14 mM pyruvate was always the lowest of all concentrations. This could be due to the cells utilising pyruvate as a nutrient source.

Overall, *E. coli* cells containing the *lldPp+lldR* construct appear very suitable for use as a lactate biosensor under the conditions used here. Further experiments showing this sensor in conditions more similar to mammalian cell culture are shown in Chapter 9 (Biosensors for bioprocessing).

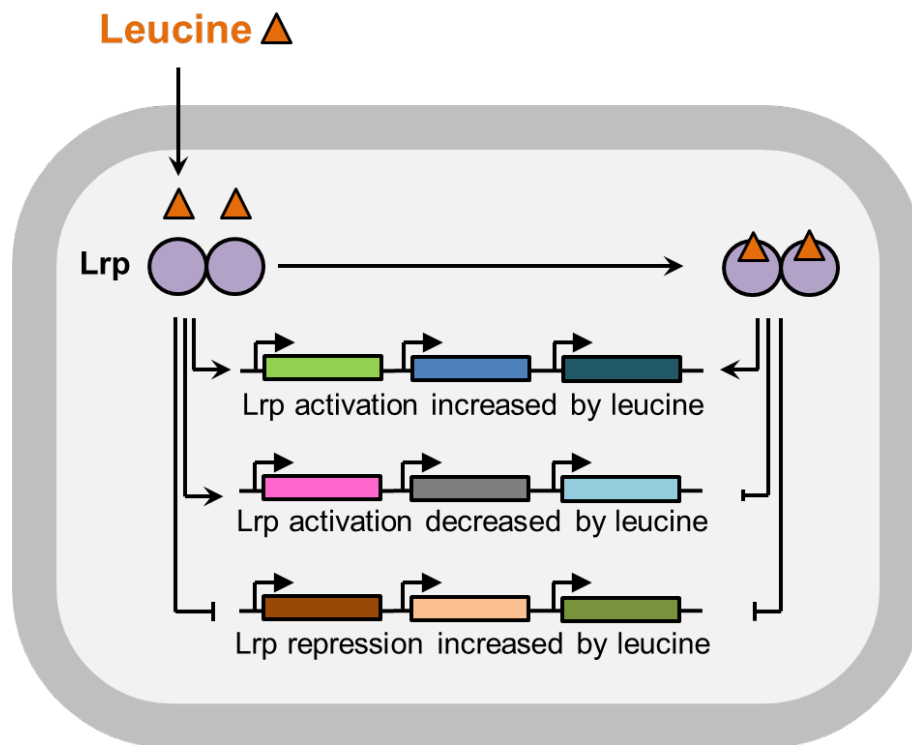
## 6 *E. coli* Lrp operon

### 6.1 Introduction

#### 6.1.1 Leucine- and alanine-responsiveness of promoters in the Lrp operon

Promoters of the Lrp regulon are controlled by the leucine-responsive protein (Lrp). Lrp acts as a global regulator in *E. coli* and regulates the expression of many genes in response to leucine and in some cases alanine (Ernsting et al., 1992; Calvo and Matthews, 1994; Newman, 1995). It directly regulates over 200 genes in *E. coli* and is present across bacteria (Newman, 1995; Hart and Blumenthal, 2011). The details of the Lrp regulon have been extensively discussed elsewhere (Calvo and Matthews, 1994; Newman, 1995). Lrp can act on cellular promoters in a number of different ways and this regulation in turn can be differently affected by leucine (Figure 6.1(A)). The Lrp operon is interlinked with other metabolic control operons such as *Ntr* and *argR* (Ernsting et al., 1992).

In order to make a library of known promoters, six *E. coli* promoter elements, which are regulated in response to leucine (*fimBp1*, *gimBp2*, *gltBp*, *ilvIHp1*, *leuLp* and *livJp*) were chosen to be characterised. From the literature, it is possible to make predictions about the behaviour of these promoter under relevant experimental conditions (Table 6.1).



**Figure 6.1:** Lrp system in cells. Lrp regulation affects different operons in different ways (see Table 6.1). This diagram was made using information from [ecocyc.org].

**Table 6.1:** Expected promoter response to changing Lrp, leucine and alanine concentrations based on the literature. Alanine can have a similar effect to leucine, but less so and in some cases it has no effect.

Promoter	Natural context	Predicted promoter behaviour			Reference
		+Lrp	+Leu	+Ala	
fimBp1	type I pili	Activation	Activation	Activation	(Calvo and Matthews, 1994; Newman, 1995); check: (Kiryu et al., 2005; Berezhnoy et al., 2006)
fimBp2	type I pili	Activation	Activation	Activation	(Calvo and Matthews, 1994; Newman, 1995)
gltBp	glutamate synthase	Activation	Repression	No known effect	(Newman, 1995), check: (Martinez-Vaz et al., 2010)
ilvIHp1	isoleucine and valine biosynthesis	Activation	De-activation	De-activation	(Calvo and Matthews, 1994; Newman, 1995; Chen et al., 2005); check: (Kaltenbach et al., 1998)
leuLp	leucine biosynthesis	Activation	Repression	No known effect	(Newman, 1995); check: (Selvamani et al., 2013)
livJp	leucine transport	Repression	Repression	Repression	(Ernsting et al., 1992; Calvo and Matthews, 1994; Newman, 1995); check (also for other promoters) (Barker et al., 2001)

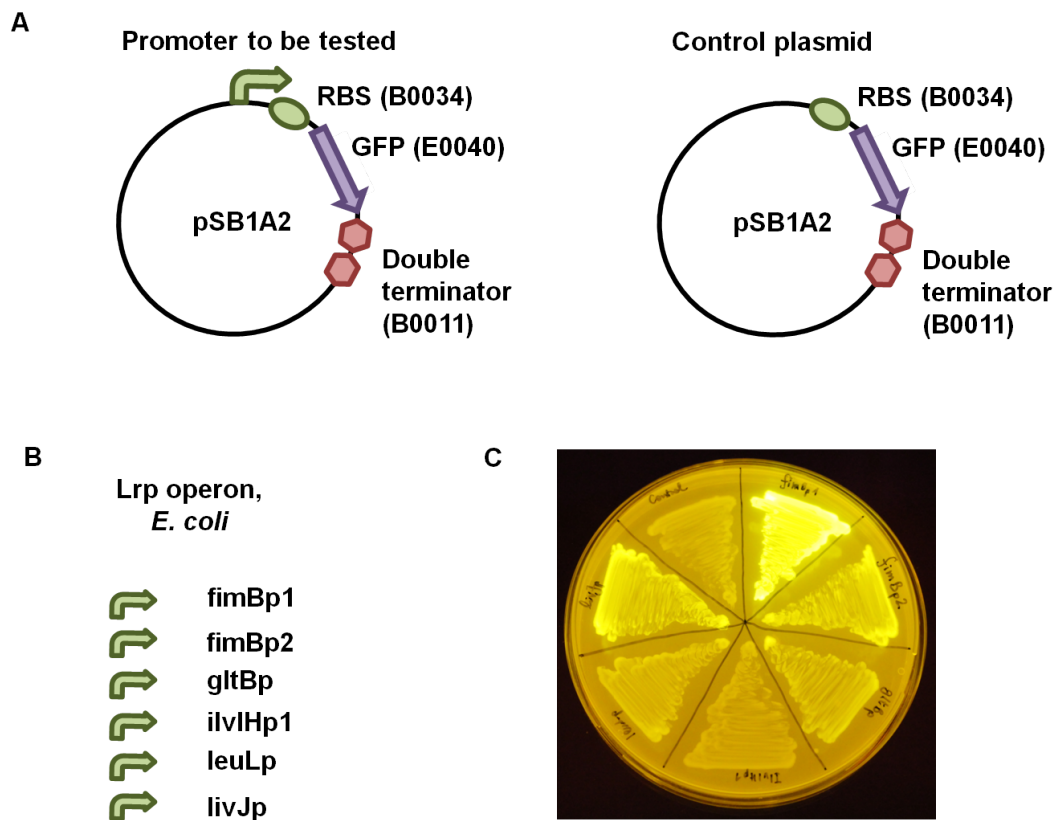
## 6.2 Results

### 6.2.1 Biosensor construct assembly

Alanine is an interesting target in hybridoma bioprocessing, as it is secreted by mammalian cells in response to ammonium stress (see Introduction). In order to make a whole-cell leucine- or alanine-biosensor six *E. coli* promoter elements, which are regulated by the leucine/alanine-responsive Lrp regulator, fimBp1, gimBp2, gltBp, ilvIHp1, leuLp, livJp (Figure 6.2(B)) were chosen to be tested for their function using the gene circuit shown in Figure 6.2(A). Unlike the lactate sensor construct shown in chapter 5, the sensor constructs here do not overexpress the transcription factor that controls the promoter, *i.e.* Lrp. Undergraduate student Harold Taylor attempted to overexpress the Lrp protein in *E. coli*, but found that cell colonies did not grow after transformation, indicating that Lrp overexpression may be toxic to these cells.

The promoters were successfully amplified from the *E. coli* genome, transformed into BioBricks and transformed into *E. coli* DH $\alpha$  cells. After transformation of the constructs into cells, the cells showed different levels of fluorescence on LB agar plates with the fimBp-containing cells showing the highest level of fluorescence (Figure 6.2(C)). All the constructs were verified by DNA sequencing.





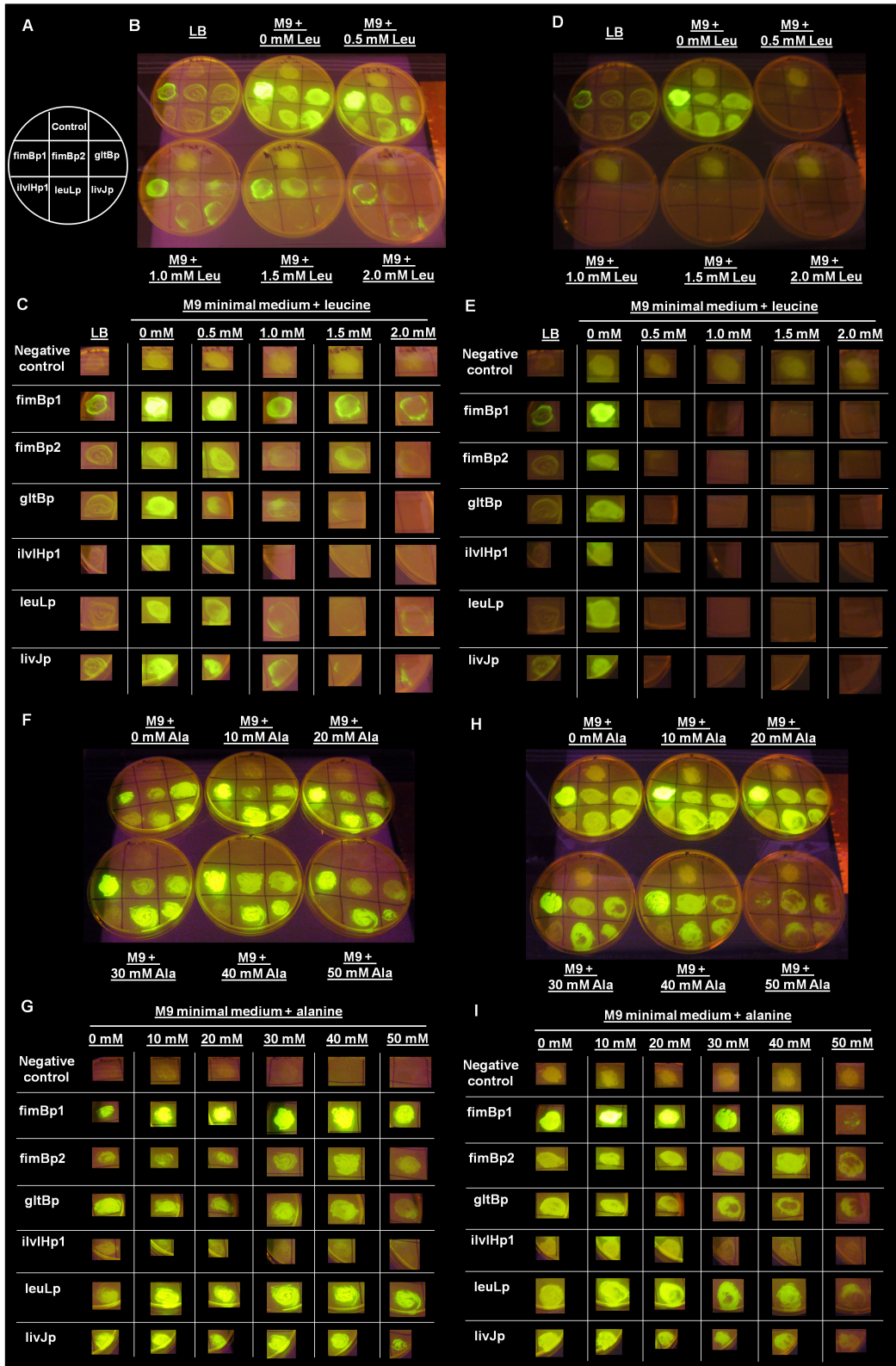
**Figure 6.2: Lrp operon promoter assembly.** (A) Characterisation and control gene circuit. (B) List of lrp operon promoters tested here. (C) LB agar plates with *E. coli* DH $\alpha$  transformed with the constructs from (A) containing six different Lrp promoters.

## 6.2.2 Biosensor characterisation on agar plates

As it is known that growth conditions can affect the behaviour of the Lrp regulon (Cho et al., 2008), it was important to characterise the promoters in a number of different conditions and to clearly define each particular condition. Initially, biosensors were tested qualitatively on M9 minimal medium plates, supplemented with varying concentrations of leucine. Leucine concentrations ranged from 0-2.0 mM, which approximately covered leucine solubility range in this medium. (Figure 6.3). To test the effect of different level of nitrogen availability, different concentrations of  $\text{NH}_4\text{Cl}$  were also tested. Plates contained either 19 mM (Figure 6.3(B)-(C)) or 37 mM  $\text{NH}_4\text{Cl}$  (Figure 6.3(D)-(E)).  $\sim 19$  mM is the commonly used concentration of  $\text{NH}_4\text{Cl}$  in M9 medium and  $\sim 37$  mM is double that concentration.

Generally, increasing leucine concentrations correlated with decreasing cell fluorescence. However, non-control cells grown on higher leucine concentrations also showed decreased growth. Thus the decreased fluorescence is likely to be a side-effect of decreased growth. Control cells did not show growth inhibition. Leucine has been shown to be toxic to *E. coli* cells at high concentrations (Quay et al., 1977; Tavori et al., 1981; Ernsting et al., 1992). Addition of 10 mM leucine has been shown to slow *E. coli* growth (Ernsting et al., 1992). The concentrations used here, however, were lower than 10 mM. The results suggest that leucine toxicity is only displayed here when cells are forced to overproduce GFP from the characterisation construct. It is possible that the additional metabolic burden of overexpression causes the toxicity in sensor cells, but not control cells. However, the effect of leucine toxicity was more pronounced under high  $\text{NH}_4\text{Cl}$  conditions.

Response to alanine was also tested. From the literature it is predicted that alanine has a similar (though smaller) effect to leucine on many of these promoters (Table 6.1). The promoters *gltBp*, *ilvIHp1* and *livJp* showed repression, *leuLp* showed activation and *fimBp1* showed very little to no effect (Figure 6.3(F)-(I)). Results were similar in high and low  $\text{NH}_4\text{Cl}$  conditions, although the centre of cell streaks were less fluorescent in high  $\text{NH}_4\text{Cl}$  conditions.

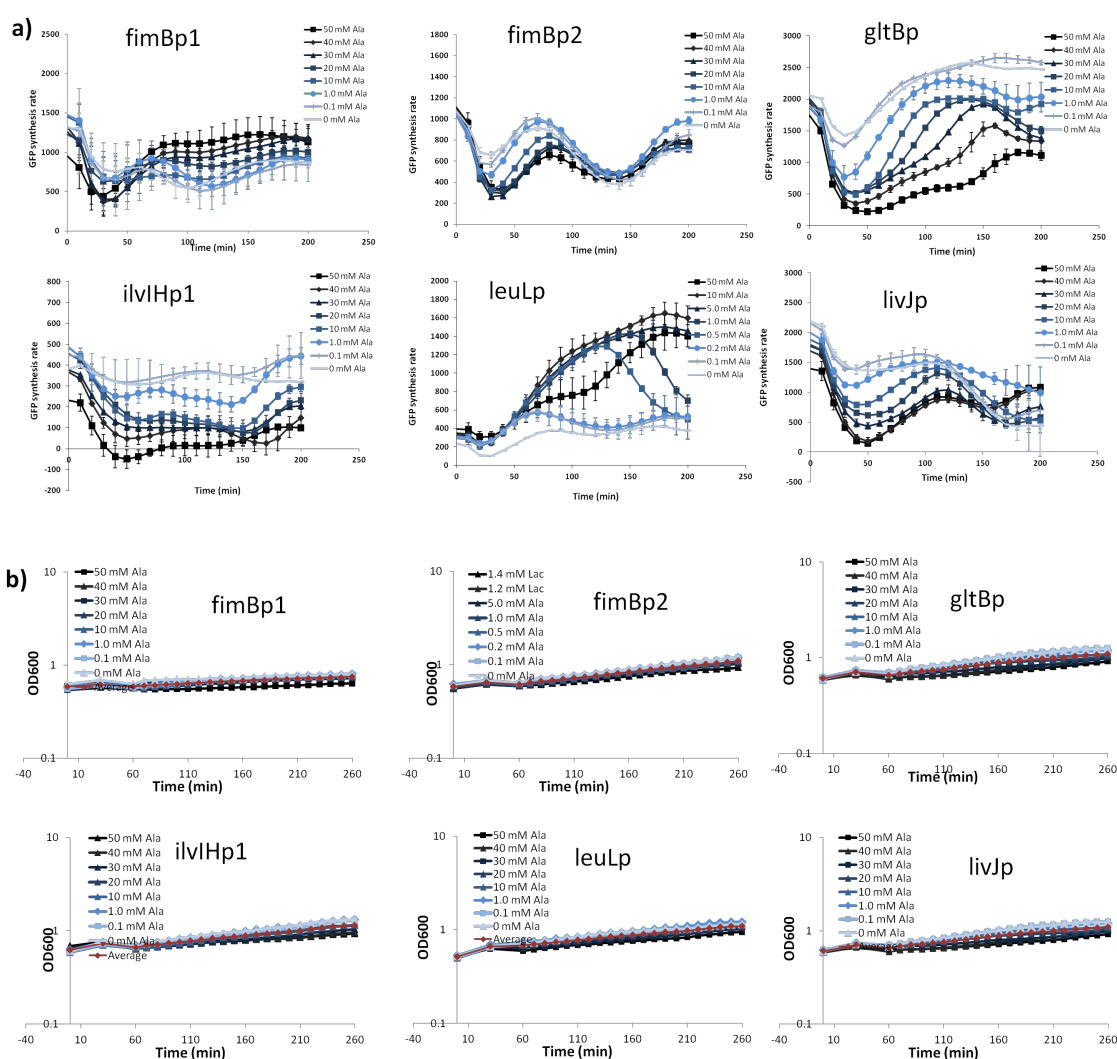


**Figure 6.3:** Promoter characterisation on solid medium. (A) Plate layout. (B)-(E) Response to leucine with plates containing (B)-(C) 19 mM NH<sub>4</sub>Cl or (D)-(E) 37 mM NH<sub>4</sub>Cl. (F)-(I) Response to alanine with plates containing (F)-(G) 19 mM NH<sub>4</sub>Cl or (H)-(I) 37 mM NH<sub>4</sub>Cl. Plates are shown placed on a UV-illuminator. Image was taken after ~1.5 days at 37°C.

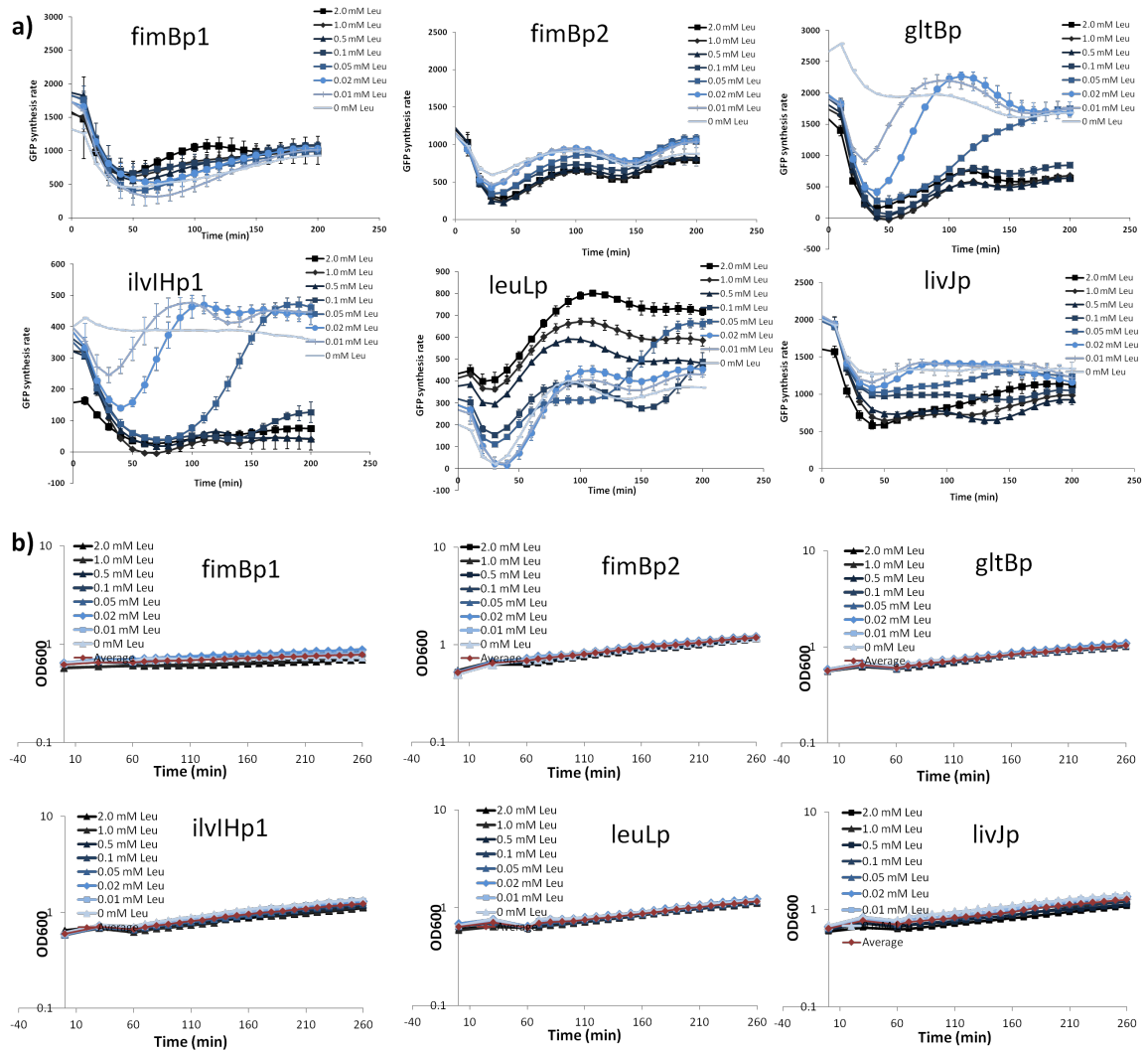
### 6.2.3 Biosensor characterisation in liquid media

These potential leucine/alanine-responsive biosensors were then tested in liquid medium and 96-well plate format using a robotic platform with an integrated plate reader. Promoters were characterised for their response to alanine (Figure 6.4) and leucine (Figure 6.5). Cell growth during these experiments is also shown.

fimBp2, gltBp, ilvIHp1 and livJp showed repression by alanine and leucine; fimBp1 and leuLp showed activation by alanine and leucine. In each case, magnitude and dynamics of responses differ between the different promoters and also between the two inducers.



**Figure 6.4:** (a) GFP synthesis rate of Lrp promoters at different alanine concentrations in M9 medium. Error bars represent the standard deviation of three biological replicates. (b) Growth of cells during the assay. Error bars represent the standard deviation of three biological replicates.



**Figure 6.5:** (a) GFP synthesis rate of Lrp promoters at different leucine concentrations in M9 medium. Error bars represent the standard deviation of three biological replicates. (b) Growth of cells during the assay. Error bars represent the standard deviation of three biological replicates.

**Table 6.2:** Observed promoter responses compared to expectations from the literature.

Promoter	Observed promoter behaviour				Commentary
	+Leu solid medium	+Leu liquid medium	+Ala solid medium	+Ala liquid medium	
fimBp1	growth inhibition	small activation	repression/ no effect	small activation	predicted to be activated
fimBp2	growth inhibition	repression	no effect	repression	predicted to be activated
gltBp	growth inhibition	repression	repression	repression	not previously known to be responsive to alanine
ilvHbp1	growth inhibition	repression	repression	repression	as expected
leuLp	growth inhibition	activation	activation	activation	predicted to be repressed
livJp	growth inhibition	repression	repression	repression	as expected
<b>Legend</b>	could not determine	prediction fulfilled	prediction partially fulfilled	prediction not fulfilled	

These results show different levels of biosensing, but also different agreements with the existing literature. Table 7.2 summarises how the results shown here compare with what was expected from the literature (Table 6.1). The results are mixed. *ilvHbp1* and *livJp* behave as expected. *gltBp* shows repression by alanine, though previous studies could not find a response. *fimBp1*, *fimBp2* and *leuLp* do not behave as expected from the literature. *fimBp2* have been shown to be activated by leucine, whereas here there is a small repressive effect. This may be as this promoter becomes less active in *E. coli* laboratory strains (Iida et al., 2001). *leuLp* should be activated by *Lrp*, but repressed by leucine. In this work, increasing leucine concentration activated *leuLp*. In some cases there are differences between solid and liquid medium results. Liquid and solid media present quite different growth conditions to cells (Dubey and Ben-Yehuda, 2011; Dalchau et al., 2012), which explains why the promoters behave differently in the two conditions. The cells are thought to be in stationary phase on agar plates. There are also edge effects to consider on plates, as cells at the edge of a growth zone are exposed to different conditions than those near the centre. It can be difficult to relate observations on solid medium to cell concentration.

## 6.3 Summary of results

Promoters of the *E. coli* Lrp operon were assembled into constructs to be characterised for their response to alanine and leucine. The promoters showed a range of responses and different dynamics, which in some cases changed with growth conditions. In some cases the responses differed from those predicted from the existing literature.

Biosensor cells were characterised for their alanine and leucine response on agar plates. Leucine caused growth inhibition of the cells. In response to alanine, cells exhibited a range of responses, including both activation and repression (Table 7.2). Biosensor cells were then characterised for their response to alanine and leucine in liquid M9 medium. Under these conditions, several of the promoters showed responses to leucine and alanine (Table 7.2).

Undergraduate Harold Taylor attempted to overexpress Lrp in these cells, but did not succeed, possibly because the overexpression construct was toxic to the cells. Lrp overexpression has many effects on general cell metabolism (Newman, 1995). Elevated protein concentrations could also lead to non-physiological cross-talk even where it normally does not exist (Ninfa et al., 2007). This is one of the challenges in synthetic biology when working with systems that are based on transcription factors that are integral to cellular metabolism instead of only controlling one promoter. Therefore, in the sensors shown here, Lrp was not overexpressed in these constructs. Instead, the constructs used the endogenous Lrp. The effect of overexpressing the promoters on plasmids is predicted to be equivalent to lowering the concentration of Lrp (Table 6.1). It is possible that the dynamics of these parts could be improved in terms of signal amplitude and repeatability if Lrp were successfully overexpressed.

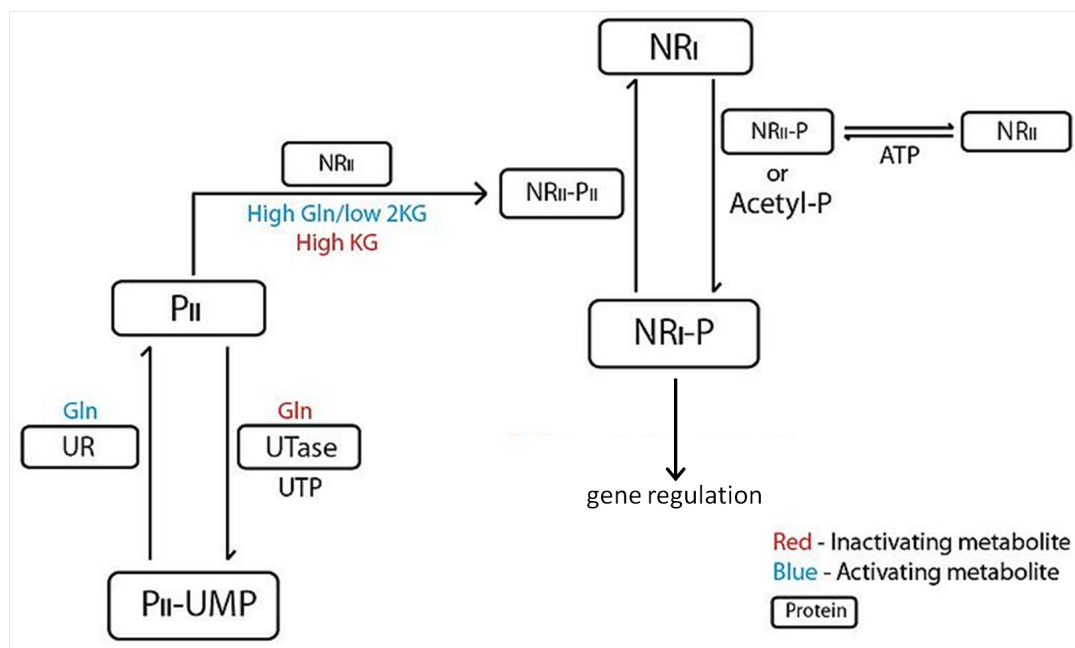
These promoters are now characterised bioparts under these conditions that could be used by the synthetic biology community. *gltBp*, *ilvHp1*, *leuLp* and *livJ* show responses to alanine and leucine, and could in theory be used as biosensors for these compounds. However, since the promoters respond to both these amino acids, they could not currently be used to *e.g.* determine the alanine concentration in a sample that also contains leucine (as would be the case with most cell culture samples). It is possible that protein engineering of Lrp could be used to increase specificity to either alanine to leucine.

## 7 *E. coli* Ntr regulon

### 7.1 Introduction

#### 7.1.1 Glutamine-responsiveness of the Ntr system

Promoters in the Ntr (nitrogen-responsive) regulon in *E. coli* are regulated in response to nitrogen availability (Reitzer, 2003; Mutalik and Venkatesh, 2007). The two-component system NtrB/NtrC (NRI/NRII) controls gene expression from many promoters in response to cellular nitrogen status (Maheswaran and Forchhammer, 2003). Glutamine feeds into this system as an indicator of nitrogen status (Figure 7.1). NRI-P activates gene expression from several promoters when cells enter nitrogen starvation. High concentrations of glutamine cause a decrease of NRI-P and increase of NRI.



**Figure 7.1:** Ntr system in *E. coli*. Figure taken from [[http://commons.wikimedia.org/wiki/File:Ntr\\_system.jpg](http://commons.wikimedia.org/wiki/File:Ntr_system.jpg)].

Promoters from the Ntr regulon were researched in the literature. Based on previous knowledge of this system from the literature, it is possible to make predictions for how these promoters should behave when exposed to different concentrations of glutamine (Table 7.1).



**Table 7.1:** Promoters of the Ntr regulon and their predicted response to glutamine.

Promoter	Gene context	Predicted promoter behaviour		Reference
		+NRI-P	+Gln	
argTp	lysine/arginine/ ornithine ABC transporter periplasmic binding protein	Activation	Repression?	Ecocyc
astCp2	enzymes of arginine succinyltransferase (AST) pathway	Repression	Activation	Ecocyc
glnAp1	glutamine synthetase	Repression	Activation	Ecocyc
glnAp2	glutamine synthetase	Activation	Repression	Ecocyc
glnHp2	glutamine ABC transporter	Activation or repression depending on other factors	Activation or repression depending on other factors	Ecocyc
glnKp	nitrogen regulatory protein PII-2	Activation	Repression	Ecocyc
glnLp	NtrB (NRII)	Repression	Activation	Ecocyc
nac	Nac DNA-binding transcriptional dual regulator	Activation	Repression	Ecocyc

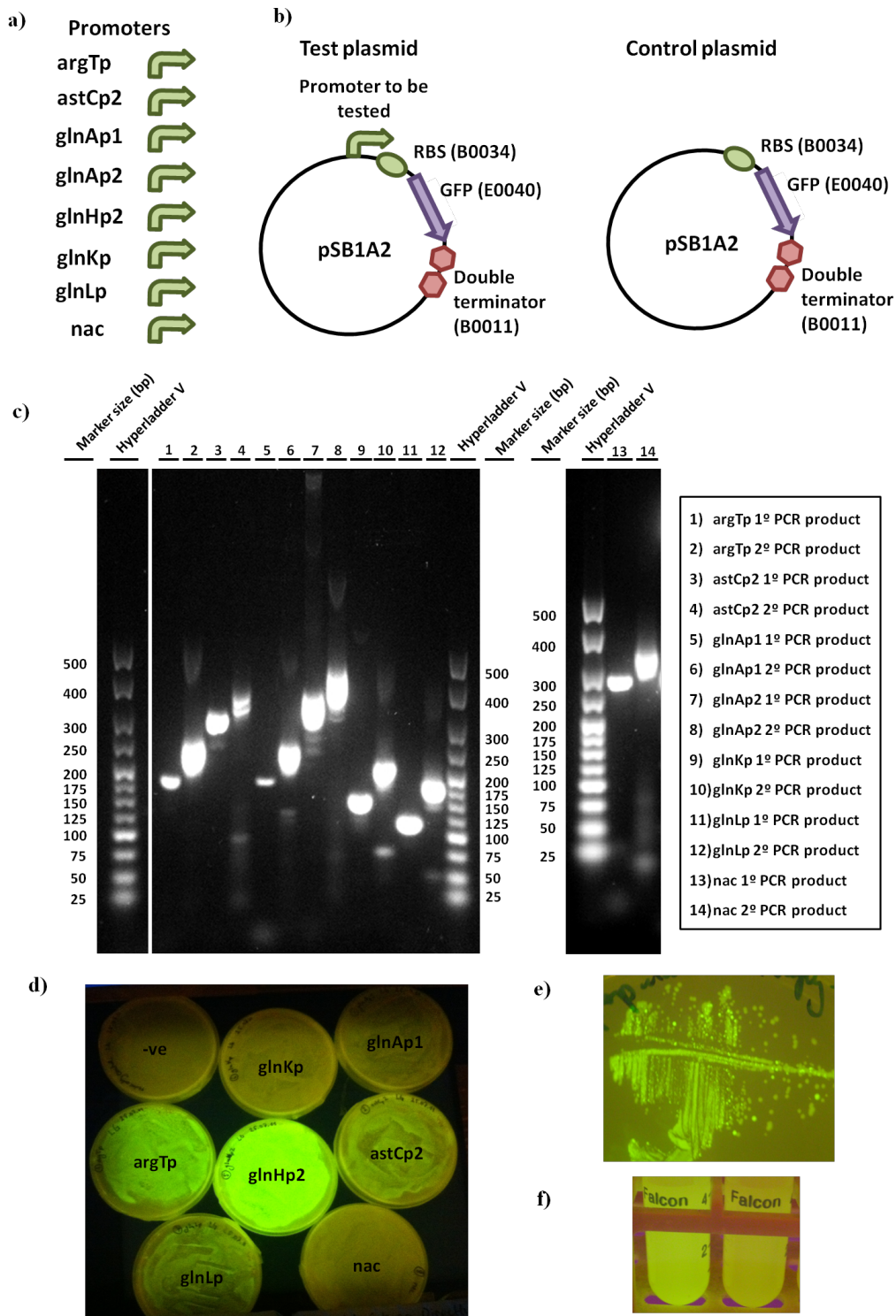
The Ntr operon has been previously used in synthetic biology. Components of the system have been used to create oscillatory behaviour (Atkinson et al., 2003), attempts have been made to rewire signalling through this network by adding different receivers (Wang, Barahona, Buck and Schumacher, 2013) and the downstream region of the glnAp2 promoter has been used as part of a bioreporter by fusing it with a xylene-responsive promoter (Carmona et al., 2005; van der Meer and Belkin, 2010).

## 7.2 Results

### 7.2.1 Biosensor construct assembly

In order to make a whole-cell glutamine biosensor, eight *E. coli* promoter elements, which are regulated by the glutamine-responsive NtrC regulator, argTp, astCp2, glnAp1, glnAp2, glnHp2, glnKp, glnLp and nac (Figure 7.2(A)), were chosen to be tested for their function using a characterisation gene circuit (Figure 7.2(B)). The promoters were successfully amplified from the *E. coli* genome and converted into BioBricks via two PCR reactions (Figure 7.2(C)) and assembled into the characterisation circuit. After initial transformation of the constructs into *E. coli* DH $\alpha$  cells, cells showed different levels of fluorescence on LB agar plates (Figure 7.2(D)). This indicates different expression levels from the various Ntr promoters under the conditions on LB agar plates. All eight constructs were shown to be correctly assembled and verified by DNA sequencing.

Colonies transformed with the astCp2 promoter in the characterisation construct showed fluorescence heterogeneity (Figure 7.2(E)). This was found to be caused by an insertion in the construct present in some of the colonies. The more fluorescent cells contained a TAGCGGCCGCTACTAG insertion in the TACTAG<sup>^</sup>AG BioBrick scar site between the promoter and the ribosome binding site. Both versions of the astCp2 construct were included in subsequent characterisation experiments.

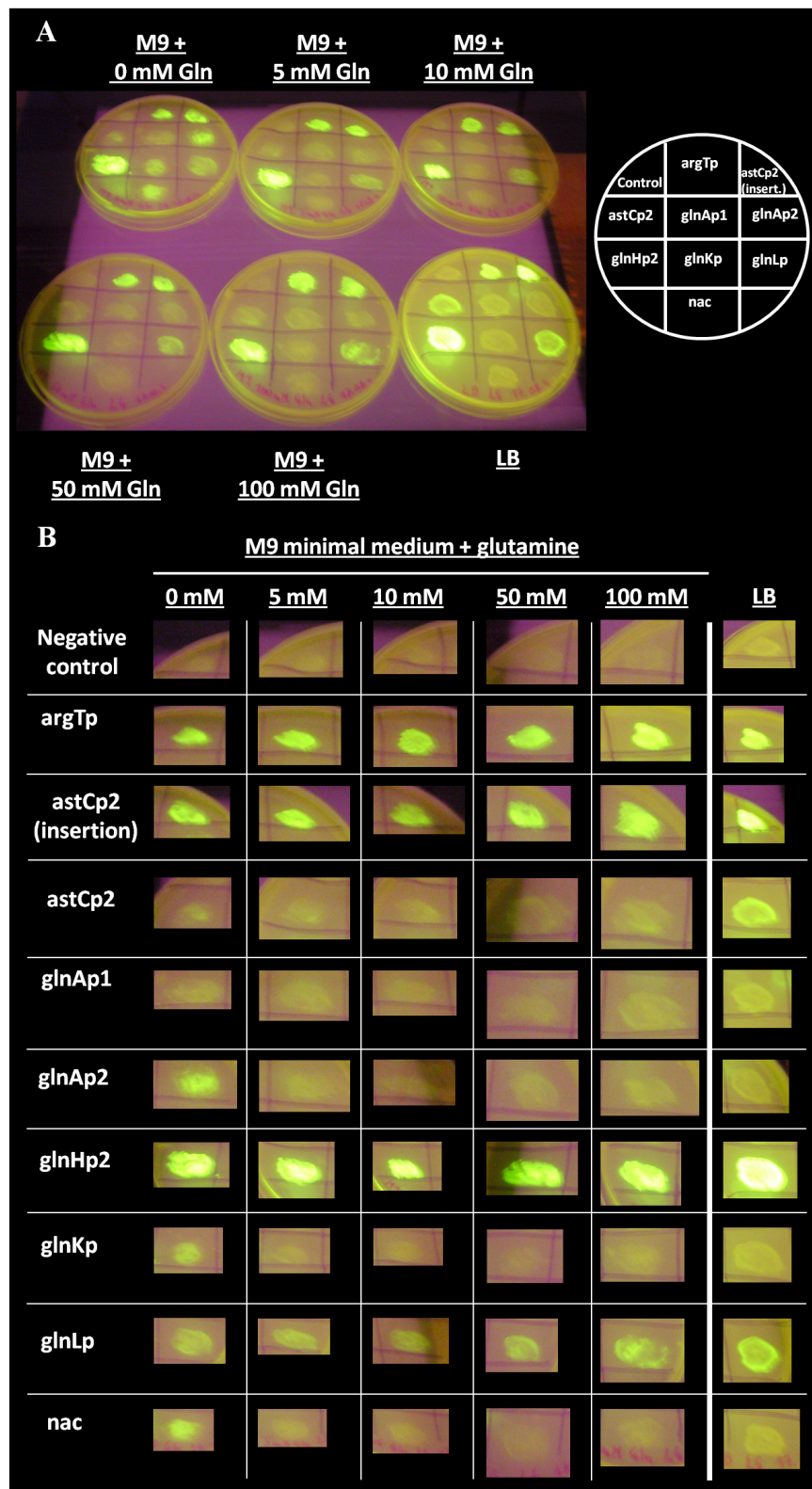


**Figure 7.2:** Biosensor construct assembly. **a)** Genetic circuit used to characterise promoters of interest. The BioBrick name of each part is shown in brackets. **b)** List of Ntr promoters tested here. **c)** Agarose gel showing successful amplification of *E. coli* glutamine-responsive promoters as indicated in the legend. PCR products were run on two separate gels, and one lane on the left-hand gel was omitted from the figure for clarity. **d)** LB agar plates with *E. coli* DH $\alpha$  transformed with the characterisation construct containing different promoters. **e) - f)** Fluorescence heterogeneity in colonies transformed with the astCp2 promoter in the characterisation construct in **e)** colonies and **f)** cultures.

## 7.2.2 Biosensor characterisation on agar plates

Once the promoter constructs were correctly assembled, the next step was to characterise them in terms of their dose response and dynamic response. A qualitative end-point characterisation test was carried out in which *E. coli* DH $\alpha$  cells containing the verified promoter constructs were streaked out onto M9 minimal medium plates supplemented with varying concentrations of glutamine. Cells were left to grow overnight and photographed the next day (Figure 7.3). While it is difficult to draw firm conclusions from these results, general trends can be seen. By comparing the behaviour of the promoters in different glutamine concentrations in M9, biosensor responses can be discerned. Comparing the fluorescence of the cells on M9 minimal medium with that in rich LB medium, shows how these promoters change behaviour between minimal and rich conditions. However, this is difficult to interpret on Petri dishes with solid medium, as the cells may also show increased growth in rich medium. Also, fluorescence levels or changes may not be great enough to see by eye. The promoters *glnAp2*, *glnKp* and *nac* show repression with increasing glutamine concentration. *glnLp* shows some activation. *argTp*, *astCp2*, *glnAp1* and *glnHp2* do not show a visible effect. *glnAp2*, *glnKp*, *glnLp* and *nac* do therefore behave as predicted from the literature (Table 7.1).

Growth conditions can have large effects on BioPart function (Dalchau et al., 2012) and so it is important to test these under a number of different conditions to see where they fail and work and any differences in function.



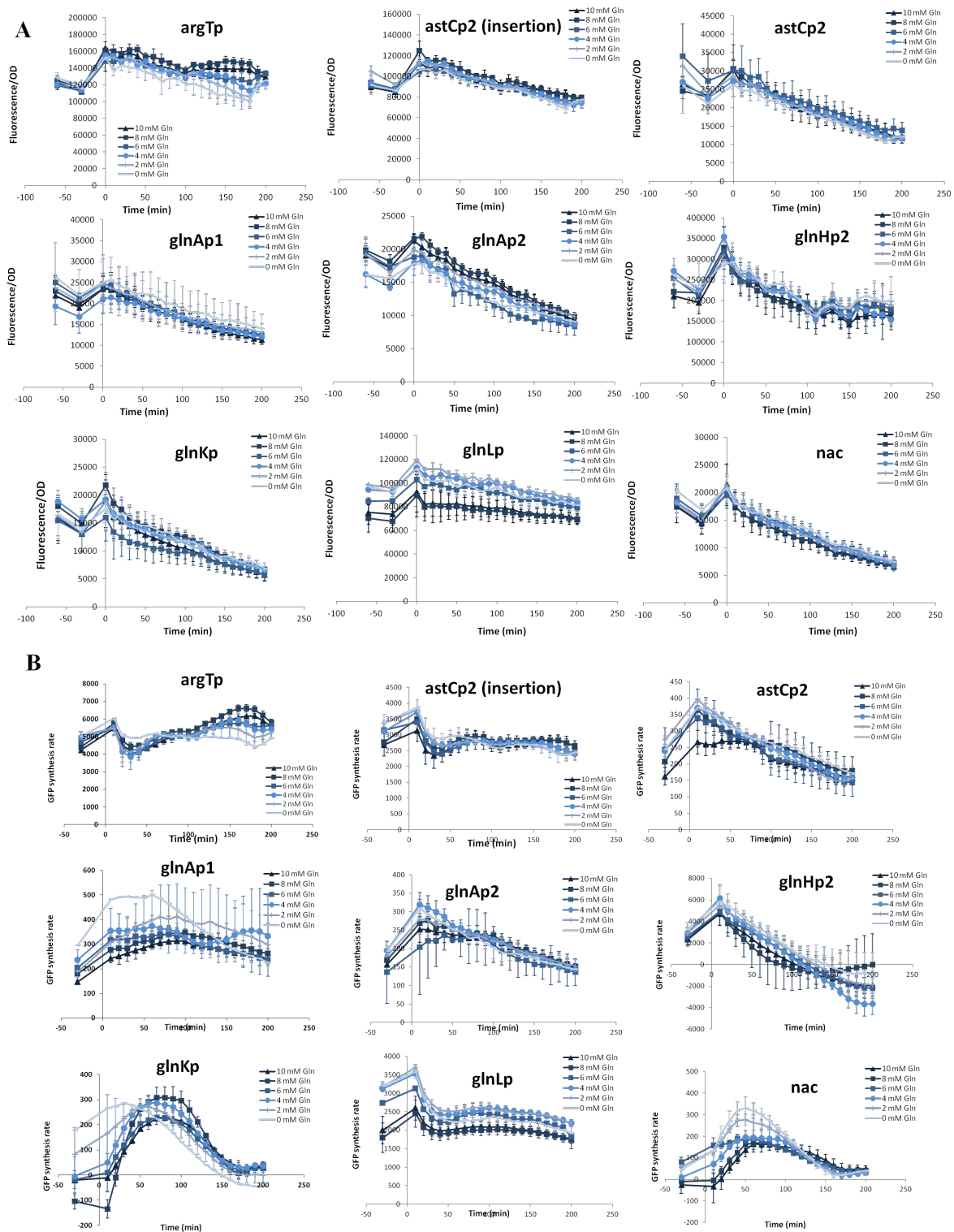
**Figure 7.3:** Ntr promoter characterisation on solid medium. *E. coli* DH $\alpha$  cells containing the various NtrC promoter characterisation constructs were plated out on plates containing different concentrations of glutamine. Plates are shown placed on a UV-illuminator. (A) Arrangement of cells on the plates. (B) Cells in re-arranged order for better comparison.

### 7.2.3 Biosensor characterisation in liquid medium

Subsequent time-resolved characterisation experiments were carried out on a robotic platform using a plate reader to aid high-throughput processing and repeatability. Figure 7.4 shows fluorescence and GFP synthesis rate from these characterisation experiments. Several of the promoters show synthesis rate trends that mark them as possibly suitable biosensors. Specifically arpTp, glnAp1, glnKp and nac. However, the responses to glutamine are all minor. Upon initial inspection of the data in Figure 7.4, ArgTp seemed to be the most promising biosensor. However, data analysis and close inspection of the OD data for these experiments (Figure 7.5), it was found that the response was largely caused by the OD values, as the cells grew differently in different glutamine concentrations.

In Figure 7.4 many of the sensors seem to react to glutamine concentration during the first few time points and then level off. This might suggest that the reaction to glutamine in terms of different protein expression rates happens very quickly and only for a short time. Alternatively, it could be that the cells very quickly use up any available glutamine and so the reaction to any glutamine “sensed” would decrease over time. This possibility was further investigated by testing some of the promoters at higher glutamine concentrations in Figure 7.6. No response to glutamine was seen in the fluorescence output, however, synthesis rate data again showed some initial response to glutamine. This could indicate a brief response in gene expression changes to changing glutamine concentration, followed by quick adaptation or re-equilibration.

The results of the promoter characterisation in this chapter are summarised in Table 7.2.



**Figure 7.4:** Ntr promoter characterisation in liquid medium. (A) Fluorescence of Ntr promoters at different glutamine concentrations in M9 medium measured by robot. Error bars represent the standard deviation of three biological replicates. (B) GFP synthesis rate of Ntr promoters at different glutamine concentrations in M9 medium measured using an integrated robotic platform. Error bars represent the standard deviation of three biological replicates.

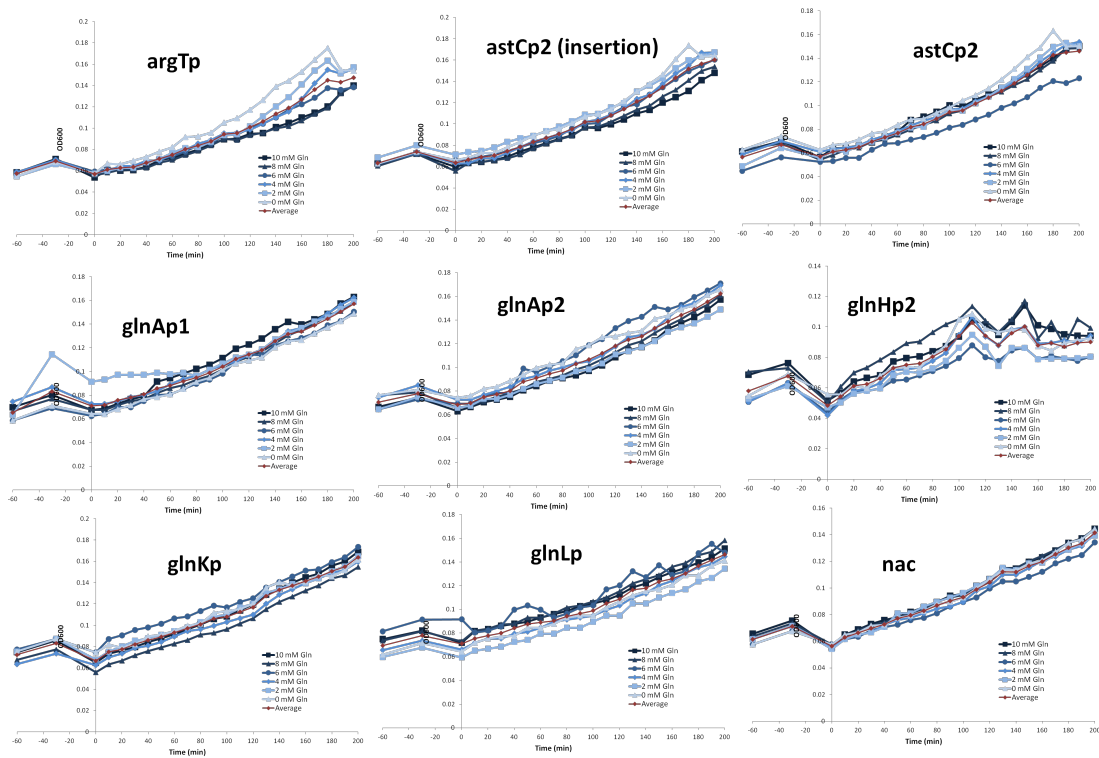


Figure 7.5: Cell growth from experiments shown in Figure 7.4.

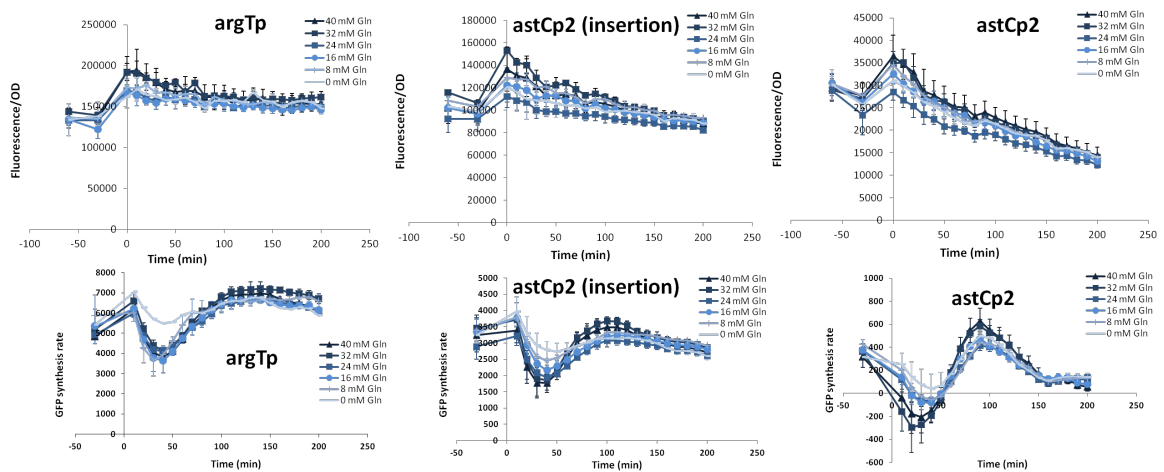


Figure 7.6: GFP fluorescence and synthesis rate of argTp, astCp2 (insertion) and astCp2 Ntr promoters at higher glutamine concentrations in M9 medium. Error bars represent the standard deviation of three biological replicates.



**Table 7.2:** Observed Ntr promoter responses to glutamine.

Promoter	Observed promoter behaviour	
	+Glutamine solid medium	+Glutamine liquid medium
argTp	No response	No response
astCp2	No response	No response
glnAp1	No response	No response
glnAp2	Repression	No response
glnHp2	No response	No response
glnKp	Repression	No response
glnLp	Activation	No response
nac	Repression	No response
<b>Legend</b>	Promoter behaves as expected	Promoter does not behave as expected

### 7.3 Discussion

There are a number of factors that may make the Ntr system unsuitable as a basis for bacterial whole-cell glutamine biosensors for bioprocessing contexts. The system reacts to intracellular glutamine availability (Schumacher et al., 2013), which may not be representative of extracellular glutamine. Internal concentration is related to external through processes like transport, synthesis, degradation and consumption of glutamine. Undergraduate student Kai Jiang investigated the possibility of overexpressing the glnHPQ *E. coli* glutamine import complex. However, it was not possible to overexpress these proteins, which may suggest that such overexpression is toxic to the cells. Nohn et al. (1986) have previously cloned the proteins using a low copy number plasmid. As NtrC-type proteins are very widely distributed in bacteria it would be difficult to find a chassis where this system would be completely orthogonal (Ninfa et al., 2007). A known intracellular glutamine biosensor could be used in cells exposed to known extracellular glutamine concentrations to observe the relationship between intracellular and extracellular glutamine concentrations and to compare to the output of the Ntr biosensors. An example of such a sensor that could be used is one presented by Behjousiar et al. (2012). The *Bacillus subtilis* glutamine sensor (see Chapter 8) is thought to be extracellular, and therefore should be more suitable.

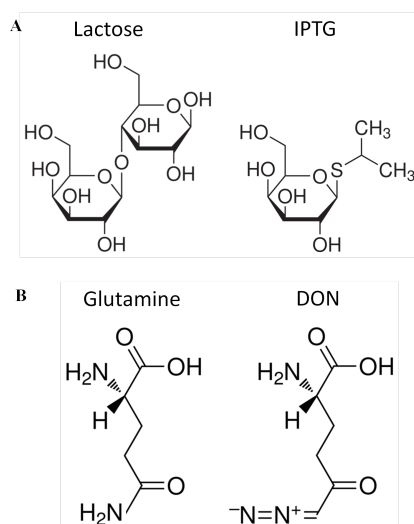
Another factor is that the Ntr system processes a number of inputs in addition to intracellular glutamine availability such as carbon availability and nitrogen availability in general (Schumacher et al., 2013). Therefore, promoter output may not be specific to glutamine concentration and changes in other metabolites can change promoter output (Willis, 1975). Even more so, as the promoters tested here control expression of glutamine uptake and level of glutamine synthetase. The relation between intracellular and extracellular glutamine is therefore influenced by availability of not just glutamine, but also other nitrogen sources and even carbon source. These promoters therefore influence their own expression in very complex ways. Higher glutamine concentrations as used in the experiment shown in Figure 7.6, may therefore not have the predicted effect, because there are complex feedback systems in play. The output may also be influenced by concentration of ammonia, which might be different for different experimental wells depending on previous dilutions steps. In addition to this, *E. coli* has several glutamine uptake systems which possibly underlie different kinds of regulation, making the level of glutamine uptake is difficult to predict.  $\alpha$ -ketoglutarate, an intracellular indicator of carbon status can override glutamine signalling to NtrC under certain conditions (Schumacher et al., 2013).

Finally, what might influence the results is that glutamine is used up by the cells as well as sensed. The same is true for a number of sensor systems that have shown good responses, *e.g.* the arabinose operon [[http://partsregistry.org/Part:BBa\\_I0500](http://partsregistry.org/Part:BBa_I0500)] and lactose operon. However, in those two cases, the operon encodes the genes needed for the breakdown of the inducer, so there is expected to be a time lag between sensing and breaking down, which may not be true for a glutamine sensor, as glutamine is always used up and the enzymes for glutamine breakdown are always present. Also, *E. coli* cells preferentially use glucose over lactose and arabinose.

Non-metabolisable glutamine analogues could be used to tackle this challenge and make glutamine biosensors more versatile in general. While the promoters shown here may not be suitable as glutamine sensors under the conditions used, they may yet be useful as inducible promoters. For a bacterial cell to act as a whole cell biosensor, it is useful if the inducer is 1) specifically detected by the biosensor mechanism, 2) does not interfere with other cellular function in any way and 3) is not metabolised by the cell so that its concentration remains unchanged. This standard is difficult or impossible to achieve when working with inducers that are staple cellular metabolites, products or nutrient sources, such as glutamine, leucine, alanine or lactate. Non-metabolisable analogues for metabolite biosensor inducers provide a partial solution, insofar as they can be used to characterise a biosensor and act as inducers for inducible biosensors. These analogues could assist the development

process and help separate interfering factors from the sensing mechanism.

A very commonly used metabolite analogue is the lactose analogue Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (Figure 7.7(A)), which is used to induce expression from the pLac promoter. This system works well because lactose is not an essential metabolite in the presence of other carbon sources such as glucose and IPTG cannot be metabolised by *E. coli* cells. IPTG is used in the concentration range 100  $\mu$ M-1mM. IPTG, however, does have toxic effects on cells at high concentrations (mM range), but this is outside the range where it is used as an inducer. It has been shown that IPTG imposes a metabolic burden cells, possibly caused by utilisation of proton pumps during uptake (Malakar and Venkatesh, 2012). Presence of IPTG might also impact the utilisation of other carbon sources by *E. coli*.



**Figure 7.7:** Chemical structures of lactose and IPTG. Chemical structures of glutamine and DON. Figure modified from [www.sigmaaldrich.com](http://www.sigmaaldrich.com).

There exists a non-metabolisable glutamine analogue called 6-Diazo-5-oxo-L-norleucine (DON). This has been used to study mechanisms of glutamine utilisation (see Fig. 7.7(B)). This compound could be used when characterising glutamine biosensors and avoid the effects of glutamine metabolism. DON was first isolated from *Streptomyces* and has been used as a tumour inhibitor (Dion et al., 1956). Many investigations show its potential use for tumour treatment (Shelton et al., 2010). The compound DON can for example be used to distinguish between effects of glutamine due to its role as a substrate of metabolism and effects of glutamine due to its other roles (Wischmeyer et al., 1997). DON can inhibit amination reactions, where an amide nitrogen is transferred from glutamine to an acceptor molecule, so it is also called glutamine antagonist (Coggin and Martin, 1965; Hartman, 1971). It can be used to determine presence of glutaminase activity in an enzyme and number of active sites (Hartman, 1971). It forms covalent bonds with active sites. When

used in *E. coli*, DON inhibits *E. coli* ATCC 9637 by inhibiting purine, hexosamine and cell wall synthesis, as these involve such reactions (Coggin and Martin, 1965; Khedouri et al., 1966). Adding glutamine could not revert the inhibition, but adding inosine and glucosamine can bypass the inhibitory effects of DON and cells could acquire resistance.

## 7.4 Summary of results

Promoters from the *E. coli* Ntr regulon (*argTp*, *astCp2*, *glnAp1*, *glnAp2*, *glnHp2*, *glnK*, *glnL*, *nac*) were cloned and assembled into characterisation constructs to test them for their suitability as glutamine biosensors. Characterisation experiments were carried out on agar plates and in M9 liquid medium. The biosensors showed no clear response to glutamine in liquid medium. However, several of the promoters showed responses on solid medium. Where promoters showed a response to glutamine, it confirmed predictions from the literature.

Suggestions are made as to why these promoters may not reliably function as glutamine biosensors under these conditions. These include the fact that this system is an intracellular sensor and that expression is affected by several factors in addition to glutamine, including carbon source. Work on an extracellular glutamine sensor is shown in Chapter 8.

Improvements to the characterisation procedure that were made after the data in this chapter were obtained could improve the quality of the characterisation data in this chapter.

## 8 *Bacillus subtilis* GlnK-GlnL system

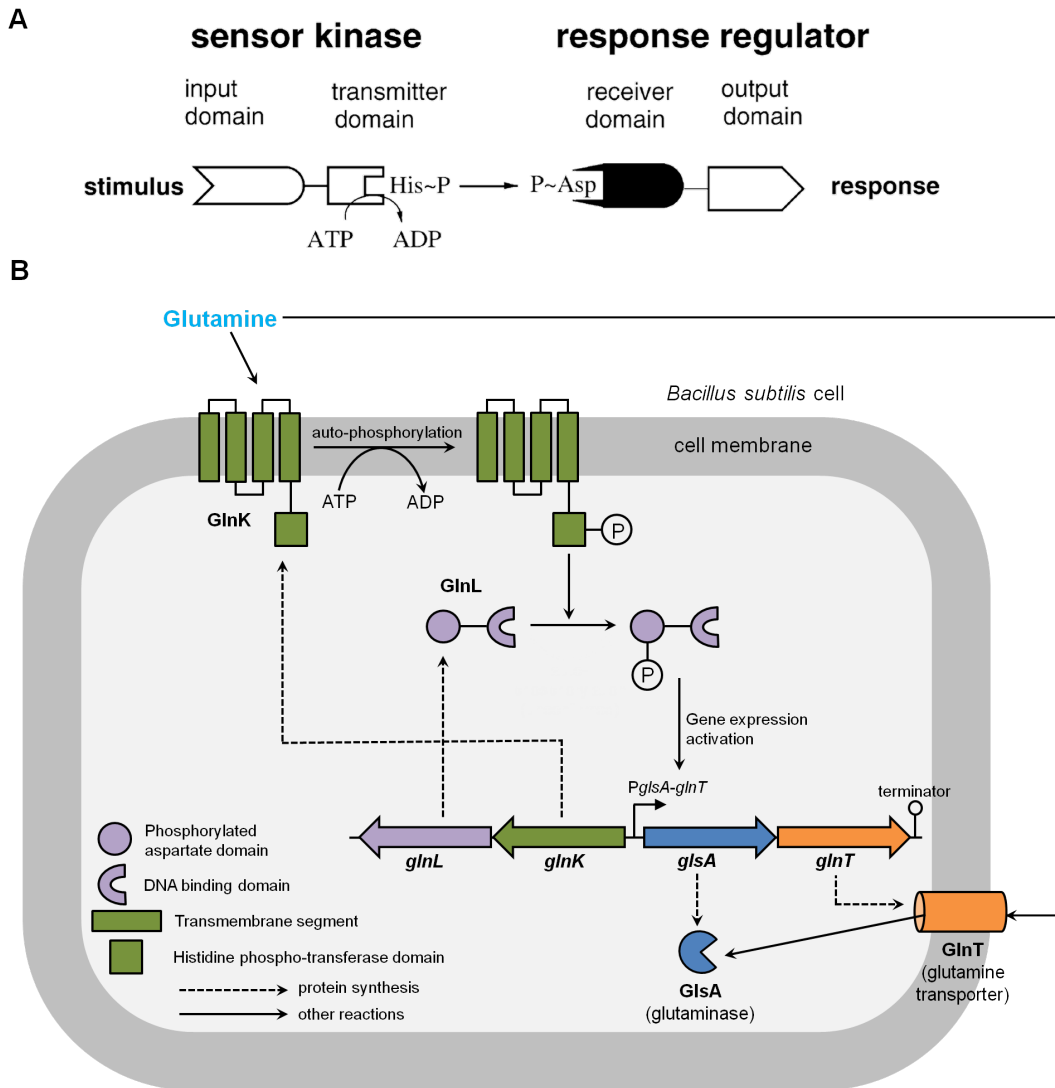
### 8.1 Introduction

#### 8.1.1 Glutamine-responsiveness of the GlnK-GlnL system

The promoter element PM8J2-706 (P<sub>glsA-glnT</sub>) from *B. subtilis* is naturally under the control of the glutamine-responsive two-component system GlnK-GlnL (Figure 8.1).

The adjacent genes *glnK* and *glnL* were identified during the sequencing of the *B. subtilis* genome as parts of a two-component system of unknown function (Kunst et al., 1997). These genes were initially named *ycbA* and *ycbB* to emphasise that their function had not yet been ascertained (Kunst et al., 1997). The *B. subtilis* two-component system kinases were found to fall into five classes based on sequence analysis of the regions around the phosphorylated histidine, of which YcbA fell into group IV together with three other systems (Fabret et al., 1999). Structure prediction from the sequence was used to predict that YcbA has four transmembrane segments (Figure 8.1) (Fabret et al., 1999). The YcbA-YcbB system as well as several other systems in group IV had unknown function and were known to be nonessential (Fabret et al., 1999). Out of the four kinase-regulator pairs in group IV, three have been reported to sense tricarboxylic acid cycle intermediates (Satomura et al., 2005). DNA microarray analysis suggested that YcbA-YcbB regulates the expression of 19 genes, 5 of which were upregulated and 14 were downregulated Kobayashi et al. (2001). However, in work by Satomura et al. (2005) only one of the positively regulated candidate gene targets (*ybgH*) was confirmed and the others were found to be false positives. *YbgH* is located adjacently to the *ycbAB* genes and was found to constitute an operon with *ycbJ*. *YbgH* was shown to encode a glutaminase and renamed *glsA*. *ycbJ* was shown to encode a glutamine transporter and renamed *glnT*. YcbA-YcbB was found to be responsive to extracellular glutamine and renamed GlnK-GlnL. This nomenclature is somewhat unfortunate as it leads to confusion with the PII-like GlnK protein (previously named NrgB) involved in regulation of *B. subtilis* nitrogen metabolism (Forchhammer, 2007). GlnK has also been called GlnJ ([www.ecocyc.org](http://www.ecocyc.org)). Some of these results were later confirmed using time-resolved transcriptome analysis (Ye et al., 2009). The *glsA-glnT* operon was induced 25/50-

fold 5 minutes after glutamine addition (Ye et al., 2009). Relatively little is known about the system. The exact mechanism is unknown (see Fig. 8.1).



**Figure 8.1:** *B. subtilis* GlnK/GlnL two-component system. (A) General schematic of two-component signal transduction systems. Figure taken from Kremling et al. (2004). (B) Schematic showing a proposed model of the *B. subtilis* GlnK/GlnL glutamine-responsive two-component system. Previous gene and protein names: *glnK*/GlnK - *ycbA*/YcbA; *glnL*/GlnL - *ycbB*/YcbB; *glsA*/GlsA - *ybgJ*/YbgJ; *glnT*/GlnT - *ybgH*/YbgH. Based on information from Fabret et al. (1999); Kobayashi et al. (2001); Satomura et al. (2005); Forchhammer (2007) and Ye et al. (2009).

## 8.2 Results

### 8.2.1 Biosensor construct assembly

The PM8J2-706 promoter element was chosen to be tested for its suitability as a glutamine biosensor. The promoter was integrated into a suitable characterisation construct that would allow for integration into the *B. subtilis* genome (Figure 8.2(A)). The promoter was amplified from the *B. subtilis* genome by PCR and transformed into a BioBrick (Figure 8.2(B)). The constructs were assembled in *E. coli* and verified by sequencing and subsequently transformed into *B. subtilis* cells. Successful integration of the correct constructs into the AmyE locus was verified using an amylase test (Figure 8.2(C)), colony PCR and DNA sequencing.

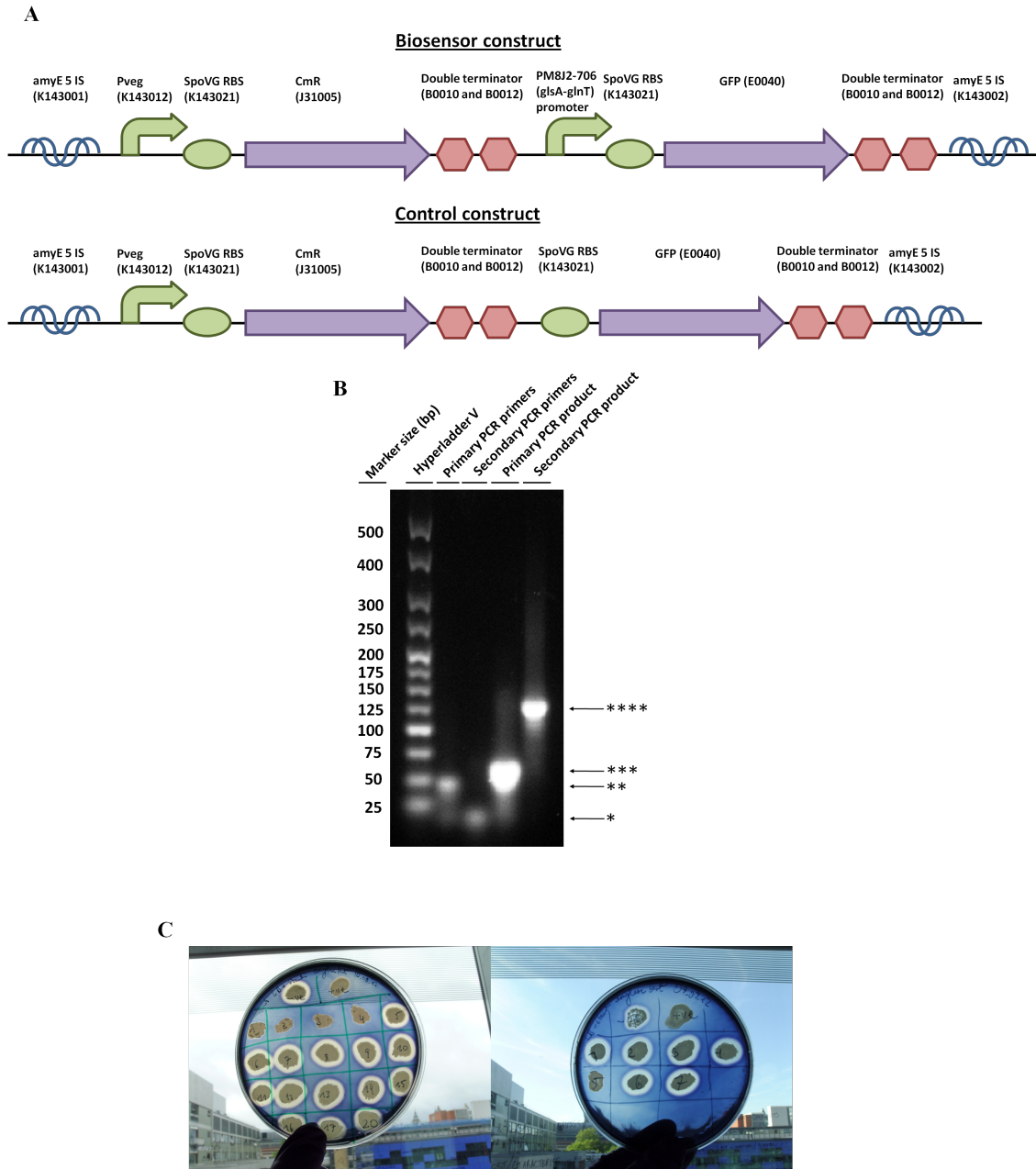
### 8.2.2 Biosensor characterisation

The next step was to confirm the glutamine-responsive behaviour of the PM8J2-706 promoter under conditions identical or similar to those used previously. Satomura et al. (2005) carried out experiments in MM medium, which is the standard minimal medium used by the “Functional analysis of the Bacillus subtilis genome in Japan and Europe” consortium. Ye et al. (2009) used an adapted minimal medium.

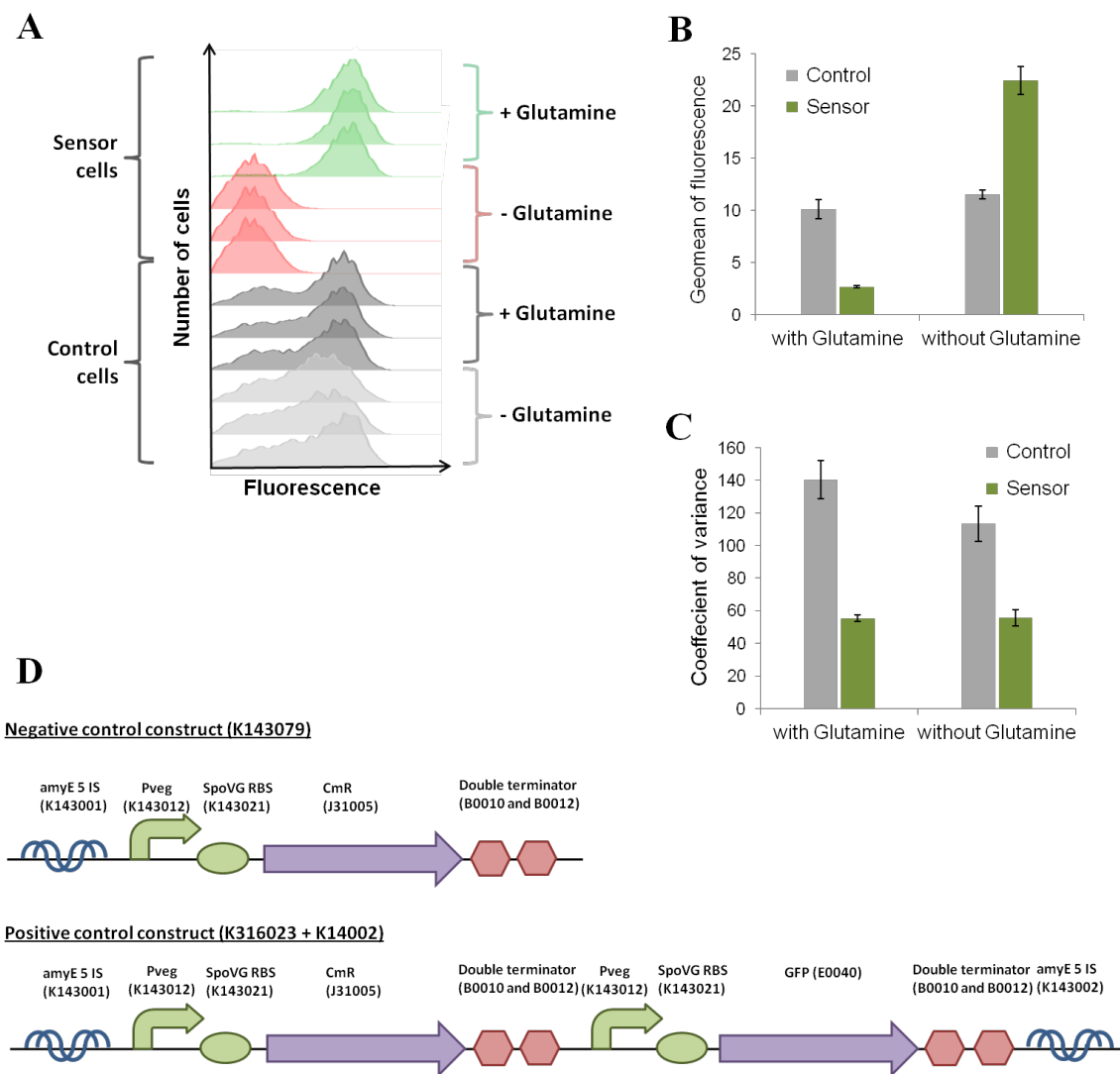
Initial characterisation experiments were carried out in MM medium and using flow cytometry as opposed to the plate reader format used in the previous chapters. This was done because the protocol developed in Chapter 4 was optimised for *E. coli* and modifications would have to be made to optimise the set-up for *B. subtilis*.

Cells were grown overnight in LB and then used to seed MM cultures with or without glutamine. Samples for flow cytometry were taken after several hours of growth (Figure 8.3(A-C)). There was some precipitation in the MM medium, probably iron from the medium. Fluorescence of the control cells did not change much between absence and presence of glutamine (10.12 to 11.5), whereas the sensor cell fluorescence changed greatly (2.68 to 22.46). Also, the coefficient of variance for sensor cells is much lower than for control cells. Unexpectedly, the fluorescence level of the control cells is higher than for the sensor cells in absence of glutamine. The control cells do not contain a promoter in front of the GFP gene, however the local gene sequence must be acting as a promoter, allowing leaky gene expression of the GFP gene in the control cells. It was decided that, ideally, a further two cell lines should be established, one containing a positive and one containing a negative control circuit for future characterisation (Figure 8.3(D)).





**Figure 8.2: *B. subtilis* glutamine sensor assembly.** (A) Genetic constructs that will be used to characterise the *B. subtilis* PM8J2-706 (glsA-glnT) promoter. The control construct is identical to the biosensor construct but missing the PM8J2-706 (glsA-glnT) promoter. (B) Agarose gel showing successful amplification of the *B. subtilis* PM8J2-706 (glsA-glnT) promoter. (\*) Blunt primers used for primary PCR, see Table 2.4) PM8J2-706-F and PM8J2-706-R. (\*\*) BioBrick extension primers used for secondary PCR, see Table 2.4) PM8J2-706-BB-F and PM8J2-706-BB-R. (\*\*\*) Primary PCR product, 67 bp. (\*\*\*\*) Secondary PCR product, 126 bp. (C) LB agar starch plate showing results of amylase test for the sensor and control constructs. Top row shows positive and negative control colonies. Subsequent rows show test colonies.



**Figure 8.3: *B. subtilis* glutamine biosensor characterisation in MM medium.** (A) Fluorescence of cells in absence or presence of glutamine measured through flow cytometry. (B) Fluorescence data from (A) shown as bar chart. (C) Coefficient of variance. (C) Additional control constructs for testing of the *B. subtilis* glutamine sensor. Error bars represent the standard deviation of three biological replicates.

## 8.3 Summary of results

The PM8J2-706 promoter controlled by the *B. subtilis* GlnK-GlnL system was used to create a glutamine biosensor. Characterisation experiments in MM medium and using flow cytometry measurements confirmed responsiveness to extracellular glutamine. This confirms the results from the literature about this system under the conditions used here. These results make the construct made in this work promising as a potential glutamine biosensor that could be used in a bioprocessing context. The *B. subtilis* glutamine sensor shown in this chapter is a more promising basis for a glutamine biosensor than the *E. coli* constructs shown in Chapter 7.

However, further characterisation of this system would be necessary before it could be used as a glutamine biosensor. This characterisation would involve adapting the protocol from Chapter 4 for *B. subtilis*. This would involve doing characterisation in M9 medium suitable for *B. subtilis* and adapting the work flow to the growth rate of these cells under such conditions. Also, the constructs from Figure 8.3(D) would need to be made to further investigate the behaviour of this sensor.

# 9 Towards using bacterial biosensors in bioprocessing

## 9.1 Introduction

Once a number of metabolite biosensors had been built and characterised (*i.e.* in terms of response to the target metabolite, dynamic range, dose-response and interfering factors), the next step was to test the biosensors in the mammalian bioprocessing context. Chapters 4-7 discussed the assembly and testing of metabolite biosensors for lactate, leucine/alanine and glutamine. The sensors were characterised for their response to the target metabolites in defined bacterial growth medium. To test the biosensors in a mammalian bioprocessing context requires an understanding of the growth characteristics of bacteria in standard culture medium as well as an assessment of how the biosensor performance is affected by the addition of samples in complex growth medium. These experiments are shown in this chapter.

## 9.2 *E. coli* and *B. subtilis* growth on DMEM

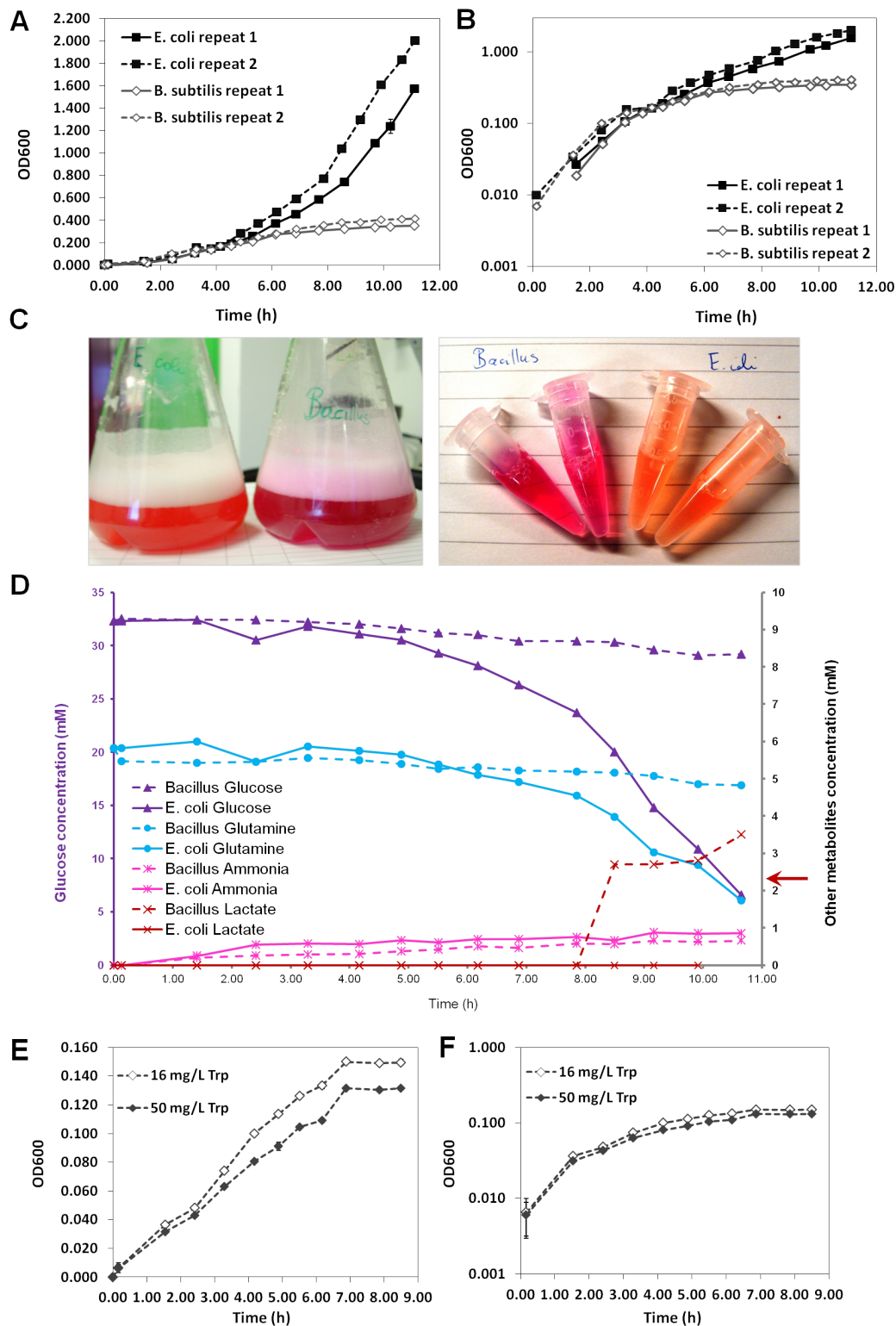
If the biosensors in this work are to be used to monitor mammalian cell cultures, the bacterial cells would be exposed to at least some amount of mammalian cell culture medium. To test the possibility of co-culturing bacterial and hybridoma cells in a shared growth medium, the bacterial growth characteristics in DMEM growth medium were explored. The DMEM used in Chapter 4 (Biosensor characterisation protocol) was free of glutamine, serum and phenol red and contained  $\text{NH}_4\text{Cl}$  as an additional nitrogen source. The DMEM used here does contain glutamine, serum and phenol red but no  $\text{NH}_4\text{Cl}$  and so is the exact same medium as used in the hybridoma cell experiments. *E. coli* and *B. subtilis* were cultured in DMEM and extracellular medium samples were collected at each time point for analysis (Figure 9.1). The growth rate of *E. coli* in DMEM was higher than that of *B. subtilis* (Figure 9.1(A) and (B)). While *E. coli* cells grow to a high density ( $\sim 2.0$ ), *B. subtilis* growth almost ceases after  $\sim 6$  h, reaching an eventual level of  $\sim 0.400$ . As expected, bacterial cell growth rate is much higher than hybridoma cell growth rate. This presents a challenge for bacterial-mammalian cell co-culture, as the bacterial

cells would form the dominant population and consume nutrients needed by the mammalian cells. Some form of population control would be necessary in a co-culture scenario, at least for *E. coli* cells. These growth curves were not done under conditions of high CO<sub>2</sub> and yet no precipitation in the medium was observed as had been seen previously. This could be due to the added serum.

Photos taken of the culture flasks and culture samples at a late stage of the culture showed a stronger colour change of phenol red in the *E. coli* culture than in the *B. subtilis* culture (Figure 9.1(C)). Phenol red is a pH indicator that is red or pink at pH > 7 and orange or yellow at pH < 7. Bacterial growth in DMEM causes the medium to acidify, more so for *E. coli* than for *B. subtilis*. This may simply be reflective of the different growth rates of these species. This would likely negatively affect hybridoma cells in a co-culture. Separating the bacterial and mammalian cells and their respective growth media to some extent will probably be necessary for a viable co-culture, at least for *E. coli* cells.

The next question was whether the bacterial cells would affect the levels of specific metabolites relevant for hybridoma cells. Extracellular medium samples were analysed for metabolites (Figure 9.1(D)). *E. coli* cells rapidly deplete both glucose and glutamine, while the levels of these metabolites are only slowly decreasing in *B. subtilis* cultures, probably reflective of the *B. subtilis* growth rate. The most important carbon source for hybridoma cells is glucose and the most important nitrogen source is glutamine. Therefore, for co-cultures of any significant time-scale, *E. coli* cells would need to be provided with a secondary nutrient feed to avoid nutrient depletion for the hybridoma cells. Both *E. coli* and *B. subtilis* cultures show slowly increasing levels of ammonia. Ammonia has a negative impact on hybridoma growth. While the maximum level of ammonia reached in this experiment was relatively low, the time-scale of the bacterial cultures is much shorter than for mammalian cell experiments. At typical mammalian culture time-scales, the ammonia could reach inhibitory levels. However, the depletion of glutamine and glucose by *E. coli* is a much more significant factor than the ammonia production for co-culture viability. This is because glutamine and glucose would be depleted long before ammonia from the bacteria had reached a significant level. *E. coli* cells do not seem to produce lactate at levels above the detection limit during the culture. *B. subtilis* do produce lactate, reaching ~3.5 mM at the end of the culture. It should be noted that the sudden increase in lactate for the *B. subtilis* culture after 8 h is an artefact of the lower detection limit for lactate of the Nova BioProfile Analyzer as indicated (Figure 9.1(D)). The lactate sensor presented in this work, however, can be used to monitor lactate in that concentration range.

It was suggested that the lower growth rate of *B. subtilis* in DMEM was caused by lack of tryptophan. DMEM contains 16 mg/L tryptophan, whereas *B. subtilis* growth media are often supplemented with 50 mg/L tryptophan. Dependency of a cell population on a particular compound can be used to control population growth (Chuang et al., 2010), which would be very useful for a co-culture. Growth of *B. subtilis* in DMEM supplemented with additional tryptophan compared to growth in DMEM without additional tryptophan was monitored (Figure 9.1(E) and (F)). *B. subtilis* cells cultured in higher levels of tryptophan did not show increased growth. The growth rate was in fact lowered by added tryptophan, suggesting that *B. subtilis* cells in DMEM are not limited by tryptophan levels.



**Figure 9.1: Bacterial growth in DMEM.** Optical density of *E. coli* and *B. subtilis* cultures grown in DMEM growth medium shown on a (A) linear and a (B) logarithmic scale. (C) Photos showing culture medium colour in flasks and culture samples. (D) Metabolite analysis of extracellular medium samples using a Nova BioProfile Analyzer. Red arrow indicates lower concentration detection limit of lactate (E) and (F) Optical density of *B. subtilis* cultures grown in DMEM growth medium with and without added tryptophan shown on a (E) linear and a (F) logarithmic scale. Error bars represent standard deviation of O.D. measurement on two repeat samples.

## 9.3 Testing of biosensors in cell culture relevant conditions

The lactate *lldp+lldR* sensor is the most responsive, reliable and well-characterised sensor in this work. This sensor was therefore chosen for further testing in conditions approximating the testing of cell culture samples.

### 9.3.1 Lactate sensor characterisation in DMEM and CD CHO growth media

*lldp+lldR* sensor cells were grown and characterised for their lactate response directly in DMEM medium and also CD CHO medium (common growth medium used for Chinese hamster ovary, CHO cells). However, the growth and fluorescence data collected under these conditions were inconsistent and did not allow for reliable characterisation data to be collected (data not shown). This may partially due to similar reasons as for LB medium (see Chapter 5: *E. coli lldPRD* lactate-responsive operon). These media contain many complex molecules and in the case of CD CHO medium the composition is not publically available. It is possible that any of these molecules may be interfering with the sensing mechanism of the biosensor.

Because of this, it was decided to grow cells in M9 medium (with glycerol as the main carbon source) (100  $\mu$ l) and add small amounts of cell culture samples (25  $\mu$ l) for testing. As there may still be interference and other issues from the components and properties of the cell culture medium, a number of experiments were carried out using increasingly complex conditions to verify the functioning of the biosensor at each stage.

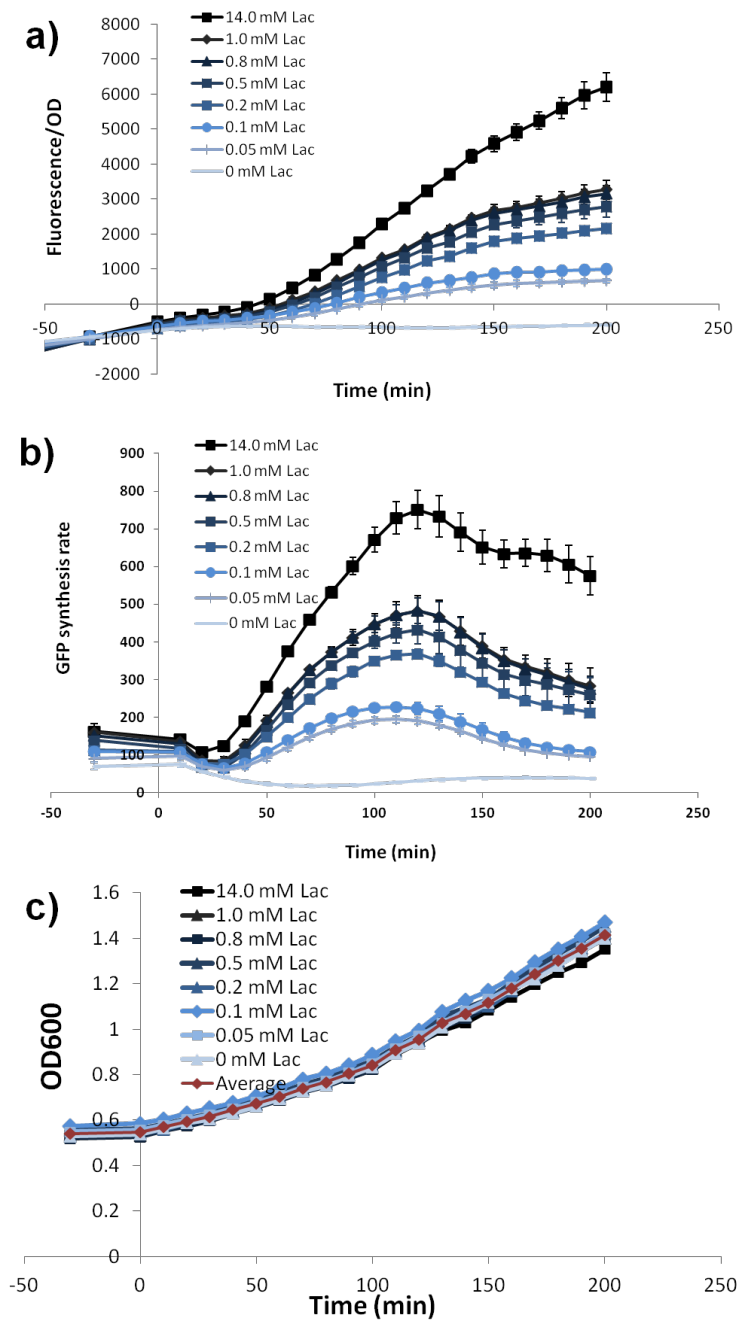
### 9.3.2 Simulating cell culture samples (DMEM)

#### 9.3.2.1 Standard lactate concentrations in DMEM

*lldp+lldR* sensor cells were grown in 100  $\mu$ l M9 medium and 25  $\mu$ l of DMEM containing known lactate concentrations were added (Figure 9.2). The results show that the lactate sensor shows good lactate responsiveness under these conditions. Although the exact shape of the fluorescence and synthesis rate graphs changes slightly in each run based on small cell growth and fluorescence variations, the overall dynamics are consistent. Even in the presence of all the potentially interfering



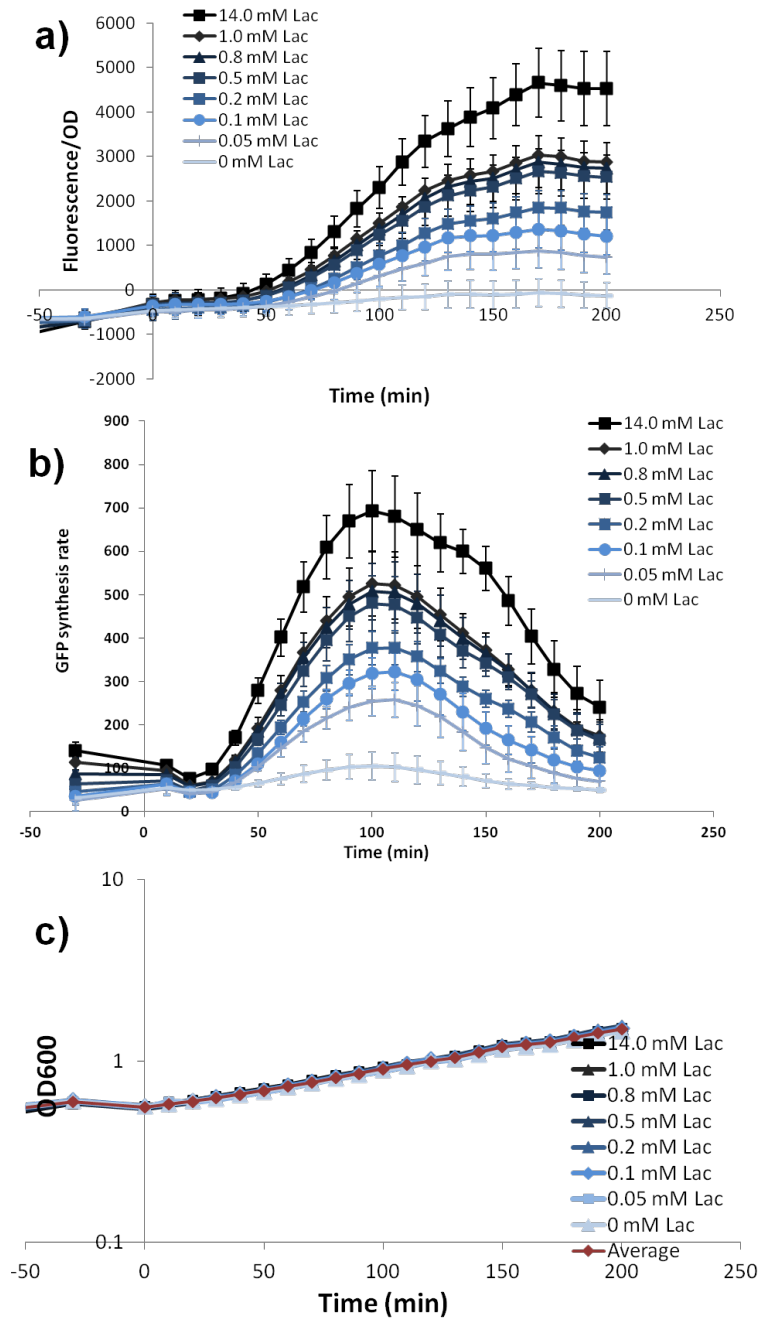
compounds found in DMEM, there is clear differentiation of GFP synthesis rate by lactate concentration.



**Figure 9.2:** Testing standard lactate concentrations in DMEM with sensor cells grown in M9. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. Error bars represent the standard deviation of three biological replicates.

### 9.3.2.2 Standard lactate concentrations in DMEM supplemented with serum

With the exception of the bacterial growth experiment shown at the beginning of this chapter, all biosensor characterisation experiments in this work used DMEM that did not contain any supplements. Here, a characterisation experiment was carried out using standard lactate concentrations in DMEM, where the DMEM was supplemented with 10 % (v/v) fetal bovine serum, which is a commonly used supplement for hybridoma cells. Serum is a complex mix of compounds that have the potential to interfere with the mechanism of the lactate sensor. It was found that the sensor showed a lactate-response under these conditions (Figure 9.3). The data was more variable than in the conditions where serum was not present. This could be due to the fact that the presence of serum presents a more variable and complex environment for the sensor to function. These results mean that the sensor will likely be able to function if used to test real mammalian cell culture samples using this medium.

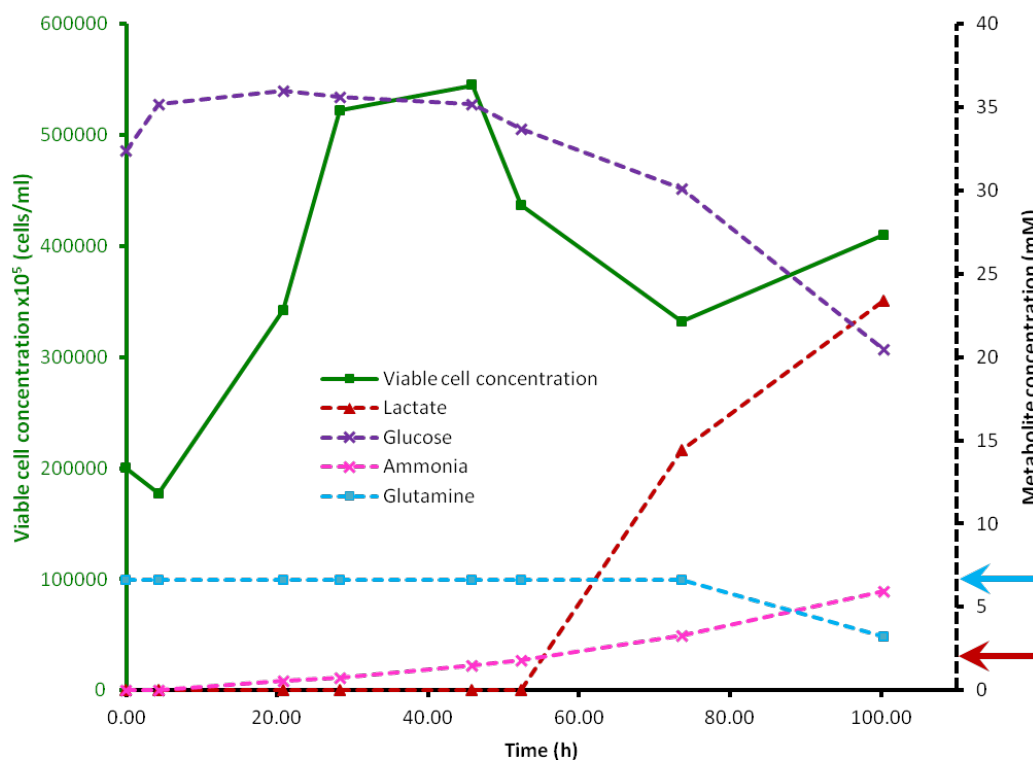


**Figure 9.3:** Testing standard lactate concentrations in DMEM (supplemented with serum) with cells grown in M9. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. Error bars represent the standard deviation of three biological replicates.

## 9.4 Mammalian cell culture samples

### 9.4.1 CHO cell culture

During the course of this work, the opportunity for collecting samples from a CHO culture in CD CHO medium was presented. The specific cells were CHO cells transformed with a construct made by Antony Constantinou (CSynBI, Imperial College). CD CHO was supplemented with 8 mM glutamine and HT supplement. The cells were grown for four days. Supernatant samples were collected at regular intervals during the culture and spent medium was collected at the end of the culture run. Supernatant samples were analysed for metabolite concentrations (Figure 9.4).



**Figure 9.4:** CHO culture supernatant metabolites analysis using a Nova BioProfile Analyzer. Arrows indicate lower or upper concentration detection limits. Data are based on a single culture.

### 9.4.2 Simulating cell culture samples (CD CHO medium)

Before analysing these CHO cell culture samples using the lactate biosensor, the biosensor was tested under conditions resembling cell culture samples.

#### **9.4.2.1 Standard lactate concentrations in CD CHO**

As previously done for DMEM growth medium, lactate sensor cells were grown in M9 medium and known lactate concentrations in fresh CD CHO medium were added to simulate CD CHO cell culture samples (Figure 9.5). CD CHO was supplemented with 8 mM glutamine and HT supplement. This was done to test if lactate sensing from CD CHO culture samples would work with this biosensor. The results show that the lactate sensor shows lactate-responsiveness under these conditions. At the highest lactate concentration (14 mM), the data shows high variability. This could be due to the complex CD CHO medium affecting the function of the sensor at this lactate concentration range. The GFP synthesis graphs also show a dip in synthesis rate at around 150 min. This could be due to the bacterial sensor cells reacting to components in the CD CHO medium or any other change in conditions during this run. Overall, the results indicate that the sensor could be used under these conditions, as there is differentiation in fluorescence and GFP synthesis rate for different lactate concentrations.

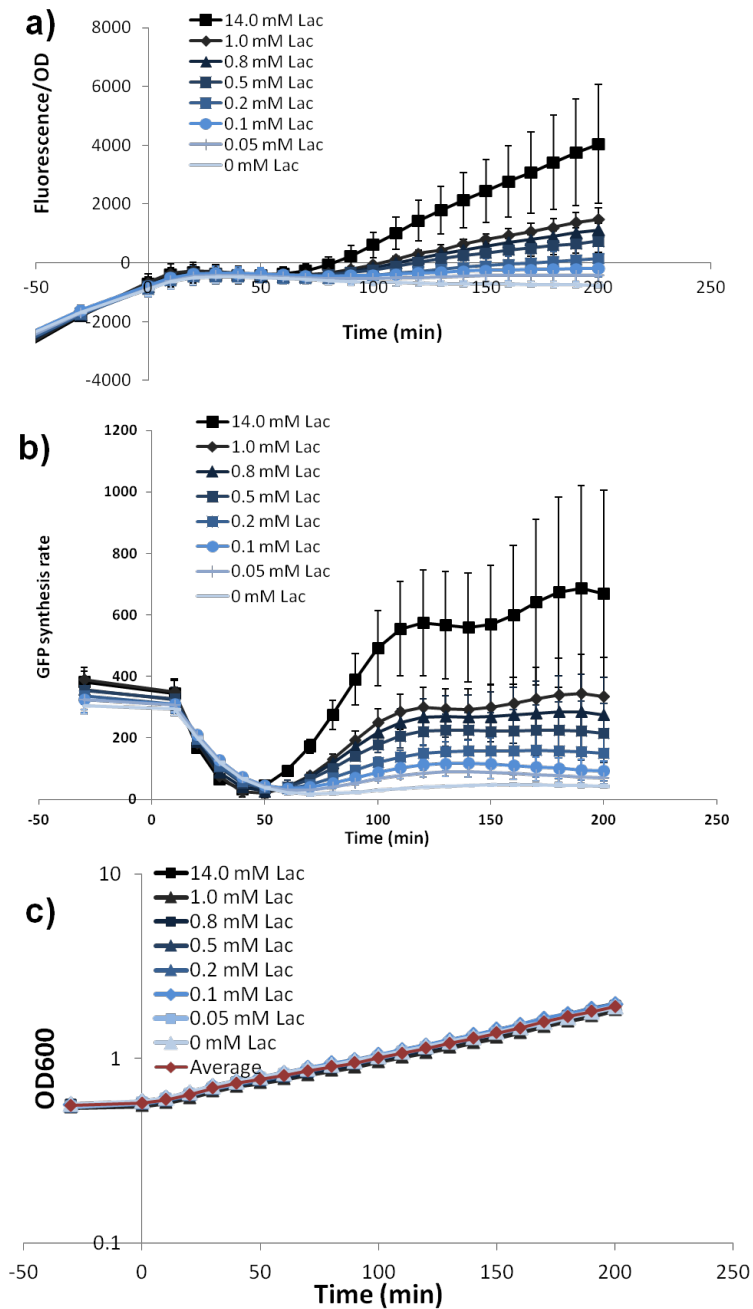
#### **9.4.2.2 Diluted spent CD CHO medium samples**

During a cell culture, mammalian cell may secrete many molecules into the culture medium. When cells die they may burst, emptying their contents into the supernatant. All these substances have the potential to interfere with the lactate biosensing that is being tested here. In order to create a more realistic test for using the biosensor on cell culture samples, collected spent medium was diluted in different ratios with fresh CD CHO medium and the resulting samples tested using the biosensor (Figure 9.6-9.7). As in the experiment using the standard lactate concentrations in CD CHO, the variability at 14 mM lactate is relatively high. The sensor cells that were exposed to the spent medium dilutions show decreases in fluorescence at various times after 100 min. Overall, the sensors shows lactate-responsiveness under these conditions.

The lactate concentration in the spent medium dilution samples was determined in parallel using the biosensor and the bioprofiler (Figure 9.7). The lactate concentrations determined through the two different methods are comparable.

#### **9.4.2.3 Samples from CHO culture**

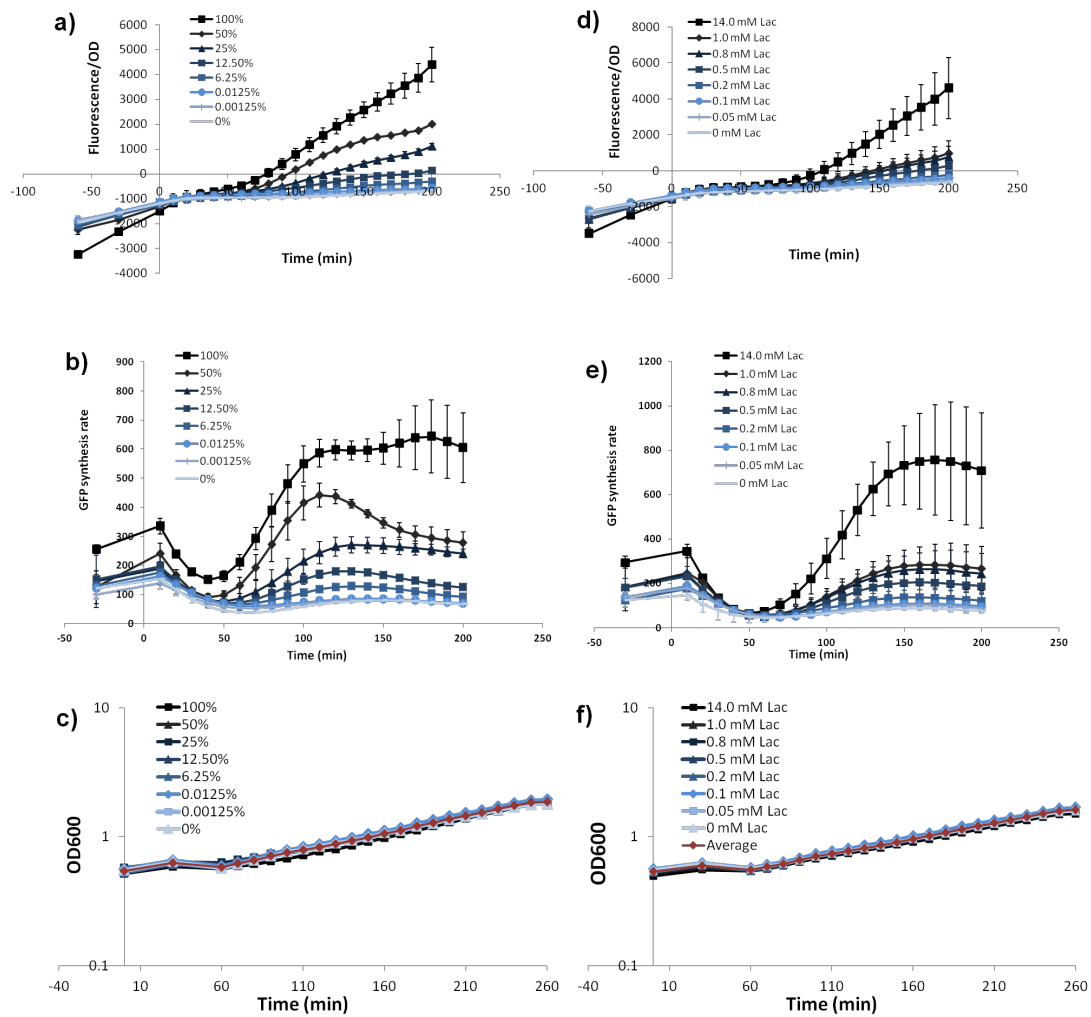
Finally, the samples from the CHO cell culture were analysed for lactate concentration using the lactate biosensor (Figure 9.8-9.9). The biosensor cells that



**Figure 9.5:** Testing standard lactate concentrations in CD CHO with sensor cells grown in M9. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. Error bars represent the standard deviation of three biological replicates.

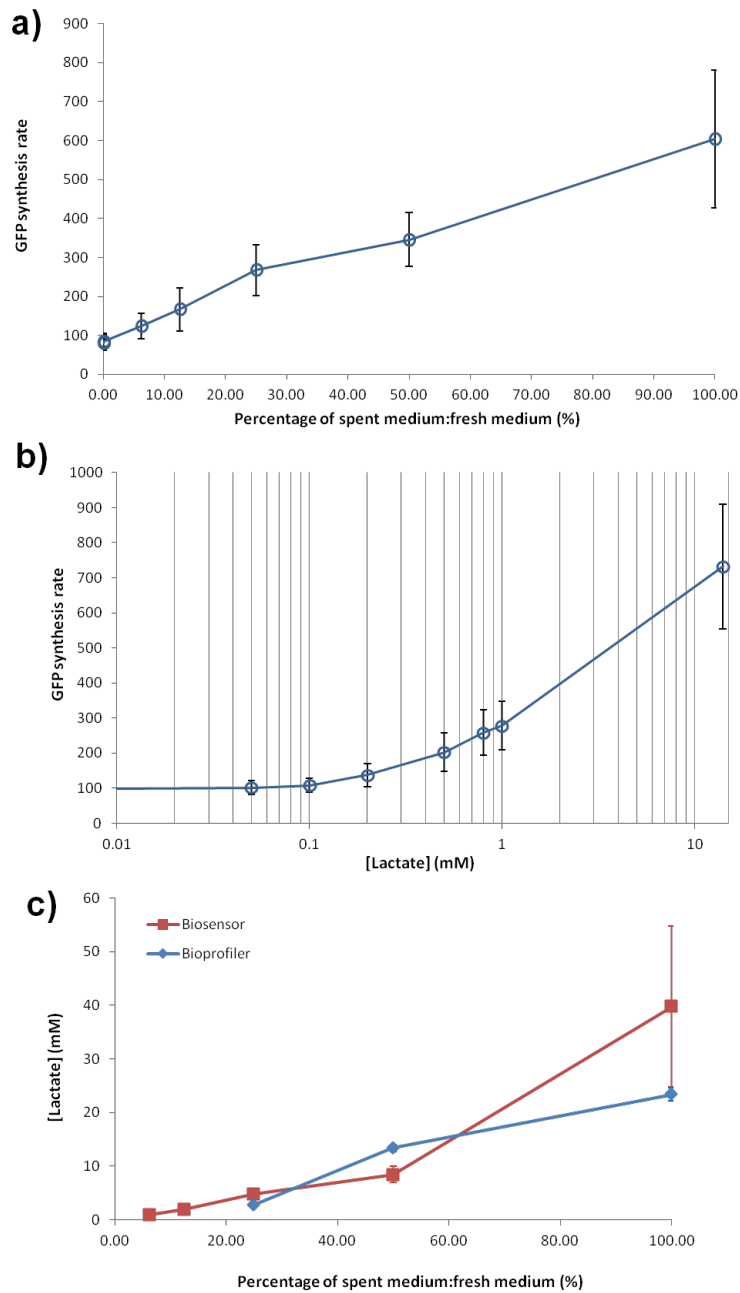
were exposed to cell culture samples showed a dip in fluorescence that is especially pronounced with the cell culture samples from later time points. A similar, though smaller, dip is seen for the biosensor cells exposed to standard lactate concentrations in CD CHO.

Only the last two cell culture samples contained lactate at concentrations that were high enough to be detected by the bioprofiler (Figure 9.9). The lactate



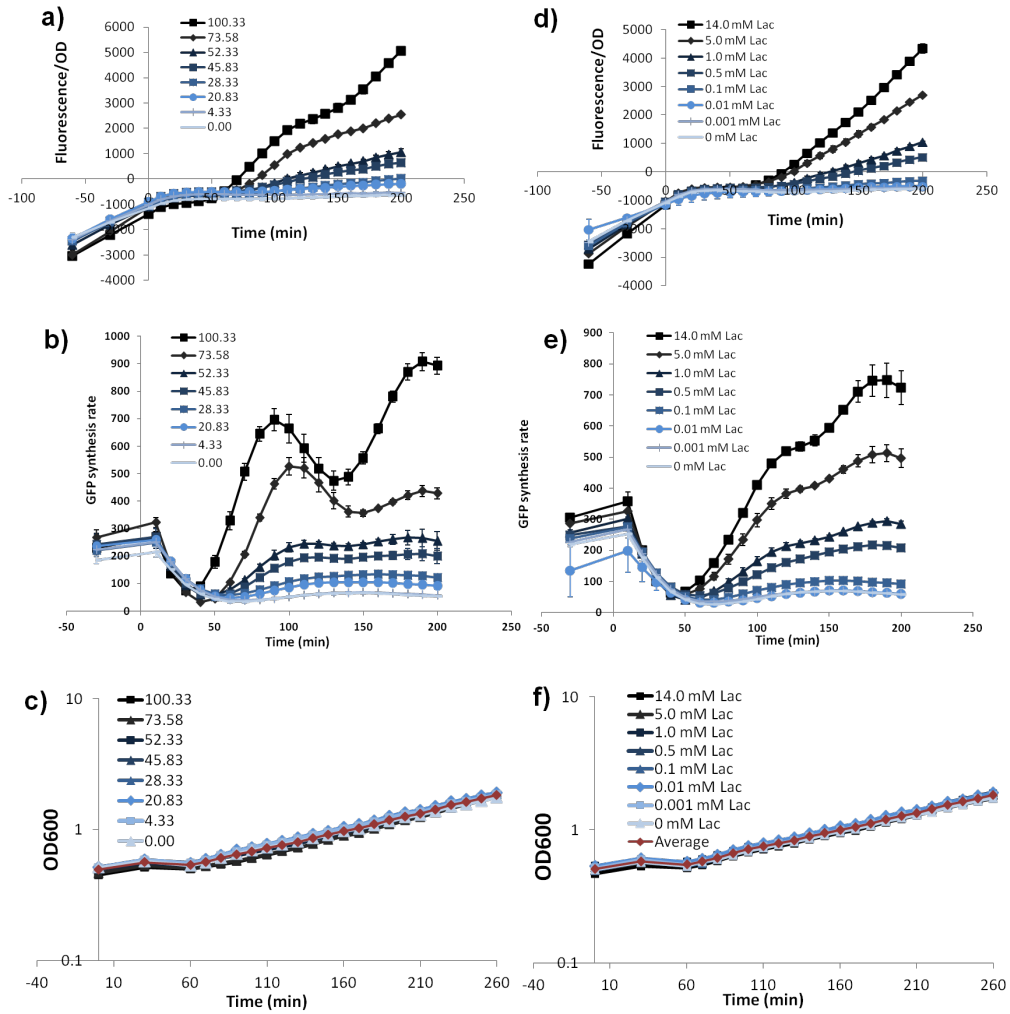
**Figure 9.6:** Using the lactate biosensor to determine the lactate concentration in spent CD CHO medium dilutions. **a) - c)** Spent medium dilutions. **d) - f)** Standard lactate concentrations in CD CHO. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. **d)** Corrected fluorescence. **e)** GFP synthesis rate. **f)** Cell growth. Error bars represent the standard deviation of three biological replicates.

concentrations in the sample collected after 73.58 h of cell culture were similar whether determined by biosensor (12.83 mM) or bioprofiler (14.4 mM). However, for the sample collected after 100.33 h, the lactate concentrations determined by the two methods differ strongly (biosensor: 140.46 mM; bioprofiler: 23.4 mM). This could be due to a number of reasons, including the variability seen in the sensor response for these samples.

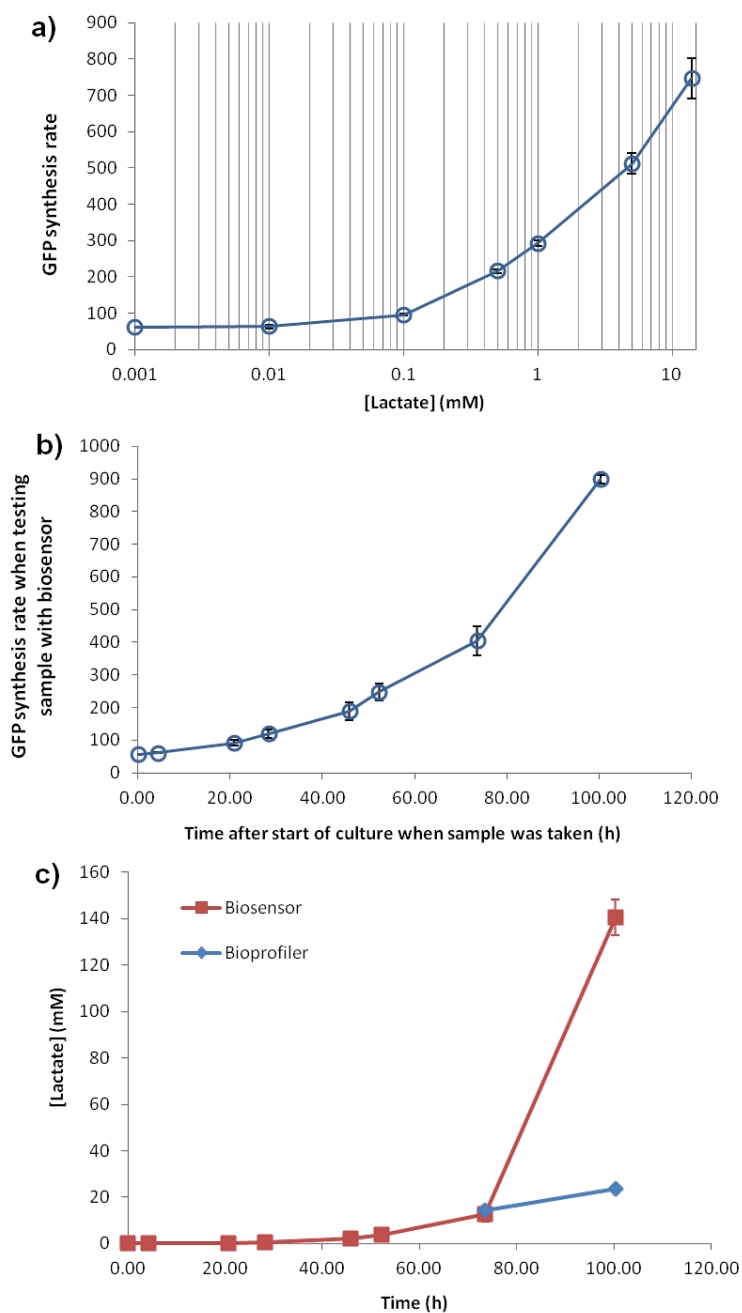


**Figure 9.7:** Using the lactate biosensor to determine the lactate concentration in spent CD CHO medium dilutions. **a)** Dose-response curve for spent medium dilutions. **b)** Dose-response curve for standard lactate concentrations. **c)** Lactate concentrations in spent culture medium dilutions according to biosensor and bioprofiler. Missing data points for the bioprofiler graph are due to lactate concentration being lower than the detection limit of the machine. Error bars for biosensors represent standard deviations of three biological replicates. Error bars for bioprofiler data represent imprecision of bioprofiler measurements.





**Figure 9.8:** Using the lactate biosensor to determine the lactate concentration in CHO cell culture samples. **a) - c)** CHO cell culture samples. **d) - f)** Standard lactate concentrations in CD CHO. **a)** Corrected fluorescence. **b)** GFP synthesis rate. **c)** Cell growth. **d)** Corrected fluorescence. **e)** GFP synthesis rate. **f)** Cell growth. Error bars represent the standard deviation of three biological replicates.



**Figure 9.9:** Using the lactate biosensor to determine the lactate concentration in CHO cell culture samples. **a)** Dose-response curve for spent medium dilutions. **b)** Dose-response curve for standard lactate concentrations. **c)** Lactate concentrations in spent culture medium dilutions according to biosensor and bioprofiler. Missing data points for the bioprofiler graph are due to lactate concentration being lower than the detection limit of the machine. Error bars for biosensors represent standard deviations of three biological replicates. Error bars for bioprofiler data represent imprecision of bioprofiler measurements.

## 9.5 Results summary

Experiments were carried out towards using the metabolite biosensors created in this work to determine metabolite concentrations in mammalian cell cultures. Growth of *E. coli* and *B. subtilis* cells in DMEM growth medium was characterised. *E. coli* cells had healthy growth characteristics, while *B. subtilis* cells could only be grown to a low optical density.

Testing of the *lldp+lldR* lactate sensor in DMEM or CD CHO media did not give usable characterisation data. It was therefore decided to keep M9 as the main growth medium for the biosensor cells and add small amounts of cell culture samples to these cultures. The biosensor showed good lactate-responsiveness for standard lactate concentrations in DMEM, DMEM supplemented with serum, and CD CHO medium.

The lactate biosensor was used to determine the lactate concentration in diluted spent CD CHO medium samples and cell culture supernatant samples. These samples were also analysed for lactate concentration using a bioprofiler. For the diluted spent medium samples, the results from the two methods were comparable. For the cell culture samples, the lactate concentrations matched closely for one of the data points and differed strongly for another data point. This may be caused by variability in the sensor response seen for these samples. Improvements to the analysis method for determining lactate concentrations could address this issue. In presence of CD CHO, the biosensor cells often show a dip in fluorescence over the time course. When picking a time point to determine lactate concentrations, this dip may affect the results.

Overall, the lactate sensor showed promising results for use in mammalian cell cultures, although the exact process for determining lactate concentrations may need to be optimised further.

## 10 Discussion

The aim of this project was to engineer novel bacterial *in vivo* biosensors for mammalian bioprocessing applications. Growth dynamics of a hybridoma cell line were investigated to learn more about the context in which biosensors would be used, and to suggest possible targets for biosensing. Based on these results and literature searches of interesting mammalian cell culture parameters and bacterial sensing systems, a number of systems were chosen for further investigation:

The *E. coli* lldPRD operon that is responsive to lactate. Lactate is produced by mammalian cells during culture and can inhibit cell growth.

The *E. coli* Lrp operon that is responsive to leucine and alanine. Both these amino acids are present in mammalian cell culture media. Alanine is secreted by certain mammalian cells in response to ammonia stress.

The *E. coli* Ntr system as well as the *B. subtilis* GlnL/GlnK system which are both responsive to glutamine. Glutamine is the major nitrogen source in many mammalian cell cultures.

Promoters from these systems were cloned into DNA constructs using GFP as the reporter and characterised for their response to the metabolite of interest and hence their suitability as biosensors.

### 10.1 Engineering biosensors for common metabolites

#### 10.1.1 Lactate biosensors based on the *E. coli* lldPRD system

Three biosensor constructs were investigated in this work. *lldPp + lldR + lldP* was found to show reduced growth compared to the other sensors and could not be characterised. This may be due to the additional metabolic burden or toxicity from the overexpression of the permease. *lldPp* contains the lldPp promoter linked to a GFP gene. *lldPp + lldR* contains all the parts of the *lldPp* construct as well as an overexpression construct for the lldR transcription factor. *lldPp + lldR* showed

lactate-responsiveness under all conditions tested. *lldPp* was also responsive to lactate, though less usefully than *lldPp + lldR*.

The results shown here support the current model of how this operon is regulated. When the *lldR* transcription factor is overexpressed, fluorescence in absence of lactate is very low. When *lldR* is not overexpressed (with only the genomic copy of the gene present), fluorescence baseline in absence of lactate is much higher. Specifically, the high basal expression prevents the ability to measure the small changes in expression that result from the addition of small amounts of lactate. A much more differentiated response to lactate is seen for *lldPp + lldR* than for *lldPp*. These results support the model stating that *lldR* acts as a repressor of gene expression from the *lldPp* promoter in absence of lactate and as an activator in the presence of lactate. In the presence of overexpressed *lldR*, the fluorescence in absence of lactate is lower than when *lldR* is not overexpressed. Also, the relative increase in fluorescence in response to increasing concentrations of lactate is greater in presence of overexpressed *lldR* than in absence.

The difference between *lldPp* and *lldPp + lldR* agrees with a theory discussed in Dehli et al. (2012). This theory states that the higher the unregulated activity the less induction can be achieved with an activator. Thus in general activator-operated promoters show low unregulated activity while repressor operated promoters show high unregulated activity. This is referred to as an activation ceiling. *lldPp* shows high unregulated activity, indicating a repressor operated promoter.

Presence of glucose in the growth medium lowers gene expression from the *lldPp* promoter, but does not change qualitatively the overall dynamics of the lactate-response. The results shown here confirm previous results from the literature showing that this system is also responsive to D-lactate, though to a lesser extent than L-lactate. Characterisation of the response of this sensor to pyruvate was inconclusive, as increasing concentrations of pyruvate lead to increased fluorescence, but the highest concentration used (14 mM) showed the lowest fluorescence. This may be caused by the way that the cells utilise pyruvate in ways not connected to the biosensor construct. The presence of large concentrations of pyruvate may change the flux in *E. coli* metabolism.

Overall, *lldPp + lldR* was found to act as a reliable lactate biosensor in a concentration range  $\sim 0-14$  mM, which was the concentration range tested here. The exact dynamic range of the sensor changes with extracellular conditions. Undergraduate student Katarzyna Roguska (Roguska, 2012), who tested the sensor in LB medium using a different protocol to the one used here found responses up to 100 mM under certain conditions, although this required measuring over a much

longer time scale.

### 10.1.2 Leucine/alanine biosensors based on the *E. coli* Lrp system

Six promoters controlled by the Lrp protein in response to leucine and alanine were incorporated into DNA constructs using GFP as a reporter and characterised for their response to, and their potential to be used as biosensors for, these metabolites.

Biosensors were characterised on solid and in liquid media. Biosensor responses are summarised in Table 7.2. The biosensor cells showed some differences in behaviours between the two media. High concentrations of leucine caused growth inhibition on solid medium but not in liquid medium. Some biosensors showed responses in liquid media but not on solid media. However, that could be due to the fact that fluorescence on solid media were assessed by eye. Solid and liquid media are very different growth conditions, especially in terms of diffusion, which can explain differences in cell behaviour (Dalchau et al., 2012). Different behaviours between plates and liquid medium have been shown for *E. coli* and *B. subtilis* (Dubey and Ben-Yehuda, 2011).

Every promoter tested showed a response to either alanine or leucine in at least one of the conditions tested. Some promoters showed activation in response to alanine and leucine and some showed repression. Not all the responses seen agree with what was predicted from the previous literature on these promoters. *fimBp1* and *fimBp2* were predicted to be activated by leucine/alanine, but showed little to no response here. *gltBp* showed repression by leucine, which was expected from the literature. It also showed repression by alanine, to which it was not previously known to be responsive. *ilvIHp1* and *livJp* both behaved as predicted. *LeuLp* showed activation in response to leucine/alanine under the conditions used here, but it was predicted to be repressed. These results are useful for commenting on the utility of these promoters as biosensors. They also shows how these systems behave under the conditions used here, which teaches us about the how this operon works.

The Lrp transcription factor was not overexpressed in these constructs. Overexpression of Lrp is known to have a range of effects on *E. coli* metabolism. Undergraduate Harold Taylor attempted to overexpress the Lrp protein in *E. coli* cells. However, while he was able to transform a plasmid containing the Lrp gene and a terminator, this was not possible when a promoter and RBS was added to the front of the gene. This may suggest that the Lrp gene cannot be overexpressed in *E. coli* cells (at least not in a straightforward way). Because the promoters were overexpressed

on high copy plasmids, whereas the Lrp protein is only present in one genomic copy, the effect is equivalent to lowering the concentration of Lrp relative to the promoters compared to wild type cells. If possible, overexpression of Lrp might increase responsiveness of the biosensor in the same way that overexpression of the transcription factor improves function of the lactate sensor. However, the “dilution” of Lrp may not have that great an effect considering that Lrp is present at relatively high copy number (3000 dimers) in *E. coli* cells. The exact concentration of Lrp within cells changes with growth conditions.

When switching from rich to poor media, Lrp concentration can increase 10-fold (Newman, 1995). This may again explain why an Lrp-dependent response to leucine/alanine is seen here in minimal medium without the overexpression of Lrp. However, the fact that Lrp concentration changes with growth conditions and presence of certain amino acids (Newman, 1995), makes this system a complicated basis for synthetic biology biosensors. If Lrp is not easily overexpressed in *E. coli* cells then the system may be difficult to transfer between cells trains while maintaining predictable behaviour.

For these reasons, systems such as the lldPRD operon that are more orthogonal to cellular metabolism than the Lrp system are generally more suitable for synthetic biology systems like biosensors. However, the Lrp promoters shown here do represent usable bioparts and some of them act as leucine/alanine biosensors.

### **10.1.3 Glutamine biosensors based on the *E. coli* Ntr system**

Eight promoters that are controlled by the Ntr operon in response to glutamine were incorporated into DNA constructs using GFP as the reporter and characterised for their response to, and their potential to be used as biosensors for, glutamine.

The promoters were characterised on solid and liquid medium. Several of the promoters showed responses to glutamine concentration on solid media, but none of the promoters showed a significant response to glutamine when characterised in liquid medium.

As discussed in detail in Chapter 7, there are a number of reasons that this system may not be suitable for glutamine-sensing under the conditions used here. The system reacts to intracellular glutamine concentration, which may not equilibrate with extracellular glutamine concentration. Glutamine is not the only input for the Ntr system.  $\alpha$ -ketoglutarate is also detected as an indicator of cellular carbon status (Schumacher et al., 2013). Glutamine is also used up by cells as it enters the cells.

Overall, the Ntr promoters shown in this work did not act as glutamine biosensors under the conditions used here.

#### 10.1.4 Glutamine biosensor based on the *B. subtilis* GlnK-GlnL system

The PM8J2-706 promoter that is controlled by the glutamine-responsive *B. subtilis* GlnK-GlnL two-component system was cloned into a characterisation construct that was integrated into the *B. subtilis* genome at the AmyE locus. The construct linked the promoter to GFP as a reporter. These cells were characterised for their response to glutamine and their utility as biosensors.

In an experiment using flow cytometry, biosensor cells grown in MM medium with glutamine showed higher fluorescence than those grown in MM medium without glutamine. Control cells did not show a fluorescence shift in response to glutamine. However, control cell fluorescence was consistently at the same level as biosensor cells in presence of glutamine. there are several possible explanations for this. The control construct is identical to the sensor characterisation construct except that it does not contain the relevant promoter. The region in front of the GFP gene in the control construct may act as a cryptic promoter leading to leaky uncontrolled GFP expression. The GFP expression may also be caused by read-through from the antibiotic resistance cassette that is located upstream of the GFP gene. Finally, the control of the PM8J2-706 promoter by the GlnK-GlnL two-component system may work through repression of expression in absence of glutamine rather than activation of expression in presence of glutamine as previously thought. Further characterisation constructs could be constructed to investigate these dynamics more closely (see Chapter 8).

Unlike the *E. coli* sensors shown in this work, which are based on high-copy plasmids, the *B. subtilis* sensor is based on a single genomic copy of the characterisation construct. Signal strength of the response could be amplified by having the sensor based on a plasmid. The *glnL* and *glnK* genes could also be overexpressed in this context. This, however, could prove challenging as *glnK* is a transmembrane protein. The characterisation protocol used for the *E. coli* sensors in this work would have to be adapted for use on this *B. subtilis* sensor. This would involve studying the growth dynamics of the *B. subtilis* cells under the chosen conditions more closely and adapt incubation and measurement times accordingly.

The results shown here confirm previous results from the literature showing that the system is responsive to extracellular glutamine. This sensor is the only sensor shown



in this work that directly responds to the extracellular concentration of the target metabolite instead of the intracellular concentration. As the aim of this work is to engineer biosensors that can be used to monitor metabolite concentrations in the culture medium this represents a major advantage for a biosensor. No assumptions have to be made about whether the intracellular concentration equilibrates with the extracellular concentration.

Overall, this system is promising as the base for a glutamine biosensor but further characterisation is required before it can be put to use in a mammalian cell culture context.

## 10.2 Metabolite biosensors and synthetic biology

The aim of this work was to engineer metabolite biosensors for mammalian cell bioprocessing contexts. Several functioning biosensors were created as discussed above. This work therefore presents an overall strategy for engineering such biosensors. Many challenges were encountered during this work that ultimately taught us about the biological process underlying these biosensors. Some of these topics were already discussed above for the relevant biosensors.

Attempts were made in this project to follow the synthetic biology design cycle process (see Introduction) (Freemont and Kitney, 2012). Initially, the context in which the biosensors were to be used (*i.e.* mammalian cell culture) was analysed to derive specifications for how the biosensors needed to function. Natural bacterial sensing systems were researched and several suitable candidates chosen for characterisation. Characterisation was mostly done in liquid M9 medium using 96-well format on a robot platform that integrated an incubator and a plate reader.

There exist different kinds of data used to represent the output of GFP-based biosensors when using plate reader-based measurements of fluorescence and optical density: GFP synthesis rate (Ronen et al., 2002; Canton et al., 2008), relative promoter units (RPU) (Kelly et al., 2009) and normalised or corrected fluorescence (Lee et al., 2011).

The measures used in this work were corrected fluorescence (fluorescence/OD) and GFP synthesis rate. Both of these are calculated from measures of OD and fluorescence over time (see details in Materials and Methods). Corrected fluorescence is the fluorescence from a given time point divided by the cell OD from that time point and therefore roughly represents fluorescence per cell. GFP synthesis rate calculated the fluorescence change between two time points and therefore represents

the amount of GFP produced or broken down in that time. Both of these measures have been shown for various biosensors here and GFP synthesis rate data was used to determine the lactate concentrations in cell culture samples. However, corrected fluorescent data could also have been used. Synthesis rate is more sensitive and an indicator of what is directly happening in the system. However, as it is more sensitive, it is also much more affected by noise as well as cell state. This means that when comparing systems with vastly different growth rates, corrected fluorescence may be the more suitable measurement. An additional complication is that both synthesis rate and fluorescence/OD can be calculated differently, with a number of alternate ways of accounting for control measurements. Which method is most suitable will depend on the individual experimental set-up and aims.

The control cell used in this work contained a construct that was identical to the biosensor constructs, except lacking the potentially responsive promoter. In some cases, subtraction of control cell fluorescence from biosensor cell fluorescence gave negative corrected fluorescence values. This may be caused by run-through expression from the control constructs. Different kinds of control circuits (see Introduction) could be used instead to prevent this problem.

Overall, all these different methods are based on certain assumptions that may not hold true under all conditions and they all come with advantages and disadvantages which will depend on the situation. In many cases, biosensor output will be reported in vague terms such as “reporter signal intensity” (van der Meer and Belkin, 2010). Therefore, the real important factor is complete reporting of metadata to make data from different labs comparable.

A lot of the systems used in this work are “non-orthogonal”. This means that the sensor system is not entirely separated from cellular metabolism. The sensor may respond to inducers that also interact with other cellular pathways or may respond to more than one input. In all the systems used here, the biosensor was composed of elements that were already present in the cells, *i.e.* the natural operon is there and working, which could cause cross-talk.

The metabolites that act as biosensor targets in this work are metabolisable and in some cases fulfill many cellular functions. This means that the metabolites sensed could be produced or used up by the bacterial cells, which could interfere with the sensing mechanism, *e.g.* by causing noise in the sensing or preventing the sensing output. This is unlike the properties of commonly used inducers such as IPTG, which are non-metabolisable by cells.

Overall, orthogonality of a system from the cellular machinery makes the system

more suitable for use in synthetic biology. In this work, the more orthogonal systems (IldPRD and GlnK-GlnL) are more promising as biosensors than the less orthogonal systems (Ntr and Lrp). However, even highly non-orthogonal systems can be used as biosensors or other bioparts as long as the limitations are known. This means that during characterisation point of failure need to be identified. This work has shown that natural promoters can be used as biosensors to sense non-orthogonal extracellular metabolites.

While most of the characterisation was carried out in liquid M9 medium, some characterisation experiments were carried out on solid media or in different liquid media. Generally, the biosensors characterised here often showed context-dependency, *i.e.* they function differently in different conditions. This could be due to factors specific to the biosensor system. Some biosensors react to more than one target. The presence or absence of these alternate targets in different media could cause differences in biosensor response. Alternatively, the differences could be caused by processes that alter larger parts of cellular metabolism instead of just the biosensor system. For instance, cells grow differently on solid and liquid media.

With the exception of the *B. subtilis* glutamine sensor, all the sensors presented here respond to intracellular metabolite concentrations. As the parameters we are ultimately interested in are extracellular metabolite concentrations in mammalian cell culture, an assumption in this work is that the intracellular and extracellular concentrations will equilibrate. It is assumed that intracellular concentration is sufficiently representative of extracellular concentration. However, this may not always be the case and is dependent on available cellular transport systems.

Standardisation was attempted at several levels in this work. All parts and constructs used the BioBrick standard DNA assembly method and can thus be used by the wider synthetic biology community. As far as possible the same control constructs and general characterisation conditions were used for characterisation of all the biosensors. However, it is not always possible to keep all constructs and experiments in the same format. This is due to differences between the biological processes underlying the different systems. Overall, it is important to appreciate that not every experimental technique or analysis method is suitable for every kind of biosensor. While standards and standard methods are very useful, they need to be flexible to some extent to be able to incorporate most contexts. However, this fact makes extensive reporting of characterisation and analysis methods all the more important. Datasheets, which are already used in many areas of synthetic biology (Canton et al., 2008) are an example of this. However, better reporting standards for publications should also be implemented.

It has been stated that synthetic biology is less like highly modular and standardised electrical engineering and more like civil and mechanical engineering in that it requires optimisation and appreciation of whole-system stresses (Church et al., 2014).

## 10.3 Using bacterial biosensors in mammalian cell culture

After a number of biosensors were engineered and characterised under standard conditions, the next step was to test these biosensors in a mammalian cell culture context (see chapter 9).

Initially, the growth of *E. coli* and *B. subtilis* cells in DMEM medium was investigated. During future applications, the bacterial biosensors would be exposed to samples of mammalian cell culture medium or even be used in a co-culture set-up with the mammalian cells. Therefore, it was important to know if these cells could grow in presence of mammalian growth media. Growth of *E. coli* in DMEM in 96-well plate format was already shown in chapter 4. In that format, precipitation of medium components was seen, which interfered with optical density measurements needed for biosensor characterisation. This was not seen when *E. coli* and *B. subtilis* cells were grown in DMEM also containing serum and based in a flask (chapter 9). There are many differences between the conditions in a 96-well plate and in a culture flask, such as the surface to volume ratio and aeration through shaking. These differences could explain why precipitation was seen in one case and not the other. The presence of serum and the different measurement methods (direct measurement of well using a plate reader and cuvette-based sample measurements) could also have an effect. This experiment showed that *E. coli* and *B. subtilis* cells can grow in DMEM and that *E. coli* cells have a higher growth rate than *B. subtilis* cells and grow to a higher final optical density.

It was decided to carry out the tests of biosensors in a cell culture context using the *lldPp + lldR* lactate sensor, as this was the sensor showing the most reliable response of all the sensors shown in this work. The lactate biosensor was characterised for lactate response in CD CHO medium. However, this did not give usable data. Due to this, as well as the precipitation issues in DMEM in 96-well plate format, it was decided to initially use the biosensor by growing cells in M9 medium and adding small volumes of mammalian cell culture samples. The lactate sensor was found to be responsive to lactate when testing standard lactate concentrations in DMEM, DMEM+serum and CD CHO medium.

A CHO cell batch culture was carried out to collect samples that could be tested using the lactate biosensor as well as using a bioprofiler machine (the current industry standard for cell culture sample metabolite analysis). There was a good match between lactate concentrations in spent culture medium dilutions determined

through the two methods. When the same approach was applied to cell culture samples, the results from the two methods were similar for one time point, but differed significantly for a later time point.

There are a number of things that need to be tested for novel sensing or monitoring methods (Derfus et al., 2010): Equivalence of measurement to previous methods, reproducibility of measurements, length of sampling process, sensitivity, specificity, required sample volume, interference, ability to give quantitative versus qualitative information and measurement window over which quantification is possible (*i.e.* dynamic range).

The Bioprofiler 400 Analyzer used in this work can monitor lactate in the approximate concentration range 2-50 mM. The lactate biosensor presented in this work can detect lactate in a lower concentration range of approximately 0.01 mM to 14+ mM, though the exact dynamic range can change with cellular growth conditions and presence of other compounds in the samples. It is therefore possible to determine the lactate concentration in samples that would be below the detection limit using the Bioprofiler. Furthermore, as samples get diluted 1:5 when added to the bacterial biosensor culture, the protocol used here would allow samples with higher lactate content to be tested as well. The level of dilution could be modified to some extent for different cell cultures if needed. Overall, the dynamic range of lactate sensor and testing protocol presented here can cover the lactate concentration range found in mammalian cell culture samples.

As the dynamic range of the lactate biosensor is so much lower than for the Bioprofiler, it was difficult to use the Bioprofiler for validation here. Only a small number of samples showed lactate concentrations that could be accurately detected by both methods. An alternative established method for lactate analysis could be used to validate the results of the biosensor in the lower concentration range. Examples of possible methods include enzymatic assays (*e.g.* the lactate oxidase assay), HPLC or mass spectrometry-based methods.

The lactate sensor presented here can be used to quantitatively determine lactate concentration in cell culture samples grown in DMEM and CD CHO growth media. The biosensor cells are very low cost compared to the reagent packs that need to be purchased for the Bioprofiler. As the sensor is based on the transcription machinery of living cells, the output of the biosensor could in future be linked to a more complex biological response by the bacterial cells. For instance, the bacterial cells could produce nutrients for the mammalian cells as needed or deplete waste compounds. Alternatively the bacteria could produce compounds to induce mAb production by the mammalian cells.

However, as can be seen from the large difference in the last cell culture samples, there are ways in which the use of the biosensor needs to be improved and factors that may affect the function of the sensor. The large difference shown for the late time point could be due to a number of reasons.

Mammalian cell culture is a very complex biological context. Mammalian growth media contain many complex molecules that have the potential to interfere with biosensor function. Undergraduate student Harry Taylor showed that glutamate, galactose and mannose can all disrupt the sensing function of the biosensor under certain conditions (data not shown, unpublished data).

As mentioned above, in the current protocol cell culture samples are diluted 1:5 during addition to the biosensor culture. While this can be advantageous, it also means that small errors in calculating the concentration will be amplified 5-fold when calculating back to the original sample.

The lactate concentration in cell culture samples in this work was calculated using the information from a lactate standard curve. The way this curve was constructed was by picking a particular time point to compare the standard and sample results. This introduces a source of error and bias. Variances in any given time-point will affect the result. A better way of doing this analysis would be to use an average over several time points. Alternatively, integration over the whole time course could be used, thus greatly reducing bias of selecting a particular end-point and error due to point-to-point variation. GFP synthesis rate rather than normalised fluorescence was used. But other options could be investigated.

There are always small differences in bacterial cell growth between different runs of a characterisation experiment. For this reason, use of the biosensors in this work for analysis of cell culture samples currently always needs to include a set of standard known lactate concentrations to create a dose-response curve for a given experiment. However, this is also the case for most current methods used for metabolite analysis, including the BioProfiler, enzyme assay kits and HPLC methods. Because of the growth differences, it can be complicated to combine datasets collected in separate experimental repeats. There is ongoing work by Catherine Ainsworth (CSynBI, Imperial College London) to address this problem that occurs in many areas of synthetic biology by using novel data analysis methods.

For these biosensors to be used in industrial contexts, changes to the process technology would need to be considered as well as any regulations from governing bodies concerning the use of bacterial cells in this context. The system would be an example of contained use of genetically engineered organisms.

Many of the sensors shown here work well as biosensors and with some further characterisation and optimisation could be used in many contexts including mammalian cell culture. All the parts shown also represent bioparts that have been characterised under specific sets of conditions and that could be used by the synthetic biology community. The biosensors presented in this work (for lactate, leucine, alanine and glutamine) contribute to the field of biosensors and bioprocess monitoring.

Proteins and peptides are involved in many biological processes and represent many important biopharmaceuticals. Amino acids are the monomers of these proteins. This is why ways to calculate the concentration ranges of amino acids in cell culture are of vital importance (Mustafi et al., 2011; Rollié et al., 2012; Kyriakopoulos, 2014). While the use of *in vivo* biosensors in complex contexts is still a nascent technology, it offers exciting prospects for the future (Bracewell and Polizzi, 2014).



## 10.4 Future work

There are many further lines of investigation that were opened up by the results shown in this investigation. These are discussed below. The main themes are that the biosensors that are shown this work could be progressed to a fully functional stage for monitoring mammalian cell cultures and that the biosensors could be developed towards more complex applications.

The biosensors presented here could be progressed to a stage of being fully usable for monitoring mammalian cell cultures. While the lactate biosensor was the most promising biosensor in this work in terms of the sensing characteristics, the *B. subtilis* glutamine sensor and several of the Lrp promoters also showed promising results. Further work on the characterisation could lead to these sensors also being used on cell culture samples.

Many further cell culture samples could be analysed using the sensors shown here. There are currently plans for a CHO cell culture in collaboration with Cher Goey (Chemical Engineering, Imperial College London) during which supernatant samples will be collected. These samples will be analysed using the Bioprofile Analyzer as well as the lactate biosensor presented here. This will allow for further cross-validation of the biosensor. This culture will use a bioreactor as opposed to the cell cultures shown in this work, which were based in flasks. This will make the culture conditions more relevant to industrial contexts. As the bioreactor represents a very different cellular environment compared to flasks, the dynamics seen in these samples may be very different to those shown in this study.

During the glutamine requirement experiment (see Chapter: Hybridoma cell culture characteristics) culture supernatant samples were collected and stored. These samples will be analysed using the biosensors in this work.

The biosensor systems could be modelled mathematically, which is an important part of the synthetic biology design cycle. Understanding the underlying mechanisms of the biosensors well enough to allow for prediction would greatly improve the applicability of the sensors. Preliminary attempts have been made to write a mathematical model describing the mechanism of the lactate sensor. The main limitation here is knowledge of biochemical parameters for the different systems.

As discussed above, there are a number of ways the statistical data analysis for determining the lactate concentration in cell culture samples could be improved. For instance, by using integration over the GFP synthesis rate graphs to create the dose-response curve instead of just picking an end-point value. Different possible

analysis methods will need to be tested and compared to select the most accurate option.

There is also ongoing work by Catherine Ainsworth (CSynBI, Imperial College London) for how to properly combine datasets collected in separate experimental repeats. Currently this can be complicated due to slight differences in cell growth in different runs. Once this work is at a usable stage, it would be interesting to apply it to the data shown herein.

Instead of collecting cell-culture samples for off-line analysis, it would be good to be able to co-culture the mammalian cells with the bacterial biosensor cells for on-line monitoring of cell cultures. In addition, only in a co-culture system could the bacterial biosensor function be linked to a biological response that could feed information back into the mammalian cell culture. There are many factors that need to be considered to create such a system (Goers et al., 2014). According to Mulchandani and Bassi (1995), any scheme for online monitoring needs: 1) a biosensor; 2) a way of making contact between sensor and culture medium; and 3) control system to implement a suitable control strategy.

As mentioned above, the biosensors may not work in certain complex media. Steps would need to be taken to keep the biosensor cells in appropriate conditions. As an example, bacterial and mammalian cells would need to be separated, *e.g.* using a semi-permeable membrane and kept in optimised growth medium. In a system akin to flow injection analysis, the bacterial biosensors could be exposed to small volumes of mammalian cell culture volume. Therefore, while this project concentrated on the biological aspects of the system, the results can inform the kind of bioreactor set-up that will be required *e.g.* to prevent contamination of mammalian cells with bacterial cells. The fact that *B. subtilis* cells show reduced growth in DMEM compared to *E. coli* cells could be useful here for controlling bacterial growth.

Mammalian cell culture is only one of many possible contexts where the metabolite biosensors presented here could be applied to answer important biological questions. The possible examples are countless, including industrial processed and fundamental biology. There are already plans to use the lactate biosensor and a number of the leucine/alanine biosensors to monitor chemical reactions involving these compounds.

Similarly, there are currently plans and ongoing work to expand the use of the biosensors used here. This includes work towards using the sensors in a cell-free *in vitro* context or directly inside mammalian cells.

The strategy used in this work for engineering metabolic biosensors could be applied to other natural sensing systems, for example those listed in Table 1.3 (see Chapter:

Introduction). Over the course of this work, the procedure used for engineering biosensors was optimised extensively. This means that if this procedure were applied to a new potential biosensor system, results could likely be obtained much faster than was the case in this study. However, there are some ways in which the procedure used in this work to create biosensors could be further improved in order to get results faster. For instance, instead of the BioBrick method, faster DNA assembly methods could be used, such as the Gibson method.

The functions of several biosensors could be combined. Mammalian cell culture is a complex environment. There may not be a single limiting factor to cell growth and productivity, but several parameters may need to be considered. Using synthetic biology logic gates based on DNA constructs (Miyamoto et al., 2012) it is possible to detect several metabolites and integrate the information into a cellular response. Statistical methods such as principal component analysis (PCA) could be used to determine suitable combinations of targets.

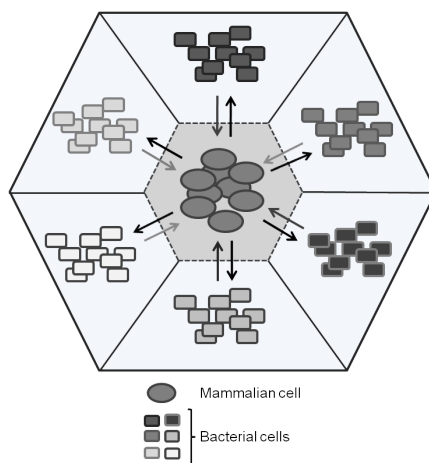
Results may also suggest ways to design artificial metabolite-responsive promoters of predictable strength by combining certain transcription factor binding sites in the same promoter. This has previously been done for the *glnAp2* promoter (Bulter et al., 2004).

By combining the function of several biosensors, certain limitations could be overcome. For example the fact that the *Lrp* biosensors shown here are responsive to both leucine and alanine. If either a specific leucine or alanine biosensor could be constructed then both metabolites could be monitored.

By monitoring several parameters, very complex biological processes can be studied (Goers et al., 2013). Correlation analysis and other statistical methods could be used to find a suitable combination of parameters for a given biological process to be studied.

Finally, these whole-cell bacterial biosensors have great potential. By linking the detection step to a transcriptional output, the bacterial cells could directly respond to the information by changing culture conditions. This could lead to a low-cost artificial symbiosis system.

If successful, the artificial symbiosis system could lead to a new kind of modular bioreactor, in which different bacterial cell cultures can be used to provide nutrients or remove waste products on demand for a mammalian cell culture (see Fig. 10.1).



**Figure 10.1:** Possible future bioreactor design using mammalian-bacterial artificial symbioses. In this system, each of the different bacterial populations produce nutrients for the mammalian cells in response to a target population-specific signal

## References

- Aguilera, L., Campos, E., Gimenez, R., Badia, J., Aguilar, J. and Baldoma, L. (2008), ‘Dual role of LldR in regulation of the lldPRD operon, involved in L-lactate metabolism in *Escherichia coli*’, *J. Bacteriol.* **190**(8), 2997–3005.
- Aleksic, J., Bizzari, F., Cai, Y., Davidson, B., De Mora, K., Ivakhno, S., Seshasayee, S., Nicholson, J., Wilson, J. and Elfick, A. (2007), ‘Development of a novel biosensor for the detection of arsenic in drinking water’, *IET Synthetic Biology* **1**(1), 87–90.
- Altamirano, C., Illanes, A., Casablanco, A., Gámez, X., Cairó, J. J. and Gòdia, C. (2001), ‘Analysis of CHO cells metabolic redistribution in a glutamate-based defined medium in continuous culture’, *Biotechnology Progress* **17**(6), 1032–1041.
- Altamirano, C., Paredes, C., Cairó, J. and Gòdia, F. (2000), ‘Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine’, *Biotechnology Progress* **16**(1), 69–75.
- Altamirano, C., Paredes, C., Illanes, A., Cairó, J. J. and Gòdia, F. (2004), ‘Strategies for fed-batch cultivation of t-PA producing CHO cells: substitution of glucose and glutamine and rational design of culture medium’, *Journal of Biotechnology* **110**(2), 171–179.
- Ames, T. D. and Breaker, R. R. (2011), ‘Bacterial aptamers that selectively bind glutamine’, *RNA Biology* **8**(1), 8.
- Andersen, D. C. and Goochee, C. F. (1995), ‘The effect of ammonia on the O-linked glycosylation of granulocyte colony-stimulating factor produced by chinese hamster ovary cells’, *Biotechnology and Bioengineering* **47**(1), 96–105.
- Atkinson, M. R., Savageau, M. A., Myers, J. T. and Ninfa, A. J. (2003), ‘Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli*’, *Cell* **113**(5), 597–607.
- Ausländer, S. and Fussenegger, M. (2013), ‘From gene switches to mammalian designer cells: present and future prospects’, *Trends in Biotechnology* **31**(3), 155–168.
- Bacchus, W. and Fussenegger, M. (2013), ‘Engineering of synthetic intercellular

- communication systems', *Metabolic Engineering* **16**(0), 33–41. (doi: 10.1016/j.ymben.2012.12.001).
- Barker, M. M., Gaal, T., Josaitis, C. A. and Gourse, R. L. (2001), 'Mechanism of regulation of transcription initiation by ppGpp. i. effects of ppGpp on transcription initiation *in vivo* and *in vitro*', *Journal of Molecular Biology* **305**(4), 673–688.
- Barngrover, D., Thomas, J. and Thilly, W. (1985), 'High density mammalian cell growth in Leibovitz bicarbonate-free medium: effects of fructose and galactose on culture biochemistry', *J Cell Sci* **78**(1), 173–189.
- Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H. and Weiss, R. (2005), 'A synthetic multicellular system for programmed pattern formation', *Nature* **434**(7037), 1130–1134.
- Beck, A., Wurch, T., Bailly, C. and Corvaia, N. (2010), 'Strategies and challenges for the next generation of therapeutic antibodies', *Nat Rev Immunol* **10**(5), 1474–1733.
- Behjousiar, A., Kontoravdi, C. and Polizzi, K. (2012), 'In situ monitoring of intracellular glucose and glutamine in CHO cell culture', *PLoS ONE* **7**(4).
- Belkin, S. (2003), 'Microbial whole-cell sensing systems of environmental pollutants', *Current Opinion in Microbiology* **6**(3), 206–212.
- Bell, S. L., Bebbington, C., Scott, M. F., Wardell, J. N., Spier, R. E., Bushell, M. E. and Sanders, P. G. (1995), 'Genetic engineering of hybridoma glutamine metabolism', *Enzyme and Microbial Technology* **17**(2), 98–106.
- Berezhnoy, A. Y., Shckorbatov, Y. G. and Hisanori, K. (2006), 'Regularities in the *E. coli* promoters composition in connection with the DNA strands interaction and promoter activity', *Journal of Zhejiang University SCIENCE B* **7**(12), 969–973.
- Bonarius, H. P. J., Hatzimanikatis, V., Meesters, K. P. H., de Gooijer, C. D., Schmid, G. and Tramper, J. (1996), 'Metabolic flux analysis of hybridoma cells in different culture media using mass balances', *Biotechnology and Bioengineering* **50**(3), 299–318.
- Bracewell, D. G. and Polizzi, K. M. (2014), 'The future for biosensors in biopharmaceutical production', *Pharmaceutical Bioprocessing* **2**(2), 121–124.
- Bulter, T., Lee, S.-G., Wong, W. W., Fung, E., Connor, M. R. and Liao, J. C. (2004), 'Design of artificial cell-cell communication using gene and metabolic networks',

*Proceedings of the National Academy of Sciences of the United States of America* **101**(8), 2299–2304.

Burleigh, S., van de Laar, T., Stroop, C., van Grunsven, W., O'Donoghue, N., Rudd, P. and Davey, G. (2011), 'Synergizing metabolic flux analysis and nucleotide sugar metabolism to understand the control of glycosylation of recombinant protein in CHO cells', *BMC biotechnology* **11**(1), 95.

Butler, M. (1987), *Growth limitations in microcarrier cultures*, Vertebrate Cell Culture I.

Butler, M. and Christie, A. (1994), 'Adaptation of mammalian cells to non-ammoniaogenic media', *Cytotechnology* **15**(1), 87–94.

Calvo, J. M. and Matthews, R. G. (1994), 'The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*', *Microbiol. Mol. Biol. Rev.* **58**(3), 466–490.

Canton, B., Labno, A. and Endy, D. (2008), 'Refinement and standardization of synthetic biological parts and devices', *Nature Biotechnology* **26**(7), 787–793.

Cariati, F., Morazzoni, F., Zanderighi, G. M., Marcotrigiano, G. and Pellacani, G. C. (1977), 'Chelating properties of lactate anion. perturbing effect of additional ligands on bis(dl-lactato)-metal(ii) complexes', *Inorganica Chimica Acta* **21**(0), 133–140.

Carmona, M., Fernandez, S., Rodriguez, M. J. and de Lorenzo, V. (2005), 'm-xylene-responsive pu-pnifh hybrid 54 promoters that overcome physiological control in *Pseudomonas putida* kt2442', *Journal of Bacteriology* **187**(1), 125–134.

Carter, P., Kelley, R. F., Rodrigues, M. L., Snedecor, B., Covarrubias, M., Velligan, M. D., Wong, W. L. T., Rowland, A. M., Kotts, C. E., Carver, M. E., Yang, M., Bourell, J. H., Shepard, H. M. and Henner, D. (1992), 'High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment', *Nature Biotechnology* **10**(2), 163–167.

Cattaneo, M. V., Luong, J. H. T. and Mercille, S. (1992), 'Monitoring glutamine in mammalian cell cultures using an amperometric biosensor', *Biosensors and Bioelectronics* **7**(5), 329–334.

Cattaneo, M. V., Male, K. B. and Luong, J. H. T. (1992), 'A chemiluminescence fiber-optic biosensor system for the determination of glutamine in mammalian cell cultures', *Biosensors and Bioelectronics* **7**(8), 569–574.

- Chang, Y., Grodzinsky, A. and Wang, D. (1995), 'In situ removal of ammonium and lactate through electrical means for hybridoma cultures', *Biotechnology and Bioengineering* **47**(3), 308–318.
- Chappell, J., Jensen, K. and Freemont, P. S. (2013), 'Validation of an entirely *in vitro* approach for rapid prototyping of DNA regulatory elements for synthetic biology', *Nucleic Acids Research* .
- Charbit, A. (1996), 'Coordination of carbon and nitrogen metabolism', *Research in Microbiology* **147**(6-7), 513–518.
- Checa, S. K., Zurbriggen, M. D. and Soncini, F. C. (2012), 'Bacterial signaling systems as platforms for rational design of new generations of biosensors', *Current Opinion in Biotechnology* **23**(5), 766–772.
- Chen, S., Iannolo, M. and Calvo, J. M. (2005), 'Cooperative binding of the leucine-responsive regulatory protein (Lrp) to DNA', *Journal of Molecular Biology* **345**(2), 251–264.
- Cho, B.-K., Barrett, C. L., Knight, E. M., Park, Y. S. and Palsson, B. Ø. (2008), 'Genome-scale reconstruction of the Lrp regulatory network in *Escherichia coli*', *Proceedings of the National Academy of Sciences* **105**(49), 19462–19467.
- Choi, Y., Lee, D., Kim, I., Kang, S., Ahn, K., Kim, H., Jeong, Y., Chun, G., Park, J. and Kim, I. (2000), 'Ammonia removal using hepatoma cells in mammalian cell cultures', *Biotechnology Progress* **16**(5), 760–768.
- Christie, A. and Butler, M. (1994a), 'Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase', *Journal of Biotechnology* **37**(3), 277–290.
- Christie, A. and Butler, M. (1994b), 'Growth and metabolism of a murine hybridoma in cultures containing glutamine-based dipeptides', *Focus* **16**, 9–13.
- Chuang, J. S., Rivoire, O. and Leibler, S. (2010), 'Cooperation and Hamilton's rule in a simple synthetic microbial system', *Molecular Systems Biology* **6**. (doi: 10.1038/msb.2010.57).
- Church, G. M., Elowitz, M. B., Smolke, C. D., Voigt, C. A. and Weiss, R. (2014), 'Realizing the potential of synthetic biology', *Nat Rev Mol Cell Biol* **15**(4), 289–294.
- Coco-Martin, J. M. and Harmsen, M. M. (2008), 'A review of therapeutic protein expression by mammalian cells.', *BioProcess International* **6**(28).



- Coggin, JH, J. and Martin, W. (1965), '6-Diazo-5-Oxo-L-Norleucine inhibition of *Escherichia coli*', *J Bacteriol.* **89**(5), 1348–1353.
- Dalchau, N., Smith, M. J., Martin, S., Brown, J. R., Emmott, S. and Phillips, A. (2012), 'Towards the rational design of synthetic cells with prescribed population dynamics', *Journal of the Royal Society Interface* **9**(76), 2883–2898. (doi: 10.1098/rsif.2012.0280).
- Darwent, M. J., Paterson, E., McDonald, A. J. S. and Tomos, A. D. (2003), 'Biosensor reporting of root exudation from *Hordeum vulgare* in relation to shoot nitrate concentration', *Journal of Experimental Botany* **54**(381), 325–334.
- Dattelbaum, J. D. and Lakowicz, J. R. (2001), 'Optical determination of glutamine using a genetically engineered protein', *Analytical Biochemistry* **291**(1), 89–95.
- Daunert, S., Barrett, G., Feliciano, J. S., Shetty, R. S., Shrestha, S. and Smith-Spencer, W. (2000), 'Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes', *Chemical Reviews* **100**(7), 2705–2738.
- De Lorimier, R. M., Smith, J. J., Dwyer, M. A., Looger, L. L., Sali, K. M., Paavola, C. D., Rizk, S. S., Sadigov, S., Conrad, D. W., Loew, L. and Hellinga, H. W. (2002), 'Construction of a fluorescent biosensor family', *Protein Science* **11**(11), 2655–2675.
- Dehli, T., Solem, C. and Jensen, P. R. (2012), *Tunable promoters in synthetic and systems biology*, Springer, pp. 181–201.
- del Val, I., Kontoravdi, C. and Nagy, J. (2010), 'Towards the implementation of quality by design to the production of therapeutic monoclonal antibodies with desired glycosylation patterns', *Biotechnology Progress* **26**(6), 1505–1527.
- Deparis, V., Durrieu, C., Schweizer, M., Marc, I., Goergen, J. L., Chevalot, I. and Marc, A. (2003), 'Promoting effect of rapeseed proteins and peptides on Sf9 insect cell growth', *Cytotechnology* **42**(2), 75–85.
- Derfus, G. E., Abramzon, D., Tung, M., Chang, D., Kiss, R. and Amanullah, A. (2010), 'Cell culture monitoring via an auto-sampler and an integrated multi-functional off-line analyzer', *Biotechnology Progress* **26**(1), 284–292.
- Desai, S. K. and Gallivan, J. P. (2004), 'Genetic screens and selections for small molecules based on a synthetic riboswitch that activates protein translation', *Journal of the American Chemical Society* **126**(41), 13247–13254.
- Dion, H. W., Fusari, S. A., Jakubowski, Z. L., Zora, J. G. and Bartz, Q. R. (1956),

- '6-diazo-5-oxo-l-norleucine, a new tumor-inhibitory substance. ii. isolation and characterization', *Journal of the American Chemical Society* **78**(13), 3075–3077.
- Dong, J., Taylor, J., Latour, D., Iuchi, S. and Lin, E. (1993), 'Three overlapping lct genes involved in L-lactate utilization by *Escherichia coli*', *Journal of Bacteriology* **175**(20), 6671–6678.
- Dubey, G. P. and Ben-Yehuda, S. (2011), 'Intercellular nanotubes mediate bacterial communication', *Cell* **144**(4), 590–600. 10.1016/j.cell.2011.01.015.
- Dwyer, M. A. and Hellinga, H. W. (2004), 'Periplasmic binding proteins: a versatile superfamily for protein engineering', *Current Opinion in Structural Biology* **14**(4), 495–504.
- Eagle, H. (1956), 'The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid', *Journal of Biological Chemistry* **218**(2), 607–616.
- Ellis, T., Adie, T. and Baldwin, G. S. (2011), 'DNA assembly for synthetic biology: from parts to pathways and beyond', *Integrative Biology* **3**(2), 109–118.
- Ernsting, B., Atkinson, M., Ninfa, A. and Matthews, R. (1992), 'Characterization of the regulon controlled by the leucine-responsive regulatory protein in *Escherichia coli*', *Journal of Bacteriology* **174**(4), 1109–1118.
- Europa, A. F., Gambhir, A., Fu, P.-C. and Hu, W.-S. (2000), 'Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells', *Biotechnology and Bioengineering* **67**(1), 25–34.
- Fabret, C., Feher, V. A. and Hoch, J. A. (1999), 'Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world', *Journal of Bacteriology* **181**(7), 1975–1983.
- Fehr, M., Ehrhardt, D. W., Lalonde, S. and Frommer, W. B. (2004), 'Minimally invasive dynamic imaging of ions and metabolites in living cells', *Current Opinion in Plant Biology* **7**(3), 345–351.
- Ferguson, B. S., Hoggarth, D. A., Maliniak, D., Ploense, K., White, R. J., Woodward, N., Hsieh, K., Bonham, A. J., Eisenstein, M., Kippin, T. E., Plaxco, K. W. and Soh, H. T. (2013), 'Real-time, aptamer-based tracking of circulating therapeutic agents in living animals', *Science Translational Medicine* **5**(213), 213ra165.
- Fernandez, E. J., Mancuso, A. and Clark, D. S. (1988), 'NMR spectroscopy studies

- of hybridoma metabolism in a simple membrane reactor', *Biotechnology Progress* **4**(3), 173–183.
- Forchhammer, K. (2007), 'Glutamine signalling in bacteria', *Frontiers in Bioscience* **12**, 358–370.
- Freemont, P. S. and Kitney, R. I. (2012), *Synthetic Biology: A Primer*, World Scientific.
- French, C. E., de Mora, K., Joshi, N., Elfickssible, A., Haseloff, J. and Ajioka, J. (2011), 'Synthetic biology and the art of biosensor design'.
- French, C. and Gwenin, C. (2012), *Biosensors in bioprocess monitoring and control*, CRC Press-Taylor & Francis Group, Boca Raton, FL, pp. 369–402.
- Gao, C., Hu, C., Zheng, Z., Ma, C., Jiang, T., Dou, P., Zhang, W., Che, B., Wang, Y. and Lv, M. (2012), 'Lactate utilization is regulated by the FadR-type regulator LldR in *Pseudomonas aeruginosa*', *Journal of Bacteriology* **194**(10), 2687–2692.
- Gao, Y.-G., Suzuki, H., Ito, H., Zhou, Y., Tanaka, Y., Wachi, M., Watanabe, N., Tanaka, I. and Yao, M. (2008), 'Structural and functional characterization of the LldR from *Corynebacterium glutamicum*: a transcriptional repressor involved in L-lactate and sugar utilization', *Nucleic Acids Research* **36**(22), 7110–7123.
- Gardner, T. S., Cantor, C. R. and Collins, J. J. (2000), 'Construction of a genetic toggle switch in *Escherichia coli*', *Nature* **403**(6767), 339–342.
- Genzel, Y., Ritter, J. B., Konig, S., Alt, R. and Reichl, U. (2005), 'Substitution of glutamine by pyruvate to reduce ammonia formation and growth inhibition of mammalian cells', *Biotechnology Progress* **21**(1), 58–69.
- Georgi, T., Engels, V. and Wendisch, V. F. (2008), 'Regulation of L-lactate utilization by the FadR-type regulator LldR of *Corynebacterium glutamicum*', *Journal of Bacteriology* **190**(3), 963–971.
- Glacken, M., Fleischaker, R. and Sinskey, A. (1986), 'Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells', *Biotechnology and Bioengineering* **28**(9), 1376–1389.
- Goers, L., Freemont, P. and Polizzi, K. M. (2014), 'Co-culture systems and technologies: taking synthetic biology to the next level', *Journal of the Royal Society Interface* **11**(96).
- Goers, L., Kylilis, N., Tomazou, M., Yan Wen, K., Freemont, P. and Polizzi,

- K. (2013), *Engineering microbial biosensors*, Vol. Volume 40, Academic Press, chapter 5, pp. 119–156.
- Gu, X., Trybilo, M., Ramsay, S., Jensen, M., Fulton, R., Rosser, S. and Gilbert, D. (2010), ‘Engineering a novel self-powering electrochemical biosensor’, *Systems and synthetic biology* **4**(3), 203–214.
- Hägström, L. (1991), *Energetics of glutaminolysis - a theoretical evaluation*, Butterworth-Heinemann Ltd., Oxford, pp. 79–81.
- Harms, P., Kostov, Y. and Rao, G. (2002), ‘Bioprocess monitoring’, *Current Opinion in Biotechnology* **13**(2), 124–127.
- Hart, B. R. and Blumenthal, R. M. (2011), ‘Unexpected coregulator range for the global regulator Lrp of *Escherichia coli* and *Proteus mirabilis*’, *Journal of Bacteriology* **193**(5), 1054–1064.
- Hartman, S. C. (1971), *Glutaminases and  $\gamma$ -glutamyltransferases*, Vol. IV - Hydrolysis; Other C-N bonds, phosphate esters, 3rd edn, Academic Press, Inc., chapter 4, pp. 79–98.
- Harwood, C. and Cutting, S. (1990), *Molecular biological methods for Bacillus*, Wiley, Chichester, UK.
- Hassell, T. and Butler, M. (1990), ‘Adaptation to non-ammoniogenic medium and selective substrate feeding lead to enhanced yields in animal cell cultures’, *Journal of Cell Science* **96**(3), 501–508.
- Hermann, T. (2003), ‘Industrial production of amino acids by *Coryneform* bacteria’, *Journal of Biotechnology* **104**(1-3), 155–172.
- Hirst, C. D. (2014), Automated BioPart characterisation for synthetic biology., PhD thesis.
- Hu, W. S. and Aunins, J. G. (1997), ‘Large-scale mammalian cell culture’, *Current Opinion in Biotechnology* **8**(2), 148–153.
- Hu, W.-S. and Himes, V. (1989), *Stoichiometric considerations of mammalian cell metabolism in bioreactors.*, Springer-Verlag, Berlin/Weis York, pp. 33–45.
- Hua, Q., Joyce, A. R., Palsson, B. Ø. and Fong, S. S. (2007), ‘Metabolic characterization of *Escherichia coli* strains adapted to growth on lactate’, *Applied and environmental microbiology* **73**(14), 4639–4647.
- Iida, K.-I., Mizunoe, Y., Wai, S. N. and Yoshida, S.-I. (2001), ‘Type 1 fimbriation

- and its phase switching in diarrheagenic *Escherichia coli* strains', *Clinical and diagnostic laboratory immunology* **8**(3), 489–495.
- Iio, M., Moriyama, A. and Murakami, H. (1985), *Effects on cell proliferation of metabolites produced by cultured cells and their removal from culture in defined media*, Springer-Verlag, New York, pp. 437–442.
- Inaba, Y., Mizukami, K., Hamada-Sato, N., Kobayashi, T., Imada, C. and Watanabe, E. (2003), 'Development of a D-alanine sensor for the monitoring of a fermentation using the improved selectivity by the combination of D-amino acid oxidase and pyruvate oxidase', *Biosensors and Bioelectronics* **19**(5), 423–431.
- Iuchi, S., Aristarkhov, A., Dong, J., Taylor, J. and Lin, E. (1994), 'Effects of nitrate respiration on expression of the Arc-controlled operons encoding succinate dehydrogenase and flavin-linked L-lactate dehydrogenase', *Journal of Bacteriology* **176**(6), 1695–1701.
- Jayapal, K., Wlaschin, K., Hu, W. and Yap, M. (2007), 'Recombinant protein therapeutics from CHO cells – 20 years and counting', *Chemical engineering progress* **103**(10), 40–47.
- Jenkins, H. A., Butler, M. and Dickson, A. J. (1992), 'Characterization of glutamine metabolism in two related murine hybridomas', *Journal of Biotechnology* **23**(2), 167–182.
- Jeong, Y. and Wang, S. (1995), 'Role of glutamine in hybridoma cell culture: effects on cell growth, antibody production, and cell metabolism', *Enzyme and Microbial Technology* **17**(1), 47–55.
- Jin, T. and Inouye, M. (1993), 'Ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the signaling domain of the hybrid *Escherichia coli* transmembrane receptor, Taz1', *Journal of molecular biology* **232**(2), 484–492.
- Juurlink, B. H. J. (1987), 'Effects of medium glutamine, glutamate, and ammonia on glutamine synthetase activity in cultured mouse astroglial cells', *Neurochemical Research* **12**(12), 1053–1058.
- Kaltenbach, L., Braaten, B., Tucker, J., Krabbe, M. and Low, D. (1998), 'Use of a two-color genetic screen to identify a domain of the global regulator Lrp that is specifically required for pap phase variation', *Journal of Bacteriology* **180**(5), 1224–1231.

- Kampf, M. M. and Weber, W. (2010), ‘Synthetic biology in the analysis and engineering of signaling processes’, *Integrative Biology* **2**(1), 12–24.
- Kaper, T., Looger, L. L., Takanaga, H., Platten, M., Steinman, L. and Frommer, W. B. (2007), ‘Nanosensor detection of an immunoregulatory tryptophan influx/kynurenine efflux cycle’, *Plos Biology* **5**(10), e257.
- Kelly, J. R., Rubin, A. J., Davis, J. H., Ajo-Franklin, C. M., Cumbers, J., Czar, M. J., de Mora, K., Glielberman, A. L., Monie, D. D. and Endy, D. (2009), ‘Measuring the activity of BioBrick promoters using an *in vivo* reference standard’, *J Biol Eng* **3**, 4.
- Khedouri, E., Anderson, P. M. and Meister, A. (1966), ‘Selective inactivation of the glutamine binding site of *Escherichia coli* carbamyl phosphate synthetase by 2-amino-4-oxo-5-chloropentanoic acid’, *Biochemistry* **5**(11), 3552–3557.
- Kiryu, H., Oshima, T. and Asai, K. (2005), ‘Extracting relations between promoter sequences and their strengths from microarray data’, *Bioinformatics* **21**(7), 1062–1068.
- Kitney, R. and Freemont, P. (2012), ‘Synthetic biology – the state of play’, *FEBS Letters* **586**(15), 2029–2036. (doi: 10.1016/j.febslet.2012.06.002).
- Kobayashi, K., Ogura, M., Yamaguchi, H., Yoshida, K.-I., Ogasawara, N., Tanaka, T. and Fujita, Y. (2001), ‘Comprehensive DNA microarray analysis of *Bacillus subtilis* two-component regulatory systems’, *Journal of Bacteriology* **183**(24), 7365–7370.
- Kondragunta, B., Han, J., Joshi, B. H., Brorson, K. A., Puri, R. K., Uplekar, S., Moreira, A. R. and Rao, G. (2012), ‘Genomic analysis of a hybridoma batch cell culture metabolic status in a standard laboratory 5 L bioreactor’, *Biotechnology Progress* **28**(5), 1126–1137.
- Kontoravdi, C. (2007), An integrated modelling/ experimental framework for protein-producing cell cultures, PhD thesis.
- Kontoravdi, C., Asprey, S. P., Pistikopoulos, E. N. and Mantalaris, A. (2007), ‘Development of a dynamic model of monoclonal antibody production and glycosylation for product quality monitoring’, *Computers and Chemical Engineering* **31**(5-6), 392–400.
- Kontoravdi, C., Wong, D., Lam, C., Lee, Y. Y., Yap, M. G. S., Pistikopoulos, E. N. and Mantalaris, A. (2007), ‘Modeling amino acid metabolism in mammalian cells-

- toward the development of a model library’, *Biotechnology Progress* **23**(6), 1261–1269.
- Krebs, H. A. (1980), *Glutamine metabolism in the animal body.*, Academic Press, New York, NY, p. 319329.
- Kremling, A., Heermann, R., Centler, F., Jung, K. and Gilles, E. D. (2004), ‘Analysis of two-component signal transduction by mathematical modeling using the KdpD/KdpE system of *Escherichia coli*’, *Biosystems* **78**(13), 23–37.
- Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Cummings, N. J., Daniel, R. A., Denizot, F., Devine, K. M., Dusterhoft, A., Ehrlich, S. D., Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S. Y., Glaser, P., Goffeau, A., Golightly, E. J., Grandi, G., Guiseppi, G., Guy, B. J., Haga, K., Haiech, J., Harwood, C. R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M. F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S. M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medigue, C., Medina, N., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O’Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S. H., Parro, V., Pohl, T. M., Portetelle, D., Porwollik, S., Prescott, A. M., Presecan, E., Pujic, P., Purnelle, B. et al. (1997), ‘The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*’, *Nature* **390**(6657), 249–256. 10.1038/36786.
- Kurano, N., Leist, C., Messi, F., Kurano, S. and Fiechter, A. (1990), ‘Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products’, *Journal of Biotechnology* **15**(1-2), 113–128.
- Kwan, R. C. H., Hon, P. Y. T. and Renneberg, R. (2004), ‘Amperometric biosensor for rapid determination of alanine’, *Analytica Chimica Acta* **523**(1), 81–88.
- Kyriakopoulos, S. (2014), Amino acid metabolism in Chinese hamster ovary cell culture, PhD thesis.
- Kyriakopoulos, S. and Kontoravdi, C. (2013), ‘Analysis of the landscape of

- biologically-derived pharmaceuticals in europe: Dominant production systems, molecule types on the rise and approval trends', *European Journal of Pharmaceutical Sciences* **48**(3), 428–441.
- Lee, T., Krupa, R., Zhang, F., Hajimorad, M., Holtz, W., Prasad, N., Lee, S. and Keasling, J. (2011), 'BglBrick vectors and datasheets: A synthetic biology platform for gene expression', *Journal of Biological Engineering* **5**(1), 1–14.
- Lee, Y. Y., Yap, M. G. S., Hu, W.-S. and Wong, K. T. K. (2003), 'Low-glutamine fed-batch cultures of 293-HEK serum-free suspension cells for adenovirus production', *Biotechnology Progress* **19**(2), 501–509.
- Li, Y., Lee, K. K., Walsh, S., Smith, C., Hadingham, S., Sorefan, K., Cawley, G. and Bevan, M. W. (2006), 'Establishing glucose- and ABA-regulated transcription networks in *Arabidopsis* by microarray analysis and promoter classification using a relevance vector machine', *Genome Research* **16**(3), 414–427.
- Linz, M., Zeng, A.-P., Wagner, R. and Deckwer, W.-D. (1997), 'Stoichiometry, kinetics, and regulation of glucose and amino acid metabolism of a recombinant BHK cell line in batch and continuous cultures', *Biotechnology Progress* **13**(4), 453–463.
- Liu, X. and De Wulf, P. (2004), 'Probing the ArcA-P modulon of *Escherichia coli* by whole genome transcriptional analysis and sequence recognition profiling', *Journal of Biological Chemistry* **279**(13), 12588–12597.
- Ljunggren, J. and Häggström, L. (1990), 'Glutamine limited fed-batch culture reduces ammonium ion production in animal cells', *Biotechnology letters* **12**(10), 705–710.
- Ljunggren, J. and Häggström, L. (1994), 'Catabolic control of hybridoma cells by glucose and glutamine limited fed batch cultures', *Biotechnology and Bioengineering* **44**(7), 808–818.
- Lynch, A. S. and Lin, E. (1996), 'Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters', *Journal of Bacteriology* **178**(21), 6238–6249.
- Maheswaran, M. and Forchhammer, K. (2003), 'Carbon-source-dependent nitrogen regulation in *Escherichia coli* is mediated through glutamine-dependent GlnB signalling', *Microbiology* **149**(8), 2163–2172.
- Malakar, P. and Venkatesh, K. (2012), 'Effect of substrate and iptg concentrations



- on the burden to growth of *Escherichia coli* on glycerol due to the expression of lac proteins', *Applied Microbiology and Biotechnology* **93**(6), 2543–2549.
- Male, K. B., Gartu, P. O., Kamen, A. A. and Luong, J. H. T. (1997), 'A flow-injection (FI) mediated biosensor for on-line monitoring of lactate in mammalian cell culture', *Analytica Chimica Acta* **351**(1-3), 159–167.
- Mandal, M., Lee, M., Barrick, J. E., Weinberg, Z., Emilsson, G. M., Ruzzo, W. L. and Breaker, R. R. (2004), 'A glycine-dependent riboswitch that uses cooperative binding to control gene expression', *Science* **306**(5694), 275–279.
- Marchisio, M. A. and Rudolf, F. (2011), 'Synthetic biosensing systems', *The International Journal of Biochemistry and Cell Biology* **43**(3), 310–319.
- Martinez-Vaz, B. M., Makarevitch, I. and Stensland, S. (2010), 'Studying gene expression: database searches and promoter fusions to investigate transcriptional regulation in bacteria', *Journal of Microbiology & Biology Education: JMBE* **11**(1), 42.
- Marvin, J. S. and Hellinga, H. W. (1998), 'Engineering biosensors by introducing fluorescent allosteric signal transducers: construction of a novel glucose sensor', *Journal of the American Chemical Society* **120**(1), 7–11.
- Matsumura, M. and Nayve, F. R. P. (1995), 'Effects of ammonium ion removal on growth and mab production of hybridoma cells', *Cytotechnology* **18**(1), 35–50.
- Mayer, C., Frauer, A., Schalkhammer, T. and Pittner, F. (1999), 'Enzyme-based flow injection analysis system for glutamine and glutamate in mammalian cell culture media', *Analytical Biochemistry* **268**(1), 110–116.
- McDermott, R. and Butler, M. (1993), 'Uptake of glutamate, not glutamine synthetase, regulates adaptation of mammalian cells to glutamine-free medium', *J Cell Sci* **104**(1), 51–58.
- Meleady, P. (2007), 'Proteomic profiling of recombinant cells from large-scale mammalian cell culture processes', *Cytotechnology* **53**(1), 23–31.
- Miller, W., Wilke, C. and Blanch, H. (1988), 'Transient responses of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture', *Bioprocess and Biosystems Engineering* **3**(3), 113–122.
- Miyamoto, T., Razavi, S., DeRose, R. and Inoue, T. (2012), 'Synthesizing biomolecule-based boolean logic gates', *ACS Synthetic Biology* **2**(2), 72–82.
- Mulchandani, A. and Bassi, A. S. (1995), 'Principles and applications of

- biosensors for bioprocess monitoring and control’, *Critical reviews in biotechnology* **15**(2), 105–124.
- Mulchandani, A. and Bassi, A. S. (1996), ‘Determination of glutamine and glutamic acid in mammalian cell cultures using tetrathiafulvalene modified enzyme electrodes’, *Biosensors and Bioelectronics* **11**(3), 271–280.
- Mullard, A. (2011), ‘2010 FDA drug approvals’, *Nat Rev Drug Discov* **10**(2), 82–85.
- Muranaka, N. (2009), ‘Efficient design strategy for whole-cell and cell-free biosensors based on engineered riboswitches’, *Analytical letters* **42**(1), 108.
- Mustafi, N., Grünberger, A., Kohlheyer, D., Bott, M. and Frunzke, J. (2011), ‘The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids’, *Metabolic Engineering* (0).
- Mutalik, V. K. and Venkatesh, K. V. (2007), ‘A theoretical steady state analysis indicates that induction of *Escherichia coli* glnALG operon can display all-or-none behavior’, *Biosystems* **90**(1), 1–19.
- Nadeau, I., Sabati, J., Koehl, M., Perrier, M. and Kamen, A. (2000), ‘Human 293 cell metabolism in low glutamine-supplied culture: interpretation of metabolic changes through metabolic flux analysis’, *Metabolic Engineering* **2**(4), 277–292.
- Newland, M., Greenfield, P. and Reid, S. (1990), ‘Hybridoma growth limitations: The roles of energy metabolism and ammonia production’, *Cytotechnology* **3**(3), 215–229.
- Newman, E. B. (1995), ‘Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli*’, *Annual Review of Microbiology* **49**(1), 747.
- Ninfa, A. J. and Atkinson, M. R. (2000), ‘Pii signal transduction proteins’, *Trends in Microbiology* **8**(4), 172–179.
- Ninfa, A. J., Selinsky, S., Perry, N., Atkins, S., Xiu Song, Q., Mayo, A., Arps, D., Wolf, P. and Atkinson, M. R. (2007), *Using two-component systems and other bacterial regulatory factors for the fabrication of synthetic genetic devices*, Vol. 422, Academic Press, pp. 488–512.
- Nohno, T., Saito, T. and Hong, J.-s. (1986), ‘Cloning and complete nucleotide sequence of the *Escherichia coli* glutamine permease operon (glnHPQ)’, *Molecular and General Genetics MGG* **205**(2), 260–269.
- Núñez, M. F., Kwon, O., Wilson, T. H., Aguilar, J., Baldoma, L. and Lin, E. C. (2002), ‘Transport of l-lactate, d-lactate, and glycolate by the LldP and GlcA

- membrane carriers of *Escherichia coli*', *Biochemical and Biophysical Research Communications* **290**(2), 824–829.
- Núñez, M. F., Pellicer, M. T., Badía, J., Aguilar, J. and Baldomù, L. (2001), 'The gene yghK linked to the glc operon of *Escherichia coli* encodes a permease for glycolate that is structurally and functionally similar to L-lactate permease', *Microbiology* **147**(4), 1069–1077.
- O'Callaghan, P. M. and James, D. C. (2008), 'Systems biotechnology of mammalian cell factories', *Briefings in Functional Genomics and Proteomics* **7**(2), 95–110.
- Ortuño-Olea, L. and Durán-Vargas, S. (2000), 'The L-asparagine operon of *Rhizobium etli* contains a gene encoding an atypical asparaginase', *FEMS Microbiology Letters* **189**(2), 177–182.
- Ozturk, S., Riley, M. and Palsson, B. (1992), 'Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production', *Biotechnology and Bioengineering* **39**(4), 418–431.
- Palmer, A. E., Qin, Y., Park, J. G. and McCombs, J. E. (2011), 'Design and application of genetically encoded biosensors', *Trends in Biotechnology* **29**(3), 144–152.
- Paredes, C., Prats, E., Cairó, J. J., Azorn, F., Cornudella, L. and Gòdia, F. (1999), 'Modification of glucose and glutamine metabolism in hybridoma cells through metabolic engineering', *Cytotechnology* **30**(1), 85–93.
- Park, B.-G., Chun, J.-M., Lee, C.-J., Chun, G.-T., Kim, I.-H. and Jeong, Y.-H. (2000), 'Development of high density mammalian cell culture system for the production of tissue-type plasminogen activator', *Biotechnology and Bioprocess Engineering* **5**(2), 123–129.
- Park, M., Tsai, S.-L. and Chen, W. (2013), 'Microbial biosensors: engineered microorganisms as the sensing machinery', *Sensors* **13**(5), 5777–5795.
- Partridge, J. D., Scott, C., Tang, Y., Poole, R. K. and Green, J. (2006), '*Escherichia coli* transcriptome dynamics during the transition from anaerobic to aerobic conditions', *Journal of Biological Chemistry* **281**(38), 27806–27815.
- Patterson Jr, M. (1979), 'Measurement of growth and viability of cells in culture', *Methods in enzymology* **58**, 141–152.
- Pinchuk, G. E., Rodionov, D. A., Yang, C., Li, X., Osterman, A. L., Dervyn, E., Geydebekht, O. V., Reed, S. B., Romine, M. F. and Collart, F. R. (2009),

- ‘Genomic reconstruction of *Shewanella oneidensis* MR-1 metabolism reveals a previously uncharacterized machinery for lactate utilization’, *Proceedings of the National Academy of Sciences* **106**(8), 2874–2879.
- Pittard, J., Camakaris, H. and Yang, J. (2005), ‘The TyrR regulon’, *Molecular Microbiology* **55**(1), 16–26.
- Purnick, P. E. and Weiss, R. (2009), ‘The second wave of synthetic biology: from modules to systems’, *Nat Rev Mol Cell Biol* **10**(6), 410–22.
- Quay, S. C., Dick, T. E. and Oxender, D. L. (1977), ‘Role of transport systems in amino acid metabolism: leucine toxicity and the branched-chain amino acid transport systems’, *Journal of Bacteriology* **129**(3), 1257–1265.
- Rehm, N. (2010), Biochemische und molekularbiologische Untersuchungen zur Verstoffwechslung von Ammonium und Glutamin in *Corynebacterium glutamicum*, PhD thesis.
- Reichert, J. M., Rosensweig, C. J., Faden, L. B. and Dewitz, M. C. (2005), ‘Monoclonal antibody successes in the clinic’, *Nat Biotech* **23**(9), 1073–1078.
- Reitzer, L. (2003), ‘Nitrogen assimilation and global regulation in *Escherichia coli*’, *Annual Reviews in Microbiology* **57**(1), 155–176.
- Rigali, S., Derouaux, A., Giannotta, F. and Dusart, J. (2002), ‘Subdivision of the helix-turn-helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies’, *Journal of Biological Chemistry* **277**(15), 12507–12515.
- Roguska, K. (2012), Development of a whole-cell biosensor for L-lactate., Master thesis.
- Rohm, I., Genrich, M., Collier, W. and Bilitewski, U. (1996), ‘Development of ultraviolet-polymerizable enzyme pastes: bioprocess applications of screen-printed L-lactate sensors’, *Analyst* **121**(6), 877.
- Rollié, S., Mangold, M. and Sundmacher, K. (2012), ‘Designing biological systems: systems engineering meets synthetic biology’, *Chemical Engineering Science* **69**(1), 1–29. (doi: 10.1016/j.ces.2011.10.068).
- Ronen, M., Rosenberg, R., Shraiman, B. I. and Alon, U. (2002), ‘Assigning numbers to the arrows: Parameterizing a gene regulation network by using accurate expression kinetics’, *Proceedings of the National Academy of Sciences* **99**(16), 10555–10560.
- San Martín, A., Ceballo, S., Ruminot, I., Lerchundi, R., Frommer, W. B. and Barros,

- L. F. (2013), 'A genetically encoded FRET lactate sensor and its use to detect the Warburg effect in single cancer cells', *Plos One* **8**(2), e57712.
- Satomura, T., Shimura, D., Asai, K., Sadaie, Y., Hirooka, K. and Fujita, Y. (2005), 'Enhancement of glutamine utilization in *Bacillus subtilis* through the GlnK-GlnL two-component regulatory system', *J. Bacteriol.* **187**(14), 4813–4821.
- Schlatter, S., Stansfield, S., Dinnis, D., Racher, A., Birch, J. and James, D. (2005), 'On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells', *Biotechnology Progress* **21**(1), 122–133.
- Schneider, M., Marison, I. and Von Stockar, U. (1996), 'The importance of ammonia in mammalian cell culture', *Journal of Biotechnology* **46**(3), 161–185.
- Schoen, R., Bentley, K. and Klebe, R. (1982), 'Monoclonal antibody against human fibronectin which inhibits cell attachment', *Hybridoma* **1**(2), 99–108.
- Schumacher, J., Behrends, V., Pan, Z., Brown, D. R., Heydenreich, F., Lewis, M. R., Bennett, M. H., Razzaghi, B., Komorowski, M., Barahona, M., Stumpf, M. P. H., Wigneshweraraj, S., Bundy, J. G. and Buck, M. (2013), 'Nitrogen and carbon status are integrated at the transcriptional level by the nitrogen regulator ntrc in vivo', *mBio* **4**(6).
- Selvamani, R. S. V., Friehs, K. and Flaschel, E. (2013), 'Extracellular recombinant protein production under continuous culture conditions with *Escherichia coli* using an alternative plasmid selection mechanism', *Bioprocess and Biosystems Engineering* pp. 1–13.
- Selvarasu, S., Karimi, I., Ghim, G. and Lee, D. (2010), 'Genome-scale modeling and *in silico* analysis of mouse cell metabolic network', *Molecular Biosystems* **6**(1), 152–161.
- Selvarasu, S., Kim, D. Y., Karimi, I. A. and Lee, D.-Y. (2010), 'Combined data preprocessing and multivariate statistical analysis characterizes fed-batch culture of mouse hybridoma cells for rational medium design', *Journal of Biotechnology* **150**(1), 94–100.
- Selvarasu, S., Wong, V., Karimi, I. and Lee, D. (2009), 'Elucidation of metabolism in hybridoma cells grown in fed-batch culture by genome-scale modeling', *Biotechnology and Bioengineering* **102**(5), 1494–1504.
- Sheikh, K., Frster, J. and Nielsen, L. (2005), 'Modeling hybridoma cell metabolism using a generic genome scale metabolic model of *Mus musculus*', *Biotechnology Progress* **21**(1), 112–121.

- Shelton, L. M., Huysentruyt, L. C. and Seyfried, T. N. (2010), ‘Glutamine targeting inhibits systemic metastasis in the VM-M3 murine tumor model’, *International journal of cancer. Journal international du cancer* **127**(10), 2478–2485. Research Support, N.I.H., Extramural.
- Shetty, R. P., Endy, D. and Knight, T. F., J. (2008), ‘Engineering BioBrick vectors from BioBrick parts’, *J Biol Eng* **2**, 5.
- Siedler, S., Schendzielorz, G., Binder, S., Eggeling, L., Bringer, S. and Bott, M. (2013), ‘SoxR as a single-cell biosensor for NADPH-consuming enzymes in *Escherichia coli*’, *ACS Synthetic Biology* **3**(1), 41–47.
- Spiess, C., Merchant, M., Huang, A., Zheng, Z., Yang, N.-Y., Peng, J., Ellerman, D., Shatz, W., Reilly, D., Yansura, D. G. and Scheer, J. M. (2013), ‘Bispecific antibodies with natural architecture produced by co-culture of bacteria expressing two distinct half-antibodies’, *Nat Biotech* **31**(8), 753–758.
- Stansen, C., Uy, D., Delaunay, S., Eggeling, L., Goergen, J.-L. and Wendisch, V. F. (2005), ‘Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production’, *Applied and environmental microbiology* **71**(10), 5920–5928.
- Street, J., Delort, A., Braddock, P. and Brindle, K. (1993), ‘A  $1\text{H}/15\text{N}$  nmr study of nitrogen metabolism in cultured mammalian cells’, *Biochemical Journal* **291**(Pt 2), 485–492.
- Stricker, J., Cookson, S., Bennett, M. R., Mather, W. H., Tsimring, L. S. and Hasty, J. (2008), ‘A fast, robust and tunable synthetic gene oscillator’, *Nature* **456**(7221), 516–9.
- Tavori, H., Kimmel, Y. and Barak, Z. (1981), ‘Toxicity of leucine-containing peptides in *Escherichia coli* caused by circumvention of leucine transport regulation’, *Journal of Bacteriology* **146**(2), 676–683.
- Tercé-laforge, T., Carrayol, E., Cren, M., Desbrosses, G., Hecht, V. and Hirel, B. (1999), ‘A strong constitutive positive element is essential for the ammonium-regulated expression of a soybean gene encoding cytosolic glutamine synthetase’, *Plant Molecular Biology* **39**(3), 551–564.
- Tjio, J. and Puck, T. (1958), ‘Genetics of somatic mammalian cells: II. chromosomal constitution of cells in tissue culture’, *The Journal of Experimental Medicine* **108**(2), 259–268.
- Tolosa, L., Ge, X. and Rao, G. (2003), ‘Reagentless optical sensing of glutamine

- using a dual-emitting glutamine-binding protein', *Analytical Biochemistry* **314**(2), 199–205.
- Toyoda, K., Teramoto, H., Inui, M. and Yukawa, H. (2009), 'The *ldhA* gene, encoding fermentative L-lactate dehydrogenase of *Corynebacterium glutamicum*, is under the control of positive feedback regulation mediated by LldR', *Journal of Bacteriology* **191**(13), 4251–4258.
- van Berkel, P. H. C., Gerritsen, J., Perdok, G., Valbjørn, J., Vink, T., van de Winkel, J. G. J. and Parren, P. W. H. I. (2009), 'N-linked glycosylation is an important parameter for optimal selection of cell lines producing biopharmaceutical human IgG', *Biotechnology Progress* **25**(1), 244–251.
- van der Meer, J. R. and Belkin, S. (2010), 'Where microbiology meets microengineering: design and applications of reporter bacteria', *Nat Rev Micro* **8**(7), 511–522.
- Veterayan, S. (2011), Construction of a whole-cell biosensor for L-lactate., Bachelor thesis.
- Vitreschak, A. G., Lyubetskaya, E. V., Shirshin, M. A., Gelfand, M. S. and Lyubetsky, V. A. (2004), *Attenuation regulation of amino acid biosynthetic operons in proteobacteria: comparative genomics analysis*, Vol. 234.
- Vogl, T., Hartner, F. S. and Glieder, A. (2013), 'New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*', *Current Opinion in Biotechnology* **24**(6), 1094–1101.
- Voigt, C. A. (2012), 'Synthetic biology: Bacteria collaborate to sense arsenic', *Nature* **481**(7379), 33–34. 10.1038/481033a.
- Vriezen, N., Romein, B., Luyben, K. and van Dijken, J. (1997), 'Effects of glutamine supply on growth and metabolism of mammalian cells in chemostat culture', *Biotechnology and Bioengineering* **54**(3), 272–286.
- Vriezen, N. and van Dijken, J. P. (1998), 'Fluxes and enzyme activities in central metabolism of myeloma cells grown in chemostat culture', *Biotechnology and Bioengineering* **59**(1), 28–39.
- Wang, B., Barahona, M. and Buck, M. (2013), 'A modular cell-based biosensor using engineered genetic logic circuits to detect and integrate multiple environmental signals', *Biosensors and Bioelectronics* **40**(1), 368–376.
- Wang, B., Barahona, M., Buck, M. and Schumacher, J. (2013), 'Protein engineering:

- New approaches and applications: Rewiring cell signalling through chimaeric regulatory protein engineering', *Biochemical Society Transactions* **41**(Pt 5), 1195.
- Wang, Q., Wu, J., Friedberg, D., Plakto, J. and Calvo, J. M. (1994), 'Regulation of the *Escherichia coli lrp* gene', *J. Bacteriol.* **176**(7), 1831–1839.
- Wang, W.-D., Chen, Z.-T., Kang, B.-G. and Li, R. (2008), 'Construction of an artificial intercellular communication network using the nitric oxide signaling elements in mammalian cells', *Experimental cell research* **314**(4), 699–706. (doi: 10.1016/j.yexcr.2007.11.023).
- Weber, W. (2009), 'A synthetic metabolite-based mammalian inter-cell signaling system', *Molecular Biosystems* **5**(7), 757. (doi: 10.1039/B902070P).
- Weber, W., Baba, M. and Fussenegger, M. (2007), 'Synthetic ecosystems based on airborne inter- and intrakingdom communication', *Proceedings of the National Academy of Sciences of the United States of America* **104**(25), 10435–10440. (doi: 10.1073/pnas.0701382104).
- Weber, W. and Fussenegger, M. (2002), 'Artificial mammalian gene regulation networks - novel approaches for gene therapy and bioengineering', *Journal of Biotechnology* **98**(23), 161–187.
- Weber, W. and Fussenegger, M. (2011), 'Molecular diversity – the toolbox for synthetic gene switches and networks', *Current Opinion in Chemical Biology* **15**(3), 414420.
- Weber, W., Lienhart, C., Baba, M. D. E. and Fussenegger, M. (2009), 'A biotin-triggered genetic switch in mammalian cells and mice', *Metabolic Engineering* **11**(2), 117–124.
- Weiner, L. M., Surana, R. and Wang, S. (2010), 'Monoclonal antibodies: versatile platforms for cancer immunotherapy', *Nat Rev Immunol* **10**(5), 317–327.
- White, S. F., Turner, A. P. F., Bilitewski, U., Schmid, R. D. and Bradley, J. (1994), 'Lactate, glutamate and glutamine biosensors based on rhodinised carbon electrodes', *Analytica Chimica Acta* **295**(3), 243–251.
- Willis, R. C. (1975), 'Regulation of glutamine transport in *Escherichia coli*', *Journal of Bacteriology* **122**(3), 1032.
- Wilmore, D. and Rombeau, J. (2001), 'Introduction to the symposium proceedings', *Journal of Nutrition* **131**(9), 2447S.
- Wischmeyer, P. E., Musch, M. W., Madonna, M. B., Thisted, R. and Chang,



- E. B. (1997), ‘Glutamine protects intestinal epithelial cells: role of inducible HSP70’, *American Journal of Physiology - Gastrointestinal and Liver Physiology* **272**(4), G879–G884.
- Wong, D. C. F., Tin Kam Wong, K., Tang Goh, L., Kiat Heng, C. and Gek Sim Yap, M. (2005), ‘Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures’, *Biotechnology and Bioengineering* **89**(2), 164–177.
- Wong, D. C. F., Wong, N. S. C., Goh, J. S. Y., May, L. M. and Yap, M. G. S. (2010), ‘Profiling of N-glycosylation gene expression in CHO cell fed-batch cultures’, *Biotechnology and Bioengineering* **107**(3), 516–528.
- Xie, L. and Wang, D. (1994), ‘Fed batch cultivation of animal cells using different medium design concepts and feeding strategies’, *Biotechnology and Bioengineering* **43**(11), 1175–1189.
- Ye, B.-C., Zhang, Y., Yu, H., Yu, W.-B., Liu, B.-H., Yin, B.-C., Yin, C.-Y., Li, Y.-Y., Chu, J. and Zhang, S.-L. (2009), ‘Time-resolved transcriptome analysis of *Bacillus subtilis* responding to valine, glutamate, and glutamine’, *Plos One* **4**(9), e7073.
- Yee, J. C., de Leon Gatti, M., Philp, R. J., Yap, M. and Hu, W.-S. (2008), ‘Genomic and proteomic exploration of CHO and hybridoma cells under sodium butyrate treatment’, *Biotechnology and Bioengineering* **99**(5), 1186–1204.
- Yeomans, C. V., Porteous, F., Paterson, E., Meharg, A. A. and Killham, K. (1999), ‘Assessment of lux-marked *Pseudomonas fluorescens* for reporting on organic carbon compounds’, *FEMS Microbiology Letters* **176**(1), 79–83.
- Yoshida, K.-i., Ishio, I., Nagakawa, E., Yamamoto, Y., Yamamoto, M. and Fujita, Y. (2000), ‘Systematic study of gene expression and transcription organization in the gntZywaA region of the *Bacillus subtilis* genome’, *Microbiology* **146**(3), 573–579.
- Young, J. D. (2013), ‘Metabolic flux rewiring in mammalian cell cultures’, *Current Opinion in Biotechnology* .
- Zaslaver, A., Kaplan, S., Bren, A., Jinich, A., Mayo, A., Dekel, E., Alon, U. and Itzkovitz, S. (2009), ‘Invariant distribution of promoter activities in *Escherichia coli*’, *PLoS Comput Biol* **5**(10), e1000545.
- Zhou, M., Crawford, Y., Ng, D., Tung, J., Pynn, A. F. J., Meier, A., Yuk, I. H., Vijayasankaran, N., Leach, K., Joly, J., Snedecor, B. and Shen, A. (2011), ‘Decreasing lactate level and increasing antibody production in Chinese hamster

ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases', *Journal of Biotechnology* **153**(12), 27–34.