Enhanced bioethanol production by Zymomonas mobilis in response to the quorum sensing molecules AI-2

YANG, JUNG,WOO

Enhanced bioethanol production by *Zymomonas mobilis* in response to the quorum sensing molecules AI-2

By

Jungwoo Yang B. Sc.

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Abstract

The depletion of non-renewable energy resources, the environmental concern over the burning of fossil fuels, and the recent price rises and instability in the international oil markets have all combined to stimulate interest in the use of fermentation processes for the production of alternative bio-fuels. As a fuel, ethanol is mainly of interest as a petrol additive, or substrate, because ethanol-blended fuel produces a cleaner, more complete combustion that reduces greenhouse gas and toxic emissions. As a consequence of the surge in demand for biofuels, ethanol producing microorganisms, such as the bacterium *Zymomonas mobilis*, are of considerable interest due to their potential for industrial-scale bioethanol production.

Although bioethanol has traditionally been produced in batch fermentation with the yeast *Saccharomyces cerevisiae*, there are advantages in using *Z. mobilis* as an alternative for bioethanol production. In comparison to yeast, *Z. mobilis* grows and ferments rapidly, without the requirement for the controlled supply of oxygen during fermentation, and has a significantly higher ethanol product rate and yield. Most importantly, it has a high tolerance for ethanol.

Bacteria communicate with one another using chemical signalling molecules. In general, chemical communication involves producing, releasing, detecting, and responding to small hormone-like molecules termed autoinducers (AI). This process allows bacteria to monitor the environment for other bacteria and to alter behaviour on a population-wide scale in response to changes in the number and/or species present in a community.

Currently, there are three well-defined classes of molecules that serve as the paradigms for chemical signaling in bacteria: oligopeptides, AI-1 (AHLs) and AI-2. Oligopeptide signalling is the predominant signal used by Gram-positive bacteria, and AHLs (acyl-homoserine lactones) are for species-specific communication in Gram-negative bacteria. Finally, the LuxS/AI-2 pathway is generally considered as involved in interspecies communication because the *luxS* gene, which is responsible for AI-2 production, is found in various bacteria.

Many physiological functions in bacteria such as toxin, virulence factor and bacteriocin production, biofilm formation, bioluminescence, type III secretion, have been shown to be under the control of AI-2 quorum sensing. In *Z. mobilis*, *in vitro* synthesized and *in vivo* produced AI-2 treatment enhanced ethanol production by this bacterium up to a maximum of 50% in comparison with untreated control cells. This appears attributable to the overproduction of the glycolytic enzymes, enolase and pyruvate carboxylase, which are only rarely found in bacteria and the key enzymes for ethanol production.

From the perspective of interspecies communication, enhanced ethanol production in *Z. mobilis*, under the control of the AI-2 signalling molecules, could represent a good example of a bacterium that does not produce AI-2, but responds to it.

Another interesting finding is that two extracellular proteins from *Z. mobilis*, ZMO0994 and ZMO0134 which were originally induced by AI-2, were secreted when they were cloned, transformed and expressed in *E. coli* strain BL21 DE3; since it is generally accepted that nonpathogenic strains of *E. coli*, particularly derivatives of K12, do not

secrete proteins under routine growth conditions. Presumably, these proteins possess signal sequences for secretion that could be used to provide a strategy for their use as carriers of recombinant proteins produced in *E. coli* K12. The merit of this system is that there would be few contaminant cytoplasmic proteins, and could possibly solve problems in protein purification, such as protease activity, protein misfolding and inclusion body formation.

Finally, the discovery that the metabolic pathway leading to ethanol production is regulated by AI-2 is of considerable biotechnological importance because it will provide a basis for further engineering of strains for more efficient ethanol production. Indeed, engineering *Z. mobilis* by introducing the genes that encode Pfs and LuxS to produce AI-2, would be a means to stimulate increased ethanol production.

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Abbreviations

Acyl-ACP	acyl-acyl carrier protein
AHL	N-acyl homoserine lactone
BSA	bicinchoninic acid
CBP	calcium binding protein
CF	cystic fibrosis
DPD	4,5-dihydroxyl-2,3-pentanedione
ED	Entner-Doudoroff pathway
EMP	Embden-Meyerhof-Parnas pathway
IPTG	isopropyl-D-thiogalactopyanoside
kDa	kilo dalton
LEA	late embryogenesis abundant
LuxS	S-ribosylhomocysteine lyase
MALDI-TOF	matrix assisted laser desorption/ionization- time of flight
MWCO	molecular weight cut-off
OMP	outermembrane protein
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
PEP	phosphoenolpyruvate
Pfs	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase
SAH	S-adenosylhomocystein
SAM	S-adenosylmethionine
SDS-PAGE	sodium dodacyl sulfate-polyacrylamide gel electrophoresis
SRH	S-ribosylhomocysteine
TFA	tri-fluoro acetic acid
TLC	thin-layer chromatography
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Chapter 1 Introduction

1.1 The biology of *Zymomonas mobilis*

1.1.1 The Cell: history, morphology and taxonomy

Alcoholic beverages in the Western world are usually made by yeast fermentation. In many tropical areas of South and Central America, Africa, and Asia, *Z. mobilis* is a common organism used for natural fermentation of sugar cane, agave sap and palm sap. In Europe, *Z. mobilis* is occasionally responsible for spoilage of fruit juices such as apple cider and pear juice (perry) and is also a constituent of the bacterial flora of spoiled beer (Swings and De Ley, 1977). As a beer spoilage microorganism, the growth of *Z. mobilis* correlates with the production of large quantities of acetaldehyde and/or H_2S and a marked turbidity of the beverage rendering it undrinkable (Ingledew, 1979; Jespersen and Jakobsen, 1996). In ciders, *Z. mobilis* was shown to be responsible for cider sickness in English ciders (Barker and Hillier, 1912), and for 'framboise' spoilage in French ciders (Coton and Coton, 2003).

The *Z. mobilis* bacterium is a Gram-negative rod, 2-6 microns long and 1-1.5 micron wide, that occurs singly but mostly in pairs (Figure 1.1). *Z. mobilis* has no resting stages, so there are no spores, capsules, intracellular lipids or glycogen. Even if motility is not an essential feature of *Z. mobilis*, its mobility is by means of 1 to 4 lophotrichous flagella, which form a tuft, or group, either at one or both ends of the cell (Carr, 1974; Swings and De Ley, 1977).

From a taxonomical point of view, *Z. mobilis* belongs to the family of Sphingomonadaceae

(White *et al*., 1996; Kosako *et al*., 2000),Group 4 of the alpha-subclass of the class Proteobacteria.

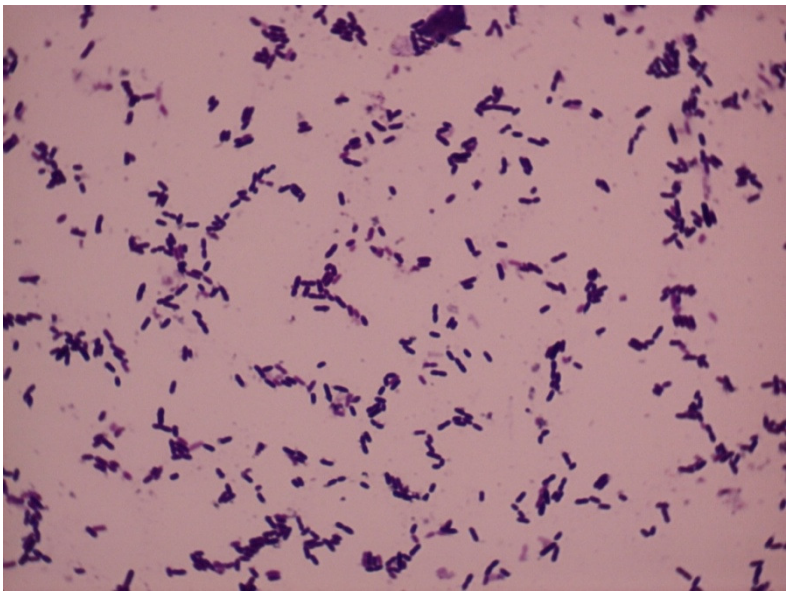


Figure 1.1 Photomicrograph of *Zymomonas mobilis* ZM4. After crystal violet staining, samples were examined under the commonly used light microscope (x3000).

Zymomonas shows phylogenic affiliation with *Pseudomonas*, but *Zymomonas* is distinguished from *Pseudomonas* by its fermentative metabolism, microaerophilic to anaerobic nature, oxidase negativity, and other molecular taxonomic characteristics. The genus *Zymomonas* was originally subclassified into two species as *Z. mobilis* and *Z. anaerobia* according to their ability to utilize sucrose (Carr, 1974), but Swings and De Ley in 1976 reclassified the genus *Zymomonas* into only one species with two subspecies as *Z. mobilis* subsps. *mobilis* and *Z. mobilis* subsps. *promaceae.* Recently, a rapid method for the identification, involving amplified ribosomal DNA restriction analysis, of strains of *Z. mobilis* at the subspecies level was developed and shown to be more reliable and faster than the conventional method based on physiological tests (Coton *et al*., 2005).

1.1.2 The central metabolic routes: Entner-Doudoroff (ED) pathway

Z. mobilis is an obligate fermentative microorganism. It ferments glucose, fructose and sucrose via the Entner-Doudoroff (ED) pathway (Figure 1.2) in conjunction with the enzymes, pyruvate decarboxylase and alcohol dehydrogenase, producing ethanol and carbon dioxide in equimolar amounts (Gibss and DeMoss, 1954; Dawes *et al*., 1970). The Embden-Meyerhof-Parnas (EMP) pathway does not operate in this bacterium because the gene for phosphofructokinase is lacking in *Z. mobilis* (Seo *et al*., 2005). Likewise, most enzymes of the pentose phosphate pathway are missing (Seo *et al*., 2005).

The enzymes, α-ketoglutarate dehydrogenase, succinyl thiokinase, succinate dehydrogenase and fumarase (Dawes *et al*., 1970), and malate dehydogenase (Bringer-Meyer and Sahm, 1989), are lacking. Accordingly, the genes for α-ketoglutarate dehydrogenase and malate

dehydrogenase have not been found in the genome sequence (Seo *et al*., 2005). A pyruvate dehydrogenase complex has been purified and characterized, and the sequence and localization of the corresponding genes have been analysed (Neveling *et al*., 1998). Two anaplerotic enzyme activities, those of phosphoenolpyruvate (PEP) carboxylase and malic enzyme, have been found in cell free extracts (Bringer-Meyer and Sahm, 1989). The *Z. mobilis* genome also contains genes for PEP carboxylase, citrate lyase, malic and fumarate dehydratase (Seo *et al*., 2005)

The glycolytic pathway in *Z. mobilis* appears to function with minimal allosteric control. Unlike yeast, it lacks allosterically regulated pyruvate kinase and phosphofructokinase, typical of EMP glycolysis (Barrow *et al*., 1984; Strohhackter *et al*., 1993; Snoep *et al*., 1996). Allosteric inhibition by PEP has been demonstrated for the second enzyme of the ED pathway, glucose-6-phosphate dehydrogenase (Scopes, 1997). Notably, the same enzyme appears to exert a considerable control over the glycolytic flux. The flux control coefficient of glucose-6-phosphate dehydrogenase for the early stages of batch growth was found to be 0.4, or even higher (Snoep *et al*., 1996). Glucokinase and the glucose transporter might also contribute to the flux control under these conditions. On the other hand, when ethanol is present at high concentrations (around 10% w/w), which is typical for the late fermentation stages, the flux control is shifted to enolase and phosphoglycerate mutase (Barrow *et al*., 1984; Strohhacker *et al*., 1993; Snoep *et al*., 1996).

Carbohydrate metabolism in *Z. mobilis* operates as a true “catabolic highway” (Springer, 1996). The rate at which cells of *Z. mobilis* convert glucose into ethanol and CO_2 is three to five times faster than observed in yeast and 1.2-1.5 times faster than in the Gram-positive

obligately fermentative *Streptococcus bovis*, which serves as another example of the uncoupled growth phenomenon among bacteria (Cook and Russell, 1994).

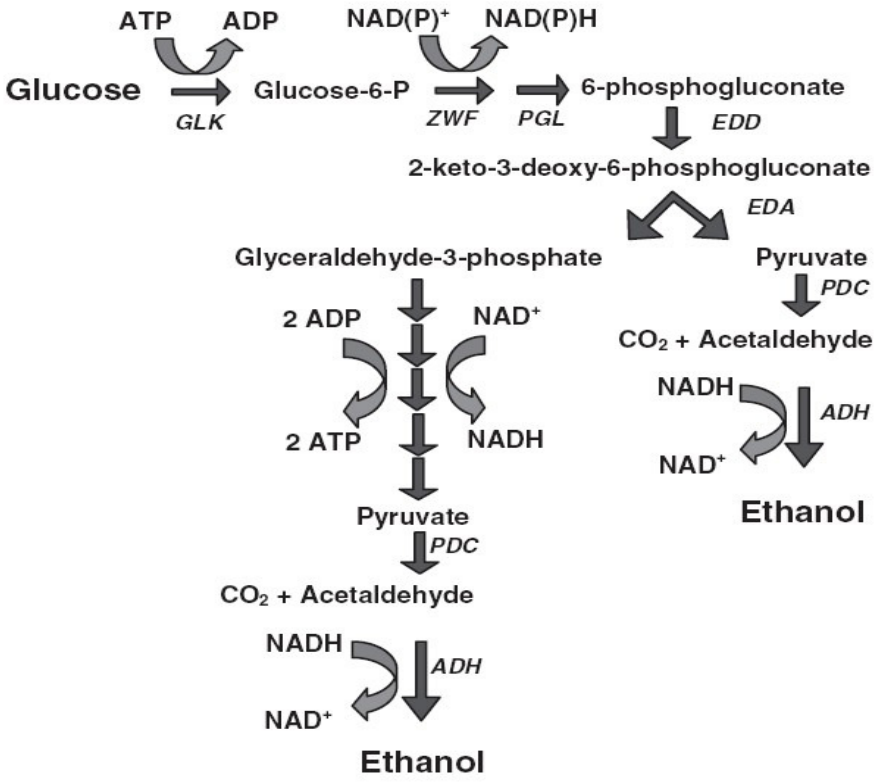


Figure 1.2 The Entner-Doudoroff (ED) pathway and ethanologenesis. The branch from glyceraldehyde-3-phosphate to pyruvate is identical to the Embden-Meyerhof-Parnas (EMP) pathway. The key enzyme of the ED pathway is pyruvate decarboxylase (PDC), which is only rarely found in bacteria. The ED pathway produces only 1 mole of ATP per mole of consumed glucose. Abbreviations: GLK, Glucokinase; ZWF, glucose-6-phosphate dehydrogenase; PGL, phosphogluconolastonase; EDD, 6-phosphogluconate dehydratase; EDA, 2-keto-3-deoxy-gluconate adolase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase (Adapted from Uldis, 2006)

Unlike glycolysis, which can theoretically generate two moles of ATP for each mole of glucose fermented to ethanol, the ED pathway has a net yield of only one ATP per mole of glucose (Conway, 1992). In combination, the low ATP yield of the ED pathway and the abundant expression of fermentative enzymes certainly help to explain the high catabolic rate in *Z. mobilis* (Dien *et al*., 2003). In the second step of the ED pathway, inhibition of glucokinase by glucose-6-phosphate and that of glucose-6-phosphate dehydrogenase by ATP are known to be weak. Thus, it is reported that in comparison to yeast, the ATP pool during the log phase of growth in *Z. mobilis* is constant, and glycolysis and the ATP demand are uncoupled in *Z. mobilis* (Lazdunski and Belaich, 1972; Uldis, 2006). Considering that the ATP pool only decreases when glucose is limiting, glycolysis may not be the main consumer in *Z. mobilis* (Lazdunski and Belaich, 1972). In other words, energy production and energy consumption are always well balanced in the log phase of growth. The high specific rate of ATP synthesis must be balanced by an equally rapid utilization and obviously, cell-biomass synthesis is not the main consumer of ATP in *Z. mobilis* (Zikmanis *et al*., 2001). Apparently, ATP spilling would permit glycolysis to proceed without a concomitant biomass synthesis under conditions when essential growth factors are absent. Scopes (1997) suggested that *Z. mobilis* is able to adjust its glucose consumption to match its ATP demand, through the combined controls of glucokinase and glucose-6-phosphate dehydrogenase.

1.1.3 Applications in science and industry

1.1.3.1 Antibacterial effects by *Z. mobilis* and therapeutic use

Traditionally, people in South America are used to having “Aguamiel”, a beverage made with

Zymomonas for use in cases of renal and metabolic diseases. Lindner (1928) reported that this fresh and concentrated juice of Agave would be the tropical counterpart of a probiotic drink in a Western country; and he further reported on the successful application of *Zymomonas mobilis* against several diseases in cattle and against purulent furuncles and wounds in man. The therapeutic applications of *Zymomonas* cultures are listed in Table 1.1. However, although it has been known that *Zymomonas* has antibacterial activity since 1900s, there was no evidence that *Zymomonas* produce a bacteriocin-like peptide or chemicals. Nevertheless, the antagonistic effect of *Zymomonas* against a number of bacteria and filamentous fungi has been examined by many scientists since 1900s (Lima *et al*., 1972; Skotnicki *et al*., 1984; Haffie *et al*., 1985).

Table 1.1 Therapeutic use of *Z. mobilis*

Disorder	Percentage of full recovery	Treatment
Chronic entrocolitis	100%	300ml of liquid cultures
Chronic cystis	100%	300ml of liquid cultures
Vaginitis	100%	Tampons impregnated with cells
Gynecological infection	100%	Liquid culture and agar jellies

(Adapted and modified from Swing and De Lay, 1977)

1.1.3.2 Bioethanol production

The depletion of non-renewable energy resources, the environmental concern over the burning of fossil fuels, and instability in the international oil markets have all combined to stimulate interest in the use of fermentation process for the production of alternative biofuels. Biofuel is any fuel from carbonaceous material. Typically though, the term biofuels is used in

the context of liquid transportation fuels or alternatives to gasoline and diesel. As an alternative energy source, ethanol is a colourless, water soluble, non-toxic and biodegradable liquid. The most important use of ethanol is as a fuel or fuel additive (Farrell *et al*., 2006). In addition to ethanol, biogas is produced by anaerobic digestion of organic materials (e.g. crops, organic wastes) and oils, in the form of straight or wasted vegetable oil, can be used directly in diesel engines. In comparison to fossil fuel, biofuel produced by lignocellulosic biomass could have the following advantages. Firstly, energy sources are geographically more evenly distributed than fossil fuels. Secondly, the raw material is less expensive than conventional agricultural feedstock. Thirdly, biofuels from lignocelluloses generate low net greenhouse gas emission by recycling. Finally, the biofuels industry may provide future employment especially in developing countries. For these reasons, both developed and developing countries have investigated these processes with a view to obtaining advances in the techniques for biofuel production. Lignocellulosic biomass refers to plant biomass that is composed of cellulose, hemicellulose and lignin. The carbohydrate polymers (cellulose and hemicelluloses) are tightly bound to the lignin. Cellulose is a polymer of glucoses, whilst hemicellulose is heteropolysaccharide which consist of hexoses, pentoses and glucoronic acid (Hahn-ha *et al*., 2006; Himmel *et al*., 2007; Ragauskas *et al*., 2006).

The main techniques for bioethanol production are industrial fermentation processes based on batch/continuous fermentation and co-culture fermentation, exploiting the bacterial strains modified by genetic manipulation. For the latter, many scientists have focused on engineering numerous microorganisms to selectively produce ethanol. For the application of engineered microorganism, the following conditions, set out in Table 1.2, should be satisfied. Importantly *Z. mobilis* which was screened in nature almost fits these conditions. Although *Z. mobilis*

produces ethanol at high yields, it only ferments glucose and fructose. In addition to *Z. mobilis*, bacteria such as *E. coli* and *Klebsiella oxymora*, which can naturally use a wide spectrum of sugars have been engineered to selectively produce ethanol. Although *E. coli* has the ability to use a wide spectrum of sugars, *E. coli* has several disadvantages, such as a narrow pH growth range (pH 6-7) and its cultures are less hardy compared to yeast. Thus, *S. cerevisiae* and *Z. mobilis* are still regarded as industrially suitable microorganism.

Table 1.2 Important traits for ethanol production

Traits	Requirements
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40g/L
Ethanol productivity	>1g/L in hour
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperature

(Adapted from Dien *et al*. 2003)

1.1.3.2.1 *Saccharomyces cerevisiae*

S. cerevisiae is widely regarded as the prime species for ethanol production, although many microorganisms have been exploited for this process. Traditionally ethanol has been produced in batch fermentation with yeast strains; and under aerobic conditions, it metabolize glucose to ethanol primarily by way of the Embden-Meyerhof pathway (Figure 1.3). Typical yeast fermentations require that the temperature be maintained at 30-35℃ (D'Amore *et al*., 1989). Lignocellulosic biomass contains a complex of carbohydrates, mainly glucose and xylose. *S. cerevisiae* takes up xylose poorly owing to the low affinity of its native nonspecific hexose-

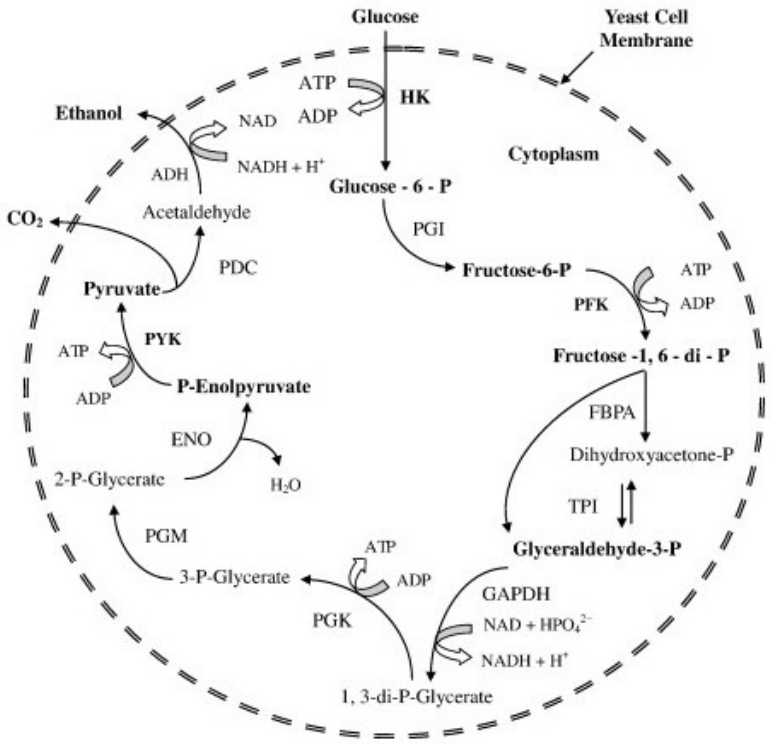


Figure 1.3 Embden-Meyerhof pathway (EMP) in yeast. In contrast to ED pathway, 2 ATP and 2 NADH are produced via EMP pathway. Abreviations: HK, hexokinase; PGI, Phosphoglucoisomerase; PFK, phosphofructokinase; FBPA, fructose bisphosphate; TPI, triose phosphate isomerase; GAPDH, glyceraldehydes-3phosphate dehydorgenase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; ENO, enolase; PYK, pyruvate kinase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase. (Adapted from Bai *et al*., 2008).

-transport system for xylose. For this reason, most yeast metabolic engineering for ethanol production from xylose has focused on improving sugar uptake and the initial assimilation steps (Van Vleet and Jeffries, 2009). Figure 1.4 schematically illustrates the metabolic pathways for pentose metabolism in yeast.

Katahira *et al*. (2008) showed that transformation of xylose-utilizing *S. cerevisiae* with *sut1*, encoding the Sut1 xylose transporter, from *Pichia stipitis* enhanced ethanol production. Similarly, transformation of *S. cerevisiae* strains with the genes for *Gxf1* (glucose/xylose facilitator) and *Gxs1* (glucose/xylose proton symporter) from *Candida intermedia* led to a 10-fold higher affinity for glucose than xylose. This result suggested that yeast has a metabolic selection, when glucose and xylose are mixed (Leandro *et al*., 2008). Heterologous expression of the *At5g59250* and *At5g17010* genes, which encode two xylose transporters from *Arabidopsis thaliana* in yeast increased xylose uptake and consumption by up to 2.5-fold and during the xylose/glucose co-consumption phase cells had up to a 70% increase compared to the control strain in ethanol production (Hector *et al*. 2008).

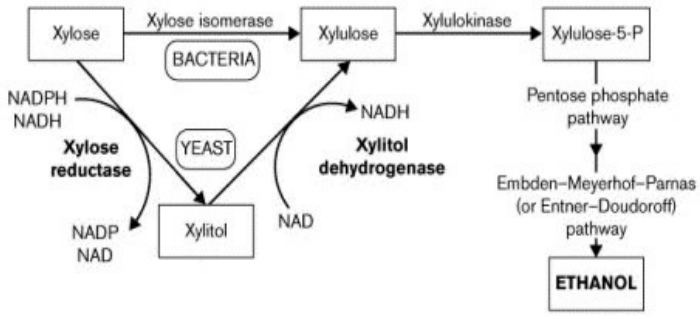


Figure 1.4 Outline of xylose metabolism in yeast and bacteria. (Adapted from Aristidou and Penttila, 2000)

Other target genes for xylose utilization introduced into yeast include xylose reductase (XR) and xylitol dehydrogenase (XDH). These enzymes require cofactors, NADPH for XR and NADH for XDH. Watanabe *et al*., (2007) showed that cloning of NADH-preferring enzymes, such as PsXR from *Pichia stipitis*, gave a 20% increase in ethanol productivity. However, co-expression of XR and XDH resulted in a low ethanol yield, but cells were capable of growth and ethanol production from xylose (Viitanen *et al*., 2008). The eukaryotic pathway for xylose metabolism initiates from xylose reductase, whilst in most bacteria xylose is directly isomerized to xylulose by xylose isomerase (XI) (Figure 1.4). Heterologous expression of XI in yeast was unsuccessful mainly due to protein misfolding (Moes *et al*. 1996; Sarthy *et al*. 1987). Although Walfridsson *et al*. (1996) were able to obtain functional expression of XI from *Thermus thermophilus* in yeast, maximal growth conditions for *S. cerevisiae* could not be attained, resulting in poor xylose fermentation. In addition, adaptation of *S. cerevisiae* strains for efficient xylose metabolism (Sauer, 2001), and more recently improved *S. cerevisiae* strains for ethanol productivity and tolerance were screened by genome shuffling methods (Shi *et al*., 2009).

1.1.3.2.2 *Zymomonas mobilis*

The overall net chemical reaction of fermentation involves the production of 2 moles of ethanol (1 mole of C_6-glucose to 2 mole of C_3-ethanol), but the yield of ethanol attained in practical fermentations however does not usually exceed 90% of theoretical in yeast. This is partly due to the requirement for some nutrient to be utilized in the synthesis of new biomass and cell maintenance related reactions in yeast. In addition to that, yeast is highly susceptible to ethanol inhibition. A concentration of 1-2% ethanol is sufficient to retard microbial growth,

and at 10% alcohol, the growth nearly stops. These characteristics of yeast necessitated the development of strains which are suitable for the fermentation process. *Zymomonas mobilis*, a Gram negative bacterium, is considered as an alternative organism in large-scale ethanol fuel production (Roger, 1982). It tolerates up to 120g/L (e.g. 12%) ethanol and ferments glucose to an ethanol content of 10%. According to Gunasekaran (1999) and Springer (1996), *Zymomonas mobilis* possesses advantages over *S. cerevisiae* with respect to higher sugar uptake and ethanol yield, lower biomass production, higher ethanol tolerance, no requirement for the controlled addition of oxygen during fermentation, and amenability to genetic manipulations. In addition, *Z. mobilis* produces byproducts, such as sorbitol, acetoin, glycerol, acetic acid and levan polymer which are useful for diabetes, pharmacological application and chemical refinement. However, wild type strains of *Z. mobilis* can only use glucose, fructose and sucrose as carbon substrates. To overcome this problem, genetic manipulation of *Z. mobilis* has been undertaken in an attempt to broaden the utilizable range of substrates. The major techniques to achieve this aim were either transformation of *Z. mobilis* with genes of interest acquired from other organisms, or to transform *Z. mobilis* genes with the required characteristics into other bacteria.

In *E. coli*, ethanol is produced from pyruvate formate lyase (PFL). This fermentation pathway is unbalanced because 2 NADH are required for 1 pyruvate fermentation, while 1 NADH is generated for each pyruvate from sugar. In contrast to *E. coli*, yeast and *Z. mobilis* are homoethanol fermentative, with only 1 NADH required for 1 pyruvate fermentation by pyruvate decarboxylase. Thus, the genes *pdc* (pyruvate decarboxylase) and *adhII* (alcohol dehydrogenase) of *Z. mobilis* have been transferred to other bacterial strains to make ethanologenic novel strains. Ingram *et al*. (1987) showed that expression of *Z. mobilis pdc*

and *adhII* genes, via the pLOI295 plasmid, in *E. coli* increased the cell growth and ethanol productivity. In pLOI295 *pdc* and *adhII* genes were controlled by a *lac* promoter, leading to their operon being referred to as the *PET* (Production of Ethanol) operon (Ingram *et al*., 1987). These findings have encouraged the use of the *PET* plasmid for use in developing new genetically modified strain and have been used for transforming a number of bacteria (Ingram *et al*., 1987; Hespell *et al*., 1996; Talarico *et al*., 2005).

Successful fermentation of lignocellulosic biomass depends on xylose utilization, because xylose is the second most abundant sugar in cellulose and hemicellulose. As mentioned, *Z. mobilis* has a very narrow spectrum of sugar utilization. Thus recent research has focused on development of *Z. mobilis* recombinant strains capable of using pentose sugars for conversion of cheaper lignocellulosic hydrolysates to ethanol and the improvement of the fermentation process (Yanase *et al*., 2005; Stephanopoulos, 2007). In analogy to the genetic engineering of yeast to utilize xylose, *Z. mobilis* has also been genetically engineered to utilize and undertake xylose fermentation (Figure 1.5). Zhang *et al*. (1995) were the first researchers to attempt to introduce a pathway for pentose metabolism in *Z. mobilis*; by insertion of the genes encoding xylose isomerase (*xylA*) and xylulokinase (*xylB*) for pentose metabolism. However, this had its limitation and it soon became evident that such failures were due to the absence of detectable transketolase and transaldolase activities in *Z. mobilis*. Initially xylose is transported through the membrane of *Z. mobilis* by the Glf (glucose facilitator protein) transporter (Saier and Crasnier, 1996). The Glf protein, which belongs to the major facilitator superfamily (e.g. lactose permease, LacY from *E. coli*) can transport fructose, xylose and glucose, but it has been shown that the xylose transport activity of Glf transporter was totally inhibited in the presence of glucose in *E. coli (*Chen *et al., 2009*). This could be the reason

that glucose is preferred to xylose by *Z. mobilis* in mixed medium conditions. After uptake, XylA catalyses the conversion of xylose to xylulose and XylB phosphorylates xylulose to form xylulose 5-phosphate, which is then, converted to the intermediates of the ED pathway by transketolase and transaldolase (Figure 1.5).

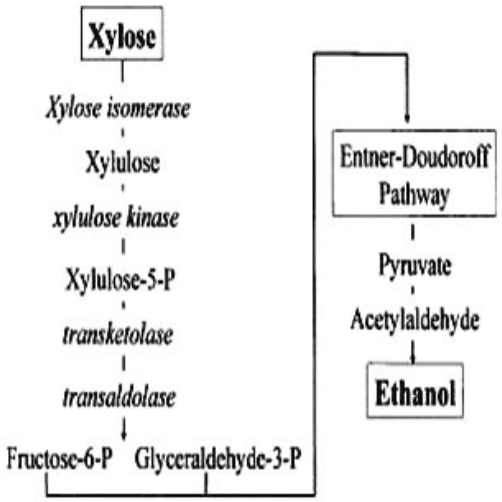


Figure 1.5 Strategy of ethanol production in *Z. mobilis* engineered for xylose metabolism. Target genes for *Z. mobilis* introduction are Xylose isomerase (*xylA*), Xylulose kinase (*xylB*), transketolase (*tktA*) and transaldolase (*tal)*. (Adapted from Dien *et al*., 2003)

A chimeric shuttle vector (pZB5) that carries two independent operons: the first encoding the *E. coli xylA* and *xylB* genes and the second expressing transketolase (*tktA*) and transaldolase (*tal*), again from *E. coli*, were introduced into *Z. mobilis* for xylose utilization (Zhang, 2003). The two operons, which included the four xylose assimilation and non-oxidative pentose phosphate pathway genes, were expressed successfully in the *Z. mobilis* ZM4 or CP4 strain. The recombinant strain was capable of fast growth on xylose as the sole carbon source, and

moreover, it efficiently converted glucose and xylose to ethanol with 86 and 94% of the theoretical yield from xylose and glucose, respectively (Zhang, 2003). In addition to the xylose fermentation gene introduction into *Z. mobilis*, α-amlyase of *E. coli* for starch (Yanase *et al*., 1991), α-galactosidase of *E. coli* for raffinose (Yanase *et al*., 1990), and five other genes, encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transaldolase (*talB*), and transketolase (*tktA*), of *E. coli* for arabinose (Deanda *et a*., 1996) have been introduced into *Z. mobilis* in an attempt to extend the carbon utilization.

The celluloses are composed of long chains of sugar molecules. During cellulose hydrolysis via enzymatic and chemical means, the cellulose chains are broken down to glucose for microbial fermentation (Figure 1.6). In an attempt to introduce cellulose degradation properties on to *Z. mobilis*, cellulase-encoding genes have been cloned and expressed in *Z. mobilis*. The endoglucanase gene (*eglX*) from *Pseudomonas fluorescens* subsp. *cellulosa* (Lejeune *et al*., 1988) and the endo-β-1,4-glucanase from *Bacillus subtilis* (Yoon *et al*. 1988; Brestic-Goachet *et al*., 1990) were introduced into *Z. mobilis* and *Z. anaerobia*, respectively. However, both genes were poorly expressed in *Zymomonas* and there was no activity for these exo-cellulases in the supernatant of the transformants. In contrast, the endoglucanase gene (*celZ*) of *Erwinia chrysanthemi* and carboxymethyl gene (*celC*) of *Acetobacter xylinum* were successfully expressed in *Z. mobilis*, with maximal gene expression yielding up to 35% and 75% of the cellulose degradation, respectively during the exponential phase (Brestic-Goachet *et al*., 1990; Okamoto *et al*., 1994). Despite these successful trials, there is a limitation in the direct use of recombinant *Z. mobilis* for simultaneous saccharification and fermentation (SFF) because of the typical enzymatic hydrolysis condition; pH 4.5 and 55℃.

Generally, lignocellulose is degraded either by chemically or enzymatically at pH < 4.5 and at a temperature >45℃ (Lin, Y., and Tanaka, 2006).

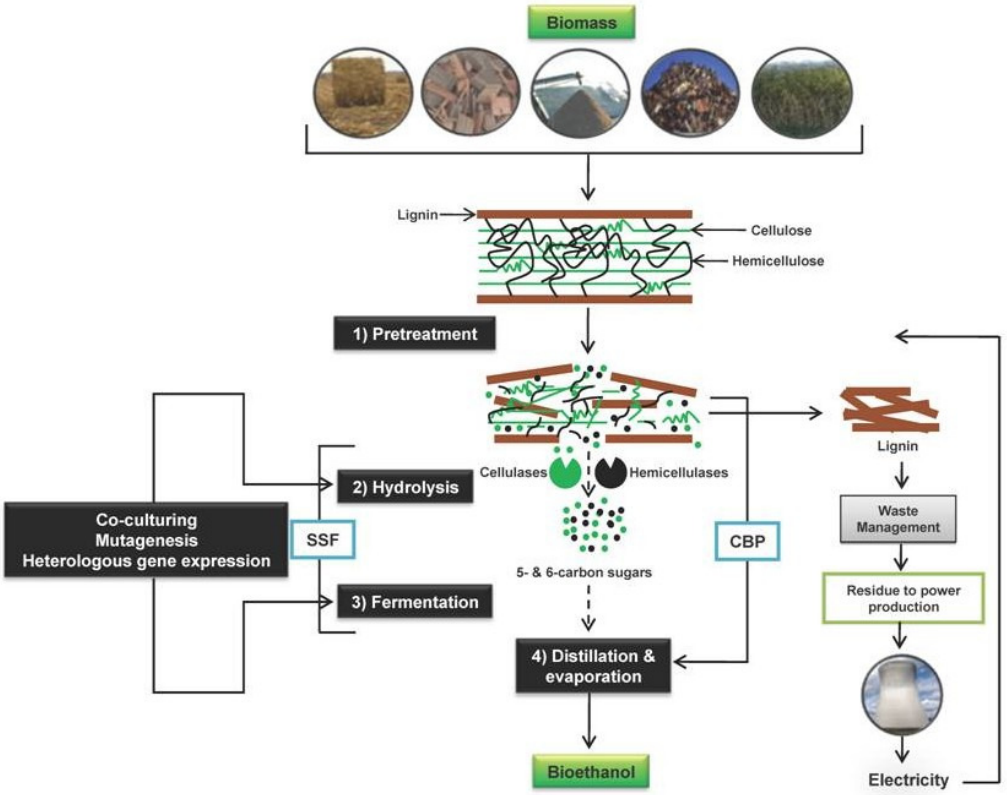


Figure 1.6 Conversion of lignocellulose to ethanol. Crystalline cellulose, the largest (50%) and most difficult fraction, can be hydrolyzed by a combination of acid and enzymatic processes. During these steps 95–98% of the xylose and glucose are recovered. These monosaccharides are subsequently converted to ethanol by appropriate microorganisms (Adapted from Dashtban *et al*., 2009). Because lignin is highly resistance to chemical and enzymatic degradation it cannot be converted into ethanol by fermentation. Thus, this portion of lignocellulose is alternatively burned for efficient heating and electrical production (Nevoigt, 2008).

1.2 Quorum sensing: cell-to-cell communication in bacteria

Bacteria communicate with one another using chemical signalling molecules. In general, chemical communication involves producing, releasing, detecting and responding to small hormone-like molecules termed autoinducers. This process, termed quorum sensing, allows bacteria to monitor the environment for other bacteria and to alter its behaviour on a population-wide scale in response to changes in the number and/or species present in a community (Schauder and Bassler, 2001). The phenomenon of bacterial communication was first stated by Tomasz in 1965, who reported that a hormone-like extracellular peptide helped regulate competence in *Streptococcus pneumoniae*. Later the signal, termed a bacterial autoinducer, was first discovered in the luminescent marine bacterium *Vibrio fischeri* and its free living relative *Vibrio harveyi* in the early 1970s (Nealson *et al*., 1970; Eberhard, 1972), since then this has been shown to be a widespread mechanism of gene regulation in bacteria (see Atkinson and Williams, 2009; Keller and Surette, 2006; Waters and Bassler, 2005; for recent reviews).

Typically, an autoinducer induces the transcription of a set of genes that includes the gene encoding the autoinducer-producing enzyme, which results in a positive feedback loop. Although autoinducers are small, diffusible and metabolically relatively inexpensive molecules, they belong to many different classes of chemicals. N-acyl-$_{L}$-homoserine lactones (AHLs) are the best-studied class of autoinducer, but are only found in Gram negative bacteria of the phylum Proteobacteria (Fuqua *et al*., 2001). Gram positive bacteria typically communicate by using oligopeptide signals that are detected by two-component phosphorelay proteins (Lyon and Novick, 2004). The more recently discovered autoinducer 2 (AI-2) is a

mixture of S-adenosylmethionine-derived fuanones in chemical equilibrium (Xavier and Bassler, 2005a).

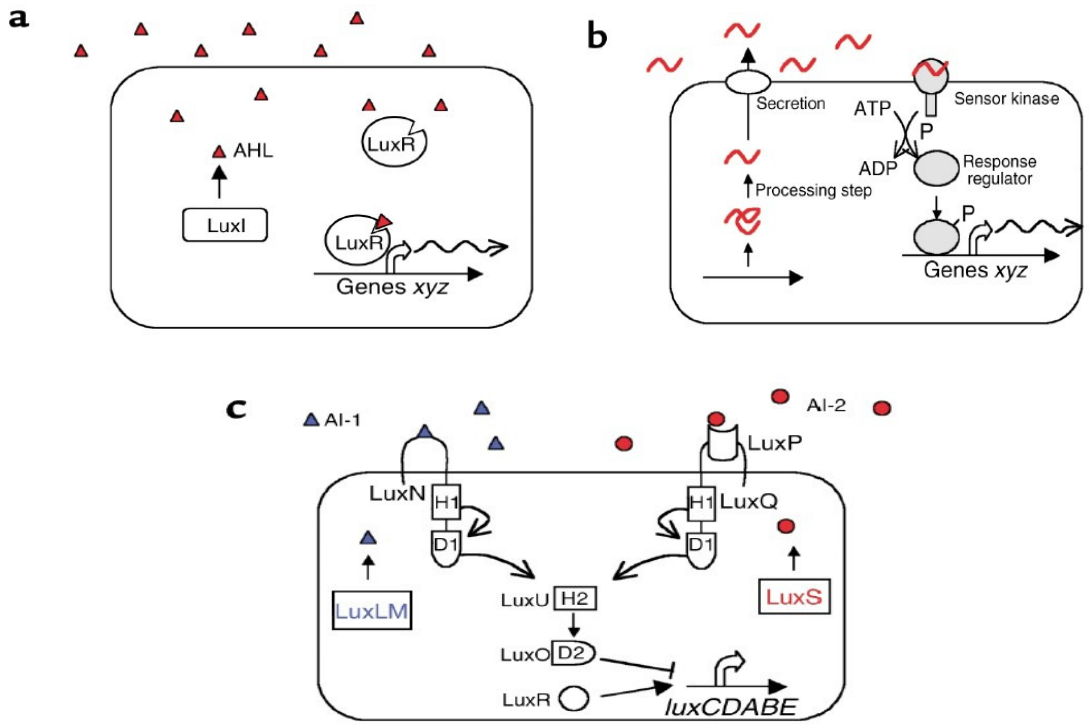


Figure 1.7 Three general quorum sensing circuits. (a) In Gram-negative bacteria, AHLs (red triangle) are produced by LuxI-type proteins and are detected by LuxR-type proteins. Upon interaction with cognate AHLs, LuxR proteins bind specific DNA promoter regions and activate transcription of target genes. (b) Oligopeptides (red waved lines) are synthesized, typically modified and actively secreted by Gram-positive bacteria. Detection occurs via a two-component signal transduction circuit, leading to the phosphorylation of a response regulator protein, which can bind promoter DNA and regulate gene expression. (c) AI-1 (blue triangle) and AI-2 (red triangle) quorum sensing in *V. harveyi*, which incorporates mechanistic aspects of both (a) and (b). (Adapted from Federle and Bassler, 2003, more details in text)

AI-2 has already been found in many different bacteria, including Gram-positive and Gram-negative, and genetic and biochemical evidence suggests it to be a universal signal for communication across species (Figure 1.7) (Henke and Bassler, 2004).

1.2.1 Quorum sensing in Gram positive bacteria

Quorum sensing in Gram positive bacteria rely on small peptide (Figure 1.8) which is produced post-transitionally modified (or cleaved) and secreted by ABC-type transporter.

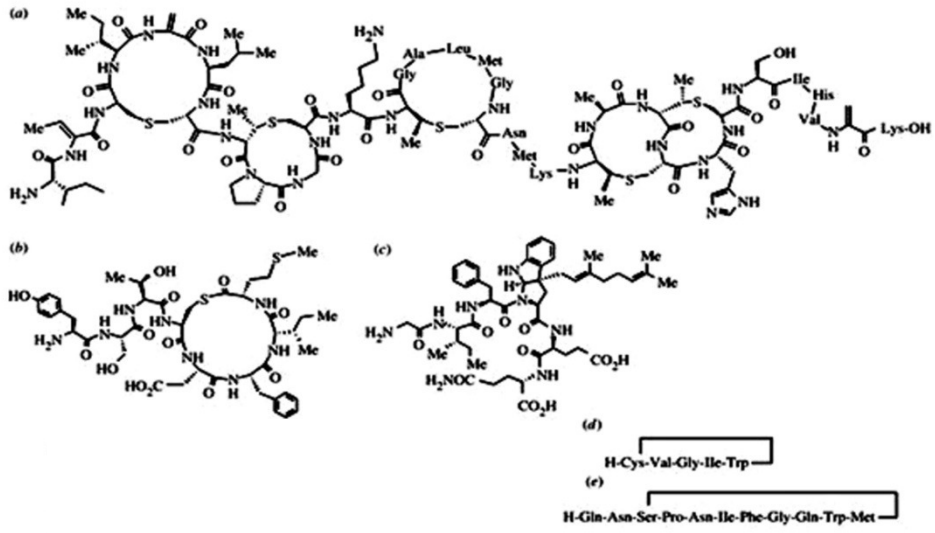


Figure 1.8 Quorum sensing oligopeptides from various Gram positive bacteria. Nisin from *Lactococcus Lactis* (a), AIP from *Staphylococcus* (b), ComX involving in competence development from *B. subtilis* (c), AIP from *Lactobacillus plantarum* (d), AIP from *E. faecalis* (e). (Adapted from Williams *et al*., 2007).

Streptococcus pneumoniae, which becomes transiently "competent" to take up environmental DNA at high cell density, is a representative example of a Gram positive system. Tomasz (1965) showed that the induction of competence resulted from an extracelluar factors known as Competence Stimulating Peptide (CSP). He further showed that the competence depends on the cell density and cells behave coordinately. CSP has since been characterized as a cationic, 17 amino acid peptide containing a gly-gly leader sequence (Håvarstein *et al*., 1995). The molecular fate of this CSP is depicted in Figure 1.9.

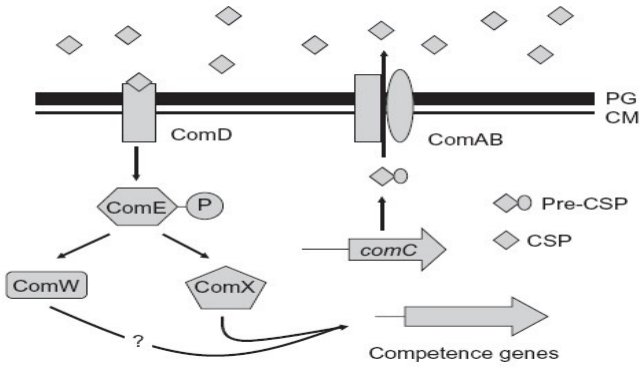


Figure 1.9 Competence development of *S. pneumoniae* by CSP. CSPs are synthesized by c*omC*, and modified and secreted by an ABC-type transporter, ComAB. The detection of CSP occurs via a two-component signal transduction circuit, composed of ComD and ComE, a signal receptor histidine kinase and cognate response regulator, respectively. The CSP-ComD complex can then bind to the DNA promoter and regulate gene expression including competence induction. PG, peptidoglycan; CM, cytoplasmic membrane. (Adapted from Antunes and Ferreira, 2009)

The development of natural competence in *S. pneumoniae* is regulated by the action of a two component signaling system encoded by *comC* and *comD*, which encode the peptide signal CSP and its cognate receptor, respectively; and *comE* encodes a response regulator for CSP (Håvarstein *et al*., 1996). This, in turn, initiates the transcription of >100 additional genes that are expressed in a temporal sequence, beginning first with genes functionally associated with regulating competence and transformation, and later to genes with functions affecting cellular stress responses, virulence, bacteriocin production, and others (Claverys and Håvarstein, 2002).

Peptide signalling by *Staphylococcus aureus* is also a well-studied quorum sensing system (Boyen *et al*., 2009; Novick and Geisinger, 2008). *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrom and abscesses, to life-threatening diseases (Kluytmans, 1997). A major problem associated with *S. aureus* infections is the rise of methicillin resistant *S. aureus* (MRSA) as well as multidrug resistant strains (MDR) (Fey *et al*., 2003). Basically there are two phenotypes. At low cell density *S. aureus* express proteins involved in attachment and colonization (adhesive) for host invasion, but as the cell density increases, it finally releases a toxin and protease (invasive) (Novick, 2003; Novick and Geisinger, 2008). This switch in phenotypes is mediated by the *agr* (accessory gene regulator) quorum sensing system. In *S. aureus* the *agr* system consists of a four gene operon, the *arg*BDCA genes. The *arg*D gene encodes the autoinducing peptide (AIP) that is processed by AgrB, which adds a thiolactone ring to the peptide, which is then transported out of the cell (Novick *et al*., 1995). The AIP is then sensed by a membrane-bound histidine sensor kinase, ArgC. Upon AIP binding to ArgC, the complex of AIP-ArgC phosphorylates and activates AgrA, a

transcription factor that regulates a small regulatory molecule, RNAIII, which in turn upregulates the expression of invasive virulence factors (Novick. 2003) (Figure 1.10). In analogy to *S. pneumoniae* and *S. aureus*, most quorum sensing systems in Gram positive bacteria incorporate a two-component regulatory system, consisting of a sensory histidine kinase and a response regulator (See Lyon and Novick, 2004; Sturme *et al*., 2002 for reviews).

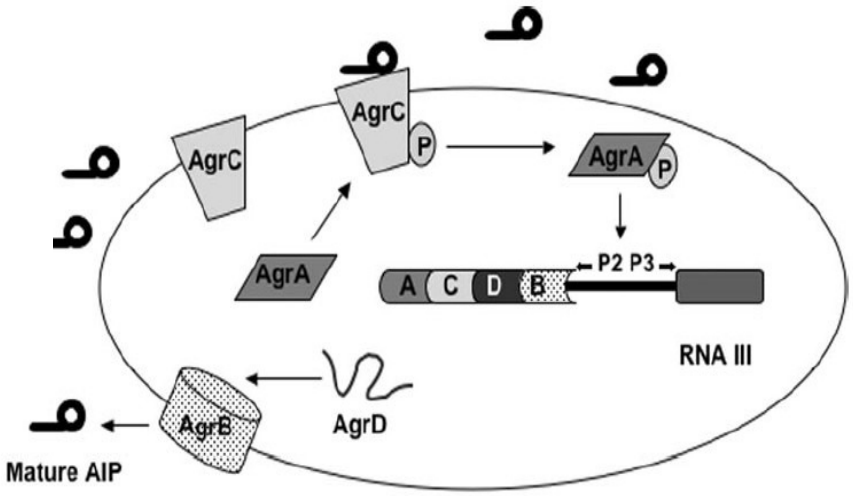


Figure 1.10 Schematic overview of the accessory gene-regulator (*agr*) system in *S. aureus*. The *agr* locus consists of two divergent operons driven by the P2 and P3 promoters, which modulate transcription of two transcripts, RNAII and RNAIII, respectively. The P2 operon contains *agrBDCA* and codes for the RNAII transcript. The signal peptide (AgrD) is processed by AgrB, integral membrane protein, resulting in secretion of a mature oligopeptide. AgrA and AgrC comprise a two-component regulatory system (a receptor histidine kinase and a response regulator, respectively) that responds to the secreted peptides. Once activated, AgrC undergoes auto-phosphorylation and activates the response regulator, AgrA, which goes on to directly activate transcription at the intergenic P2 and P3 promoters. Thus, *agr* system results in a positive feedback loop and transcription of RNAIII. RNAIII is known to act to increase the expression of secreted virulence factors and decrease the expression of several surface adhesins, including protein A and the fibronectin-binding protein. (Adapted from George and Muir, 2007)

1.2.2 Quorum sensing in Gram negative bacteria

Most common signaling molecules found in Gram negative bacteria are N-acyl derivatives of homoserine lactone (Eberl, 1999; Swift *et al*., 2001). Modulation of the physiological processes controlled by AHLs occurs in a cell density- and growth phase-dependent manner. Therefore, the term 'quorum sensing' has been coined to describe this ability of bacteria to monitor cell density before expressing specific genes for phenotypic traits

V. fischeri lives in symbiotic associations with a number of marine animal hosts. In these partnerships, the host uses the light production by *V. fischeri* for specific purpose such as attracting prey, avoiding predators, or finding a mate (Ruby, 1996; Visick and McFall-Ngai, 2000). When these bacteria are cultured in broth, the onset of exponential growth occurs without a lag but luminescence does not increase until mid-logarithmic phase, and maximum luminescence occurs in the stationary phase. Furthermore, luminescence in early-log-phase cultures can be induced by addition of cell-free fluid extract from the stationary phase. Later on, the *V. fischeri* autoinducer was identified, which turned out to be N-3-(oxohexanoyl)-homoserine lactone (Eberhard *et al*., 1981), and the regulatory circuit controlling quorum sensing in *V. fischeri* was elucidated (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984; 1987). They showed that two regulatory components are required for the process: the gene *luxR* encodes a transcription factor that positively activates the *luxICDABEG* operon, which encodes the genes for luciferase enzymes for luminescence, and the *luxI* gene encodes the enzyme for autoinducer biosynthesis (Figure 1.11).

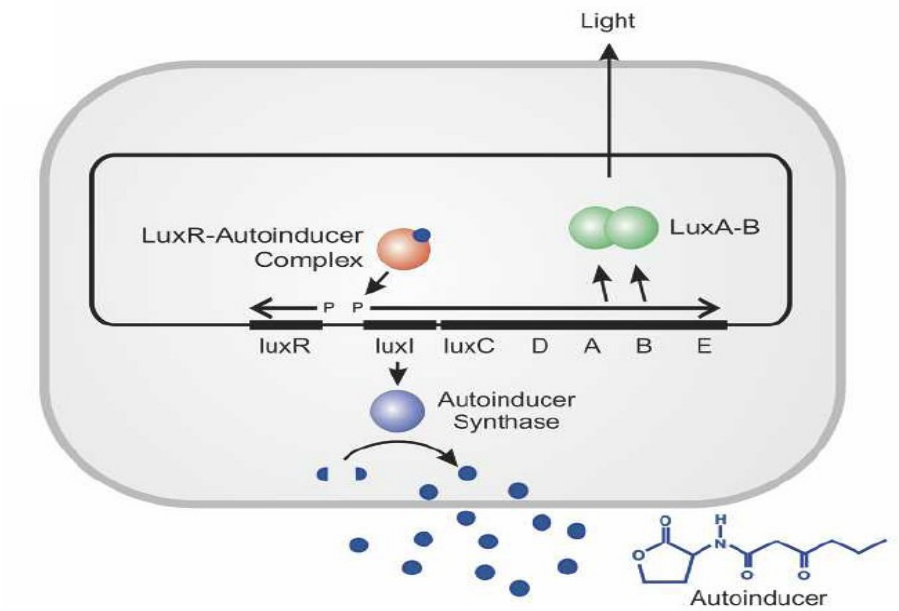


Figure 1.11 N-acyl homoserine lactone (AHL) quorum sensing in *V. fischeri.* *V. fischeri* cells express *luxI* (LuxI, light blue circle) at a basal level at low cell densities, so the concentration of OHHL (N-3-(oxohexanoyl)-homoserine lactone, small blue circle) in the medium is low. However, as the population density increases, the concentration of OHHL in the environment also increases. Once a critical concentration of OHHL has been achieved, the autoinducer can be bound by its cytoplasmic counterpart, the LuxR protein (red circle). Upon interaction with a cognate AHL, a LuxR protein binds specific DNA promoter elements and activates downstream target gene expression, resulting in emission of light. Briefly, LuxI protein of *V. fisheri* produces AHLs which are detected by AHL-dependent transcription factors, LuxR. When the autoinducer concentration reaches the threshold, its efflux from the cells becomes balanced by an influx, so that it can interact with LuxR. LuxR–autoinducer complexes bind the luciferase promoter and activate transcription (Adapted from Sprague and Winans, 2006).

1.2.2.1 LuxI/LuxR regulation in diverse bacterial species

In recent years, the microorganism on which most quorum sensing related studies have been initiated is *Pseudomonas aeruginosa*, reflecting its importance as a common opportunistic human pathogen (Alonso *et al*., 1999). The basis of the pathogenicity of *P. aeruginosa* is its ability to produce and secrete multiple extracellular virulence factors (Prithiviraj *et al*., 2005). The expression of the virulence factor elastase is under the control of the transcription activator LasR, which is a homolog of LuxR (Gambello and Igleweki, 1991; Gambello *et al*., 1993; Passador *et al*., 1993). In addition, the *luxI* homolog gene *LasI* was also identified and found to be essential for high-level expression of elastase in *P. aeruginosa* (Passador *et al*., 1993). Later on, the autoinducer N-(3-oxoxdodecanoyl)-l-homoserine lactone was identified (Pearson *et al*., 1994).

Agrobacterium tumefaciens is a pathogen that induces crown gall tumours in plants via transfer of oncogenic DNA to the nucleus of its host. It was found that a diffusible compound produced by *A. tumefaciens* can enhance conjugal transfer of *A. tumefaciens* Ti (tumour inducing) plasmid (Zhang and Kerr, 1991). The purification and structural identification of this diffusible compound showed that it is N-(3-oxooctanoyl)-L-homoserine lactone (Zhang *et al*., 1993). This small compound is very similar to *V. fischeri* autoinducer, with the same homoserine lactone but a longer acyl chain. Simultaneously, the autoinducer-dependent transcription factor TraR, a homolog of LuxR, was identified in *A. tumefaciens* (Piper *et al*., 1993). Based on these finding, it was suggested that N-acyl homoserine lactones may be widely conserved signals for gene regulation and the LuxR like regulatory mechanism may be common among different microorganisms (Zhang *et al*., 1993; Piper *et al*., 1999).

It is a similar story for the plant pathogen *Erwinia carotovora*. *E. carotovora* produces a variety of cell-wall degrading enzymes required for virulence (Hilton *et al*., 1989) and these virulence factors are activated during the late stages of bacterial growth (Williams *et al*., 1992). A search for *E. carotovora* mutants that fail to produce these exoenzymes led to the discovery of the *expI* gene, a homolog of *luxI* (Jones *et al*., 1993), and a diffusible molecule, an analog of the *V. fischeri* autoinducer, was also found to regulate exoenzyme production in *E. carotovora* (Pirhonen *et al*., 1993). Later on, *E. carotovora* was found to have two pairs of LuxR/LuxI homologs, ExpR/ExpI and CarR/CarI. Both ExpI and CarI produce the same, indeed identical, autoinducer, N-(3-oxohexanoyl)-homoserine lactone (Andersson *et al*., 2000), and it is likely that CarR and ExoR respond to the same signal to regulate production of the antibiotic carbapenem and exoenzyme, respectively. These discoveries suggest that diverse bacterial species can use a conserved mechanism, designated as quorum sensing (Fuqua *et al*., 1994) to regulate different biological functions (Table 1.3).

Table 1.3 The bacterial species utilizing N-acyl homoserine lactone (AHL) quorum sensing signal and LuxIR homologs to regulate different biological functions.

Bacterial species	AHLs signals	LuxIR homologs	Biological functions
Vibrio fischeri	N-(3-oxohexanoyl)-HSL	LuxIR	bioluminescence (Eberhard *et al*., 1981)
Aeromonas hydrophilia	N-butanoyl-HSL	AhyIR	exoprotease production (Swift *et al*., 1997;1999)
Aeromonas salmonicida	N-butanoyl-HSL	AsaIR	exoprotease production (Swift *et al*., 1997;1999)

Table 1.3 *Continued*

Bacterial species	AHLs signals	LuxIR homologs	Biological functions
Agrobacterium tumefaciens	N-(3-oxooctanoyl)-HSL	TraIR	Ti plasmid conjugal transfer (Zhang *et al*., 1993; Piper *et al*., 1993)
Burkholderia cepacia	N-octonoyl-HSL	CepIR	protease and siderpore production (Lewenza *et al*., 1999)
Chromobacterium violaceum	N-hexanoyl-HSL	CviIR	violacein pigment, hydrogen cyanide, antibiotics production (McClean *et al*., 1997)
Erwina carotovora	N-(3-oxohexanoyl)-HSL	(a) ExpIR (b) CarIR	(a) exoenzyme synthesis (b) carbapenem antibiotics synthesis (Jones *et al*., 1993; Pirhonen *et al*., 1993)
Erwina chrysanthemi	N-(3-oxohexanoyl)-HSL	ExpIR	Regulator of pectinase synthesis (Nasser *et al*., 1998)
Erwina stewartii	N-(3-oxohexanoyl)-HSL	EsaIR	capsular polysaccharide biosynthesis, virulence (Beck von Bodman *et al*., 1995)
Pseudomonas aereofaciens	N-hexanoyl-HSL	PhzIR	Phenazine antibiotic biosynthesis (Pearson *et al*., 1995)
Pseudomonas aeruginosa	(a) N-(3-oxododecanoyl)-HSL	(a) LasIR	(a) exoprotease virulence factors, biofilm formation (Pearson *et al*., 1994)
	(b) N-butyrl-HSL	(b) PhlIR	(b) rhamnolipid, stationary phase (Pearson *et al*., 1995)
Ralstonia solanacearum	N-hexanoyl-HSL N-octanoyl-HSL	SolIR	unknown (Flavier *et al*., 1998)

Table 1.3 *Continued*

Bacterial species	AHLs signals	LuxIR homologs	Biological functions
Rhizobium etili	Multiple, unconfirmed	RaiIR	restriction of nodule number (Rosemeyer *et al*., 1998)
Rhizobium leguminosarum	(a) N-hexanoyl-HSL	(a) RhiIR	(a) rhizosphere genes and stationary phase (Gray *et al*., 1996)
	(b) N-(3-hydroxyl-7-*cis*-tetradecenoyl)-HSL	(b) CinIR	(b) quorum sensing regulatory Cascade (Lithgow *et al*., 2000)
Rhodocacter sphaeroides	7,8-*cis*-N-(tetradecanoyl)-HSL	CerIR	prevention of bacteria aggregation (Puskas *et al*., 1997)
Serratia liquefaciens	N-butanoyl-HSL	SwrI/?	Swarmer cell differentiation, exprotease production (Eberl *et al*., 1996)
Vibrio anguillarum	N-(3-oxodecanoyl)-HSL	VanIR	unknown (Milton *et al*., 1997)
Yersinia entrocolitica	N-hexanoyl-HSL, N-(3-oxohexanoyl)-HSL	YenIR	unknown (Throup *et al*., 1995)
Yersinia pseudotuberculosis	(a) N-(3-oxohexanoyl)-HSL	(a) YpsIR	hierarchical quorum sensing cascade
	(b) N-octanoyl-HSL	(b) YtbIR	bacterial aggregation and motility (Atkinson *et al*., 1999)

(Adapted and from De Kievit and Iglewski, 2000 and updated)

1.2.2.2 LuxI family of N-acyl homoserine lactone (AHL) synthesis

It has been established that most AHLs are generated by the AHL Synthase LuxI family

proteins (Eberhand *et al*., 1991; Schaefer *et al*., 1996; Parsek *et al*., 1999), although some other enzymes such as AinS and LuxM were found to also synthesize AHLs (Gilson *et al*., 1995; Bassler *et al*., 1993). S-adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP) are substrates for the LuxI-type enzymes. Purification and *in vitro* studies with TraI and LuxI showed that AHL synthesis required SAM and the fatty acid biosynthetic precursors provided as acyl-ACP conjugates (More *et al*., 1996; Schaefer *et al*., 1996). The LuxI family proteins couple a specific acyl-group to SAM via amide bond formation between the acyl side chain of the acyl-ACP and the amino group of the homocysteine moiety of SAM. The subsequent lactonization of the ligated intermediate in the reaction, along with the release of methylthioadnosine, results in the formation of AHLs (Parsek *et al*., 1999).

Since AHLs differ only in their acyl chains (Figure 1.12), it seems that the specificity of different AHLs is determined by the interaction of a particular LuxI family protein with the corgnate acyl-ACP. Analysis of the structure of LuxI family proteins in different quorum sensing systems indicated that they are more conserved at the amino-terminus than at the carboxy-terminus (Hanzelka *et al*., 1997; Parsek *et al*., 1997), while the carboxy terminus could provide recognition of the different acyl chains of precursor acyl-ACPs.

1.2.2.3 LuxR family of transcriptional regulatory protein

The LuxR-type protein, a transcription factor, is the master controller in the AHLs quorum sensing regulatory scheme. The LuxR-type proteins carry out transcriptional regulation of target genes responding to accumulated AHLs through several steps: specific binding with cognate signal AHLs; followed by conformational changes and multimerization; binding to

the specific regulatory sequences upstream of target genes; and finally, activation of transcription (Parsek *et al*., 1999; Miller and Bassler, 2001).

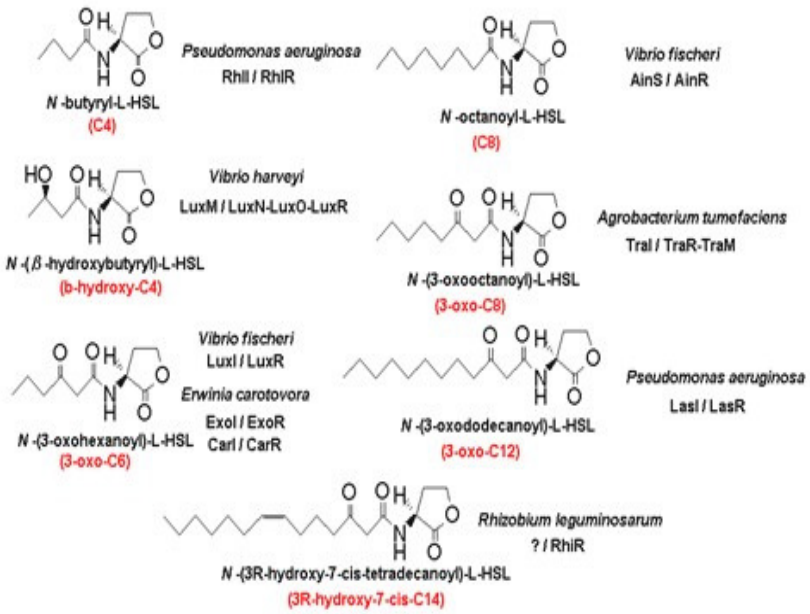


Figure 1.12 Structure of N-acyl homoserine lactone **(AHL) quorum sensing molecules from diverse bacterial species.** Each AHLs molecule incorporates a common lactone ring but only differ in acyl chains. (Adapted and modified from http://www.chem.utsunomiya-u.ac.jp/lab/bio/research_qs2.html)

Genetic and biochemical evidence indicates that LuxR-type proteins act as the receptor for AHLs. AHLs can be co-purified with LuxR-type proteins from *E. coli* cells expressing *LuxR* or *LasR* in the presence of AHLs (Adar and Ulizur, 1993; Pearson *et al*., 1997). A mutation in *LuxR* can reduce or abolish the association of 3-oxo-hexanoyl-HSL with LuxR (Hanzelka and Greenberg, 1995). Purified TraR and CarR, from *A. tumefaciens* and from *E. carotovora* respectively, also form stable complexes with their cognate AHLs, 3-oxo-octanoyl-HSL and 3-oxo-hexanoyl-HSL, respectively (Zhu and Winans, 1999; Welch *et al*., 2000). Further analysis indicated that the *V. fischeri* LuxR protein consists of two domains, the amino-terminal domain is involved in binding the AHLs, while the carboxyl-terminal domain involved in multimerization and DNA binding (Shadel *et al*., 1990; Slock *et al*., 1990; Choi and Greenberg, 1991; 1992; Stevens *et al*., 1994). It is likely based on sequence comparison that other LuxR-type proteins also interact with AHLs at their amino-terminus.

To activate target gene expression, the LuxR-AHLs complex needs to bind to the promoter region of target genes. The DNA sequence element recognized by the complex is often called a *lux* box. The *lux* box is an inverted repeated sequence ranging from 18-22 base pairs, which was originally identified upstream of the lux operon transcriptional start site in *V. fischeri* (Devine *et al*., 1989; Egland and Greenberg, 1999) and now has been found in several different bacteria (Fuqua *et al*., 1994; Gray *et al*., 1994). The sequence of *lux*-type boxes is quite conserved among diverse bacteria.

However, there are some examples that show that different promoter architectures can also be recognized by LuxR-type proteins (Fuqua *et al*., 1995; Fuqua and Winans, 1996a; Fuqua *et a l*., 1996b), suggesting that such regulatory elements could be divergent. Most LuxR-type

proteins are transcriptional activators that positively regulate target genes (Stevens and Greenberg, 1997; Zhu and Winans, 1999).

1.2.3 LuxS/autoinducer 2 (AI-2) signaling system and interspecies communication

During the past decade, research groups throughout the world have grown to understand that the behaviour of bacteria is influenced by cell-cell communication. Small molecules produced and released by bacteria can be considered as 'words' which can reach other cells and elicit 'answers'. The language used by these bacteria is chemical in nature and generally designated as quorum sensing (Bassler, 2002). As described above, these languages include N-acyl homoserine lactone (AHLs) for Gram negative bacteria and oligopeptides for Gram positive bacteria.

Beyond controlling gene expression on a global scale, quorum sensing also allows bacteria to communicate within and between species. This notion arose with the discovery and study of autoinducer 2 (AI-2), which was first discovered in *Vibrio harveyi* (Bassler *et al*., 1993). Originally, Greenberg *et al*., (1979) promoted the notion of AI-2 acting as an interspecies signal. The supernatant of the marine bacteria *Beneckea harveyi* and *Photobacterium fischeri* can induce *V. harveyi* luminescence although their own signalling cannot activate their own luciferase. Later it was revealed that the above two strains could only produce luminescence when the signalling molecules had accumulated to a critical concentration in the growth medium (Greenberg *et al*., 1979). After the finding of *luxS* in *E. coli* and *S. typhimurium* in 1999 (Surette et al., 1999), which produces AI-2, *luxS* has been shown to be conserved in roughly half of all sequenced bacterial genomes (De Keersmaecker *et al*., 2006). AI-2

production has been verified in a large number of these species, and AI-2 controls gene expression in various bacteria. Together, these findings have led to the hypothesis that bacteria use AI-2 to communicate between species (Federle and Bassler, 2003; Winzer, 2002b).

1.2.3.1 LuxS/autoinducer 2 (AI-2) quorum sensing

The first observation that bacteria could utilise multiple quorum sensing signals to communicate was in the quorum sensing system of bioluminescent marine bacterium *V. harveyi*. The *V. harveyi* quorum sensing system consists of two autoinducers and two cognate receptors functioning in parallel to channel information into a shared regulatory pathway. An AHL signal, termed AI-1, is synthesized by the protein LuxI and is detected by the two-component hybrid sensor kinase-response regulator protein LuxR (Figure 1.12). The second *V. harveyi* signal, AI-2 is synthesized by the enzyme called LuxS (Surette *et al*., 1999; Pei and Zhu, 2004). AI-2 is a furanosyl borate diester (Chen *et al*., 2002) and like AHLs, AI-2 is derived from SAM (Schauder and Bassler, 2001b). Detection of AI-2 requires two proteins: LuxP and LuxQ (Bassler *et al*., 1994). LuxP is a soluble periplasmic protein, resembling ribose binding proteins such as RsbB of *Escherichia coli*. LuxP is the AI-2 binding protein, and the LuxP-AI-2 complex interacts with the second protein required for detection, LuxQ. LuxQ is similar to LuxN and is a hybrid two-component protein containing both sensor kinase and response regulator modules. Sensory information from LuxPQ is transduced to the phosphotransferase protein LuxU, and LuxU transmits the signal to the downstream response regulator LuxO (Lilley and Bassler, 2000) (Figure 1.12). In the absence of signal (at low cell density), LuxQ has intrinsic kinase activity and phosphorylates a complex phosphorelay system, with LuxU and LuxO as intermediaries.

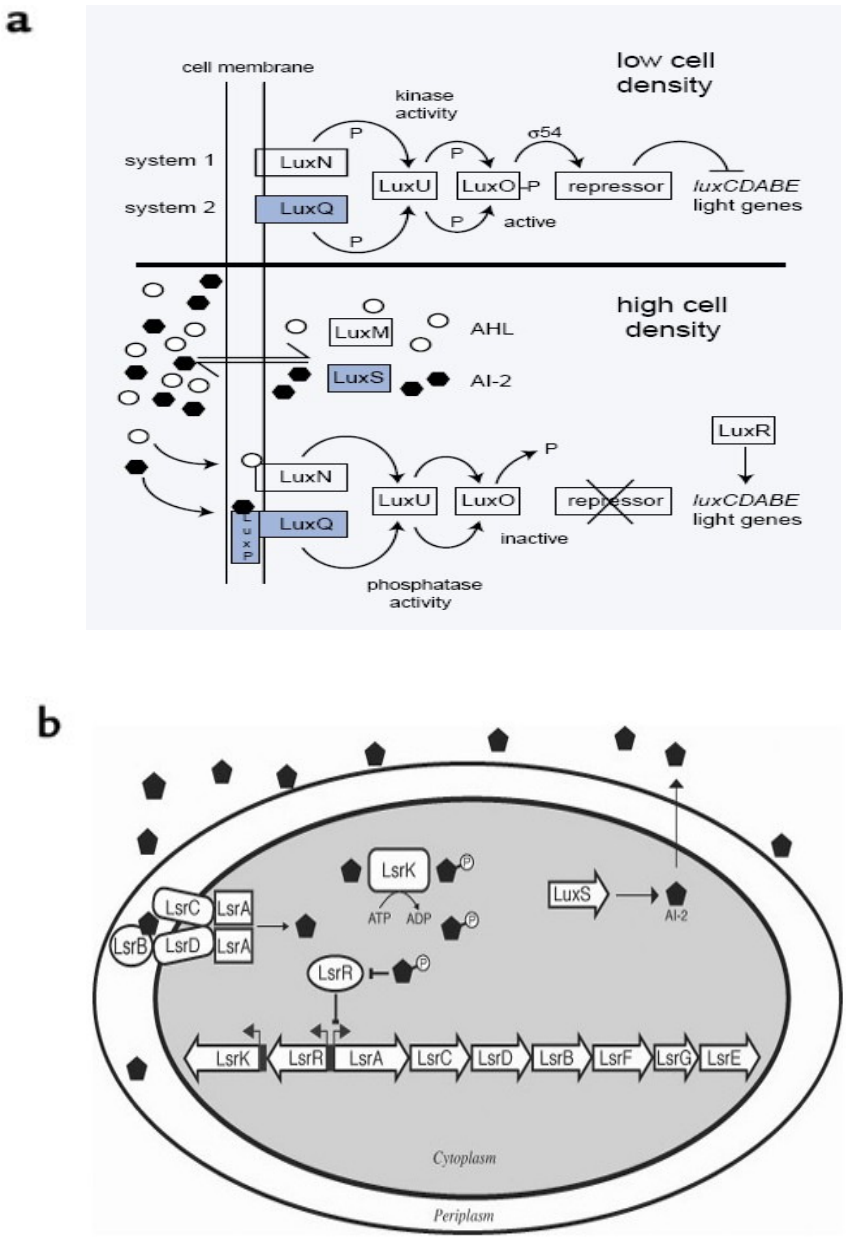


Figure 1.13 LuxS/autoinducer 2 (AI-2) quorum sensing. (a) In *V. harveyi*, at the low cell density, phosphorylated LuxO represses the luciferase operon. However, upon sensing AI-2 by LuxP, the LuxQ and LuxU kinases become phosphatase and the phosphorelay system is dephosphorylated, allowing LuxR to activate bioluminescence. (b) In *Salmonella* and *E. coli*, the AI-2 receptor is the LsrB periplasmic protein. Upon binding LsrB, AI-2 is transported inside the cell via the Lsr ABC transport system. AI-2 is then phosphorylated by LsrK, and

Figure 1.13 *Continued*

interacts with LsrR. The LsrR-AI-2 complex no longer represses *lsr* transcription. LsrR is a transcriptional repressor of the *lsr* operon (Adapted from Camara *et al*., 2002; Reading and Sperandio, 2006).

Phospho-LuxO in conjunction with σ54 then activates transcription of small regulatory RNAs (sRNA), which destabilize the mRNA for the LuxR protein, which in turn can no longer activate transcription of the luciferase operon. However, when the AI-2 signal accumulates to a level required for detection, the three sensors (e.g. LuxQ, U and O) switch from being kinases to being phosphatases, triggering dephosphorylation of these proteins. Finally, unphosphorylated LuxO cannot induce expression of the sRNA. This allows translation of *luxR* mRNA, production of LuxR, and expression of bioluminescence (Xavier and Bassler, 2003). Although a variety of bacterial species harbor the *luxS* gene and have activity as measured using a *V. harveyi* bioluminescence assay (De Keersmaecker, 2003); AI-2 binding proteins, LuxP homologs, as well as homologs from the quorum sensing cascade, have only been found in *Vibrio* species (Reading and Sperandio, 2006). However, the only genes shown to be regulated by AI-2 in other species encode an ABC transporter in *Salmonella typhimurium* named Lsr (LuxS-regulated), responsible for the uptake of AI-2 (Taga *et al*., 2001). This ABC transporter is also present in *E. coli* and shares homology with the ribose transporter. The *lsr* operon consists of the *lsrACDBGE* genes. *lsrR* encodes a protein that represses expression of the *lsr* operon in the absence of AI-2 and LsrK is a kinase that phosphorylates AI-2 upon entry into the cell (Taga *et al*., 2003). It is intriguing that the *lsr* operon that is regulated by AI-2 encodes functions resembling the ribose transporter, given the recent finding that AI-2 is derived from the ribosyl moiety of S-ribosylhomocysteine. The

difference between *Salmonella* and *E. coli*, and *Vibrio harveyi* strains in the AI-2 quorum sensing is illustrated in Figure 1.14.

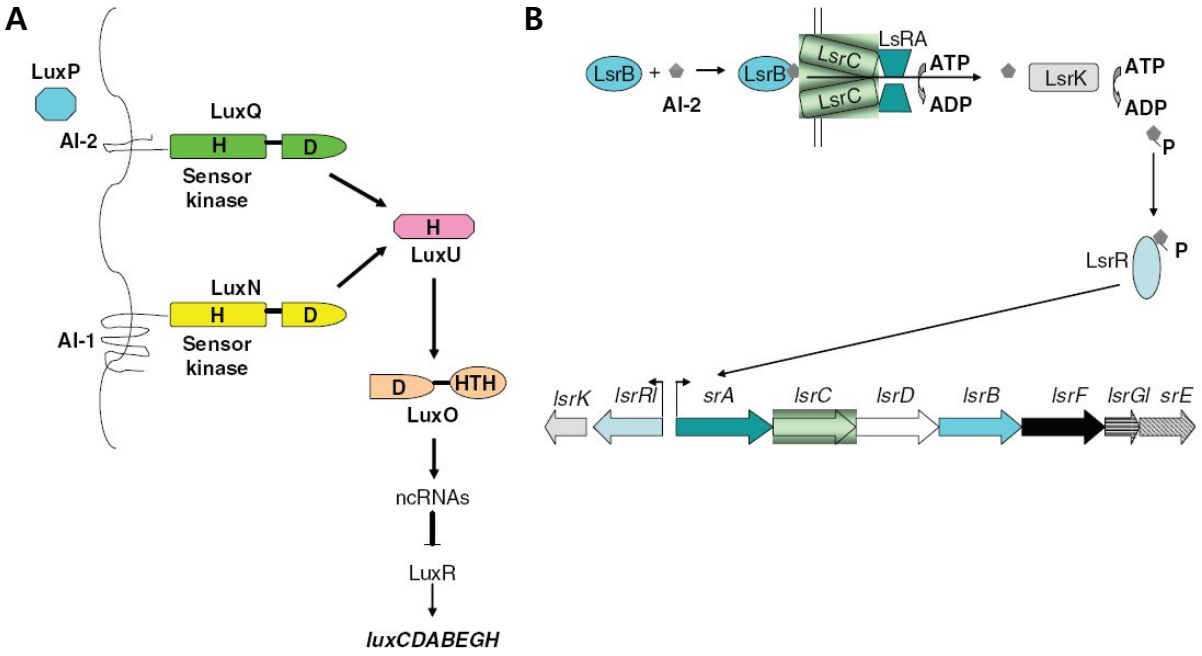


Figure 1.14 The different LuxS/autoinducer 2 (AI-2) system in *V. harveyi, E. coli* and *Salmonella*. To recognize the AI-2 quorum sensing molecules, *Vibrio harveyi* uses the LuxQ kinase, while *E. coli* and *Salmonella use LsrB*, periplasmic receptor. In *Vibrio harveyi* (A), upon sensing the signals by LuxP (receptor kinase) and LuxQ, this complex becomes a phosphatase and this complex phosphorelay system through LuxU and LuxO is dephosphorylated. Finally small regulatory RNAs no longer repress LuxR. However, In *E. coli* and *Salmonella* (B), After AI-2 binds LsrB, it is transported inside the cells via the ABC Lsr transporter. It is then phosphorylated by LsrK, resulting in activation of *lsr* transcription (Adapted from Reading and Sperandio, 2006, see more details in text).

1.2.3.2 AI-2/LuxS controlled behaviours in bacteria

The enzyme, LuxS produces AI-2 in a wide variety of bacteria and it functions as hydrolase (Figure 1.15 and 1.16). Many recent reports show that both Gram positive and Gram negative bacteria sense and regulate an assortment of 'niche-specific' gene expression using AI-2 signal molecules (Xavier and Bassler, 2003; Sprague and Winans, 2006).

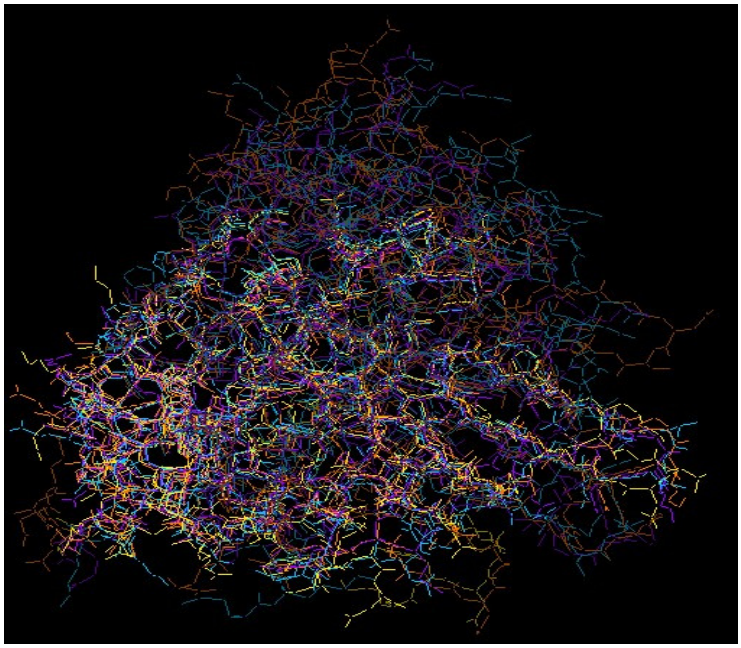


Figure 1.15 LuxS protein structure alignment from various bacterial strains. LuxS enzymes are various in bacteria species and the overlaps are conserved region of LuxS enzymes. (Adapted from homologous: alignment AL00064294: Autoinducer-2 production protein LuxS, http://sisyphus.mrc-cpe.cam.ac.uk/sisyphus/showspicejnlp.jsp?alignment=AL00064294). Bacterial strains harboring LuxS enzyme are approximately more than 100 strains including bacteria subspecies according to LuxS search in pubmed (Sep. 2011) and it consists of from 138 to 172 amino acids.

AI-2 molecules are all derived from DPD, a product of the LuxS enzyme and DPD undergoes rearrangement to produce a collection of interconverting molecules (Figure 1.16 and 1.17), some of which encode information. Presumably, AI-2 interconversions allow bacteria to respond to endogenously produced AI-2 and also to AI-2 produced by other bacteria species in the vicinity, giving rise to the idea that AI-2 represents a universal language: a "Bacterial Esperanto" (Winans, 2002). AI-2 controls a variety of traits in different bacteria ranging from bioluminescence to virulence in many clinically relevant pathogens. The functions of AI-2 that have been reported in the literature are listed in Table 1.4.

Table 1.4 Biological functions controlled by LuxS in bacteria

Bacterial species	Functions regulated by LuxS	References
Actinobacillus actinomycetemcomitans	Virulence factors: leukotoxin, iron acquisition, biofilm	Fong *et al*., 2001; 2003; Shao *et al*., 2007.
Borrelia burgdorferi	Pleotropic protein expression	Stevenson and Babb, 2002.
Campylobacter jejuni	Motility	Elvers and Park, 2002.
Clostridium difficile	Virulence genes expression	Lee and Song, 2005.
Clostridium perfringens	Virulence factors: alpha, beta and theta toxins	Ohtani *et al*., 2002.
E. coli W3110	Cell division, motility, metabolism	De Lisa *et al*., 2001.
E. coli EHEC (O157:H7)	Virulence factors: type III secretion, shiga toxin, motility, cell division	Sperandio *et al*., 1999;2002a; 2002b.
E. coli EPEC (O127:H6)	Motility (flagella expression)	Giron *et a*l., 2002.

Table 1.4 *Continued*

Bacterial species	Functions regulated by LuxS	References
Helicobacter pylori	Motility (flagella expression)	Rader *et al*., 2007
Klebsiella pneumoniae	Biofilm	Balestrino *et al*., 2005
Lactobacillus rhamnosus GG	Growth, biofilm	Lebeer *et al*., 2007
Listeria monocytogenes	Cell attachment, biofilm	Sela *et al*., 2006; Belval *et al*., 2006
Neisseria meningitidis	Bacteremic infection	Winzer *et al*., 2002b.
Photorhabdus luminescens	Antibiotic (carbapenem) synthesis	Derzelle *et al*., 2002.
Porphyromonas gingivalis	Virulence factors: protease, hemagglutinin activities, biofilm	Burgess *et al.*, 2002; Chung *et al*., 2001; McNab *et al*., 2003.
Salmonella typhi	Biofilm formation	Prouty *et al*., 2002
Salmonella typhimurium	ABC transporter expression	Taga *et al*., 2001; 2003.
Shigella flexneri	Transcription factor involving in controlling virulence	Day *et al*., 2001.
Staphylococcus epidermidis	Biofilm, virulence	Xu *et al*., 2006
Streptococcus anginosus	Biofilm, antibiotic susceptibility	Petersen *et al*., 2006; Ahmed *et al*., 2007
Streptococcus mutans	Biofilm formation, bacteriocin	Merritt *et al*., 2003; 2005.
Streptococcus pneumoniae	Virulence factor: protease (hemolysin)	Stroeher *et al*., 2003.
Streptococcus pyogenes	Virulence factor expression	Lyon et al., 2001.

Table 1.4 *Continued*

Bacterial species	Functions regulated by LuxS	References
Vibrio anguillarum	Virulence factor: metalloprotease expression	Denkin and Nelson, 2004
Vibrio cholerae	Virulence factors: Cholera toxin, toxin-coregulated pilus	Miller *et al*., 2001; Zhu *et al*., 2002
Vibrio harveyi	Luminescence, protease production, colony morphology, siderophore	Bassler *et al*., 1993; 1994; Lilley and Bassler, 2000; Mok *et al*., 2003.
Vibrio vulnificus	Virulence	Kim *et al*., 2003.

EHEC, enterohemorrhagic *E. coli;* EPEC, enteropathic *E. coli.*

(Adapted from Xavier and Bassler, 2003 and updated)

1.2.3.3 AI-2 synthesis and recycling of S-adenosylhomocystein (SAH)

The AI-2 synthesis pathway is part of the active methyl cycle, which transfers the methyl group of methionine, via S-adenosylhomocysteine, to various substrates (Surette *et al*., 1999).This process is critical for the biosynthesis of certain amino acids, nucleic acids, and proteins, and recycling of the toxic intermediate SAH (Winzer *et al*., 2002a) in all organisms (Chiang *et al*., 1996). This SAH recycling pathway is divided into a two-step conversion by the Pfs (5'methylthioadosine/S-adenosylhomocysteine nucleosidase, MTA/SAHase) and LuxS enzymes to produce adenine, homocysteine and DPD, and a one-step conversion using SAH dehydrolase (Figure 1.16). AI-2 is produced from S-adenosylmethionine (SAM) through a three-step enzymatic conversion that includes a two-step reaction (Schauder *et al*., 2001; Winzer *et al*., 2002a). SAM is an essential cofactor for processes such as DNA, RNA

and protein synthesis. SAM acts as a methyl donor and creates the toxic intermediate SAH, which is hydrolyzed by the enzyme Pfs to S-ribosylhomocysteine (SRH).

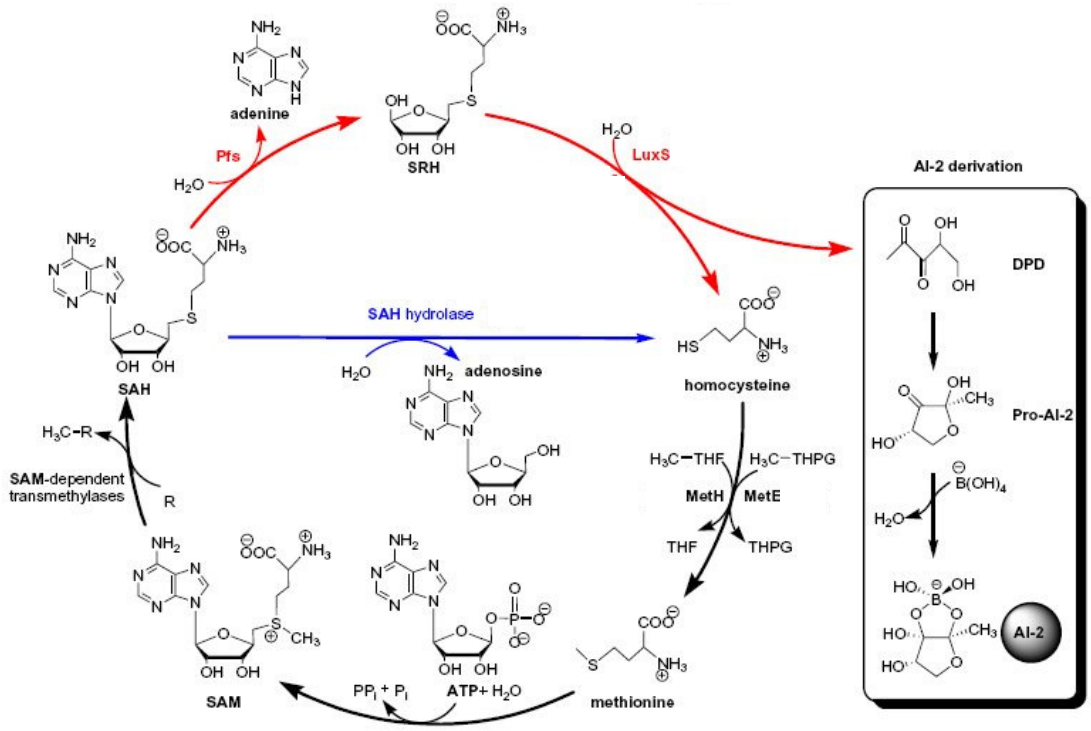


Figure 1.16 Enzymes involved in the detoxification of S-adenosylhomocystein (SAH) and synthesis of AI-2. Blue arrows represent one-step detoxification, and red arrows two-step. Abbreviations: SAM, S-adenosy-methionine; SAH, S-adenosyl-homocysteine; SRH, S-ribosyl-homocysteine; DPD, 4,5-dihydroxyl-2,3-pentanedione; Pro-AI-2, AI-2 precursor; AI-2, autoinducer 2. (Adapted from Sun *et al*., 2004)

The LuxS enzyme catalyzes the cleavage of SRH to form homocysteine and the AI-2 precursor, 4,5-dihydroxyl-2,3-pentanedione (DPD) (Chen *et al*., 2002). The LuxS enzyme responsible for the last enzymatic step of AI-2 synthesis is present in a wide phylogenic range of bacterial genera. Most Gamma-, Beta-, and Epsilonproteobacteria and firmicutes possess the Pfs-LuxS pathway, while Archaea, Eukarya, Alphaproteobacteria, Actinobacteria and Cyanobacteria prefer the SAH dehydrolase pathway (Pappas et al., 2004; Sun *et al*., 2004).

The study of several gene mutations and the *in vitro* production of AI-2 with Pfs or/and LuxS enzymes, have shown that this biosynthetic pathway leading to DPD is identical in numerous microorganisms, including *E. coli, Salmonella enteric, V. harveyi, V. cholerae, Enterococcus faecalis, Neisseria meningitides, Porphyromonas gingivalis and Staphylococcus aureus* (Schauder *et al*., 2001b; Winzer *et al*., 2002a). DPD readily undergoes spontaneous cyclization and hydration to form several furanone ring formations that freely interconvert to (2R, 4S)- and (2R, 2S)-2,4-dihydoxyl-2-methyldihydrofuran-3-one (respectively R- and S-DHMF), and (2R, 4S)- and (2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytet-rahydrofuran (respectively R- and S-THMF) (Chen *et al*., 2002; Miller *et al*., 2002; Schauder *et al*., 2001) (Figure 1.17).

The X-ray crystal structure of LuxP and LsrB of *V. harveyi* and *S. typhimurium* bound to their respective ligands reveals that these two organisms detect different DPD-derived products. *V. harveyi* detects S-THMF-borate (Chen *et al*., 2002), which can be formed by the reaction of the adjacent hydroxyl groups on the furanosyl ring of S-THMF with borate that is abundant in marine environments (Loomis and Durst, 1992), whereas *S. typhimurium* detects R-THMF (Miller *et al*., 2004).

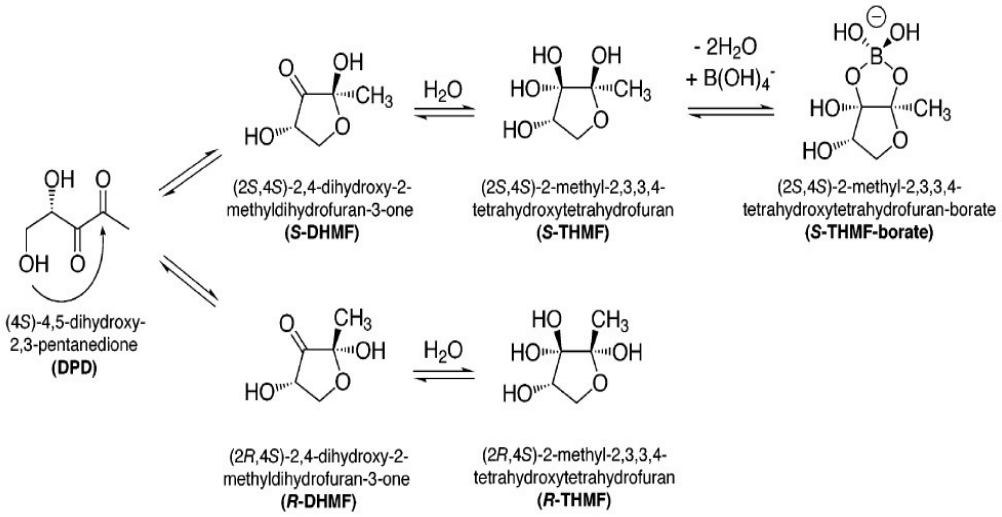


Figure 1.17 AI-2: an interconverting family of extracellular signal molecules. The precursor molecule, DPD, undergoes various rearrangements and additional reactions to form distinct biologically active AI-2 signal molecules. The *Vibrio harveyi* AI-2 (S-THMF-borate) is produced by the upper pathway, and the *Salmonella typhimurium* AI-2 (R-THMF) is produced by the lower pathway (Adapted from Miller *et al*., 2004).

Thus, these results indicate that multiple derivates of DPD are biologically active. To detoxify the intermediate SAH, the Pfs or SAH hydrolase one-step reaction is enough and it is not necessary to further convert SRH to DPD and homocysteine. Considering the SAM metabolism for methyl donor of Pfs detoxification mechanism, AI-2 is an excellent device for measuring the metabolic potential and detoxification to inform on the cell population, growth phase and prosperity (Xavier and Bassler, 2003). Thus, AI-2 is well suited to be a signal that specifies the growth phase of the population and the Pfs-LuxS pathway has the obvious advantage, of an additional product, DPD, which can contain and convey information (Xavier and Bassler, 2003).

1.2.4 The quorum sensing: evolutionary and ecological perspectives

The idea that cooperative behaviour between microorganisms is commonplace has gained widespread acceptance in recent years (Velicer 2003), and although a lot of excellent general overviews of bacterial cooperation from an ecological and evolutionary perspective have been produced by microbial ecologist, this chapter will mainly focus on interspecies bacterial communication and cheating to discuss *Z. mobilis* behaviour.

1.2.4.1 The definition of the signal

The word "signal" is widely used to define such substance in the context of quorum sensing, or communication between bacterial cells. For true communication to occur, first, one or several individuals must produce a signal that can be perceived by other organisms, and secondly, the perceivers must alter their behavior in response to this signal. Importantly, communication can be selected for and remain stable over evolutionary time only if both emitter and the receiver gain benefit from the communication (Keller and Surette, 2006). However, the natural occurrences of communication in microorganisms do not appear to coincide with this definition. For example, chemical X may be a waste product by cell A that is detected by cell B. Therefore, the definition of cue and chemical manipulation is needed to elucidate ambiguous phenomena (Diggle *et al*., 2007a; 2007b).

Chemical manipulation or coercion is chemical emission that alters the behaviour and gene expression of other organisms. However, contrary to a signal, the effect induced by chemical manipulation has a negative effect on the fitness of the receiver (Diggle et al., 2007a; 2007b).

An example of an autoinducer of one species being used for chemical manipulation by another might be the interaction between *Streptococcus gordonii* and *Veillonella atypica*, two early colonizing members of the dental plaque biofilm. *V. atypica* requires *S. gordonii* to be present to colonize dental surface because *S. gordonii* utilizes sugars and produces lactic acid, which is the preferred fermentation substrate for *V. atypica*. *V. atypica* produces a soluble chemical that induces amylase expression in *S. gordonii*, resulting in an increase of lactic acid. Apparently, there is no direct benefit for *S. gordonii* to respond to the substrate that is produced by *V. atypica* (Egland *et al*., 2004). Cue is an act, structure or chemical emission that alters the behaviour and gene expression of other organisms. However, contrary to a signal, it did not evolve specifically for that effect (Keller and Surette, 2006).

1.2.4.2 Public goods and bacterial cheating

Public goods are products manufactured by an individual that can be utilized by the individual or its neighbors (West *et al*., 2006; 2007) and cheaters are individuals who do not pay the costs of producing public goods but still exploit the benefits of public goods produced by other cells. Cheaters can disrupt cooperative systems by unfairly obtaining an excessive share of group-generated resources while making disproportionately tiny contributions. Evolutionary theory predicts that such selfish genotypes should readily invade cooperative populations in the absence of mechanisms to exclude them (Velicer, 2003). Cheaters do better when they are at lower frequencies in the population because they can exploit cooperators (West *et al*., 2007). They can become predominant in the community because they do not invest the energy required to produce public goods in the short term. Therefore, they have a high level of competitive fitness relative to wild type individuals. However, when the

cheaters in the population reach at a critical cell density, the entire population may crash because wild type cells contributing the public goods required for stable growth and maintenance becomes too low (Dunny *et al*. 2008).

One example of this nature is for siderophores, an iron-scavenging molecule in *P. aeruginosa*. When iron is limiting, the clinically screened *lasI* mutants (cheaters) can gain the benefit of sideropore production without paying the cost, and hence increase in frequency (Griffin *et al*., 2004). In quorum sensing, interspecies signalling between avirulent oropharyngeal flora (OF) and *P. aeruginosa* (mutant) within the cystic fibrosis (CF) lung resulted in an increase in virulence gene expression which was attributed to O3-C2-HSL (Duan *et al*., 2003). It is more likely that *P. aeruginosa* is able to use O3-C2-HSL as a cue, or it may be that OF bacteria coerce or manipulate *P. aeruginosa* into increased virulence which may provide them with more nutrients (Diggle *et al*., 2007c), because microorganisms in nature need to cope with extreme condition such as nutrient depletion and temperature changes, and should survive for extended periods without reproduction. In addition, cells rarely exist naturally as individuals, because one of their survival regulations is the ability to organize themselves into multicelluar communities and to differentiate into specialized cell variants (Palková, 2004).

In addition to the siderophores, Diggle *et al*., (2007c) in their elegant experiments showed that although quorum sensing can provide a benefit at the group level, exploitative individuals can avoid the cost of producing the quorum sensing signal or of performing cooperative behaviour. They further suggested that the problem of exploitation, which has been the focus of considerable attention in animal communication, also arises in bacteria. *S. aeruginosa* has two type of quorum sensing circuit, *las* and *rhl* system (Table 1.3). In the

experiments they conducted that they made use for the *lasI* mutant (signal-negative, non-producer) and *lasR* mutant (signal-blind, receptor deletion mutant) and the benefit was regarded as the protein elastase, the final product of quorum sensing, which induces rapid growth due a iron uptake specially, when iron is depleted. As expected, wild type cells grew more rapidly than signal-blind (producer, but does not respond) and -negative (non-producer, but does respond). However, the addition of an elastase led to increase of growth in mutant strains. This reflects the fact that quorum sensing public goods can provide a benefit at the population level. As they showed that the final density of mutant strains is much higher than wild type, they generalized that quorum sensing mediated cooperation is definitely costly. Finally they concluded that quorum sensing can be subject of cheating and its manner can be frequency dependent through coculture experiments with wild type and mutants.

1.2.4.3 Quorum sensing mediated bacterial interactions

Bacteria are often found in mixed population in the natural environment (e.g. biofilm). Therefore, a bacterial species may sense the information which is released by itself or other species in the communities if the language (information) is common. For example, *Chromobacterium violaceum* produces the purple pigment violacein in response to signaling molecules (e.g. C6-HSL) generated by it and supplied by another producer. This could provoke the following debate "bacterial communication, quorum sensing would be either cooperative or competitive in mixed culture condition?"

An *et al*. (2006) performed coculture experiment with two different species, *A. tumefaciens* and *P. aeruginosa*, leading them to elucidate that quorum sensing is among the mechanisms

by which bacteria compete and persist within microbial communities. *P. aeruginosa* is a Gram negative and an opportunistic pathogen that causes many nosocomial infections and is frequently associated with chronic lung infections that plague people suffering from cystic fibrosis. *P. aeruginosa* is a paradigm for the study of acyl-HSL-based quorum sensing and the formation of surface-associated communities called biofilms. *A. tumefaciens* is a Gram negative and causes crown gall disease in plants. This microbe has served as a model for horizontal gene transfer, host–microbe interactions, pathogenesis, and acyl-HSL-based signaling for many years (Zhang and Kerr, 1991). Both species are from the phylum of proteobacteria. In mixed coculture experiments, the density of *A. tumefaciens* at late stationary phase decreased in the coculture experiment with *P. aeruginosa* wild type but remains constant in coculture with a *P. aeruginosa* mutant (An *et al*., 2006). This could indicate that *P. aeruginosa* outcompete *A. tumefaciens* in coculture using the signal from *A. tumefaciens*, implying that such relationship is more likely competitive than cooperative.

Although LuxR, AHL receptor, homologues are wildly distributed in Gram negative bacteria, many can also be activated by other related substances such as autoinducer antagonist. Indeed, Hentzer *et al*., (2002) synthesized an analogue that lacks the alkyl side chain of the natural furanone and found that this compound had considerable inhibitory activity against the *P. aeruginosa* quorum sensing system (Figure 1.17). Anyway, such finding encouraged the idea for conflict through coercion, interception and chemical modification. Even though direct evidence of alternative quorum sensing explanation in nature is not significant, there were still interesting experimental trials; 1) Ahmer (2004) proved that SidA, an homologue of AHL receptors from *Salmonella enterica*, does not correspond signals from *S. enterica* but can detect and respond to signal (C6 and C8) from other species. 2) *In vivo* Riboflavin and its

derivative lumichrome from green alga *Chlamydomonas* can be detected by *P. aeruginosa* LasR (Rajamani *et al* 2008). In addition to riboflavin (or its derivative lumichrome) communication, there has been several examples of inter-kingdom species communication and this has provoked the notion of the co-evolution between prokaryotes and eukaryotes.

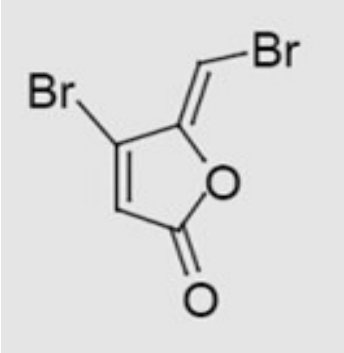


Figure 1.18 The structure of halogenated furanone. This discovery accelerated the notion that disruption of bacterial communication is as a new drug target. (Adapted from Hentzer *et al*., 2002)

1.2.4.4 Inter-kingdom communication

Xavier and Bassler (2005a) have shown that some species of bacteria can manipulate the AI-2 signalling of other competing bacteria. Similar to bacteria interspecies communication, inter-kingdom relationships, which are regulated by quorum sensing systems may occur either through bacterial invasion of host cell or protection of bacterial invasion by host cell.

This is not a one way interaction, eukaryotes also manipulate bacterial signalling networks using mimicking cytokines, hormones, and neurotransmitters (Lowery *et al*., 2008). In this point co-evolution of eukaryote and prokaryotes could occur when both exist together in symbioses.

The first evidence of quorum sensing based communication was discovered in the interaction between a marine bacterium, *Vibrio anguillarum* and green seaweed, *Enteromorpha*. Like many algae *Enteromorpha* also has sexual and asexual life cycle but both grow similar morphologically. It produces motile zoospores that explore and settle temporarily on a surface. However it will detach from the surface and continue in a planktonic lifestyle when the base is not stable. Zoospores sometimes attach to bacterial cells in a biofilm which suggests it may sense the bacterial signals (Joint *et al*., 2002). *V. anguillarum* is a Gram negative pathogen capable of causing a terminal hemorrhagic septicemia known as vibriosis in fish such as rainbow trout. It has two quorum sensing cascade called VanIR and VanMN that are responsible for C6-HSL, C10-HSL and C6-HSL production/recognition, respectively (Milton *et al*., 1997; 2001). In this study (Joint *et al*. 2002) spore settlement was measured on wild type *V. anguillarum*, mutant strains (VanI-,VanM-) and in the presence of *in vitro* synthesised AHLs. In short, settlement of zoospores was enhanced in the presence of wild type *V. anguillarum*. This result indicates that bacterial sensory system plays an important role in the development of eukaryote algae.

There are lots of examples of symbioses in nature and *Bacillus subtilis*, one of microflora in human intestine would also be a good example. These cases of symbioses will be more constructive / cooperative rather than competitive (fujiya *et al*., 2007). Numerous reports have suggested that *B. subtilis* has health-beneficial properties and has potential as a probiotic

agent. In this regards Fujiya *et al*., (2007) tested the response of the intestinal epidermal cell line $Caco_{2bbe}$ to bacterial quorum sensing molecules, known as competence and sporulation factor. When the epidermal cells were exposed to a supernatant of *B. subtilis*, heat shock proteins were induced in the $Caco_{2bbe}$ cells. The heat shock proteins, called Hsps, are essential for the maintenance of intestinal homeostasis. It is notable that many gut probiotic bacteria such as *Lactobacillus*, *Leuconostoc*, *and Bifidobacterim* are known to have quorum sensing systems. Similarly, pathogenic bacteria could use quorum sensing molecules from the host to sense the immune detection and successfully establish an infection of the host.

1.3 Aims and objectives

The original aim of the project was to identify any bacteriocins from *Z. mobilis*. In this thesis, brief introduction will be given as to the motives and rationale for the bacteriocin identification from *Z. mobilis* and then focus on the experimental efforts employed, for about a year and a half, to identify any bacteriocins produced by *Z. mobilis*. More importantly, how these efforts lead to the hypothesis that bacterial quorum sensing was involved in controlling the bioethanol production by *Z. mobilis* in Chapter 3.

As a consequence of bacteriocin identification trials, discovering that when *Z. mobilis* was co-cultured with *E. coli*, it was clearly stimulated to secrete two small proteins (with molecular masses of 26kDa). However, these proteins have no antibacterial activities. Furthermore, when the supernatant from growing *E. coli* was boiled and filtered through a 2.0µm membrane it was still able to induce secretion of these two proteins when added to growing *Z. mobilis* cells. This suggested that *Z. mobilis* responds to a small chemical secreted

by *E. coli* and the test was done whether this might be AI-2 that is produced by some strains of *E. coli* (i.e. KX1123 is an AI-2 signal producer, whilst KX1128 a *(luxS*-deletion mutant) is a non-producer). It was proven that *Z. mobilis* cannot produce, but can uptake AI-2 by *Vibrio harveyi* bioluminescence assay. Considering that *Z. mobilis* responds to, but does not produce the quorum sensing molecule AI-2, this led to wonder whether *Z. mobilis* might produce alternative quorum sensing molecules, such as AHL. However, there is no AHL detection from *Z. mobilis*.

Considering the observation that AI-2 could trigger protein secretion in *Z. mobilis*, further test was performed whether any cytoplasmic proteins/enzymes were increased their abundance after treatment of *Z. mobilis* cells with AI-2. The two overproduced cytosolic proteins were identified, by mass spectrometry finger printing, as enolase and pyruvate decarboxylase. These are sequential enzymes in the ED pathway that leads to ethanol production in *Z. mobilis*, are up-regulated in response to AI-2, led to hypothesis that AI-2 quorum sensing in *Z. mobilis* might stimulate ethanol production. To test this hypothesis enzymatically synthesized AI-2 was applied to *Z. mobilis* and a chromate assay was used to determine the ethanol content of *Z. mobilis* cultures. Consequently, ethanol concentration of *Z. mobilis* culture incubated with *in vitro* synthesized AI-2 is higher than that control without AI-2 by 58% after 24 hours.

One of the possible benefits of exploiting quorum sensing mechanisms by cheaters would be to out-compete nearby cells, because basically cheaters can get a selective advantage without paying the production cost of public goods (herein AI-2s). These benefits may include an increase in growth rate (or fitness) when signals are related to nutrient acquisition,

antibacterial substance production. Considering that 1) *Z. mobilis* has antibacterial activity against a variety of bacteria, and 2) free AI-2 stimulates *Z. mobilis* to overproduce ethanol without the cost of synthesis of AI-2; it is hypothesized that 1) when artificial AI-2 was added to a *Z. mobilis* cell culture, the growth of AI-2 treated *Z. mobilis* cells would be better than that of non-treated cells (if the AI-2 signal involves glucose acquisition, which is the first step in ethanol production in *Z. mobilis*), and 2) the killing effects of *Z. mobilis* on the *E. coli* mutant KX1186 (AI-2 producer but blind) would be better than that on the AI-2 non-producer KX1218, because enhanced ethanol production by AI-2 directly influences *E. coli* cell growth. However, there is no significant in growth and killing effects in *Z. mobilis*, assuming 1) AI-2 stimulates metabolic expression rather than growth in *Z. mobilis* and 2) toxic substances are naturally secreted and its toxicity maybe beyond the ethanol concentrations.

During the study several secreted proteins from *Z. mobilis* were identified in the presence/absence of AI-2, suggesting that they may have signal sequences that target them for secretion. Thus, the test was performed whether this might be the case by expressing these proteins in *E. coli* BL2 DE3 and determining if they appeared in the extracellular medium by Western-blotting. Whilst ZMO1147 was retained within the cell, both ZMO1034 and ZMO0994 were excreted. The secretion of these proteins from *E. coli* is an important discovery because it is generally accepted that nonpathogenic strains of *E. coli*, particularly derivatives of K12, do not secret proteins under routine growth. A major drawback of using *E. coli* for heterologous protein production is that the proteins produced need to be recovered from disrupted cells, whilst the ability to target proteins for secretion would avoid this complication. It is possible that signal sequences from ZMO1034 and ZMO0994 could be fused to other proteins to facilitate their secretion both in *E. coli* and *Z. mobilis*. In the latter

case, this might have application in engineering *Z. mobilis* for production and secretion of enzymes for degradation of biomass for release of sugars for bioethanol production.

Chapter 2 Methods and materials

2.1 Chemicals, antibiotics and solutions

All the chemical reagents and antibiotics were purchased from Sigma, Oxoid, Difco and Melford Laboratories, U.K. Reagents were weighed on a Sartorius digital balance, while smaller quantities were measured using a Mettler Toledo digital analytical balance. Microbiological growth media and solutions were prepared using MilliQ distilled/deionized water where appropriate. Solutions were sterilized either autoclaved at 121 ^{o}C for 15mins at 4 atmospheres pressure in a Priorclave electrically heated autoclave set on liquid cycle, or 0.22µm-filter sterilized, depending on their temperature stability. Recipes of the stock solutions are shown table 2.1.

Table 2.1 Stock solutions

Chemicals	Storage
Carbenicillin (Carb)	100mg/ml in distilled water. Filter sterilize and store at -20 ^{o}C. Use at 100µg/ml
Chloramphenicol (Ch)	20mg/ml in 100% ethanol. Filter sterilize and store at -20 ^{o}C. Use at 20µg/ml
Tetracycline (Tet)	10mg/ml in 100% ethanol. Filter sterilize and store at -20 ^{o}C. Use at 10µg/ml
Kanamycin (Kan)	10ml/ml in distilled water. Filter sterilize and store at -20 ^{o}C. Use at 10µg/ml
80% glycerol	Mix 80ml glycerol with 20ml distilled water. Autoclave. Store at room temperature.
1M IPTG (isopropyl-D-thiogalactop-yranoside)	2.38g IPTG in 10ml distilled water. Filter sterilize and store at -20 ^{o}C

2.2 Bacterial strains, media and plasmids

All the bacteria strains and plasmids used in this thesis are listed in Table 2.2.

Table 2.2 Bacteria strains and plasmids used in this study.

Strains / Plasmids	Description	Source/Reference
Bacterial strains		
Zymomonas mobilis ZM4	Wild type	ATCC 14990
Staphylococcus strains		
epidermidis	Wild type	ATCC 42033
aureus NTCC 8325	Wild type	Novick, 1967
aureus NTCC 8325-4	*luxS* mutant	Doherty *et al.,* 2006
E.coli strains		
KX 1123	Wild type	Xavier and Bassler, 2005a
KX 1218	*luxS* mutant	Xavier and Bassler, 2005a
KX 1186	*lsrK* mutant	Xavier and Bassler, 2005a
DH5alpha	host / *luxS* mutant	Invitrogen, U.K
Novablue	host / high-efficiency transformation of plasmid	Novagen, U.K
BL21 DE3	protein expression strain	Invitrogen,UK
C43 DE3	protein expression strain	Promega,UK
Chromobacterium violaceum CV026	AHLs detection strain	McClean *et al*., 1997
Vibrio harveyi BB170	AI-2 detection strain	Bassler *et al*., 1994

Table 2.2 *Continued*

Strains / Plasmids	Description	Source/Reference
Agrobacterium tumefaciens NT1	AHLs detection strain	Piper *et al*., 1993.
Plasmids		
pProEX-HTmtan	His-tagged pfs from *E. coli* MG1655	Winzer *et al*, 2002.
pProEX-LuxSec	His-tagged LuxS from *E. coli* MG1655	Winzer *et al*, 2002.
pGEM-T easy	Cloning vector	Promega, U.K
pGEMT-easy-ZMO0994		This study
pGEMT-easy-ZMO1034		This study
pGEMT-easy-ZMO1147		This study
pET21a(+)	Protein expression vector	Promega, U.K
pET21a(+)-ZMO0994	His-tagged extracellular ZMO0994	This study
pET21a(+)-ZMO1034	His-tagged extracellular ZMO1034	This study
pET21a(+)-ZMO1147	His-tagged ZM1147	This study

2.2.1 Preparation of stock cultures and storage of bacterial strains

A single colony was inoculated into 20ml of suitable medium in a 50ml tube with appropriate antibiotics as necessary. The incubation was shaken gently at 30 ^{o}C or 37 ^{o}C depending on the bacterial strains until the optical density reached an OD_{600}=0.5-0.6. Then, 450µl of the culture was collected and transferred to an autoclaved tube with 50µl of 80% glycerol. The

permanent stocks of bacterial strains were kept as 20% glycerol stock at -80 ^{o}C. Plasmids were kept with an appropriate (e.g. EB buffer, DNA Qiagen kits) at -20 ^{o}C.

2.2.2 Inoculation a culture from the frozen stock

A few microlitres of the frozen stock were scraped from the surface of cell stock with plastic culture loop and streaked on an agar plate or inoculated into liquid medium with appropriate antibiotics if necessary. The remainder was returned to the -80 ^{o}C freezer without thawing.

2.2.3 Culture media

The following bacterial media were prepared as describe below and sterilized by autoclaving at 121 ^{o}C for 15mins: RM medium/litre contains yeast extract 10g, $(NH_4)_2SO_4$ 1g, $MgSO_4 \cdot 7H_2O$ 1g, glucose 100g, KH_2PO_4 2g; Luria-Bertani (LB) medium/litre contains tryptone 10g, yeast extract 5g, NaCl 10g; Nutrient medium/litre contains yeast extract 2g, peptone 5g, NaCl 5g; Autoinducer Bioassay (AB) medium/litre contains NaCl 17.5g, $MgSO_4 \cdot 7H_2O$ 12.3g, 0.1M L-arginine 10ml, casamino acids 2g, 50% glycerol 20ml, 1M KH_2PO_4 10ml; SOC medium/litre contains tryptone 4g, yeast extract 1g, NaCl 0.117g, KCl 0.0373g, 1M $MgCl_2 \cdot 6H_2O$ 2ml, 1M $MgSO_4$ 2ml; 2x Yeast Tryptone (YT) Medium/litre contains tryptone 16g, yeast extract 10g, NaCl 5g. Minimal medium/litre for *Z. mobilis* contains KH_2PO_4 1g, K_2HPO_4 1g, NaCl 0.5g, $(NH_4)_2SO_4$ 1g, $MgSO_4 \cdot 7H_2O$ 0.2g, $CaCl_2 \cdot 2H_2O$ 0.2g, $Na_2MoO_2 \cdot 2H_2O$ 0.25g, $FeSO_4 \cdot 7H_2O$ 0.25g, glucose 20g and vitamin solution 1ml (calcium pantothenate 0.005g, thiamine hydrochloride 0.001g, biotin 0.001g, nicotinic acid 0.001). Agar plates were prepared by adding 1.5% (w/v) agar to liquid medium

prior to autoclaving.

2.3 Centrifugation

Routine centrifugation of materials in microcentrifuge tubes was performed in a Sigma benchtop microcentrifuge fitted with a 24 place fixed angle rotor, and a Juan CR3 refrigerated benchtop centrifuge was used where there was a requirement for cooling. Lager volumes of liquids were processed in a Beckman-Coulter Avanti J-E refrigerated centrifuge using different fixed angle rotors. A Beckman JA-10 rotor was used to process volumes up to 3 litres in 500 ml tubes and a Beckman JA-20 rotor was used to process up to 300ml in 40 ml tubes. High-speed ultracentrifugation was performed in a Beckman L8M ultracentrifuge using a Beckman Ti50 fixed angle rotor.

2.4 Co-culture experiment and purification of extracellular proteins from *Z. mobilis*

Z. mobilis ZM4 and *E. coli* KX1123 (Xavier and Bassler, 2005a) strain were grown as separate cultures until the cell density reached an OD_{600}=0.4-0.6 and OD_{600}=1.0, respectively; then 0.2% (v/v) of the mutant *E.coli* strain was placed in a dialysis bag (10kDa cut-off, SnakeSkin™, PIERCE) to avoid direct contact with *Z. mobilis*, and added to the *Z. mobilis* culture. After 12-16 hours, the supernatant from the *Z. mobilis* culture was isolated by centrifugation at 8000 rpm for 20mins at 4 ^{o}C and passed through a 2µm filter (Millipore). The pH of the supernatant was adjusted to pH 4.0 and to 1 litre of supernatant (Uteng *et al*., 2002), 10ml of SP-Sepharose™ Fast Flow resin (Amersham) was added and this mixture was gently stirred at 4 ^{o}C for 1 hour. The mixture was poured into a glass column and the

immobilized proteins were eluted with a (0-1.0M) NaCl gradient in 50mM acetic acid buffer, pH 4.0; or, alternatively, proteins were eluted with 10ml of 50mM phosphate buffer at pH 7.6 (Uteng *et al.*, 2002). Each of eluted samples was applied to a pre-cast 4-12% SDS-PAGE gel (Novex NuPAGE®). Coculture of *Z. mobilis* with *S. epidermidis* and with *S. aureus* strains was carried out in the same manner as described above.

2.5 Preparation of AI-2 induced cytoplasmic proteins from *Z. mobilis*

Z. mobilis was cultured at 30 °C to OD_{600}=0.5 and then divided into three equal aliquots to provide cells for a control experiment and two cell samples to be treated with *E. coli* supernatant. The sterile supernatant from different cell-densities (OD_{600}=0.9-1.0 and 1.4-1.5) of *E. coli* strain KX1123 were prepared by centrifugation, filtration and boiling for 10mins. To examine the effects of AI-2 on the expression of specific proteins, *Z. mobilis* cells (10 mls), previously grown to OD_{600}=0.5, were cultured for 16 hours with or without the supernatant of *E. coli* KX1123 at a 1:1 ratio (v/v) (Stevenson and Babb, 2002). The *Z. mobilis* cells were collected by centrifugation at 4000rpm for 10mins and resuspended in lysis buffer (200mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 10% glycerol, 20µl/ml protease inhibitor stock (1 tablet/ml, complete EDTA-free Roche), and DNase (5U/ml, Sigma), pH 7.3). The resuspended cells were sonificated (Soniprep 150, SANYO) three times at 15 amplitude microns to disrupt them and cell debri removed by centrifugation at 13,000rpm for 10mins at 4 °C, and the supernatant was applied to a pre-cast 4-12% SDS-PAGE gel (Novex NuPAGE®).

2.6 Visualization of proteins by SDS PAGE gel electrophoresis

The protein expression and the efficacy of protein purification procedures were routinely monitored by SDS-PAGE. All SDS PAGE experiments were performed using 4-12% NuPAGE® Novex Bis-Tris gels and XCell *SureLock* Mini-Cell (Invitrogen). The NuPAGE® LDS sample buffer (4x) (Invitrogen) was used for preparing samples for denaturing gel electrophoresis with the NuPAGE® Gels. The NuPAGE® Gel was vertically mounted. The comb was gently removed from the cassette and sample wells were rinsed with 1X NuPAGE® SDS running buffer. 20µl of protein fractions was mixed with 5µl of NuPAGE® LDS sample buffer (4x). 20µl of the mixed samples were loaded into each well of the gel. 10µl of protein molecular weight markers (SeeBlue® Pre-stained standard, Invitrogen) were loaded in one well as a standard. The gel cassette was then clamped vertically in the mini-cell such that the notched well side of the cassette faces inwards toward the buffer core. The upper and lower buffer chamber were filled with running buffer until the chambers were submerged. Samples of control and induced pellet/samples were electrophoresed at 200V for 40-50 minutes. The gel was then stained with coomassie blue for 30 minutes then destained at room temperature with constant agitation until the desired background was achieved. The gel was viewed under white light with a white box filter.

Table 2.3 SDS-PAGE buffer solutions stock

NuPAGE® LDS sample buffer (4x)	**NuPAGE® MES running buffer (20x)**
106mM Tris HCl 141mM Tris base	97.6g MES (2-(N-morpholino) ethane sulfonic acid)
0.51mM EDTA	60.6g Tris Base
0.22mM SERVA® Blue G250	10% SDS
0.175mM Phenol Red	3.0g EDTA

Table 2.3 *Continued*

NuPAGE® LDS sample buffer (4x)	NuPAGE® MES running buffer (20x)
2% LDS	To 500ml H_2O
10% Glycerol	
pH 8.5	pH 7.3

Table 2.4 Staining and destaining solutions of SDS gel

Staining solution	Destain solution
33% Acetic acid	10% Acetic acid
33% Methanol	10% Methanol
33.9% H_2O	80% H_2O
0.1% Coomassie Brilliant Blue R-250	

2.7 Mass spectrometry analysis and protein identification

Following SDS PAGE analysis, the bands corresponding to proteins of interest were cut out of the gel and sent to the Proteomics facility at Durham University (http://www.dur.ac.uk/proteomics/index.php). Separated protein bands were equilibrated in 50µl of 50mM ammonium bicarbonate and subsequently reductively alkylated with 10mM DTT and 100mM iodoacetamide; following by destaining and desiccation with acetonitrile. Gel plugs were rehydrated with 50mM ammonium bicarbonate containing 6.6% (w/v) trypsin (Promega) and digested overnight. Peptides were extracted using 50% (v/v) acetonitrile, 0.1% (v/v) TFA (tri-fluoro acetic acid) into a final volume of 50µl; and the resulting extracts were freeze-dried and resuspended in 10µl of 0.1% formic acid. Approximately 0.2µl of matrix

(e.g. α-cyano-4-hydroxy-cinnamic acid in nitrocellulose/acetone) was applied to a mass spectrometer target plate, followed by 1µl of digested sample and allowed to dry. The samples were washed *in-situ* with 0.1% TFA and left to dry again. MALDI-ToF PMF was then performed using a Voyager-DE^{TM} STR BioSpectrometry^{TM} Workstation (Applied Biosystems, Warrington, UK). De-isotoped and calibrated spectra were then used to generate peak lists, which were searched using MASCOT (www.matrixscience.com) mass spectrometry database search software to identify the proteins.

2.8 *Vibrio harveyi* BB170 bioluminescence assay

Bioluminescence assays were performed as described previously (Bassler *et al*., 1994; Xavier and Bassler, 2005). Briefly, fresh cell-free supernatant of *Z. mobilis* and *E. coli* strains were prepared by centrifugation and filtration. The *V. harveyi* BB170 reporter strain was grown for 16 hours with aeration at 30 ^{o}C in AB medium and then diluted 1:5000 into fresh AB medium. Cell-free culture fluids of test strains (*Z. mobilis, E. coli* wild/mutants) were added to the diluted *V. harveyi* culture at a 10% (v/v) final concentration in a sterile 96-well microtitre plate (Acrowell^{TM} 96 Filter Plate, PALL life Science), which was incubated at 30 ^{o}C with shaking at 175rpm. Finally, the light production was measured every hour using a liquid scintillation counter (Wallac model 1450, PerkinElmer). Enzymatically derived AI-2 was also assayed for bioluminescence using *V. harveyi* BB170 strain. In AI-2 uptake experiments, *Z. mobilis* was grown in RM media overnight, the cells pelleted by centrifugation, washed 3 times with LB medium or 50mM Tris buffer, pH 7.8 and then incubated with cell-free culture fluid (10ml) from *E. coli* KX1186 (Xavier and Bassler, 2005a) or synthetic AI-2 (10ml).

2.9 Overexpression and purification of Pfs/LuxS enzymes

E. coli DH5 alpha strains expressing 6xHis-tagged Pfs and LuxS (Winzer *et al.*, 2003) were kindly provided from Dr. Klaus Winzer and Dr. Paul Williams (University of Nottingham, UK). The following series of steps were performed for Pfs and LuxS enzyme purification: plasmid DNA purification (section 2.14.10), transformation (section 2.14.8), PCR screening (section 2.14.11.2), expression (section 2.15.1) and 6x His-tagged protein purification (section 2.15.2) from *E. coli* BL21 (DE3), and these two enzymes were used for *in vitro* AI-2 synthesis (section 2.11).

2.10 Protein buffer changes, concentration and quantification

The dialysis of proteins was essential for the removal of the imidazole from the purification procedure and to reduce the salt concentration that may interfere with functional tests. A Slide-A-Lyzer 10K MWCO Dialysis Cassette (Pierce)® was used to remove low molecular weight contaminants and perform buffer exchange. The appropriate dialysis buffer was placed in a 1 litre beaker together with a 3ml dialysis cassette, which was allowed to soak for 1 min. The air was drawn from the cassette using a sterile needle (0.8 x 40mm) and a 10ml syringe, and 3ml of each enzyme was loaded. The cassette was placed horizontally in a float and dialysed with gently stirring for 1½ hours at 4 ^{o}C. The dialysis buffer was replaced and each enzyme was dialysed for a further 1 ½ hours. After dialysis, the sample was carefully collected with a syringe from the dialysis cassette.

The Vivaspin Concentrator (Viva Science, 10K MWCO membrane) was used to concentrate

the protein samples. At first, several volumes of water were passed through the concentrator by centrifugation to remove trace amounts of glycerine and sodium azide on the membrane. The concentrator was then filled with protein samples and inserted into the rotor with the printed window of the concentrator facing towards. After the centrifugation at 4 ^{o}C until the desired concentration was achieved, the concentrator was removed and samples were recovered from the bottom of the concentrate pocket with a pipette.

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The BCA^{TM} Assay was applied as instructed in the BCA^{TM} Protein Assay Kit (Pierce). 0.1ml of each standard and unknown protein sample were transferred into a test tube and mixed with 2ml of the BSA working reagent. The tubes were then incubated at 37^{o}C for 30 minutes to allow colour development. After the incubation, reactions were cooled to room temperature and the absorbance of all the samples at 650 nm were measured against a blank. A standard curve was prepared by plotting the average blank-corrected 650 nm measurement for each BSA standard versus its concentration in µg/ml. Finally, the protein sample concentration was deduced by substituting the absorbance measured for sample into the equation y=mx+c, where y= protein concentration in µg/ml, m=gradient, x= absorbance of sample c=constant.

2.11 *In vitro* autoinducer 2 (AI-2) synthesis

In vitro synthesis of AI-2 was carried out as previously described (Winzer *et al*., 2003). His-tagged Pfs and LuxS were purified from *E. coli* BL21 (DE3) using a nickel resin (QIAGEN) according to the manufacturer's protocol (section 2.15.2); and their concentrations

determined using the BCA protein Assay kit (Pierce) (section 2.10), with bovin serum albumin as a standard. *In vitro* synthesis of AI-2 was performed with 1mM SAH (Sigma), 1mg/ml His-LuxS, and 1mg/ml His-Pfs in 50mM Tris buffer, pH 7.8, at 37 ^{o}C for 1-4h. The Pfs and LuxS enzymes were removed by filtration through a Centricon YM-10 filter (Millipore) and the presence of AI-2 confirmed by assaying for bioluminescence with *V. harveyi* BB170.

2.12 Chromate assay for detecting ethanol

Zymomonas cells were cultured overnight, then the next day, a 100µl aliquot was added to 10ml of fresh medium (1/100 dilution). When the cell density reached OD_{600}=0.5, synthesized AI-2 (different concentration) or cell supernatant (from *E. coli* wild type, OD=1.0) was added into the *Zymomonas* cell culture. To quantify ethanol production a chromate assay (Pilone, 1985) was used to monitor the increase in absorbance at 600nm that results from the reduction of Cr (IV) to Cr (III) as it catalyses the oxidation of ethanol according to the following reaction: $16H^{+} + 2CrO_7^{2-} + 3C_2H_5OH \rightarrow 4Cr^{3+} + 3CH_3COOH + 11H_2O$. Briefly; a 1M CrO_3 (Sigma, U.K) solution was added to the cell-free supernatant of a culture of *Z. mobilis* (1:10 v/v), gently stirred for 10mis at room temperature, and then the appearance of reduced Cr (III) was determined from the absorbance at 600nm. A calibration curve was constructed, using a series of ethanol standards, from which the % (v/v) ethanol in a culture could be extrapolated.

2.13 N-acyl homoserine lactone (AHL) detection from *Z. mobilis*

The detection methods of quorum sensing AHL molecules by bacterial reporter strains were

divided into several groups, such as Plate T-streak, Quantification assay, *in vivo* assay and Thin-Layer Chromatography (TLC) assay (Steindler and Venturi, 2007). In this study, *Chromobacterium violaceum* CV026 (McClean *et al*., 1997) and *Agrobacterium tumefaciens* NT1 (Piper *et al*., 1993) were used as AHL biosensors.

AHLs with an acyl chain length of 4 to 8 carbons can be detected by using Tn-5 (transposon)-generated *Chromobacterium violaceum* CV026. The exposure of strain CV026, which is able to interact with *CviR,* results in rapid production of a visually clear purple pigment. A 100µl culture of fresh *C. violaceum* CV026 reporter strain was spread on to an LB plate. Wells were then cut, to a depth of 10mm, in the LB plate with autoclaved 200µl pipette tip. Then, a serial volume of the supernatant of the *Z. mobilis* and a standard AHL were allowed to soak into these wells, and the plate was allowed to set. The plates were incubated at 28 ^{o}C for 24hr. The presence of a purple zone around the site of the wells was determined as positive.

A. tumefaciens NT1 (*TraR*, *tra::lacZ749*) displays the broadest sensitivity to AHLs (C4-C12 and 3-unsubstitued) at low concentrations (Farrand *et al*., 2002). The agar slice method basically similar to that described previously, (Dong *et al*., 2000) was used with *A. tumefaciens* NT1 to test for AHLs. Briefly, 20ml LB medium containing 50µg/ml X-gal was pooled into each petri dish as the AHL bioassay plates. The medium in the plates was cut into a separated slice (1cm in width) after solidification. Then a standard 3OC8HSL sample and cell-free culture fluid of *Z. mobilis* (5µl volume) and were added to one end of each agar slice. As a positive control, the overnight fresh culture of reporter strains was diluted to OD_{600}=0.5, then spotted (0.5 µl each spot). The plates were incubated at 28 ^{o}C for 24hr. The appearance of a blue spot was determined as positive.

2.14 General gene manipulation

2.14.1 Genomic DNA preparation from *Z. mobilis*

Small-scale isolation of genomic DNA from *Z. mobilis* ZM4 was performed using GenElute^{TM} Bacterial Genomic DNA Kit (Sigma).

RM broth (5ml) was inoculated with a single colony of *Z. mobilis*. The culture was incubated at 30 ^{o}C with gentle shaking for 16 hours. 1.5ml of overnight *Z. mobilis* broth culture was harvested at 12,000-16,000 rpm for 2 minutes and the culture medium was removed completely. The pelleted cells were resuspended in 180µl of Lysis Solution T and 20µl of the Proteinase K solution added to the cell suspension. The mixture was then incubated for 30 minutes at 55 ^{o}C. 200µl of Lysis solution C was added to the mixture and then further incubated for 10 minutes at 55 ^{o}C to achieved complete cell lysis. During the incubation period, a GenElute^{TM} Miniprep binding column was prepared by adding 500µl of the column preparation solution to each pre-assembled column, centrifugation at 12,000 rpm for 1 minute and discarding the flow-through. 200µl of ethanol was added to the lysed cells and mixed thoroughly by vortexing for 5-10 seconds to obtain a homogeneous mixture. To bind DNA to column, the entire contents of the 1.5ml tube were transferred into the binding column and centrifugated at 6500 rpm for 1 minute. After discarding of the eluate from the collection tube, a new collection tube was placed in the column. The column was washed with 500µl of washing solution 1 and 500µl of washing solution concentrate by centrifugation at 6500 rpm for 1 minute and 12-16000 rpm for 3 minutes, respectively. Because the column must be free of ethanol before eluting the DNA, the column was centrifugated for an additional 1 minute

following washing step 2. The new collection tube was placed in the column and 200µl of the elution solution was dropped directly onto the centre of the column. To increase the elution efficiency, the column was incubated for 5 minutes at room temperature after adding the elution solution, then centrifugated and the flow-through was collected. Purified pure genomic DNA was stored at -20 ^{o}C.

2.14.2 *In vitro* DNA amplification by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) technique was used for the selective amplification of a chosen region of a DNA template. PCR requires oligonucleotide primers that have been designed to anneal to the melted template DNA strands either end of the gene of interest. The NCBI data base was used in oligo design and these were synthesised by Invitrogen (Table 2.5).

Table 2.5 Oligonucleotides

Primer name	Sequence (5' to 3')
ZMO0994 Fwd	**GGATTC**CATTCGACCATGTCCAGCAACCGACTG
ZMO0994 Rev	**CTCGAG**CTTGCCGGACTTGTTACCACCC
ZMO1034 Fwd	**GGATCC**AAAGAAAAATTTTCTTTTGCGTAGTCTG
ZMO1034 Rev	**CTCGAG**TTTGGAGAGAGGTTTGGGAAATG
ZMO1147 Fwd	**GCATTC**AAGAAATTATTTGCCGGTGCAGCCTTGCTC
ZMO1147 Rev	**AAGCTT**CCGGCTGGTTGGACGACGAGC
Pfs Fwd	**CATATG**ATGAAAATCGGCATCATTGGTGCAATGGAAGAAG

Table 2.5 *Continued*

Primer name	Sequence (5' to 3')
Pfs Rev	**CTCGAG**GCCATGTGCAAGTTTCTGCACCAGTGAC
LuxS Fwd	**CCATGG**ATGCCGTTGTTAGATAGCTTCACAGTCGATCATACC
LuxS Rev	**GCGGCC**GCGATGTGCAGTTCCTGCAACTTCTCTTTCGGCAG

Incorporated restriction endonucleases sites are highlighted in bold letters and the oligonucleotide names with 'Fwd' and 'Rev' represent pairs of forward and reverse primers used for the gene amplification. The oligonucleotides were supplied on a 50nmol scale, in a desalted and lyophilized form. The oligonucleotides were suspended in high purity deionized water to a final concentration of 50pmol/µl and stored at -20^{o}C.

Table 2.6 PCR components for HotStar Taq^{TM} DNA Polymerase

Master mix Component	Volume (µl)	Final concentration
10x PCR Buffer	10	1x
dNTP mix (10mM of each)	2	200µM of each dNTP
Forward primer	1	50pmol/µl
Reverse primer	1	50pmol/µl
HotStar Taq DNA Polymerase	0.5	2.5 units/reaction
Distilled Water (dH_2O)	84.5	-
Template DNA	Variable	≤1µg/reaction
Total volume	100	-

HotStar Taq DNA Polymerase (Qiagen) is a modified form of a recombinant 94-kDa DNA polymerase, originally isolated from *Thermos aquaticus*, cloned in *E. coli*. One unit of HotStar Taq DNA Polymerase can be defined as the amount of enzyme that will incorporate 10nmol of dNTPs into acid-soluble material within 30mins at 72^{o}C. The polymerase is activated by a 15 minutes, 95^{o}C incubation step.

At first, 10x buffer, dNTP mix, Primer solution, and 25mM $MgCl_2$ (if require) was thawed in an ice bath. A master mix according to table 2.4 was prepared. The master mix was transferred to two PCR tubes in 100 µl aliquots and mixed thoroughly with pipetting. The PCR tube containing the master mix was placed in a thermal cycler (Eppendorf Matercycler Gradient). The PCR machine is fitted with heated lid to prevent evaporation of the reaction mixture during the cycles. The lid was pre-heated to 105^{o}C before thermal cycles began.

Table 2.7 The thermal cycler for the PCR reaction

Cycle	Temperature	Time
Activation of HotStar Taq DNA Polymerase	95^{o}C	15 min
Denaturation	94^{o}C	1 min
Annealing	50-68^{o}C	1 min
Extension	72^{o}C	1-1.5 min
Number of cycles	30	
Final extension	72^{o}C	10 min
Hold	4^{o}C	Up to overnight

2.14.3 DNA agarose gel electrophoresis

For positive identification of the DNA fragments, agarose gel electrophoresis analysis was carried out according to the protocol (Molecular Cloning, Handbook). 0.5-1.0% (w/v) of Agarose was mixed with 1x TAE buffer and the TAE/agarose mixture was heated in a microwave. The dissolved agarose gel was allowed to cool to approximately 50 ^{o}C, and then stained with 0.5µM/ml EtBr. Following the gentle mix of dissolved gel and EtBr, the warm agarose solution was poured into the gel tray and an appropriate comb was placed above a horizontal gel tray to form the sample wells in the gel. The gel was allowed to set for 20-30 minutes to set at room temperature. After the gel solidification, the well comb was carefully removed and gel was placed on a tray in the electrophoresis tank containing 1x TAE buffer allowing for 5mm depth over the gel. 80µl of the amplified DNA samples plus 13.5µl of a 6x Gel-loading dye, together with a DNA Marker (1000bp or 100bp DNA Ladder, Promega) were loaded into a separate well of the submerged gel. The lid of the gel tank was closed and the electrical rods were attached so that the DNA migrates toward the positive anode. The samples were electrophoresed for 30-40 minutes at 115 Volts. Progress of DNA through the gel could be monitored by the migration of Bromophenol blue. The gel was visualised on a UV transilluminator equipped with camera (Gene Genius Bio Imaging System, SYNGRNE) to capture an image of the gel.

Table 2.8 DNA gel electrophoresis running buffer (1x TAE)

Components	Volume / Weight
Tris base	4.84g

Table 2.8 *Continued*

Components	Volume / Weight
Acetic acid	1.142ml
0.5mM EDTA	2ml
dH_2O	To 1000ml

Table 2.9 DNA loading dye (6x)

Components	Volume / Weight
Bromophenol blue	0.025g
100% Glycerol	3ml
dH_2O	7ml

2.14.4 Fragment DNA extraction

The desired DNA fragments from PCR and other enzymatic reaction need to be extracted from the agarose gel following electrophoresis for further gene manipulation. A QIAquick Gel Extraction kit (Qiagen) was used to extract and purify DNA of 70bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer.

A gel slice containing DNA fragments was excised from the gel with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose and weighed in a microcentrifuge tube. 3 volumes of Buffer QG were added to 1 volume of gel (e.g. 300µl of Buffer QG to 100mg of gel). The gel slice was then incubated at 50^{o}C until completely dissolved. To help dissolve the gel, the tube was mixed by vortexing every 2-3 minutes

during incubation. A QIAquick spin column was placed in a 2ml collection tube. The DNA/Buffer QC mixture was applied to column and centrifuged at 13,000 rpm for 1 min to immobilize the DNA. When the mixture volume exceeded 800µl, additional application and centrifugation was performed. Following the discard of the flow-through; 500µl of QC was added to the column and centrifuged to remove all traces of agarose. The immobilized DNA was washed by adding 750µl of Buffer PE to the column and centrifuged for 1 minute. The eluate was discarded and additional centrifugation was done at 13,000 rpm for 1 minute to completely remove residual ethanol. The column was placed into a clean microcentrifuge tube. The DNA was eluted by the addition of 35µl of Buffer EB (10mM Tris, pH8.5) to the centre of the column. The column was incubated at room temperature for 1 minute to increase the DNA concentration, and centrifuged at 13,000 rpm for 1 minute to collect the purified DNA. The eluted DNA was stored at -20^{o}C.

2.14.5 Estimating the quantification of the extracted DNA

The quantity of a DNA fragments could be estimated by running a sample alongside a DNA Marker (Invitrogen) containing known quantities of the same-sized DNA fragment. The amount of sample DNA for a ligation and digestion could be quantitated by visual comparison of the band intensity with that of the standard on absorbance at 260nm.

2.14.6 Cloning PCR products into pGEM®-T Easy vector

The pGEM-T® Easy vector (Promega) ligation of a PCR amplified DNA allows the amplified

gene sequence to be maintained in a stable form in a heterologous *E. coli*. The vectors were added a 3' terminal thymidine to both ends. These single 3'-thymidine overhangs at the insertion site improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by HotStar Taq^{TM} DNA Polymerase (Qiagen). This polymerase often template-independently adds a single deoxyadenosine to the 3'-ends of the amplified fragments. All the components were added to a 0.5ml microcentrifuge tube in order as listed below. The ligation mixture was incubated at 4^{o}C overnight for the maximum number of transformants.

2.14.7 Preparation of competent *E. coli* cells

Chemically competent *E. coli* cells were used this study in order to produce strains for cloning, expression by transformation of recombinant genes. The competent cells, such as Novablue, DH5α, BL21 and C43, were provided from a commercial source or were produced in the laboratory.

Table 2.10 pGEM-T® Easy vector ligation reaction mixture

Component	Volume (µl)
2 x Rapid Ligation Buffer	5
pGEM®-T Easy Vector (50ng)	0.5-1.5
PCR product	3-7
T_4 Ligase (3 Weiss unit/µl)	1.0
dH_2O	variable
Total Volume = 15µl	

Table 2.11 Buffer solutions for competent *E. coli* preparation

TFB 1	TFB 2
50mM MnCl	10mM MOPS
100mM RbCl	10mM RbCl
30mM Potassium acetate	75mM $CaCl_2$
10mM $CaCl_2$	15% glycerol
15% Glycerol	-
pH 5.8, Sterile filter	pH 6.8 with KOH, Sterile filter

An *E. coli* culture was prepared by inoculating 100ml of LB/antibiotics with 1ml of overnight culture and incubating at 37°C with 250 rpm until an OD_{600} of 0.5 was reached (approximately 90-120 minutes). The cell suspension was divided into two 50ml tubes, kept on ice for 5 minutes and harvested at 4,000rpm for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended initially with 1ml of ice-cold TFB1 Buffer then an additional 29ml was added to give a final volume of 30ml. The suspension was kept on ice for 90 min and the cells were harvested at 4,000 rpm for 5 minutes at 4°C. The supernatant discarded and the pellets were resuspended in 30ml cold TFB2 Buffer and held on ice for 45 minutes. The suspension was then harvested and the cell pellets were resuspended in 2-4ml TFB2 Buffer. Competent cells were transferred to 1.5ml microcentrifuge tubes in 50µl aliquots, snap frozen in a dry ice-ethanol mix, and stored at -80°C .

2.14.8 Transformation of competent *E. coli* cells

Competent cells produced in the laboratory or obtained commercially were used for

transformation. The transformation was performed according to the following protocol (Promega, UK)

The ligation mixture was centrifuged briefly and transferred into an ice-cold sterile 1.5ml tube. An aliquot of frozen competent cells were removed from -80^{o}C and placed in an ice bath until just thawed (about 5 minutes). The cells were gently mixed with 2-10µl of the ligation reaction by flicking the tube. The transformation mixture was incubated on ice for 20-30 minutes, heat shocked in a 42^{o}C for 60 seconds and incubated on ice for a further 2 minutes. 300µl of SOC broth was added to the cells and incubated at 37^{o}C with 250 rpm for 60-90 minutes. 100-150µl of cells were spread onto LB plates containing antibiotics (optional, plus IPTG [200mg/ml and X-gal [20ml/ml]). The plates were incubated in an inverted position at 37^{o}C.

2.14.9 α-complementation: white/blue colonies selection

Successful cloning of an insert in the pGEM-T® Easy vector (Promega) disrupts the *lacZ* gene sequence coding for ß-galactosidase. Thus, Cells carrying self-ligated pGEM-T® Easy vector are capable to hydrolyse the X-gal, producing a blue pigment, whereas cells bearing recombinant plasmids are incapable to do so and remain in white colour. The white colonies were re-streaked onto an LB/antibiotics plate and incubated at 37^{o}C for PCR identification or restriction enzyme digestion screening.

2.14.10 Plasmid DNA purification

All the *E.coli* mini cultures (5-10ml LB medium) were subjected to a QIAprep Spin Miniprep Kit (Qiagen) that was used for small-scale purification of up to 20µg of high-copy plasmid.

10ml of overnight culture of *E. coli* was harvested by centrifugation at 4,000 rpm for 10 minutes at 4^{o}C. The pelleted cells were resuspended in 250µl of Buffer P1 and transferred to a 1.5ml microcentrifuge tube. 250µl of Buffer P2 added to the cell suspension for the cell lysis and 350µl of Buffer N3 was additionally mixed for neutralization. To avoid localized precipitation, the cell suspension was mixed gently but thoroughly, immediately after addition of Buffer N3. The solution became cloudy, and the insoluble and soluble parts were separated by centrifugation at 13,000 rpm for 10-15mins in a table-top microcentrifuge. The supernatant containing plasmid DNA was applied to the QIAprep spin column by decanting or pipetting. Plasmid DNA was combined on the column by centrifugation for 1 minute and the flow-through was discarded. The column was then washed by adding 750µl of Buffer PE and spinning down the buffer. This step was additionally performed to avoid residual ethanol to inhibit subsequent enzymatic reactions. The column was placed into a clean microcentrifuge tube. The DNA was eluted by the addition of 35µl of Buffer EB (10mM Tris, pH8.5) to the centre of the column. The column was incubated at room temperature for 1 minute to increase the DNA concentration, and centrifuged at 13,000 rpm for 1 minute to collect the purified plasmid DNA. The eluted DNA was stored at -20^{o}C

2.14.11 Screening transformants for inserts

2.14.11.1 Restriction enzyme digestion screening

Transformed *E. coli* colonies were selected and cultured in 10ml LB/antibiotics medium. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen). All the endonucleases enzymes were obtained from Promega and digestion reaction was performed according to manufacturer's protocol. The components, as listed below, were added to a 0.5ml microcentrifuge tube and incubated at 37^{o}C for 4 hours. The inserted sequence was analysed by agarose gel electrophoresis.

2.14.11.2 Colony PCR screening

Plasmid transformed into *E. coli* strain BL21, in contrast to *E. coli* strain Novablue or DH5α, cannot be produced in high quantity due to the typical life cycle of the *E. coli* BL21. Thus, instead of template DNA, a colony was picked from an agar plate with a 10µl tip and mixed with the PCR master mixture. HotStar Taq DNA polymerase was used to amplify the insert gene, and the insertion was analysed by agarose gel electrophoresis.

Table 2.12 Plasmid DNA restriction reaction mixture

Component	Volume (µl)
Plasmid DNA	30
10x Buffer	8
Enzyme A	3
Enzyme B	3
BSA	0.8
dH_2O	35.2
	Total volume = 80 µl

2.14.12 Cohesive end DNA cloning to produce recombinant vector

The ligation reaction was set up as described below and the master mixture was incubated at 4 ^{o}C overnight for the maximum number of transformants.

Table 2.13 Ligation reaction mixture of inserted gene and plasmid

Component	Volume (µl)
Vector DNA	1-6
Insert DNA	3-8
dH_2O	variable
The mixture was incubated at 16 ^{o}C for 1-2hours, and then added the followings.	
T_4 Ligase (10 Weiss unit/µl)	1
10x Ligation Buffer	2
	Total Volume = 15µl

2.15 6xHis-tagged proteins: expression and purification

2.15.1 Expression in *E. coli*

1ml of overnight culture of BL21 (DE3) carrying a plasmid with 6xHis-tagged coding sequence was used to inoculate prewarmed 1 litre of 2xYT/antibiotics broth medium. The culture was incubated at 37^{o}C with 200rpm until an optical density of 0.45 at 600nm was reached. At this point the culture was induced with 0.1M IPTG (1ml/L, for pET and pProEX vector) and incubated at 37^{o}C with 200rpm shaking for 4 hours. The cell culture was

harvested at 6,300rpm for 8 minutes and the pellet stored at -20^{o}C or purification steps continued.

The experiment was repeated as above but following the induction the cells were incubated overnight at 25^{o}C with 200rpm shaking.

2.15.2 Purification by Ni^{2+}-NTA chromatography

Purification of 6x-His-tagged recombinant proteins was performed by Ni-NTA affinity chromatography.

A cell pellet was resuspended in lysis buffer (typically containing 200-300mM NaCl, 50mM imidazole, 50mM Tris-HCl, pH 6.8-7.8, 1 protease inhibitor tablet (complete, EDTA-free, Roche) and 5U/ml DNase). The cells were passed 2 times through a constant cell disruptor at 20 and 15 Kpsi, ultra-centrifuged at 43,000rpm (Beckman L8-70M Ultracentrifuge) for 90 minutes at 4^{o}C and the supernatant collected into a 50ml Falcon tube. The soluble cytoplasmic component containing desired proteins was mixed with 1-2ml Ni^{2+}-NTA beads (Qiagen) and the mixture rotated at 4^{o}C for 1-2 hours. The mixture was loaded onto a 1.5ml Bio-Rad Econo-column and the flow through collected and reloaded onto column. The column was washed 1-3 times with 10ml of Wash I, 5ml Wash II and 2ml Wash III buffer, respectively, with each wash being held for 5 min. The interest His-tagged proteins were then eluted from the column, under gravity, with 1ml of Elution buffer (200-800mM imidazole concentration), held for 5 min, and the fractions collected in a 1.5ml microcentrifuge tube. The samples were stored at 20^{o}C.

2.15.3 Purification of 6xHis-tagged proteins from *E. coli* culture media by ion-exchange chromatography

The isoelectric point (IP) for the buffer was calculated using the Hendelson-Hasselbach equation on the website: http://www.expasy.ch/tools/pi_tool.html. The pH of the buffers was determined by PI value during the ion-exchange purification.

The HiTrap™ SP HP (Amersham Bioscience) column was stored in 20% ethanol. The column stopper was removed and connected to an *AKTA purifier* (Amersham Bioscience). The column was washed with 4-6 volume of distilled water. 2-4 column volumes of elution buffer were run through the column and then the column was equilibrated with 4-6 column volumes of start buffer.

The collected supernatant was loaded onto the HiTrap™ SP HP column equilibrated with the start buffer. Without washing step, the column was eluted at a flow rate of 1ml/min with a gradient of 100-1000mM NaCl.

2.16 Western-blot technique

Western-blotting was performed for identification of hexahistidine tagged protein. Two identical SDS PAGE were prepared simultaneously. After this procedure, the first gel was stained with Coomassie Blue gel as normal, but the second one was into the western blot apparatus (Figure 2.1) to compare one gel with the other.

Table 2.14 Buffer solutions for Western-blotting

Transfer buffer		Wash buffer		Phosphate solution	
Glycine	2.9g	Phosphate solution	30ml	KH2PO4	2.9g
Tris Base	5.8g	NaCl	4.38g	K2HPO4	5.8g
SDS	0.37g	Tween 20	250µl	dH_2O	to 400ml
Methanol	200ml	dH_2O	to 500ml		
dH_2O	to 1000ml				

A section of PVDF (Bio-Rad) was cut to fit the size of the gel. PVDF was put into methanol for approximately 10 seconds and left to soak in the transfer buffer for 15 minutes. Two pieces of filter paper (Whatman, type 3) were cut slightly larger than the size of the gel. The "sandwich" were assembled like Figure 2.1, and transferred to the blotting chamber. The chamber was filled with transfer buffer and run at 60 mA for 1.5 hour. The blotting gel was checked for successful transfer if the protein bands moved toward the PVDF paper. The paper was washed in wash buffer for 10 minutes, transferred to 25ml of blocking buffer (wash buffer plus 3% BSA), and then the membrane was blocked for 90minutes at room temperature with gentle agitation, alternatively, was kept at 4^oC overnight. The membrane was washed three times with Wash buffer at room temperature for 10 minutes each time. The membrane was incubated with 10µl of mouse antibody (1:3000 dilution) in 25ml blocking buffer (Wash buffer plus 0.5% BSA) at room temperature for 1 hour. The membrane was rinsed in Wash buffer three times for 10 minutes each time and incubated with 10µl of goat anti-mouse antibody (1:3000 dilutions) in 25ml blocking buffer at room temperature for 1 hour. The paper was washed in Wash buffer three times for 10 minutes each time. The membrane paper was treated with colour reagent (Immun-Blot Kit, Bio-Rad) and the place on the paper where protein/mouse antibody/goat antibody-AP is located became purple.

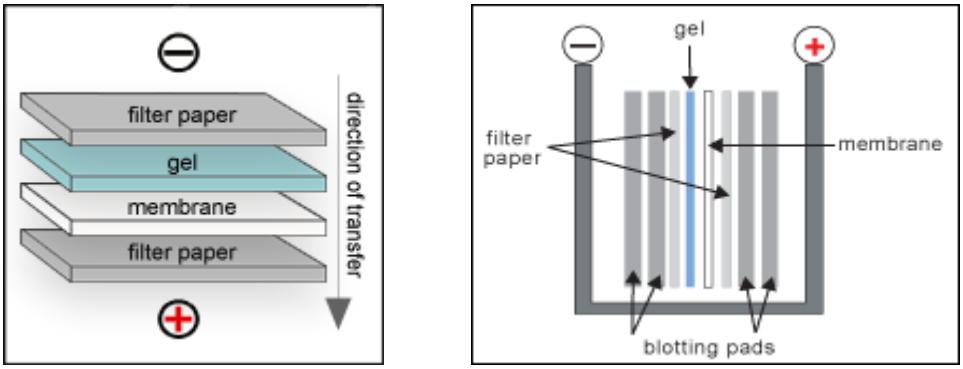


Figure 2.1 Schematic representation for setting up the Western blotting apparatus. The sandwich assembly (left), the blot chamber (right).

2.17 Bacterial competition assay

To determine the fitness of *E. coli* KX1186 (LuxK mutant) and wild type relative to *Z. mobilis*, overnight pre-conditioned cultures were mixed at a 1:1 and 1: $1:^{1}/_{10}$ (*E. coli* vs. *Z. mobilis*) in fresh RM medium. The initial cell density in each tube was averagely 1-3 x 10^{6}/ml. After incubation at 30°C for 48hours, samples were plated appropriate dilutions on modified RM (5% glucose, 1% Yeast Extract) at each time point (24h interval) to determine total CFU. Colonies were scored by phenotype by eye. Finally the relative fitness was calculated by following equations. Relative fitness = "$\frac{\ln(\frac{\text{initial}}{\text{final}}\text{cell denisty of } E.coli)}{\ln(\frac{\text{initial}}{\text{final}}\text{cell denisty of } Z.mobilis)}$.

Chapter 3 Quorum sensing of *Z. mobilis* and its applications

3.1 Identification of extracellular protein from *Z. mobilis* with bacteriocin activity.

3.1.1 Introduction

As mentioned in Chapter 1, *Z. mobilis* has received scientific interest due to its ability to produce ethanol. In addition to its use in ethanol production, there are several earlier reports of the therapeutic use of *Z. mobilis* and it has been reported to exert antagonistic effects against a wide range of Gram-negative and Gram-positive bacteria, as well as certain protozoa, yeasts and filamentous fungi (Swing and De Ley, 1977). The biochemical nature of these antagonisms is unknown. Primary metabolic products may be directly or indirectly responsible (Haffie *et al*., 1985), or *Z. mobilis* may produce compounds similar to broad-spectrum antibiotics or bacteriocins, such as are produced by the group of Proteobacteria (Parret and De Mot, 2002). For example, *Pseudomonas aeruginosa* phyogenically closely related with *Z. mobilis* is known to secrete three types of bacteriocins, termed pyocins (Michel-Briand and Baysse, 2002). This pyocin family is classified by a diversity of proteins that differ in size, microbial targets, modes of action, and immunity mechanisms (Riley, 1998). Pyocins synthesized by more than 90% of *Pseudomonas aeruginosa* strains, are also categorized by a wide range of different structures and functions (Table 3.1). R-type pyrocins has a rod-like structure and the receptor of the tail fiber is consistuted of lipopolysacchride. Once the R-type pyocin has been absorbed on its receptor, is followed by rapid contraction of the sheath and penetration of the outer membrane. F-type pyocins also resemble phage-tails, but with flexible. S-type pyrocin is colicin-like protease-sensitive protein and consists of 2

domains different from 3 domains in colicin. The large component carries the killing acitivity and small component is an immune protein protecting the host bacteria from the killing effect of the large one. The small component has sequence homology with those of colicin E2.

Although bacteriocins of other organisms are often encoded on plasmids, pyocin genes are located on the genome of *P. aeruginosa* (Kageyama, 1975). Synthesis is regulated so that only a few cells in a population actively produce pyocins. Pyocin synthesis is also inducible by treatments that cause DNA damage, such as ultraviolet irradiation (Higerd *et al*., 1967) and treatment with mitomycin C (Kageyama, 1964). Synthesis of the different pyocins begins when the mutagen induces the expression of the *recA* gene and activates RecA. Activated RecA cleaves the repressor RrtR, liberating the expression of the protein activator gene *prtN* (Figure 3.1).

Table 3.1 Various pyocins produced by *P. aeruginosa*

Type	Structure	Examples	Functions
S	Colicin E2 like structure (receptor recognition + killing domain)	S1, S2, S3, AP1	Dnase
		S4	tRNase
		S5	Pore formation
R	Non-flexible and phage tails		Depolarisation of membrane by combining with macromolecules
F	Phage tails, but with flexible		

(Summarized from Michael-Brand and Baysse, 2002; Nakayama *et al*., 2000)

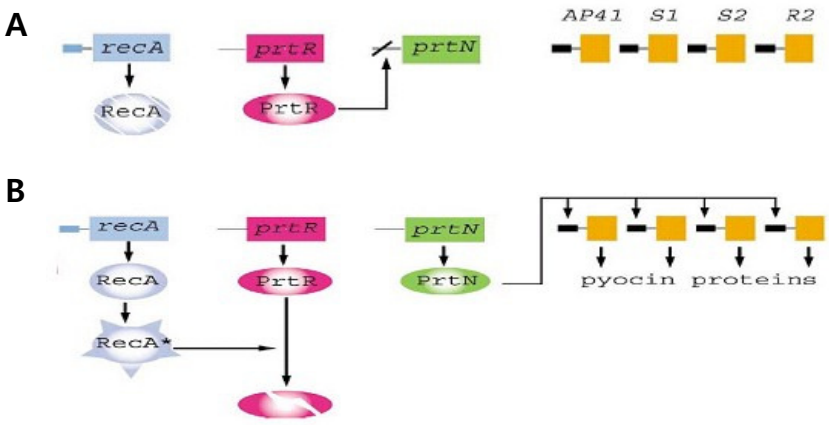


Figure 3.1 Regulation of pyocins production. The production of all types of pyocins was shown to be regulated by positive (prtN) and negative (prtR) regulatory genes. (Adapted from Michael-Brand and Baysse, 2002)

3.1.2 Optimal condition for antibacterial activity of *Z. mobilis*

Prior to the direct purification of putative bacteriocins, the optimal condition for the antimicrobial activity of *Z. mobilis* was investigated. The antibacterial activity was determined by using a well-diffusion assay (Haffie *et al*., 1985). Briefly, different initial pH-conditioned RM medium (25ml) was prepared as a base for indicator plates. Four holes were punch-prepared around the centre of the plate using a 50µl tip and each hole was filled with 50µl of RM medium. Then, preconditioned *Z. mobilis* cells (OD_{600}=0.5) were loaded in to each of the holes (at approximately 1/100 dilution). After the culture solution was solifed in the middle of the medium, pre-cultured *E. coli* or *S. epidermidis* cells (OD_{600}=0.5), which were diluted (1/100) into 0.7% soft agar were overlaid onto the RM-based plates. The plates were incubated at 30°C or 37°C overnight and the inhibition zone of *Z. mobilis* was measured

(Figure 3.2a). As previously stated, halos of growth inhibition appeared in both lawns of Gram positive and Gram negative bacteria (Figure 3.2b). The measurements of these halos are graphed in Figure 3.2c.

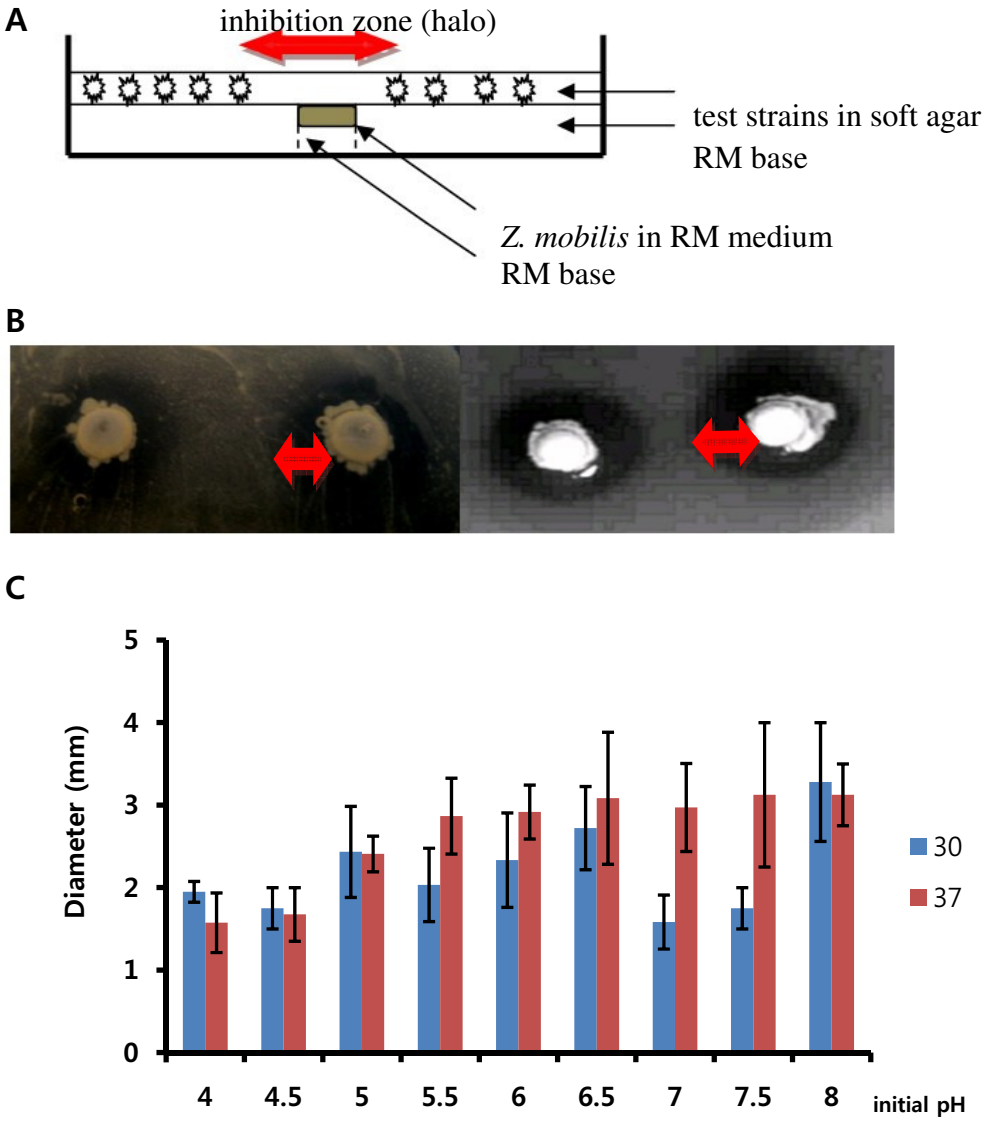


Figure 3.2 Antibacterial effects of *Z. mobilis*. (A) Diagram of a well diffusion assay. (B) Growth inhibition of *E. coli* (left) and *S. epidermidis* (right). (C) Killing effect to *E. coli* in various culture conditions by *Z. mobilis* ZM4. Data were collected from 2-6 independent replicas and each bar represents the standard error.

The maximum inhibitory activity of *Z. mobilis* occurred between pH 6 to 6.5 at 30°C and 37°C. These results were consistent with an earlier report (Swing and De Ley, 1977) about *Z. mobilis* growth rate at different conditions. Consequently, I concluded that the growth rates do correlate with the killing effects and this prompted me to use the standard RM medium (pH 6.8) for putative bacteriocin purification.

3.1.3 Putative bacteriocin purification strategies

After the determination of the optimal culture conditions for (presumed) bacteriocin production by *Z. mobilis*, an attempt at partial purification of the putative bacteriocins was performed according to the following incubation conditions and purification methods (Table 3.2). The pH of the supernatant was adjusted from pH 4 to pH 10 by adding 1M NaOH or 1M HCl and appropriate chemicals: acetate (final concentration, 50mM) for pH 4.8, malonate (50mM) for pH 5.7, BICINE (50mM) for pH 8.4, and 1,3-diaminopropane (20mM) for pH 10.5. SP and Q Sepharose™ Fast Flow resins (Amersham) were used as ion beads for cation and anion exchange chromatograph, respectively. Initial attempts to purify the extracellular proteins failed because of the low cell density of *Z. mobilis* (data not shown). However, several proteins were successfully separated from the supernatant of cells for which the incubation time was increased to at least 16 hours (approximately OD_{600}=1.5). Because the profile of extracellular proteins produced by cells from 16 and 24 hours incubation was not significantly different, gel-filtration was carefully performed with the 16 hours incubated *Z. mobilis* supernatant. Briefly, a HiLoad 16/60 Superdex 200 prep grade column (Amersham Bioscience) was used for size-exclusion chromatography to separate and purify proteins released into the supernatant from *Z. mobilis* cells grown for 16 hours. The supernatant of *Z.*

mobilis was directly loaded on to the column without treatment after equilibration of the column using elution buffer (phosphate 50mM, pH 7.2). The flow rate was controlled constantly at 1ml/min and 1ml fractions were collected over the peak area of the elution profile shown in Figure 3.3.

Table 3.2 Putative bacteriocin purification strategies and its activity

Incubation Periods	Medium temperature	Methods	Activity
4 hours			
8 hours			
16 hours	RM, 30°C/37°C		
24 hours		Cation / anion exchange	
48 hours		from *Z. mobilis* supernatant	All negative (Figure 3.4)
24 hours			
48 hours	Minimal medium 30°C/37°C		
72 hours			
16 hours	RM, 30°C	Gel filtration	
24 hours			

Cell cultures were also exposed to the following stress conditions: 1) DNA damaging ultraviolet light exposure in accordance with pyocin synthesis; 2) extension of incubation time to 72 hours for intracellular competitions within *Z. mobilis* cells. Especially for the intracellular competitions for long-term and starvation conditions, minimal medium was used. However, isolated extracelluar proteins from the above conditions did not appear to have any bacteriocin activity and extracellular proteins overexpressed in *E. coli* also did not appear at

least at the concentrations tested up to 1-1.5µg/ml.

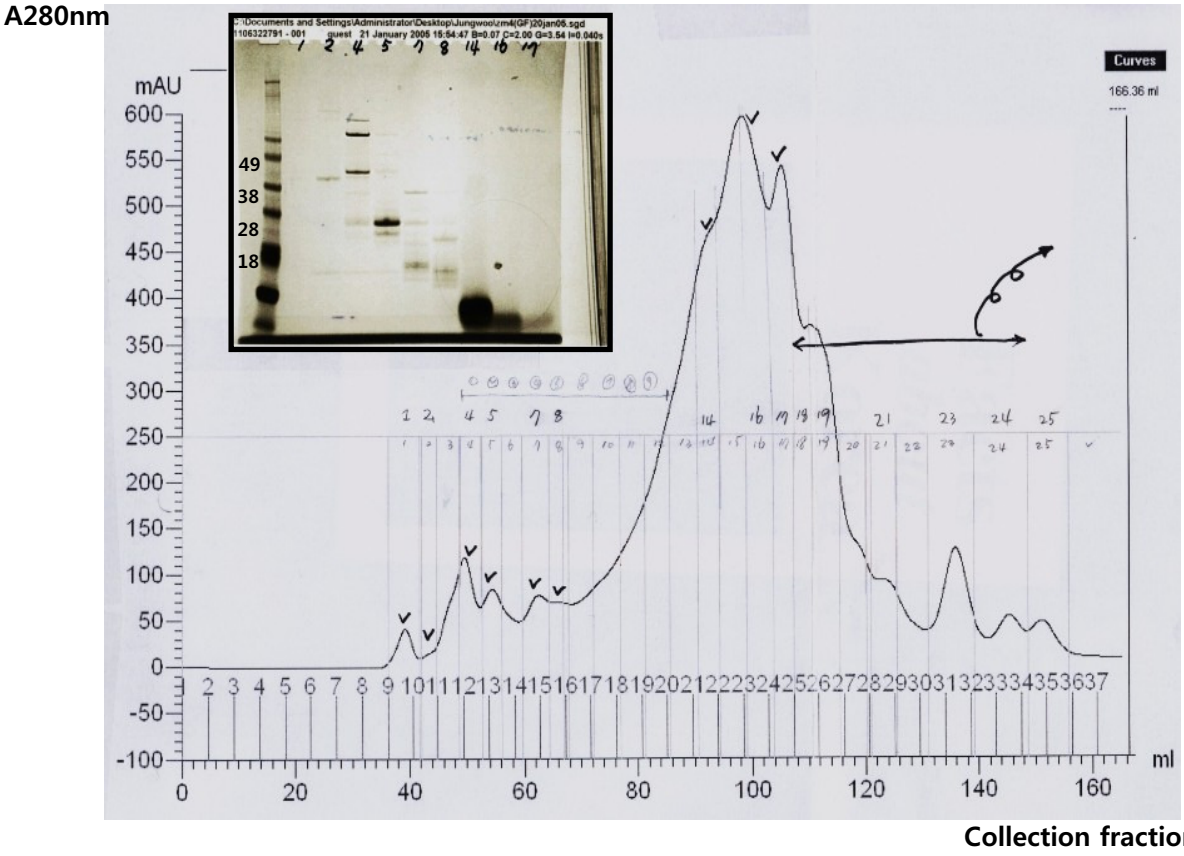


Figure 3.3 The representative extracellular proteins separation from *Z. mobilis* in various conditions. Proteins were collected from one litre of *Z. mobilis* cultured in the conditions mentioned Table 3.2: these proteins were separated by gel filtration using an *AKTA purifier* (Amersham Bioscience) with an HiLoad 16/60 Superdex 200 prep grade column. The SDS PAGE gels show a profile of partial purification of extracellular proteins from *Z. mobilis* (insert). A well diffusion assay with the eluted proteins indicated that there was no antibacterial activity (Figure 3.4).

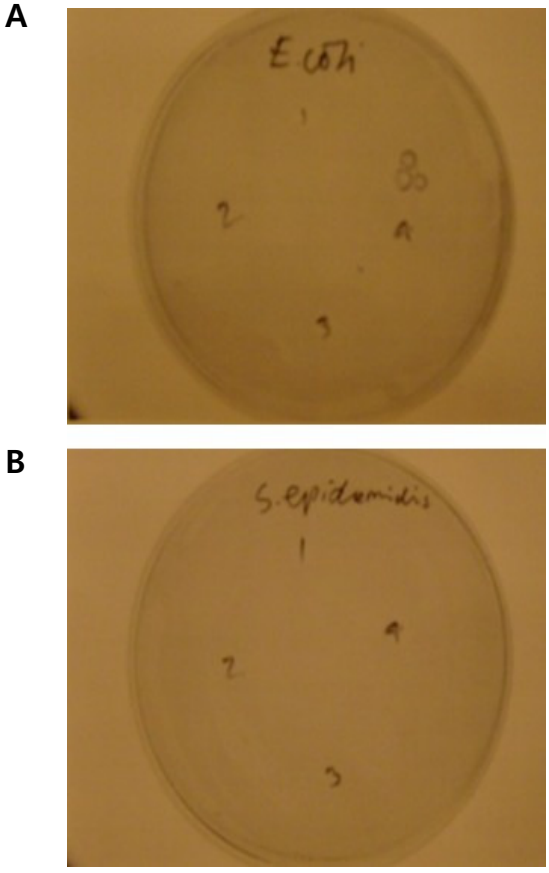


Figure 3.4 No bacteriocin activity from partially purified extracellular proteins. In order to identify conditions in which the antibacterial effect of the partially separated proteins were apparent, a range of different buffer conditions was tested (pH 4.8 – 8.4; 50mM acetate, pH 4.8, 50mM malonate, pH 5.7, 20mM L-histidine, 6.0, 20mM bis-Tris 6.5, 50mM phosphate, pH 7.2, 50mM Bicine, pH 8.4). In conclusion, there was no bacteriocin effect of the separated proteins on either *E. coli* (A) nor *S. epidermidis* (B) under these buffer conditions, because there is no halos detected, because there is no halo observed.

3.1.4 The induction of *Z. mobilis* extracellular proteins due to co-culture with *E. coli*.

Since *Z. mobilis* did not appear to produce any proteins with bacteriocin activity, but had antibacterial activity against *E.coli*, it can be hypothesized that this activity was stimulated or stress-induced by (in) direct contact with neighbouring cells, such as *E. coli*. To test this hypothesis *Z. mobilis* cells were incubated until an OD_{600} of 0.5 had been reached, then 1% (v/v) of growing *E. coli* culture (approximately OD_{600}=1.0) was directly added to the *Z. mobilis* cell culture. However, the experimental procedure and analysis was difficult to control, because 1) the *E. coli* died after incubation overnight in co-culture with *Z. mobilis*, possibly resulting in the modification of the bacteriocin or its uptake by the *E. coli* cells if bacteriocin exist; and 2) the incubation condition is a mixture of cells so that it is difficult to identify which proteins specially originated from the *Z. mobilis* cells. Thus, to avoid direct contact of the bacteria and the complication of the proteins profile, a dialysis bag was used to separate the two growing bacterial populations (Section 2.4). Under these conditions, one protein band was clearly induced and this protein induction was confirmed by more than 3 repeated experiments (Figure 3.5). However, although this protein was induced by exposure of *Z. mobilis* to the supernatant of co-cultured *E. coli*, it did not have any bacteriocin activity in a well-diffusion assay (Figure 3.4).

This research led to conclude that *Z. mobilis*, in contrast to the expectation that it would produce bacteriocins consistent with its antibacterial activity, may not produce and secrete bacteriocin. However, the studies indicated that in response to co-culture with *E. coli*, *Z. mobilis* was induced to produce extracellular proteins. This important finding caused to shift the research in a different direction: to understand how and why *Z. mobilis* responded in such

a manner. Considering that the *Z. mobilis* was separated from *E. coli* by a dialysis membrane, this indicated that it must respond to a small molecule (or peptide <12kDa) produced and released by *E. coli*. This finding prompted me to investigate if there was quorum sensing between *E.coli* and *Z. mobilis* and to identify the quorum sensing molecule(s) involved and its (their) effects on *Z. mobilis*.

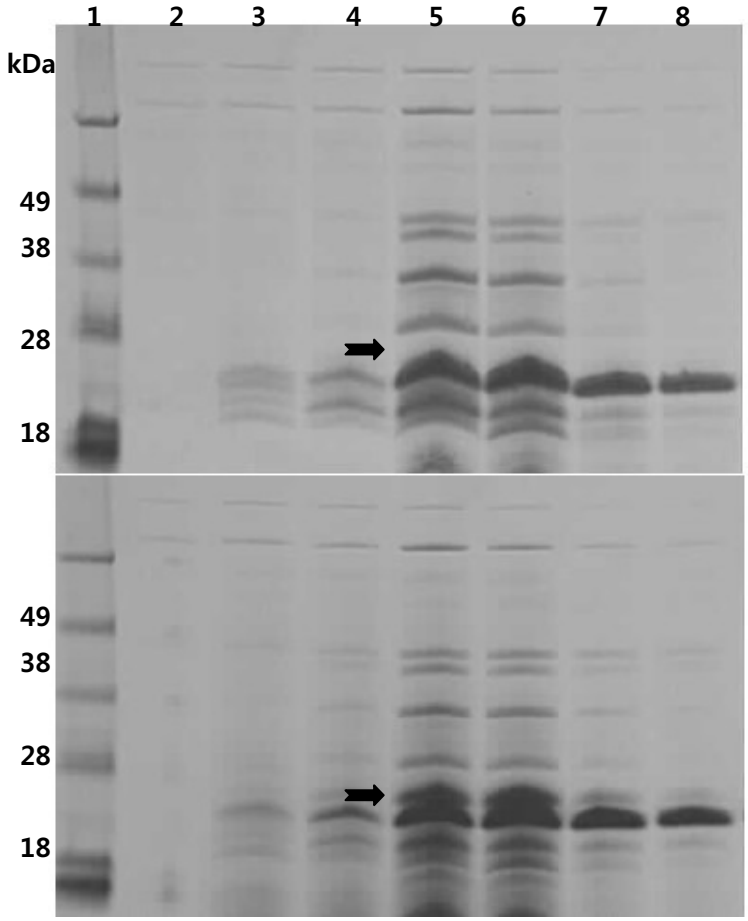


Figure 3.5 The co-culture of *Z. mobilis* with *E. coli* induces the production of extracellular proteins by *Z. mobilis*. Upper and lower gels show the extracellular protein production of *Z. mobilis* under non-induction and induction conditions, respectively. Lane 1, protein standard, Lane 2-8, elution fractions from an ion-exchange column. The arrow indicates the induced protein from a coculture experiment with *Z. mobilis* and *E. coli* K12. Because *E. coli* and *Z. mobilis* are different strains, my prediction was that there exists a small molecule signal that acts as a common language between the different strains.

3.2 Autoinducer 2 (AI-2) quorum sensing in *Z. mobilis*

3.2.1 Introduction

Many groups of bacteria are known to communicate with each other by mean of diffusible chemical molecules (known as autoinducers). This extracellular autoinducer-related behaviour is called quorum sensing. Quorum sensing is generally involved in producing, secreting, detecting and responding to signals to monitor one another's presence and to modulate gene expression in response to changes in the environment. In general, two predominant types of small-molecule autoinducer, acyl homoserine lactone (AHLs, AI-1) and modified oligopeptides are used by Gram negative and Gram positive bacteria, respectively (Figure 3.6a,b).

Bassler and her colleagues discovered a novel communication system in *Vibrio harveyi* (Bassler *et al*., 1994). Bioluminescence in *V. harveyi* is regulated by two distinct signaling molecules that are detected by independent signal-transduction systems which subsequently converge in a common pathway to regulate bioluminescence gene expression (Figure 3.6c). Autoinducer-1 (AI-1) is a well-characterized derivative of an AHL that is highly species-specific for *V. harveyi*. The LuxL and LuxM proteins are required for the synthesis of AI-1, which it is detected by the LuxN sensor protein that is associated with the cytoplasmic membrane. Molecular studies of *V. harveyi* proved that there exists a second signaling system, based on an unrelated autoinducer (AI-2), generally a cyclic derivative of dihydroxypentanedione that is non-species-specific (Xavier and Bassler, 2005b). AI-2 is synthesized by the LuxS enzyme and AI-2-mediated signal cascades involve several Lux

proteins in *V. harveyi*. LuxP is a periplasmic-binding protein that binds AI-2, and LuxQ is an inner membrane sensor kinase that detects the LuxP-AI-2 complex.

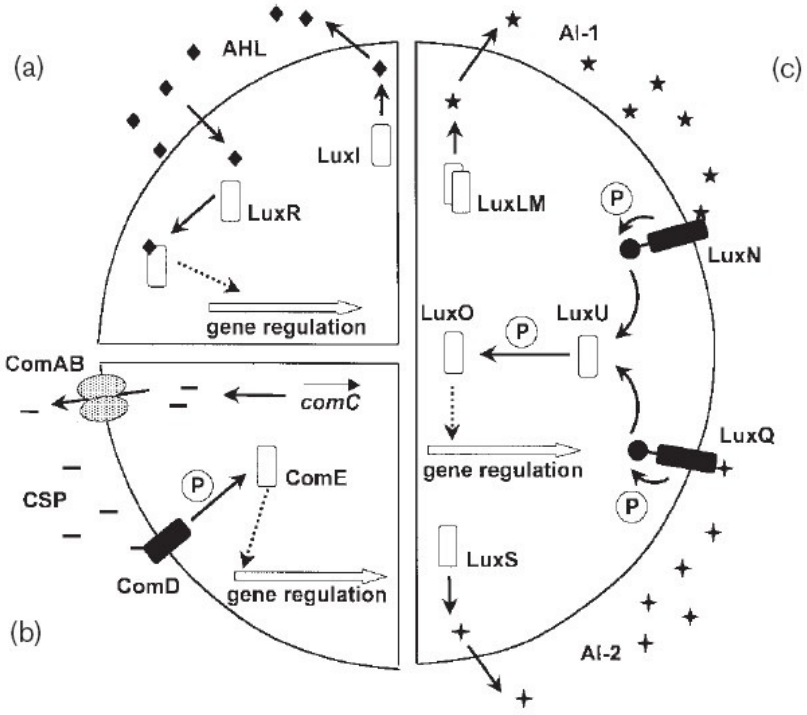


Figure 3.6 Quorum sensing circuits. (a) Acyl-homoserine lactone (AHL) signals are used for species-specific communication in most Gram negative bacteria. **(b)** Oligopeptides are the predominant signal used by Gram positive bacteria **(c)** *V. harveyi* quorum sensing circuit (Adapted from McNab and Lamont, 2003).

Upon ligand binding LuxQ changes from a kinase to a phosphatase that removes phosphate from the response regulator LuxO, via an intermediate protein, LuxU. Dephosphorylated LuxO fails to activate a hypothetical repressor of the *luxCDABE* operon with the net result being luminescence (Figure 3.6.c). In contrast to ALHs and oligopeptide autoinducers, the AI-2 biosynthetic pathway and the AI-2 molecule itself (at least, DPD) are identical in all AI-2-producing bacteria. These findings have led to the proposal that AI-2 is a universal language, which functions in interspecies cell-to-cell communication. AI-2 quorum sensing systems include those regulating bioluminescence, biofilm formation, virulence factor production and antibiotic production etc (Williams *et al*., 2007).

3.2.2 Autoinducer 2 (AI-2) signalling in *Z. mobilis*

3.2.2.1 Extracellular proteins induction in the presence of AI-2

In spite of the failure to find a bacteriocin produced by *Z. mobilis*, the finding that *Z. mobilis* produced extracellular proteins when co-cultured with an *E. coli* K12 strain lead to test whether the production of these proteins by *Z. mobilis* was in response to small signaling molecules, such as AI-2, secreted by *E. coli* strains, Thus, wild type strain (KX 1123) and a *ΔluxS* mutant (KX 1218) unable to produce AI-2 (Xavier and Bassler, 2005) was used for our co-culture experiments.

As expected, these extracellular proteins were secreted when *Z. mobilis* was co-cultured with the filter sterilized supernatant of the wild type *E.coli* KX1123 but not the *luxS*-deletion strain

KX1128 (Xavier and Bassler, 2005), indicating that they are secreted in response to AI-2 (Figure 3.7b)

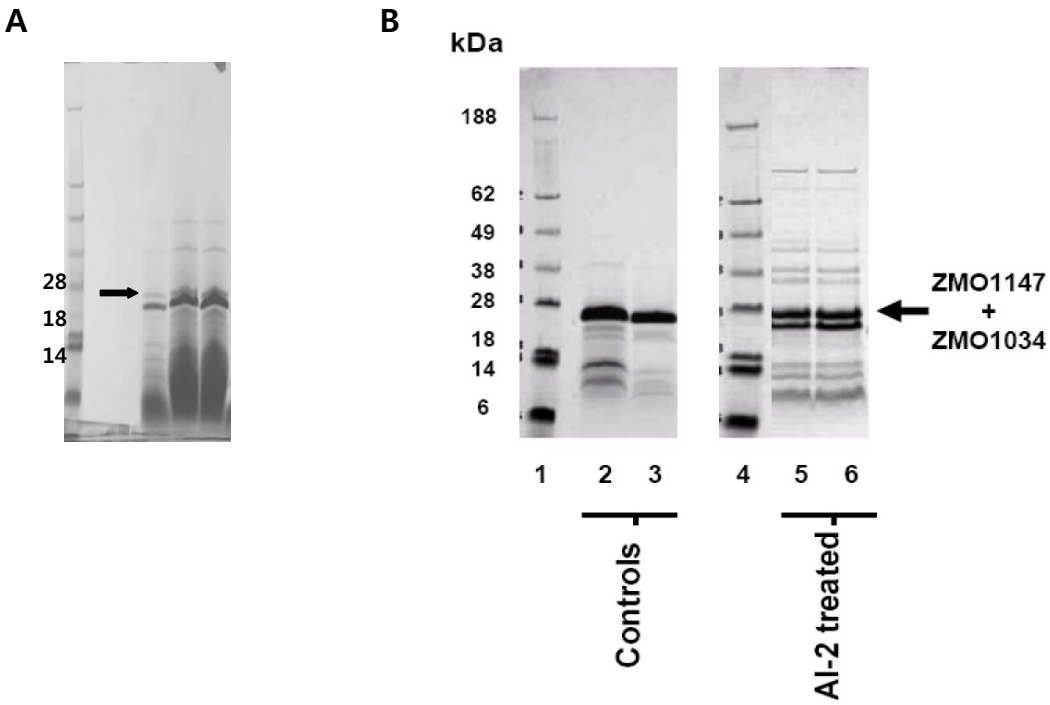
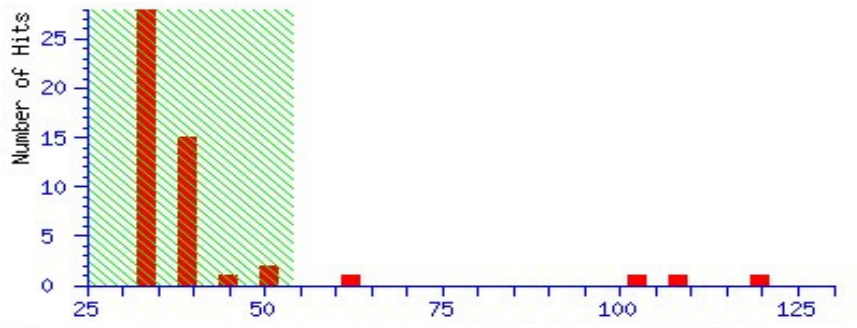


Figure 3.7 Analysis of secreted proteins overproduced in *Z. mobilis* in response to the quorum sensing molecule autoinducer-2 (AI-2). (A) Extracellular protein induction from *Z. mobilis* incubated with *E. coli* supernatant. **(B)** SDS-PAGE analysis of the proteins secreted by *Z. mobilis* co-cultured for 16 hours with the *E. coli* strain KX1128 (*ΔluxS*) (lane 2 and 3) and AI-2 producing strain KX1123 (lanes 5 and 6). For *Z. mobilis* cultured with KX1123, an extra band, with a Mr of about 26kDa, is clearly observed (lane 5 and 6). This band was subjected to *in situ* trypsin digest and the resulting oligopeptide fragments sequenced by mass spectrometry, indicating that it is composed of two proteins, ZMO1034 and ZMO1147.

To further test this hypothesis, AI-2 produced *in vitro* was used to confirm that it induced protein secretion. The two proteins secreted in response to AI-2 were unambiguously identified by mass spectrometry sequencing of typtic-fragments (Figure 3.8). The first of the secreted proteins was identified as ZMO1147, a homologue of the *E. coli* periplasmic chaperone Skp (or HlpA) (Walton and Sousa, 2004). The other secreted protein was identified as ZMO1034, with homology to a group of small EF-hand Ca^{2+}-binding proteins (Zhou *et al*., 2006). This is an important finding, because the precise role of AI-2 in other bacteria is currently a hot topic of debate and information is sparse regarding the recognition and signal transduction of this autoinducer in species other than *V. harveyi* (Straight and Kolter, 2009; West *et al*., 2006).

3.2.2.2 Induction of cytoplasmic enzyme production by *Z. mobilis* in the presence of AI-2

Considering the observation that AI-2 could trigger protein secretion in *Z. mobilis*, further test was performed whether any cytoplasmic proteins/enzymes of increased abundance could be identified after treatment of *Z. mobilis* cells with AI-2. A protein-gel analysis clearly identified two protein bands that were substantially increased after AI-2 treatment (Figure 3.9). The two overproduced cytosolic proteins were identified, by mass spectrometry finger printing, as enolase (ZMO1608, Figure 3.10) and pyruvate decarboxylase (ZMO1360, Figure 3.11). The enolase known as phosphopyruvate dehydratase and is responsible for the catalysis of the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP), the ninth step of glycolysis. The pyruvate decarboxylase is a homotetrameric enzyme that catalyses the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide in the cytoplasm. This enzyme is part of the fermentation process to produce ethanol by fermentation.



gi|56543617 Mass: 23992 Score: 120 Queries matched: 4
conserved hypothetical protein [Zymomonas mobilis subsp. mobilis ZM4]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
62	404.2410	806.4675	806.4286	0.0389	0	25	64	9	K.INAFQSK.Q
133	547.7829	1093.5512	1093.5152	0.0360	0	28	22	1	R.TQIDAYNNR.R
162	616.3207	1230.6269	1230.6204	0.0065	0	34	6.6	1	R.TALENELSQAR.D
244	692.8383	1383.6620	1383.6490	0.0129	0	32	7.8	2	R.QNQSDAGAGAAPAAR.A

gi|56543504 Mass: 22510 Score: 110 Queries matched: 11 emPAI: 0.15
conserved hypothetical protein [Zymomonas mobilis subsp. mobilis ZM4]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
183	619.3176	1236.6207	1236.5986	0.0221	0	(55)	0.049	1	K.SEFQTALANEK.N
184	619.3176	1236.6207	1236.5986	0.0221	0	(61)	0.012	1	K.SEFQTALANEK.N
185	619.3176	1236.6207	1236.5986	0.0221	0	66	0.0033	1	K.SEFQTALANEK.N
186	619.3176	1236.6207	1236.5986	0.0221	0	(38)	2.1	1	K.SEFQTALANEK.N
273	711.3463	2131.0171	2131.0222	-0.0051	0	(22)	58	1	K.TPPPPPPAGDGNTPPPPPEDGK.G
274	711.3582	2131.0527	2131.0222	0.0305	0	28	15	1	K.TPPPPPPAGDGNTPPPPPEDGK.G
275	711.3582	2131.0527	2131.0222	0.0305	0	(24)	40	2	K.TPPPPPPAGDGNTPPPPPEDGK.G
276	711.3582	2131.0527	2131.0222	0.0305	0	(24)	38	3	K.TPPPPPPAGDGNTPPPPPEDGK.G
277	711.3582	2131.0527	2131.0222	0.0305	0	(20)	1e+02	4	K.TPPPPPPAGDGNTPPPPPEDGK.G
278	711.3582	2131.0527	2131.0222	0.0305	0	(28)	17	1	K.TPPPPPPAGDGNTPPPPPEDGK.G
287	777.9000	2330.6782	2331.0293	-0.3511	1	16	2.1e+02	8	K.SEFQTALANEKNQCQAMMAK.F + Carbamidomethyl (C)

Figure 3.8 MASCOT search results from the MS-MS data generated for ZMO1034 (putative calcium binding protein) and ZMO1147 (putative chaperone) induced by AI-2.

http://www.matrixscience.com/cgi/master_results.pl?file=../data/20050721/FsnoliTT.dat#Hit1

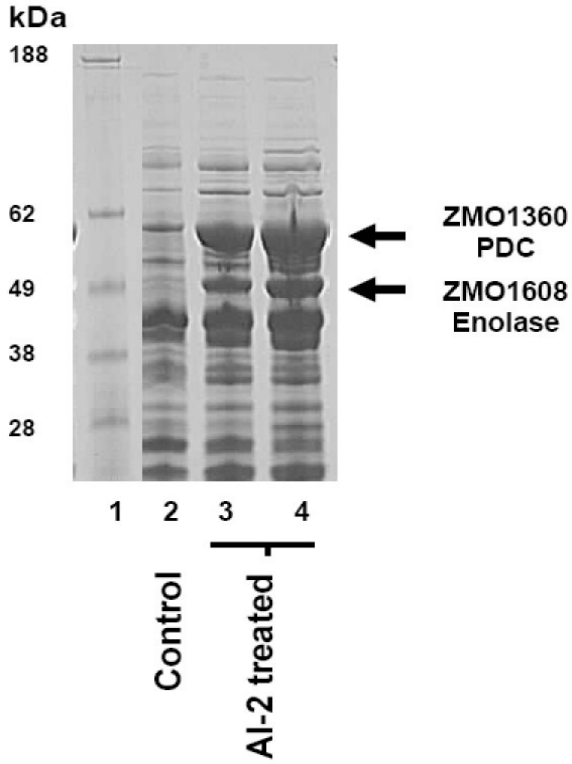
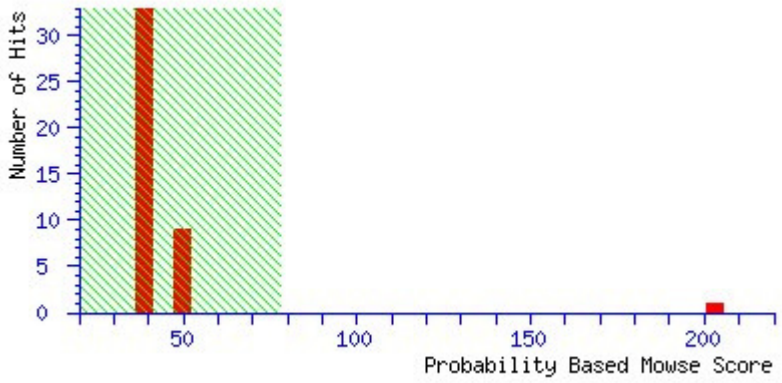
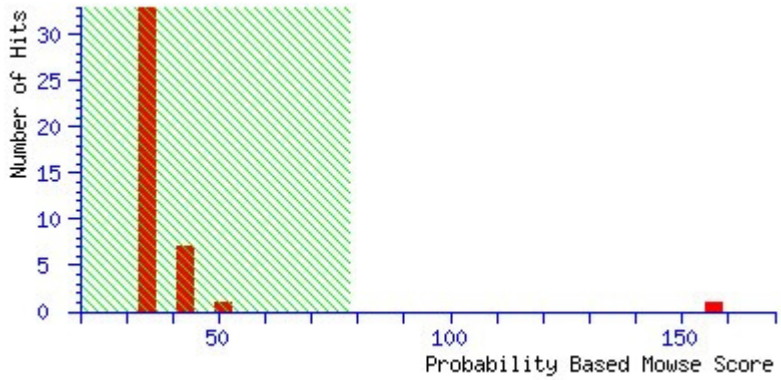


Figure 3.9 Analysis of cytosolic proteins overproduced in *Z. mobilis* in response to AI-2. SDS-PAGE analysis of the cytoplasmic proteins overproduced by *Z. mobilis* co-cultured for 16hours with the supernatant of *E. coli* KX1128 (*luxS* mutant) (lane 2) and AI-2 producing strain KX1123 (wild type) (lane 3 and 4). The *Z. mobilis* cells were incubated with the sterilized supernatant from *E. coli* cells grown to and OD_{600} of 1.0 (KX1128; lane 2), 1.0 (KX1123; lane 3) and 1.4 (KX1123; lane 4). In contrast to *Z. mobilis* cultured with the supernatant of *E. coli* KX1128 (lane 2); for *Z. mobilis* cultured with the supernatant of *E. coli* KX1123, two bands are observed that have increased abundance (lanes 3 and 4).



gi|59802545 Mass: 45885 Score: 203 Expect: 1.7e-14 Queries matched: 19
Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase)
gi|155581 Mass: 45986 Score: 155 Expect: 1.1e-09 Queries matched: 16
enolase
gi|3089611 Mass: 40336 Score: 111 Expect: 2.7e-05 Queries matched: 12
enolase [Zymomonas mobilis]
gi|5763976 Mass: 2075 Score: 47 Expect: 64 Queries matched: 3
ATP synthase beta chain [Begonia palmata]
gi|49611070 Mass: 11935 Score: 41 Expect: 2.7e+02 Queries matched: 4
hypothetical plasmid protein [Erwinia carotovora subsp. atroseptica SCRI1043]
gi|56315621 Mass: 19529 Score: 39 Expect: 4.7e+02 Queries matched: 4
hypothetical protein [Azoarcus sp. EbN1]

Figure 3.10 MASCOT search results from the MS-MS data generated for ZMO1608 (enolase). http://www.matrixscience.com/cgi/master_results.pl?file=../data/20060316/FAioSnYa.dat



gi|228768 Mass: 61155 Score: 157 Expect: 6.8e-10 Queries matched: 18
pyruvate decarboxylase
gi|48660 Mass: 61286 Score: 157 Expect: 6.8e-10 Queries matched: 18
pyruvate decarboxylase [Zymomonas mobilis]
gi|21465421 Mass: 61272 Score: 132 Expect: 2.2e-07 Queries matched: 16
pyruvate decarboxylase [Zymomonas mobilis]
gi|4388900 Mass: 61254 Score: 127 Expect: 6.8e-07 Queries matched: 16
Chain F, Pyruvate Decarboxylase From Zymomonas Mobilis
gi|155579 Mass: 61411 Score: 118 Expect: 5.4e-06 Queries matched: 15
pyruvate decarboxylase (EC 4.1.1.1)
gi|50251805 Mass: 14539 Score: 39 Expect: 4e+02 Queries matched: 4
unknown protein [Oryza sativa (japonica cultivar-group)]

Figure 3.11 MASCOT search results from the MS-MS data generated for ZMO1360 (pyruvate decarboxylase).

http://www.matrixscience.com/cgi/master_results.pl?file=../data/20060316/FAiomesn.dat

3.2.2.3 *Z. mobilis* does not produce AI-2

Considering that *Z. mobilis* was clearly responsive to AI-2, A luxS gene search by homologue searching in pubmed (http://www.ncbi.nlm.nih.gov/nuccore/NC_006526) that would be expected to be involved in AI-2 production was done. However, the genome of *Z. mobilis* does not have a discernable homologue of *luxS* that could produce AI-2 (Seo *et al.*, 2005). Furthermore, most, if not all, organisms have a pathway to recycle S-adenosylmethionine (SAM), via S-adenosylhomosysteine (SAH), either using a two-step enzymatic conversion by Pfs and LuxS to produce 4,5-dihydroxy-2,3-pentanedione (DPD); or a one-step conversion using SAH hydrolase (De Keersmaecker *et al*., 2006). A gene, encoding a SAH hydrolase (ZMO0182) was screened from *Z. mobilis*, indicating that *Z. mobilis* utilizes the one step pathway, rather than the Pfs/LuxS pathway that would lead to AI-2 production. As mentioned in Section 2.8., the cell free supernatant (20 µl) of test strains was added into growing *V. harveyi* BB170 culture (80 µl), placed in 96 well microplate, then bioluminescence in response to AI-2 was measured every 1 hour. Consquently, it was able to confirm that *Z. mobilis* does not produce AI-2 (Figure 3.12), whilst wild type of *E. coli* (KX1123) and *S. epidermidis* can produce the AI-2. However, after 5 hour timepoint *V. harveyi* strain can produce light irregardlees of the strains due to high concentration of AI-2 which is produced by it. This is an important discovery because most, if not all, bacteria that have been found to respond to AI-2 also produce AI-2. Indeed, *P. aeruginosa* is the only bacterium reported to date to respond to, but does not produce AI-2; and like *Z. mobilis*, with which it is phylogenetically related, it does not possess a *luxS* gene. AI-2 stimulates the expression of several pathogenicity determinants in *P. aeruginosa* (Duan *et al*., 2003). *P. aeruginosa* and *Z. mobilis* might possess related AI-2 signal transduction pathways, which are currently

uncharacterized.

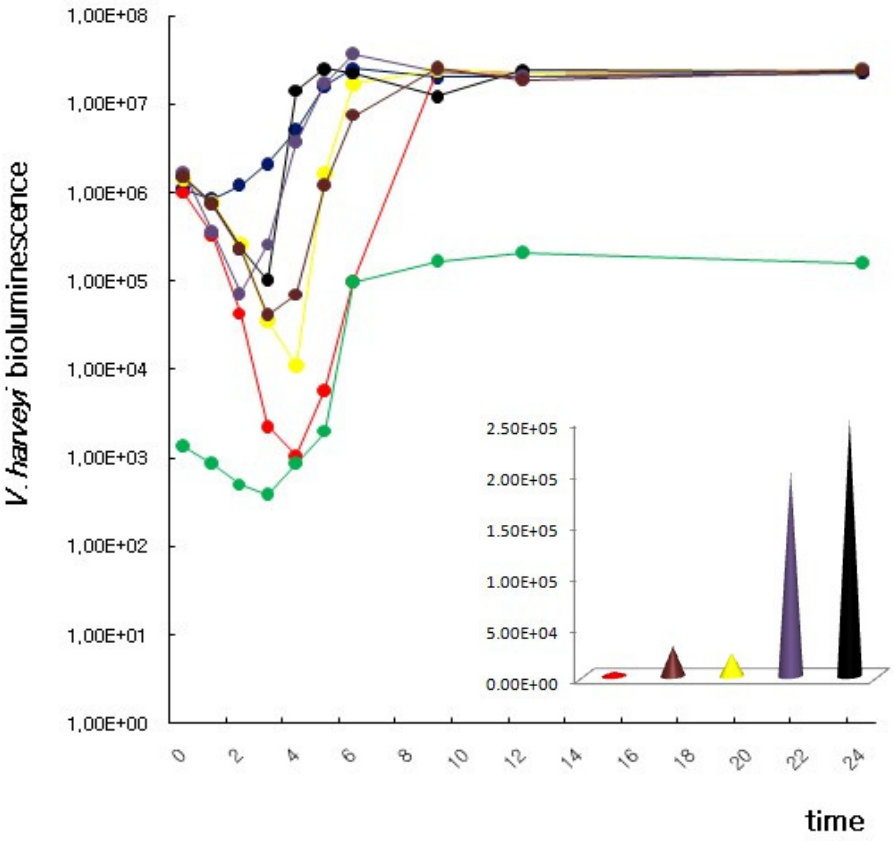


Figure 3.12 *Z. mobilis* does not produce AI-2. Light production for *V. harveyi* cultured in AB medium to which the cell free supernatant from the following strains were added: *E. coli* strains KX1123 (violet; wild type), KX1128 (yellow; LuxS mutant) and DH5α (brown); *Z. mobilis* ZM4 (red); *Staphylococcus epidermidis* ATCC 42033(black); cultured *V. harveyi* (blue) and AB medium (green) were used as positive and negative controls. The insert shows the relative light production by the different strains 4 hours after adding the supernatant from growing cultures of each strain to *V. harveyi*. From this data it is concluded that *Z. mobilis* does not produce AI-2. This graph is the representative of three independent experiments.

3.2.2.4 AI-2 consumption by *Z. mobilis*

Considering that in *Vibrio* species AI-2 is detected by a membrane bound sensory kinase, whilst in *E.coli* it is actively taken up by the cells and phosphorylated (Reading and Sperandio, 2006*)*; Further test was performed to determine the fate of AI-2 produced by *E. coli* in the presence of *Z. mobilis* using the *lsrK* mutant *E. coli* strain KX1186 that is unable to internalize AI-2 (Xavier and Bassler, 2005a). Figure 3.13 significantly shows that the bioluminescence of reporter strain *V. harveyi* BB170 significantly decreased after 2 hour when AI-2, enzymatically synthesised and produced by KX1186, was incubated with *Z. mobilis*. This result indicates that the *Z. mobilis* cells could uptake AI-2, indicating that they do not have an AI-2 detection/recognition system similar to *Vibrio* strains. This finding might suggest that *Z. mobilis* has an AI-2 signal transduction system similar to *E. coli*, but more experimentation would be needed to determine the fate of internalized AI-2. It is interesting to note though that there is no any clear homologues of the *E.coli* or *S. typhimurium* genes involved in the uptake and phosphorylation of AI-2 (e.g. *lsrACDBFGE*) (Walters and Sperandio, 2006) in *Z. mobilis*; and further considering that, in contrast to *E. coli* or *S. typhimurium*, *Z. mobilis* does not produce AI-2, might suggest that it utilizes a novel signal transduction system.

3.2.3 Investigation of N-acyl homoserine lactone (AHL) signalling in *Z. mobilis*

3.2.3.1 Introduction

The very large number of AHL quorum sensing systems identified has been rendered possible mainly via the use of bacterial biosensors that are capable of detecting the presence of AHLs.

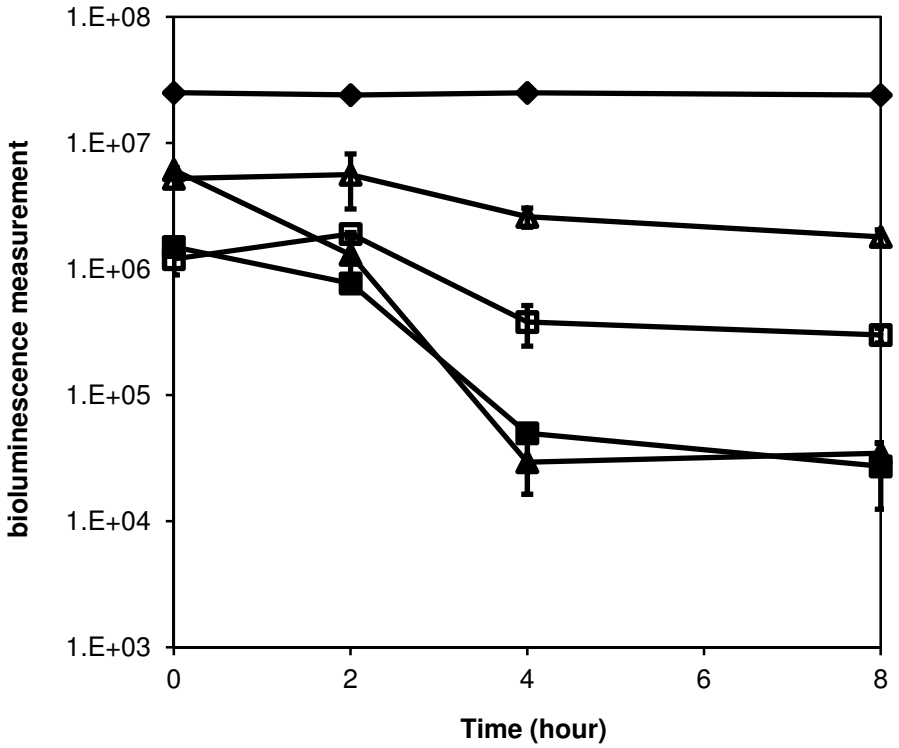


Figure 3.13 AI-2 consumption by *Z. mobilis*. Light productions of *V. harveyi* BB170 in response to the following: supernatant of *E. coli* strain KX1186 (blank triangles, LsrK mutant) that does not consume AI-2; supernatant of *Z. mobilis* cultured with supernatant of *E. coli* strain KX1186 (filled triangles); *in vitro* synthesized AI-2 (blank rectangles) as control; supernatant of *Z. mobilis* cultured in the presence of AI-2 (synthesized *in vitro*) (filled rectangle), and with *V. harveyi* BB170 as positive control (filled diamond). Clearly in the presence of *Z. mobilis* the bioluminescence decreased when AI-2 was added directly or via supernatant of *E. coli* strain KX1186, indicating that *Z. mobilis* ZM4 consumes AI-2. Values represents mean and bars represent standard errors from three independent replicas.

These biosensors, based on cells do not produce AHL contain a functional LuxR-family

protein cloned together with a cognate target promoter, which positively regulates the transcription of a reporter gene (Steindler and Venturi, 2007), such as bioluminescence, galactosidase, green-fluorescent protein and violacein pigment productions.

In this study, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NT1 were used to detect a broad range of AHLs that might be produced by *Z. mobilis*. *C. violaceum* regulates violacein production via the CviI/R quorum sensing system, which produces and responds to AHLs (McClean *et al*., 1997). The CV026 strain is a violacein and AHL-negative double miniTn5 mutant. One transposon is inserted into the *cviI* AHL synthase gene, and the other is inserted into a putative violacein repressor locus. Exposure of strain CV026 to exogenous AHLs (C4-C8), which are able to interact with CviR, results in rapid production of a visually clear purple pigmentation (McClean *et al*., 1997). The *A. tumefaciens* NT1 strain carries NT1 cured of the Ti plasmid and plasmid pZLR4. This plasmid contains the *traR* gene and one of the *tra* operons, responsible for Ti plasmid conjugal transfer, containing a *traG::lacZ* reporter fusion, the transcription of which is known to be regulated by the TraI/R quorum sensing system (Cha *et al*., 1998; Farrand *et al*., 2002). The Strain NT1 displays the broadest sensitivity to many AHLs (C4-C12 and C3-unsubstituted) at lowest concentration (Shaw *et al*., 1997). The incubation with AHLs in medium containing X-gal results in a blue zone around the site of application (Farrand *et al*., 2002).

3.2.3.2 No N-acyl homoserine lactone (AHL) production and response was detected in *Z. mobilis*

A serial concentration of various AHLs was used to investigate their effects on protein induction in *Z. mobilis*. Although *Z. mobilis* could respond to AI-2, none of the AHLs used

was able to induce extracellular / cytoplasmic proteins in an analogous manner to AI-2. The supernatant of *Z. mobilis* was also investigated as to whether it contains AHLs using the biosensor strain *C. violaceum* CV026 (Figure 3.14) and *A. tumefaciens* NT1 (Table 3.2). It was concluded that *Z. mobilis* has no activity, suggesting that it does not produce AHL (Table 3.2).

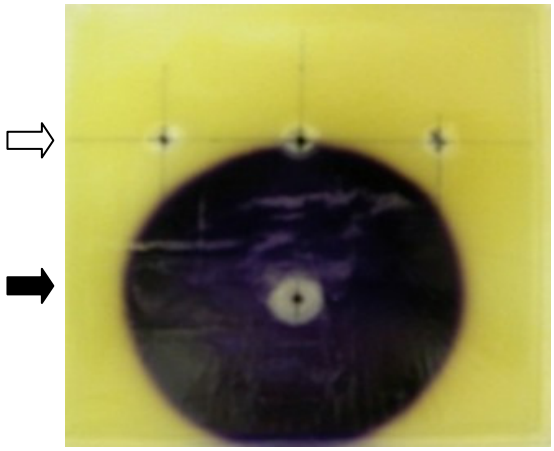


Figure 3.14 N-acyl homoserine lactone (AHL) detection using *Chromobacterium violaceum* CV026 reporter strain. The assay with *Z. mobilis* supernatant shows there is no activity of AHL (top arrow) and positive control (bottom arrow). The supernatant of *Z. mobilis* incubated for different time was loaded into well (top), and at the same time pre-cultured reporter strain (OD=0.5) was spread onto the whole plate, then plate were incubated at 30 ^{o}C for 24hours. As a positive control, 20mM C8-AHLs were used.

Table 3.3 N-acyl homoserine lactone (AHL) detection from different conditioned supernatant of *Z. mobilis*

Culture condition of *Z. mobilis*	**Test results using reporter strains**
Supernatant from 4hour	All negative
Supernatant from 8hour	
Supernatant from 16hour	
Supernatant from 24hours	
Supernatant from 48hours	
Supernatant from 72hours	

3.2.4 Ethanol production by *Z. mobilis* in response to AI-2.

3.2.4.1 Successful AI-2 synthesis

3.2.4.1.1 Transformation and colony PCR screening of *luxS* and *pfs* genes

ProEX-Htman and ProEX-LuxSec plasmids (Winzer *et al*., 2002) containing the *pfs* and *luxS* genes of *E. coli* MG1655 were obtained from Paul Williams at the University of Nottingham, UK. These two plasmids were transformed into *E. coli* strain BL21 (DE3) following purification of plasmid DNA using a QIAprep® Miniprep Kit (Qiagen). Transformed colonies were selected on the basis of vector-encoded tetracycline resistance and the colonies were screened for the presence of the inserted *pfs* and *luxS* sequence by PCR with specific primers (Figure 3.15).

3.2.4.1.2 Expression and purification of LuxS and Pfs enzymes

For the *in vitro* production of AI-2, the Pfs and LuxS enzymes were overexpressed as hexahistidine tagged proteins under the control of a T7 promoter in the vector ProEX in *E. coli* strain BL21 DE3. One litre of 2xYT containing tetracycline (10µg/ml) was inoculated with 1ml pre-grown overnight culture. The cells were grown until mid log phase (OD600=0.5-0.6) and then induced with 0.2mM isopropyl-ß-D-thiogalactopyranoside (IPTG). The temperature was then dropped to 25^{o}C and growth was continued with shaking at 200rpm overnight.

The cells were harvested by centrifugation at 8000rpm for 10min at 4^{o}C and resuspended in

Buffer A (50mM Tris, 20mM imidazole, 200mM NaCl, 20% glycerol, pH 7.8, 5 U/ml DNaseI, 1 tablet/50ml EDTA-free protease inhibitor mixture tablet). The cells were disrupted by two passages through a Constant System Cell Disruptor (20 and 15 Kpsi, Z-plus model, Constant System). Cellular debris was removed by ultracentrifugation at 43,000rpm for 90 minutes at 4^{o}C and supernatant liquid containing soluble proteins was collected in a 50ml tube. To the supernatant (appox. 100ml) was added 2ml of Ni^{2+}-NTA beads and the mixture was rotated at 4 ^{o}C for 90mins. The mixture was loaded onto a Bio-Rad Econo-column and the flow-through was collected. The column was washed with 10ml of buffer A. The enzymes were then eluted from the column, under gravity, with a 200-800mM imidazole gradient of buffer A and buffer B (50mM Tris, 200mM NaCl, 1M imidazole, 20% glycerol, pH 7.8). The fractions were collected in a 1.5ml microcentrifuge tube and loaded onto an SDS-PAGE gel for visualization (Figure 3.16). The purified enzymes were stored at -20 ^{o}C until used.

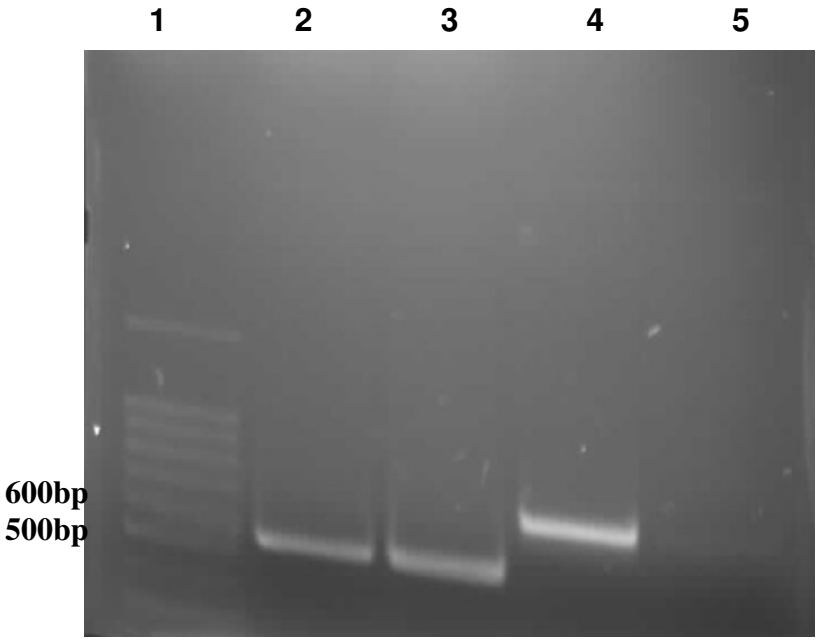


Figure 3.15 PCR screening of *pfs* and *luxS* genes. Four positive transformed colonies in LB/tetracycline medium were selected for the presence of *pfs* and *luxS* genes, and gene –

Figure 3.15 *Continued*

inserts confirmed in three of them by PCR amplification. Positively confirmed colonies were directly used for Pfs/LuxS enzyme expression and purification. Lane 1, Marker; Lane 2 and 3, positive LuxS insertion; Lane 4, positive pfs insertion; Lane 5, negative.

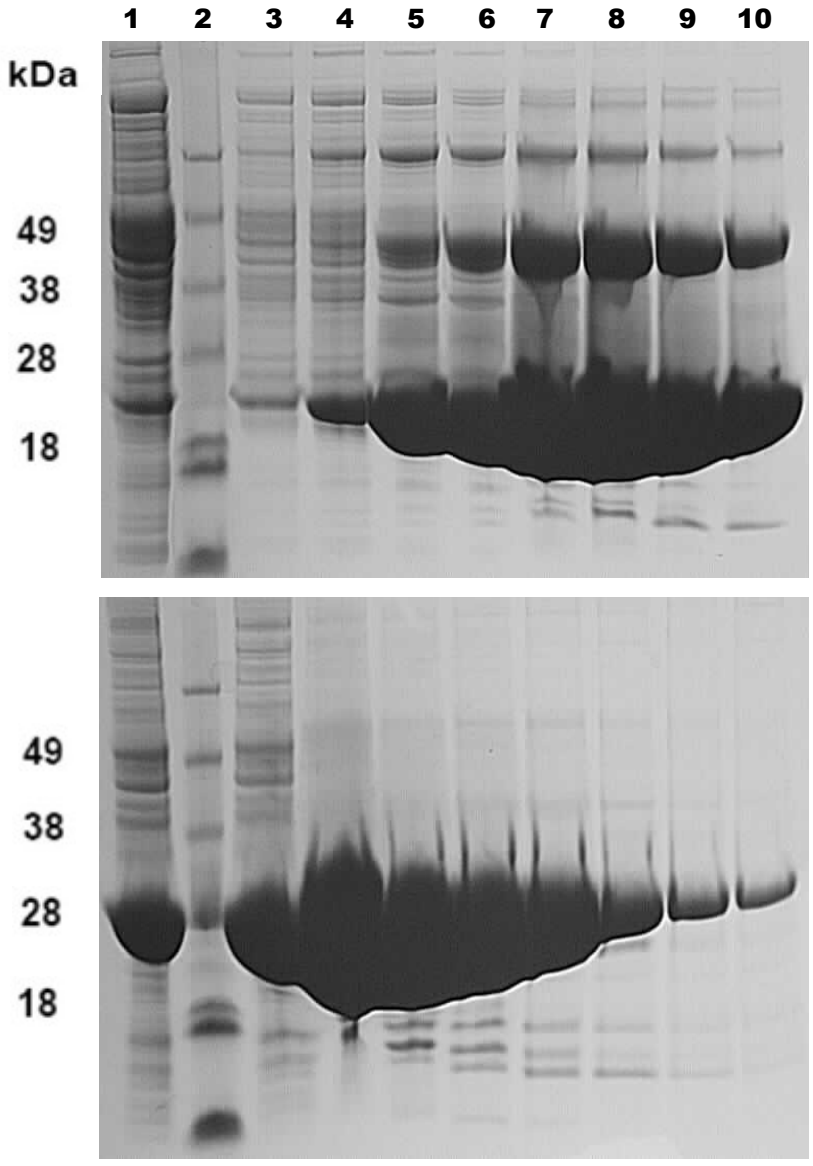


Figure 3.16 Coomassie blue stained SDS-PAGE gel of Pfs (top) and LuxS (bottom) enzymes. IMAC was used for enzyme purification. Lane 1, wash (50mM imidazole); Lane 2, SeeBlue® protein standard; lane 3-10, elution fractions with buffer containing 200-800mM imidazole.

3.2.4.1.3 Synthesized AI-2 activity

Following the dialysis of purified Pfs and LuxS enzymes in 50mM Tris buffer, pH 7.8, the concentration of each enzyme was determined using a BSA Assay Kit (Pierce). S-adenosylhomocystein (SAH) was purchased from Sigma-Aldrich (U.K). Figure 3.17 shows a standard plot for the BSA assay that was used to quantify the Pfs and LuxS enzymes which were prepared by dialysis for AI-2 production. A mixture of 1mg/ml of His-tagged Pfs, 1mg/ml of His-tagged LuxS and 1mM SAH was incubated 1-4 hours, and the conditions optimized with respect to the enzyme concentrations and the reaction time for *in vitro* AI-2 synthesis. After removing the two enzymes from the mixture by filtration through a Centricon YM filter (Millipore), a bioluminescence assay was performed to confirm the presence of *in vitro* synthesized AI-2 (Figure 3.18). Following the AI-2 production *in vitro*, different quantities of AI-2 were added directly to a growing culture of *Z. mobilis*, and the subsequent ethanol content determined.

3.2.4.2 Enhanced ethanol production by *Z. mobilis* in response to AI-2

To test if ethanol production is under the control of AI-2, a chromate assay (Figure 3.19) (Section 2.12) was used to determine the ethanol content of *Z. mobilis* cultures grown over-night in co-culture with *E. coli* strain KX1123. It was found that co-culture with the AI-2 producing *E. coli* strain KX1123 caused a marked increase in ethanol production. To confirm that this increase in ethanol production was a direct consequence of AI-2; various volume (0 - 1000 µl) of AI-2 synthesized *in vitro* was added to a growing culture of *Z. mobilis* (10 ml) and the ethanol content determined 16 hours later (Figure 3.20).

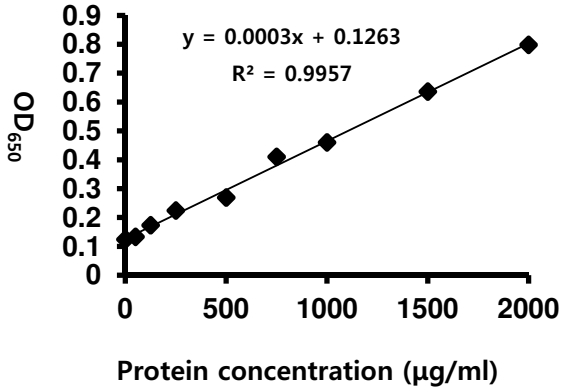


Figure 3.17 BSA standard in accordance with different concentration of proteins. The x-axe represents the OD measurement (A_{650}) and y-axe represents the concentration of the standard protein (µg/ml).

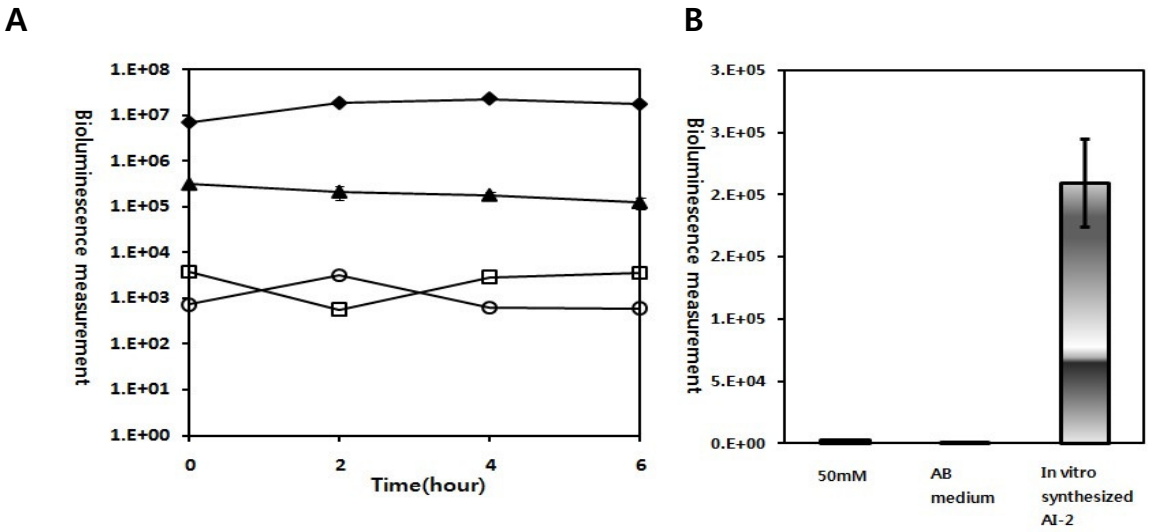


Figure 3.18 Bioluminescence assay with *in vitro* synthesized AI-2. (A) Synthesized AI-2 was left for 6 hours at 30°C incubator and bioluminescence was measured every 2hours. Each dot represents the average value of 2 independent replicas. Blank rectangle (50mM Tris buffer), blank circle (AB medium), Filled triangle (Synthesized AI-2), and filled diamond (positive control, supernatant from *V. harveyi*). **(B)** Each bar represents the average of the values for 6 hours. Graph shows that synthesized AI-2 was not decreased with time and was positively inducible of bioluminescence. *In vitro* synthesised AI-2 activity was constant with activity in AI-2 consumption experiment (Figure 3.13). Values represents mean and each bars represent standard errors.

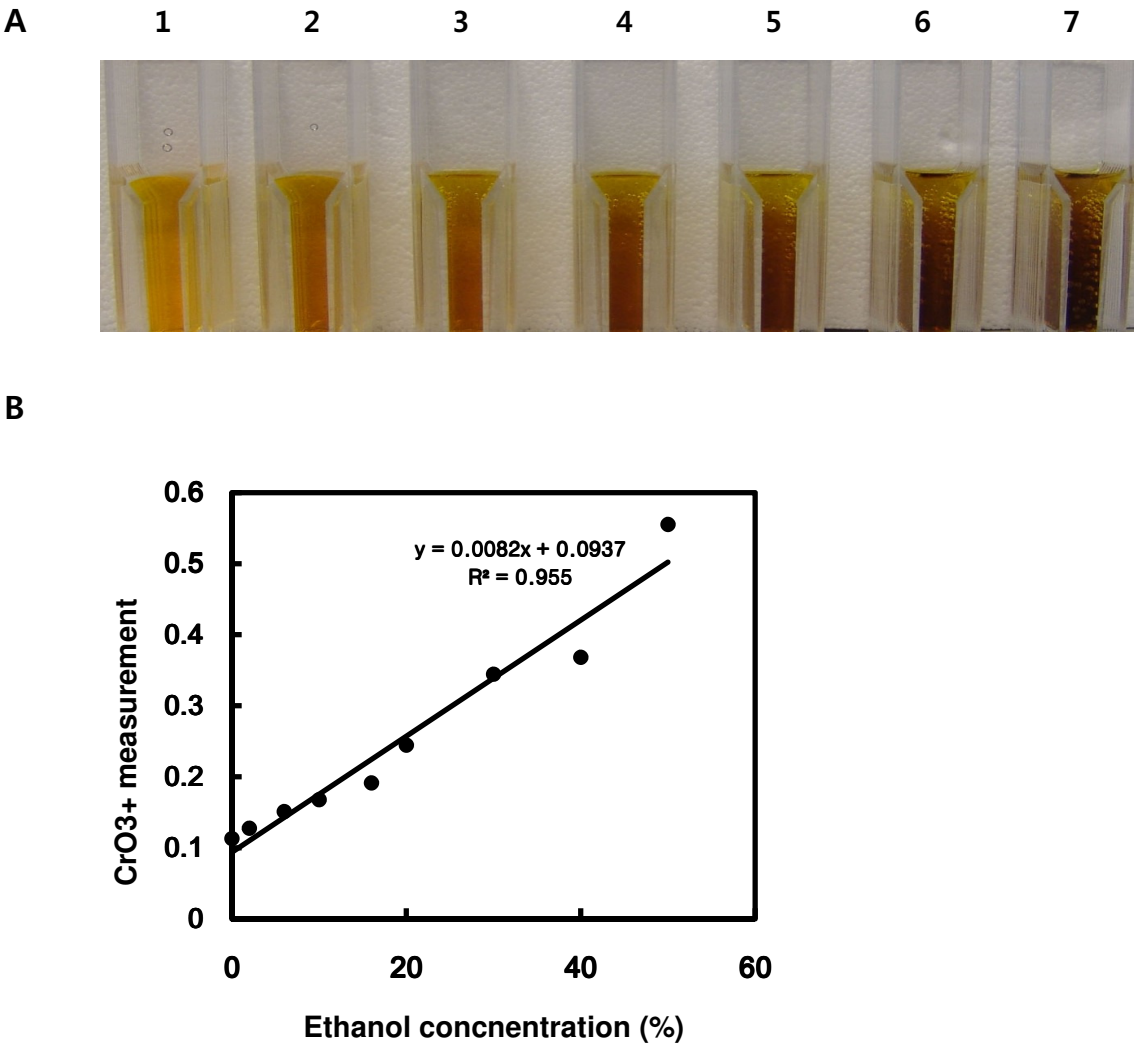


Figure 3.19 A chromate assay used to monitor the increase in absorbance at 600nm that results from the reduction of Cr (VI) to Cr (III) as it catalyses the oxidation of ethanol. **(A)** The Cr (III) color development from different concentrations of ethanol (lane 1, 0%; lane 2, 2%; lane 3, 6%; lane 4, 10%; lane 5, 16%; lane 6 20%; lane 7, 30% ethanol). **(B)** A calibration curve relating the ethanol concentration (% v/v) to the Cr (III) absorbance revealed a linear dependency that could be used to assay the ethanol content of *Z. mobilis* cultures.

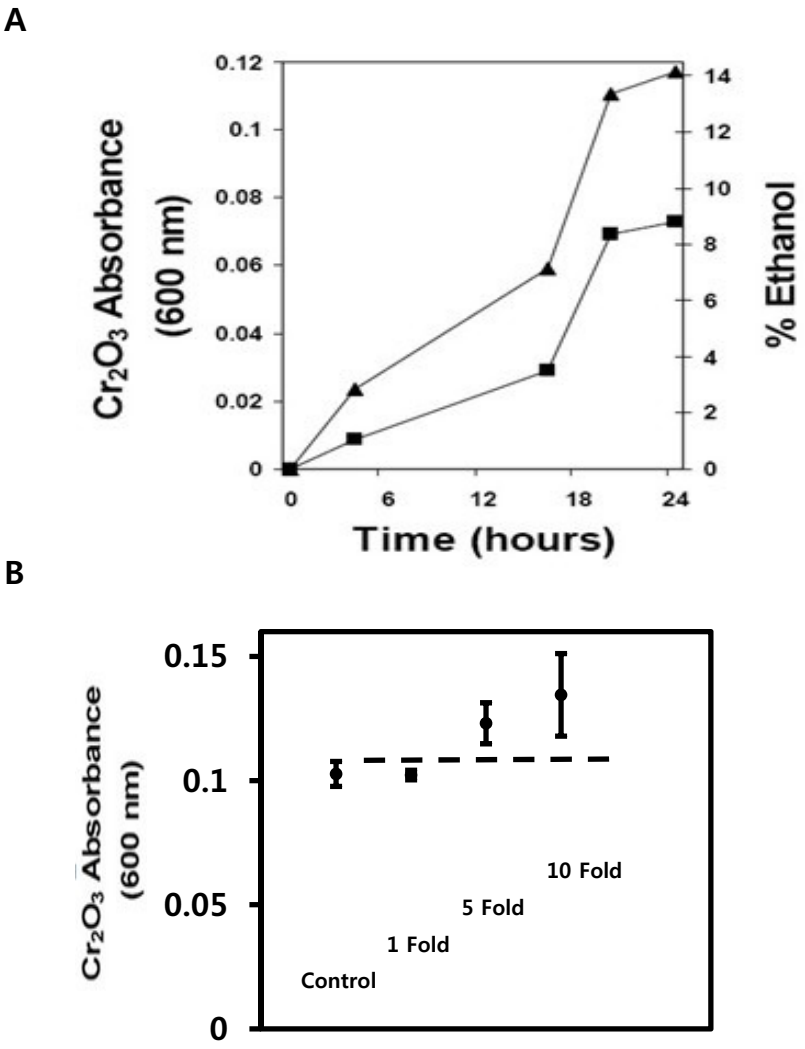


Figure 3.20 AI-2 induces the increased production of ethanol by *Z. mobilis*. **(A)** The ethanol production of *Z. mobilis* co-cultured with the supernatant of *E. coli* strain KX1128 (ΔluxS) (▲) and the AI-2 producing strain KX1123 (■). These experiments revealed that after 24 hours *Z. mobilis* co-cultured with *E. coli* strain KX1128 and KX1123 had ethanol contents of 8.9 and 14.0% respectively.

Figure 3.20 *Continued*

(B) AI-2 was synthesized *in vitro* and added (e.g. 1fold = 100µl AI-2 in 10ml of culture) directly to growing *Z. mobilis* cells and the ethanol content of the cultures determined after 24hours. For one experiment a culture of *Z. mobilis* was grown over-night and used as the inoculums for five 10ml cultures that were grown for a further 16 hours and the ethanol content determined. The bar chart shows the ethanol content of 1, 5 and 10 fold AI-2. Each bar is the average of 5 replicas (4 replica in 1 Fold) and the error bars represent the standard error of these experiments (Mean value ± standard error: Control, 0.10274 ± 0.005039; 1 fold, 0.10227 ± 0.001906; 5fold: 0.12314 ± 0.008247, 10fold, 0.13458 ± 0.016642).

These experiments indicate that ethanol production increases with the AI-2 concentration and that AI-2-treated cells repeatedly produce significantly more ethanol than control cells (i.e. 14.2% versus 9%; representing a 58% increase in ethanol production,). A T-test was used to determine if the difference in ethanol production between the samples is statistically significant. This indicate that there was a significant difference between the ethanol produced in the control and 5-10fold experiments ($P<0.08$, <0.07 respectively), but there was no difference between the control and 1fold experiment. Although this increase does not appear to be substantial, it has to be recognized that the ethanol content of the growing cultures treated with AI-2 is close to the ethanol tolerance of *Z. mobilis* (i.e about 15%, Shakirova *et al*., 2008), which acts as an upper-limit on the amount of ethanol that can be produced. Further experiments will be needed to determine if AI-2 brings about an increase in the ethanol tolerance of *Z. mobilis*, which might allow a further enhancement in ethanol production.

3.3 *Z. mobilis* quorum sensing and bacterial cheating

3.3.1 No selective benefits acquired by AI-2 quorum sensing molecules in *Z. mobilis*

Before determining the fate of *E. coli* cells grown with *Z. mobilis*, each cell type was grown in different glucose and ethanol concentrations to determine the minimal inhibition concentration of ethanol and glucose for both strains for further experiments. Figure 3.21 clearly shows that *Z. mobilis* could survive in up to 10% ethanol but growth of *E. coli* had nearly stopped at 4%. The growth of AI-2 treated *Z. mobilis* was also measured. However, there was no clear effect of AI-2 on *Z. mobilis* cell growth (Figure 3.22).

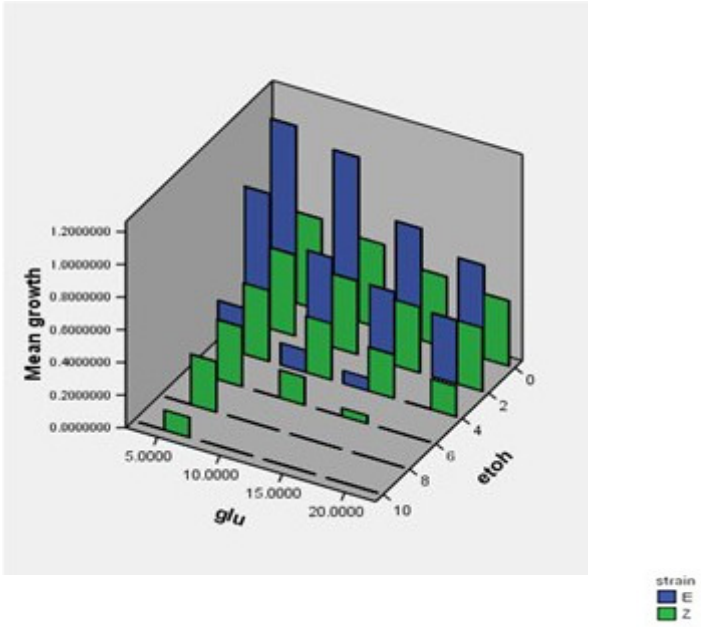


Figure 3.21 *E. coli* and *Z. mobilis* cells grown in the presence of different glucose and ethanol concentrations. Each bacteria was grown for 24 hours and each bar represents mean of the final cell density. The growth of both cells decreased with increased concentration of

Figure 3.21 *Continued*

ethanol and glucose. Growth rates of *Z. mobilis* and *E. coli* wild type were estimated with 5-fold replication in RM medium in 96-well plates and growth was assessed by measuring OD_{600} at 5-min intervals using an automated plate reader (EL808^{TM}, BioTek).

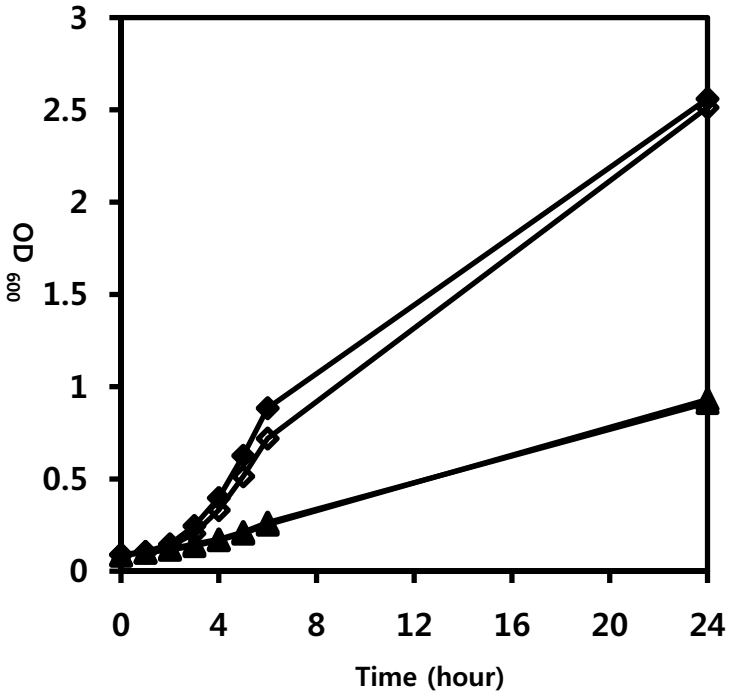


Figure 3.22 The growth of AI-2 treated or non-treated *Z. mobilis* in 0% and 8% ethanol-conditioned RM medium. Symbol of diamond and triangle represents the concentration of ethanol 0% and 8%, respectively. Blank and filled symbol indicate the growth of AI-2 non-treated and that of AI-2 treated *Z. mobilis*, respectively. The results show there was no significant growth difference between AI-2 treated and non-treated *Z. mobilis*. Thus, it is concluded that AI-2 does not give a growth benefit to *Z. mobilis* in the presence/absence of ethanol.

The molecular basis of the antibacterial effect of *Z. mobilis* is unknown. Indirectly, the previous results showed that *Z. mobilis* has a much higher tolerance to ethanol than *E.coli* strain (Figure 3.21). This might imply that enhanced ethanol production in response to AI-2 could accelerate the growth of *Z. mobilis,* potentially allowing it to outcompete *E. coli* as the ethanol is accumulated in the culture medium by *Z. mobilis* ethanol fermentation. To test this hypothesis, a competition assay (Elena and Lenski, 2003) was performed with *E.coli* KX1186 (LuxK mutant, AI-2 blind) and *Z. mobilis*. Briefly, each cell was pre-conditioned by overnight cultivation in RM medium and cells were mixed at two initial frequencies, corresponding to 1:1 and $1:^{1}/_{10}$ (*E. coli* vs. *Z. mobilis*). The initial cell density in each tube was average 2 x 10^6/ml (in ratio of 1:1). After 24 hours incubation, samples were plated with appropriate dilutions on modified RM containing 1% yeast extract and 5% glucose. The morphology of each cell can be distinguishable clearly by eye, allowing quantification of the *E. coli* and *Z. mobilis* populations in co-culture. In contrast to the expectations, the co-culture experiment of *Z. mobilis* with *E. coli* strains showed there was no clear difference in killing effect of *Z. mobilis* to *E. coli* wild type and mutant (Figure 3.23). However, as the glucose concentration was increased, the frequency of *Z. mobilis* ($\frac{\text{Cell number of } Z.mobilis}{\text{Total cell number}}$) also increased. This result could indicate that the higher the concentration of glucose that the *Z. mobilis* utilizes, the more it kills *E. coli*. In 20% glucose, the frequency of *Z. mobilis* decreased presumably owing to the increase in osmotic pressure by the glucose. The killing effect of *Z. mobilis* (1/10 of the initial) was higher than that of *Z. mobilis* mixed with *E. coli* at a 1:1 ratio (Figure 3.23). The result of this lower cell frequency effect in the mixed-population was consistent with a previous report, explaining that a lower frequency cell effectively invade the whole population (Yang *et al*., 2010).

Considering the test results (Figure 3.22 and 3.23), it is concluded that AI-2 might not be directly relevant in affording growth benefits by AI-2 (e.g. by enhancing nutrient acquisition), but based on the previous data (Figure 3.9) might involve in glucose metabolism, and the antibacterial effects to *E. coli* might be controlled by metabolic intermediates (e.g. acetic acid) or via an unrevealed bacteriocin, rather than enhanced ethanol production by AI-2 in short term.

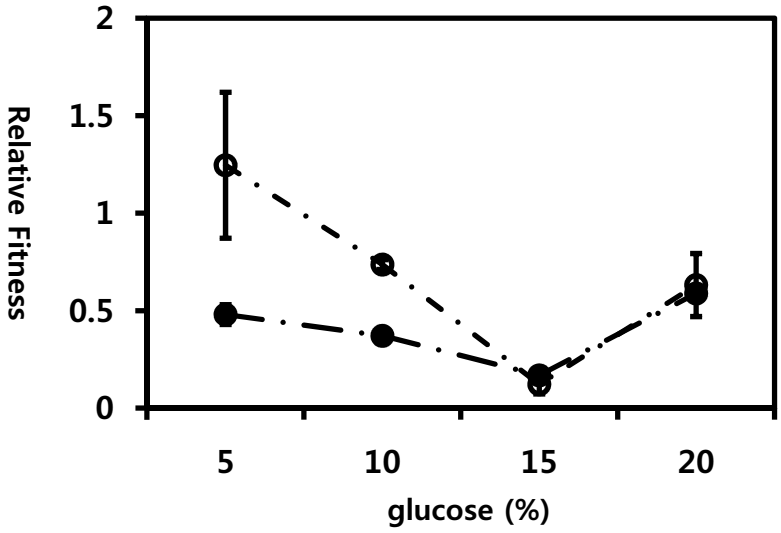


Figure 3.23 Fitness effect of *E. coli* by initial cell density of *Z. mobilis* and different glucose concentrations in culture medium. Unfortunately, there was no significant difference between fitness of *E. coli* wild type and KX 1186 mutant strain. However, it is confirmed that *Z. mobilis* invaded the population effectively when the initial cell density was low (Filled circle). In both 1:1 (Filled circle) and $1:{}^{1}/_{10}$ (blank circle) the magnitude of the relative fitness of *E. coli* declined significantly as glucose concentration increased. Relative fitness was determined by the equation "$\frac{\ln(\frac{\text{initial}}{\text{final}}\text{cell denisty of } E.coli)}{\ln(\frac{\text{initial}}{\text{final}}\text{cell denisty of } Z.mobilis)}$". All results shown as mean ± standard error from 2 independent experiments.

3.4 Extracellular proteins of *Z. mobilis* induced by AI-2 quorum sensing

3.4.1 Introduction

Expression of recombinant proteins can be approached in general by starting with a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing expression, and ending with cell lysis and SDS PAGE analysis to verify the presence of the protein. With careful choice of host strains, vectors, and growth conditions, most recombinant proteins can be cloned and expressed at high level in *E. coli*. The *E. coli* has been the 'workhorse' for the production of recombinant proteins as it is the best-characterized host with many available expression systems (Makrides, 1996). However, many polypeptide gene products expressed in *E. coli* accumulate as insoluble aggregates that lack functional activity. Other problems with protein expression may include cell toxicity, protein instability, improper processing or post-translational modification, and inefficient translation.

One approach to solve these problems is to have recombinant proteins secreted into the culture medium. The secretory production of the proteins has several advantages, such as simplicity of purification, much less protease activity, N-terminal Met extension, and a better chance of correct protein folding due to the more oxidative environment of the culture medium (Choi and Lee, 2004). For the extracellular production of recombinant proteins, periplasmic chaperones and proteases can be manipulated to improve the yields of secreted proteins. In addition to these secretion strategies, Zhang *et al*., (2006) showed that recombinant proteins (passenger) linked to the carboxyl end of YebF are efficiently secreted,

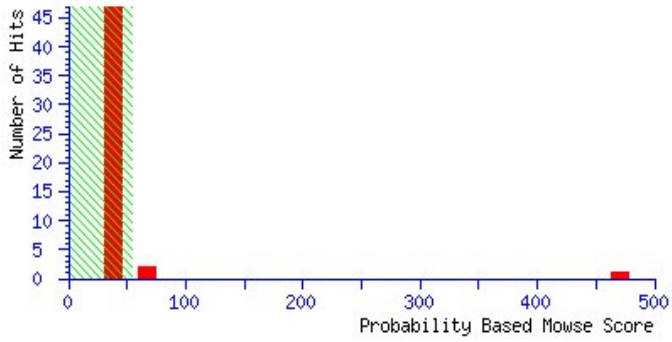
while the passenger protein itself cannot be secreted to the medium. YebF is a small (10.8kDa), soluble endogenous protein, which is excreted into the medium from a commonly used laboratory *E. coli* strain. YebF as a carrier for transgenic proteins provides a tool to circumvent toxicity and other contamination issue associated with protein production in *E. coli* (Zhang *et al*., 2006).

In Gram negative bacteria, the type II secretion system is the most widely used for protein secretion. The type II system involves a two-step process in which a premature protein with a signal sequence is exported to the periplasmic space using the Sec pathway and processed into a mature protein (Milton, 2006). The SignalP 3.0 server can be used to predict the presence and location of signal peptide sites in amino acid sequences from different organisms: Gram-positive prokaryotes, Gram-negative prokaryotes, and eukaryotes. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models (Krogh *et al*., 2001; Emanuelsson *et al*., 2007).

3.4.2 Construction of pET21 vectors incorporating the *ZMO0994, 1034* and *1147* genes

A number of extracellular proteins from *Z. mobilis* were identified by mass spectrometry analysis; these were ZMO0994 (Figure 3.24), ZMO1034 and ZMO1147 (Figure 3.8). The sequences of these proteins were analysed with SignalP 3.0, indicating a high probability of a signal occurring in ZMO0994 and ZMO1034, but no signal peptide was predicted for ZMO1147 (Figure 3.25. and 3.26). Following identification by mass spectrometry sequencing of tryptic-fragments, the sequences of ZMO0994, 1034 and 1147 genes were obtained from

NCBI database and used to design PCR primers for specific amplification of the genes from *Z. mobilis* genomic DNA.

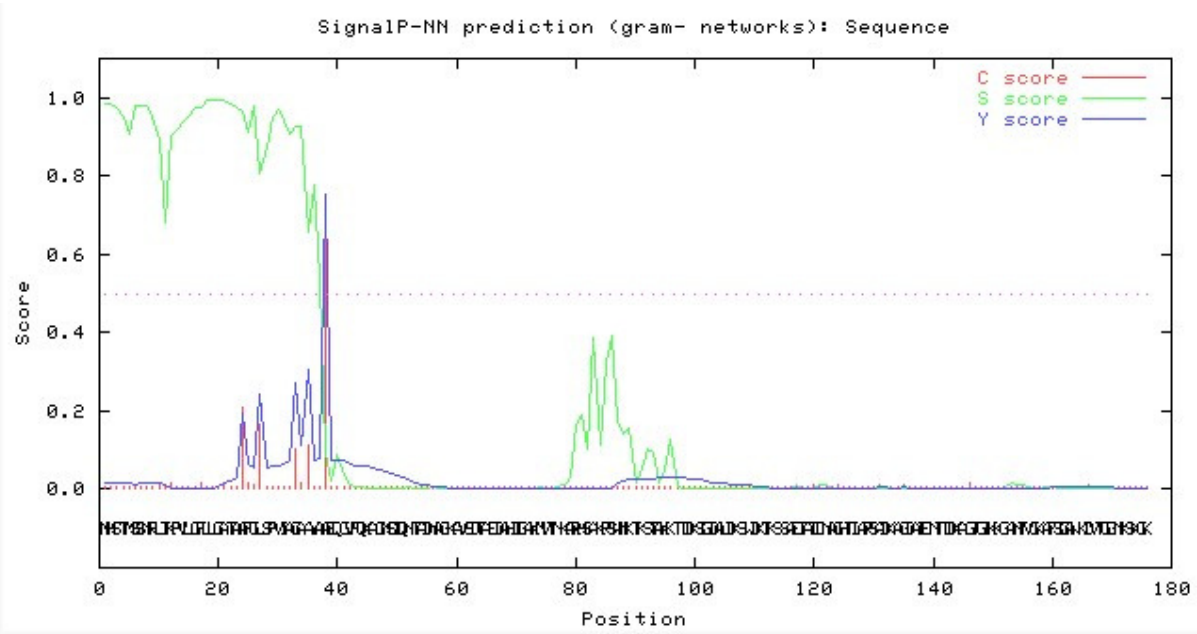


gi|56543464 Mass: 18049 Score: 471 Queries matched: 43 emPAI: 2.93
hypothetical protein ZMO0994 [Zymomonas mobilis subsp. mobilis ZM4]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
106	567.7675	1133.5204	1133.5200	0.0004	0	(50)	0.19	1	K.AGDAIENTTDK.A
107	567.7781	1133.5416	1133.5200	0.0216	0	(31)	16	1	K.AGDAIENTTDK.A
108	567.7887	1133.5628	1133.5200	0.0428	0	(42)	1.1	1	K.AGDAIENTTDK.A
109	567.7887	1133.5628	1133.5200	0.0428	0	(40)	1.9	1	K.AGDAIENTTDK.A
110	567.8099	1133.6053	1133.5200	0.0852	0	50	0.17	1	K.AGDAIENTTDK.A
116	384.2077	1149.6013	1149.5513	0.0500	1	34	6.9	2	K.TTDKSGDALDK.S
164	680.8170	1359.6194	1359.6378	-0.0184	0	(73)	0.00068	1	K.AGNSIQNTADNAGK.A
165	680.8402	1359.6658	1359.6378	0.0280	0	78	0.00026	1	K.AGNSIQNTADNAGK.A
166	680.8402	1359.6658	1359.6378	0.0280	0	(62)	0.0086	1	K.AGNSIQNTADNAGK.A
167	680.8402	1359.6658	1359.6378	0.0280	0	(77)	0.00027	1	K.AGNSIQNTADNAGK.A
168	680.8402	1359.6658	1359.6378	0.0280	0	(57)	0.031	1	K.AGNSIQNTADNAGK.A
169	680.8518	1359.6890	1359.6378	0.0512	0	(67)	0.0028	1	K.AGNSIQNTADNAGK.A
170	680.8518	1359.6890	1359.6378	0.0512	0	(75)	0.00045	1	K.AGNSIQNTADNAGK.A
172	681.3278	1360.6411	1359.6378	1.0033	0	(20)	1.4e+02	7	K.AGNSIQNTADNAGK.A
183	693.7000	1385.3854	1385.6059	-0.2204	0	(49)	0.17	1	K.AVSDTAEDAHDGAK.N
184	462.8953	1385.6641	1385.6059	0.0583	0	(61)	0.0099	1	K.AVSDTAEDAHDGAK.N
185	462.8953	1385.6641	1385.6059	0.0583	0	(55)	0.043	1	K.AVSDTAEDAHDGAK.N
186	462.8953	1385.6641	1385.6059	0.0583	0	(67)	0.0029	1	K.AVSDTAEDAHDGAK.N
187	462.8953	1385.6641	1385.6059	0.0583	0	(56)	0.031	1	K.AVSDTAEDAHDGAK.N
188	462.8953	1385.6641	1385.6059	0.0583	0	(37)	3	1	K.AVSDTAEDAHDGAK.N
189	462.8953	1385.6641	1385.6059	0.0583	0	(44)	0.59	1	K.AVSDTAEDAHDGAK.N
190	462.8953	1385.6641	1385.6059	0.0583	0	(47)	0.29	1	K.AVSDTAEDAHDGAK.N
191	462.8953	1385.6641	1385.6059	0.0583	0	(29)	19	2	K.AVSDTAEDAHDGAK.N
192	462.8953	1385.6641	1385.6059	0.0583	0	(46)	0.38	1	K.AVSDTAEDAHDGAK.N
193	463.0000	1385.9782	1385.6059	0.3723	0	72	0.00099	1	K.AVSDTAEDAHDGAK.N

Figure 3.24 MASCOT search results from the MS-MS Data generated for the ZMO0994 (unknown)

(http://www.matrixscience.com/cgi/master_results.pl?file=../data/20050721/FsnorzsS.dat)



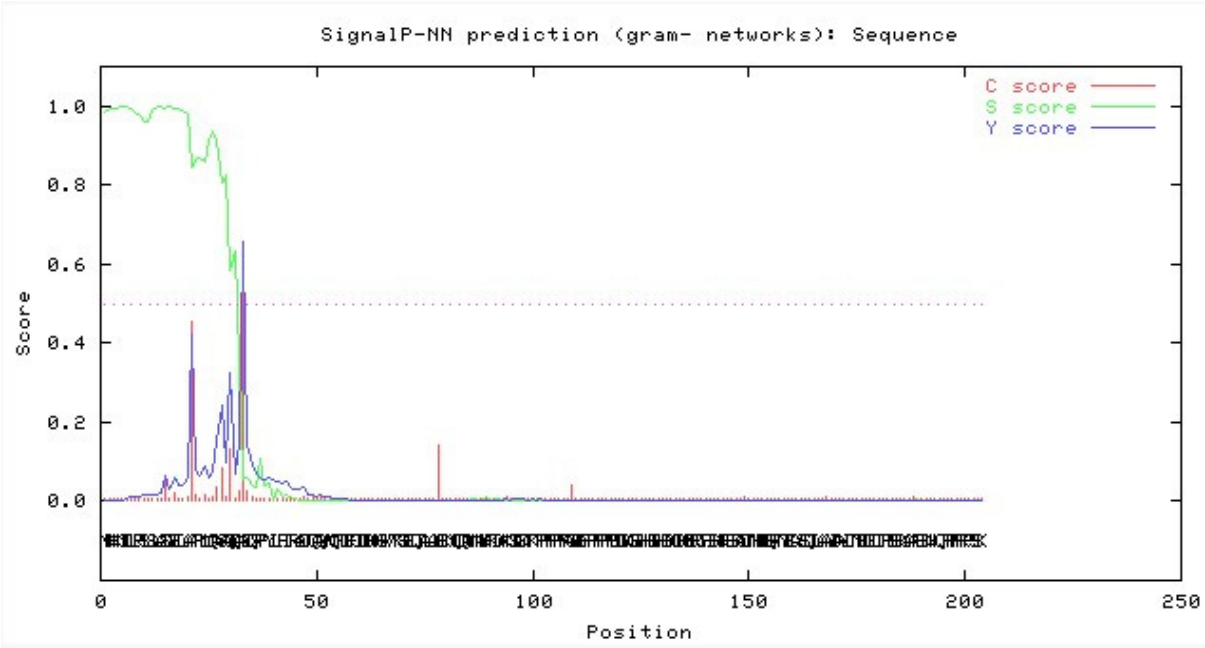

```
# data

>Sequence                length = 176
# Measure  Position  Value  Cutoff  signal peptide?
  max. C    38       0.659   0.52   YES
  max. Y    38       0.754   0.33   YES
  max. S    20       0.995   0.92   YES
  mean S     1-37    0.919   0.49   YES
       D     1-37    0.836   0.44   YES
# Most likely cleavage site between pos. 37 and 38: AYA-AE
```

MHSTMSSNRLTKPVLGFLLGATAAFGLSPVIAGAAYAAEQGVFQKAGNSIQNTADNAGKAVSDTAE
DAHDGAKNVTNKARHSAKRSWNKTKSTAKKTTDKSGDALDKSWDKTKSSAETATDNAGHTIARSAD
KAGDAIENTTDKAGTGIKKGANTVGKAFSGAWKDVTGGNKSKGK

Figure 3.25 The prediction of a signalling peptide from ZMO0994 using Signal IP 3.0.

The signal peptide of ZMO0994 is represented by the bold letters.




```
# data

>Sequence                length = 204
# Measure  Position  Value  Cutoff  signal peptide?
  max. C     33       0.535   0.52   YES
  max. Y     33       0.656   0.33   YES
  max. S     14       0.999   0.92   YES
  mean S      1-32    0.912   0.49   YES
       D      1-32    0.784   0.44   YES
# Most likely cleavage site between pos. 32 and 33: QQA-AE
```

MKKKNFLLRSLSLASGLFLAATPLMQASAQQAAEQVPPYILPITRAETQAQVKQNFDKFDLDHNGV
VTKSEFQTALANEKNQCQAMMAKFKDAKNSGKTKTPPPPPPAGDGNTPPPPPEDGKGGHHHGHHGMC
DHMDRGPISHWFERADTNHDGQVTYDEASSQILAAYDAVDTNHDGIITPEERKAAFEKWKQTHPFPKP
SSK

Figure 3.26 The prediction of a signalling peptide from ZMO1034 using Signal IP 3.0.

The signal peptide of ZMO1034 is represented by the bold letters.

Genomic DNA of *Z. mobilis* strain ZM4 was prepared using a GeneElute^{TM} Bacteria Genomic DNA Kit (Sigma). Figure 3.27 shows the successful amplification of each gene by estimating the size of the genes in comparison with the DNA Mass Marker (Invitrogen). The amplified DNA bands were cut out of the gel and the purified DNA was obtained by gel extraction. The amplified sequences were ligated into pGEM-T Easy vector and the ligation was then transformed into competent *E. coli* Novablue strain to propagate the target genes.

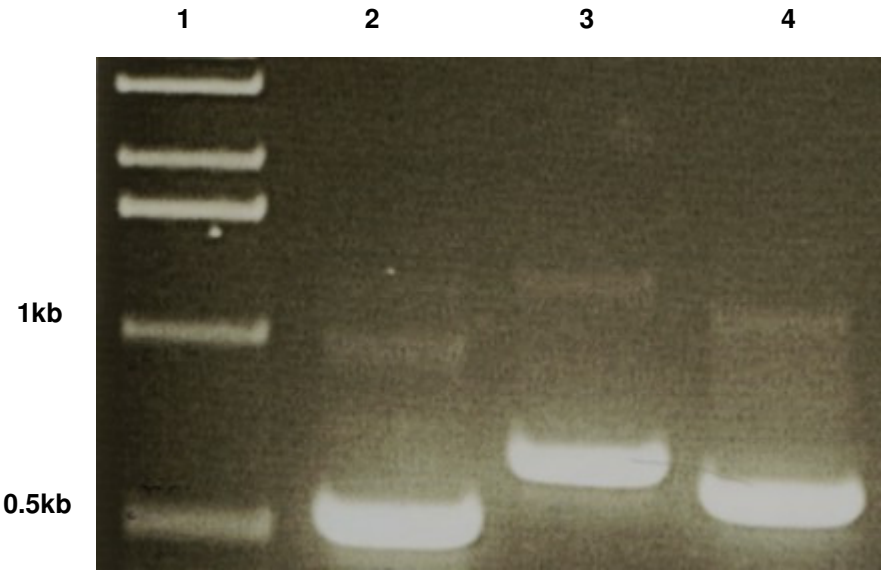


Figure 3.27 PCR amplification of *ZMO0994*, *1034* and *1147*. Lane 1, DNA Marker; Lane2, ZMO0994 (531bp); Lane 3, ZMO1147 (672bp); Lane 4, ZMO1034 (615bp)

The genes inserts could be confirmed by *EcoRI* restriction screening (Figure 3.28) and the ZMO0994, 1034 and 1147 inserts were prepared by restriction enzyme digestion of pGEM-T Easy vector with *BamHI/XhoI*, *BamHI/XhoI* and *BamHI/HindIII*, respectively. The pET21a(+) vector was obtained from a commercial source (Novagen) and the prepared gene fragments were ligated into pET21a(+) vectors, generating pET21a(+)ZMO0994, pET21a(+)ZMO1034 and pET21a(+)ZMO1147, in which the native genes were fused to a short C-terminal sequence encoding a hexahistidine tag.

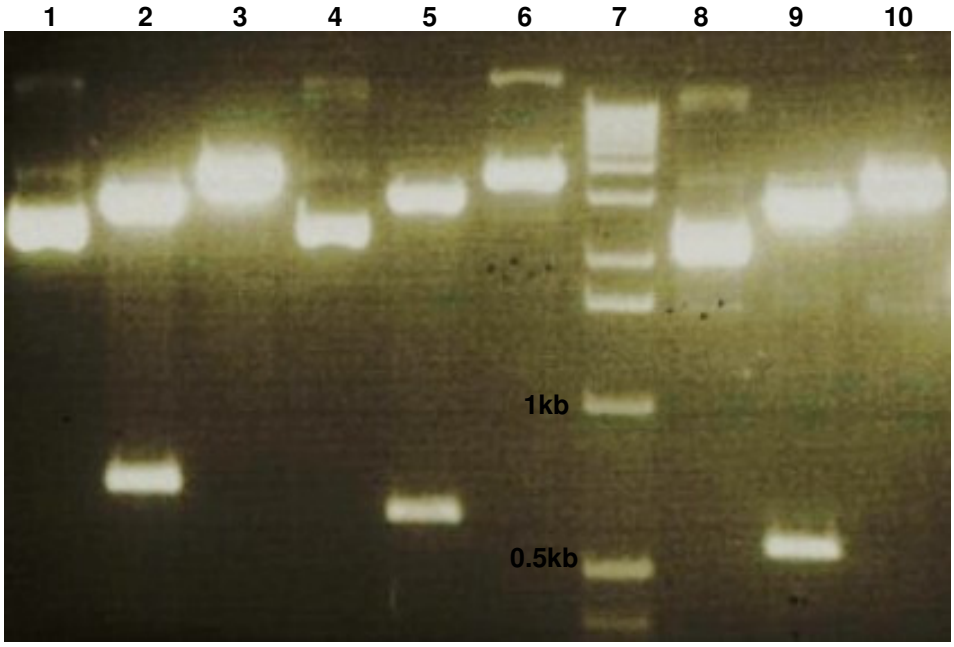


Figure 3.28 EcoRI restriction of *ZMO0994*, *1034* and *1147*, and pET plasmid restriction for each gene. Lane 1, Plasmid for ZMO1147 control; Lane 2, inserted ZMO1147; Lane 3, digested pET plasmid for ZMO1147; Lane 4, Plasmid for ZMO1034 control; Lane 5, inserted ZMO1034; Lane 6, digested pET plasmid for ZMO1034; Lane 7, DNA marker; Lane 8, Plasmid for ZMO0994 control; Lane 9, inserted ZMO0994; Lane 10, digested pET plasmid for ZMO0994.

The N-terminal ATG codon of each genes is located downstream of a T7 promoter, a *lac* operator sequence and an optimized *E. coli* ribosome binding site. PCR amplification analysis of cloned *E. coli* shows successful genes insertion in pET21a(+) construction (Figure 3.29).

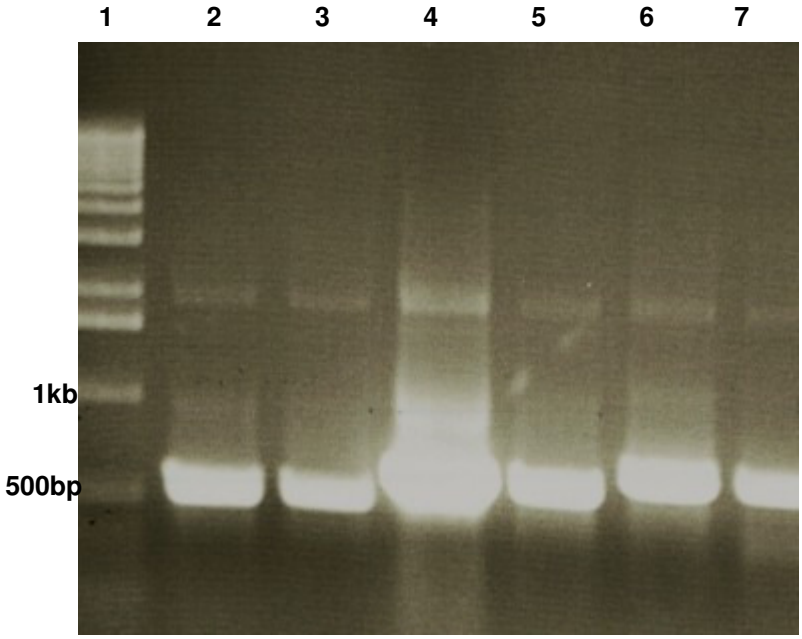


Figure 3.29 Colony PCR screening of cloned *ZMO0994*, *1034* and *1147* from *E. coli* BL21 DE3. Lane 1, DNA marker; Lane 2 and 3, inserted ZMO0994; Lane 4 and 5, inserted ZMO1034; Lane 6 and 7, inserted ZMO1147.

3.4.3 Extracellular accumulation of recombinant proteins, ZMO0994 and ZMO1034

All the recombinant proteins were overexpressed as hexahistidine tagged protein under the control of a T7 promoter in the vector pET21a(+) in the *E. coli* strain BL21 DE3. The *E. coli* cells were induced by addition of IPTG when the cell density reached mid log phase (OD_{600}=0.5-0.6). After every 2hours, 1ml of cells were collected from the medium, centrifuged at 13000rpm for 1 minute and resuspended in 50mM Tris buffer, pH7.0. The cell suspension was sonicated three times to disrupt them and cell debri removed by centrifugation at 13,000rpm for 10 minutes at 4^{o}C. The supernatant was then applied to a SDS PAGE gel electrophoresis. Figure 3.30 shows cellular accumulation of recombinant proteins, ZM0994 and ZMO1034. However, the accumulation of extracellular proteins from cell culture medium was not clear in the SDS PAGE gel as viewed by eye. Thus, cation exchange chromatography was performed in order to facilitate the purification of ZMO0994 and ZMO1034 from culture medium. The isoelectric Point (PI) value of the recombinant ZMO0994 and ZMO1034 was about 9.78 and 6.83, respectively. 500ml of the cell supernatant was collected by centrifugation at 4^{o}C and adjusted to pH 4.8. A HiTrap^{TM} SP HP column 1ml (x4) was connected to an AKTA Purifier system. The column was washed with 4 column volumes (CV) of distilled water and equilibrated with 4 CV of buffer A (50mM sodium acetate, 100mM NaCl, 10% glycerol, pH4.8). The collected supernatant was loaded onto the SP column equilibrated with buffer A. Without a further washing step, the column was eluted at a flow rate of 1ml/min with a gradient of 100-1000mM NaCl with the mixture of buffer A and buffer B (50mM sodium acetate, 1M NaCl, 10% glycerol, pH 4.8). Figure 3.31 shows the elution profile of cation exchange chromatography and SDS PAGE gel analysis of recombinant extracellular ZMO0994 and ZMO1034.

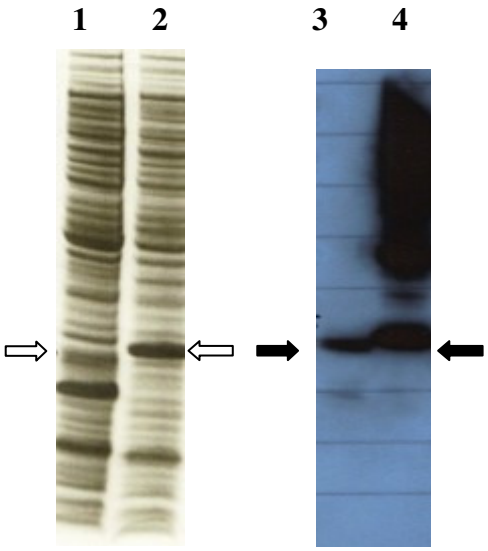


Figure 3.30 Cellular accumulation of recombinant proteins, ZMO0994 and ZMO1034. Lane 1 and 2 represent the SDS PAGE from the debris of *E.coli* cells used for the expression of the ZMO0994 and ZMO1034 recombinant proteins after induction for a short time. Lane 3 and 4 shows a Western-blot of Lanes 1 and 2, respectively, confirming the presence of the ZMO0994 and ZMO1034 recombinant proteins.

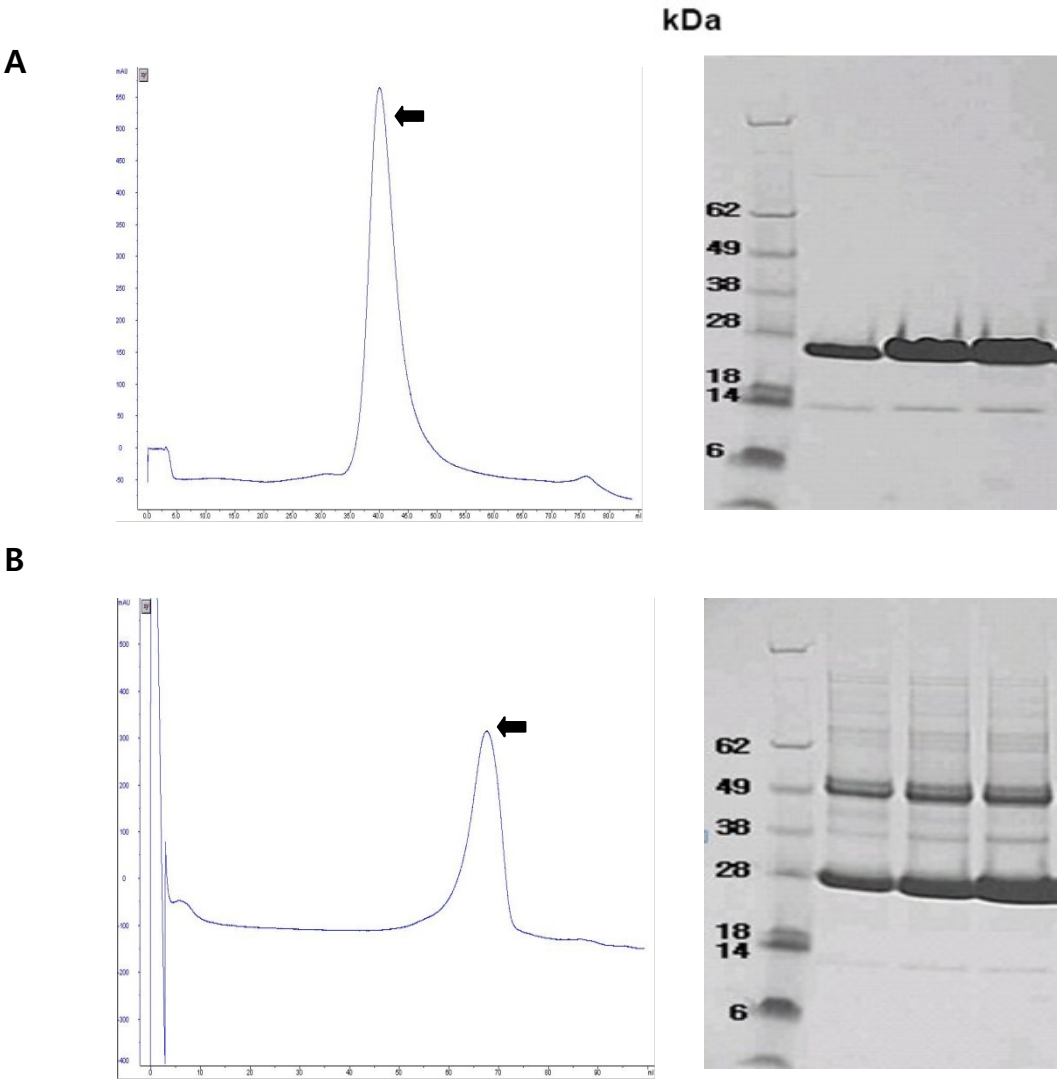


Figure 3.31 Extracellular recombinant proteins purification by ion-exchange chromatography. (A) Profile of cation exchange chromatography of ZMO0994 (left) and SDS PAGE of ZMO0994 (right). **(B)** Profile of cation exchange chromatography of ZMO1034 (left) and SDS PAGE of ZMO1034.

3.4.4 Western blot identification of extracelluar ZMO0994 and ZMO1034 in the supernatant

The samples shown in figure 3.31, plus recombinant ZMO1147, were applied to a 12% SDS-PAGE gel, transferred to a polyvinglidene difluoride (PVDF) membrane for the western blot. The transferred proteins were incubated with a mouse antibody that binds 6xhis-tagged proteins and then the blot was developed using a goat anti-mouse-AP conjugate antibody (Bio-Rad, U.K.). Figure 3.32 clearly shows that ZMO0994 and ZMO1034 were excreted from *E. coli* strain BL21 DE3 to the culture medium, while ZMO1147 was retained within the cell. It is confirmed that the candidate 24KDa and 26KDa, ZMO0994 and ZMO1034 proteins respectively, were indeed present in the cell supernatant.

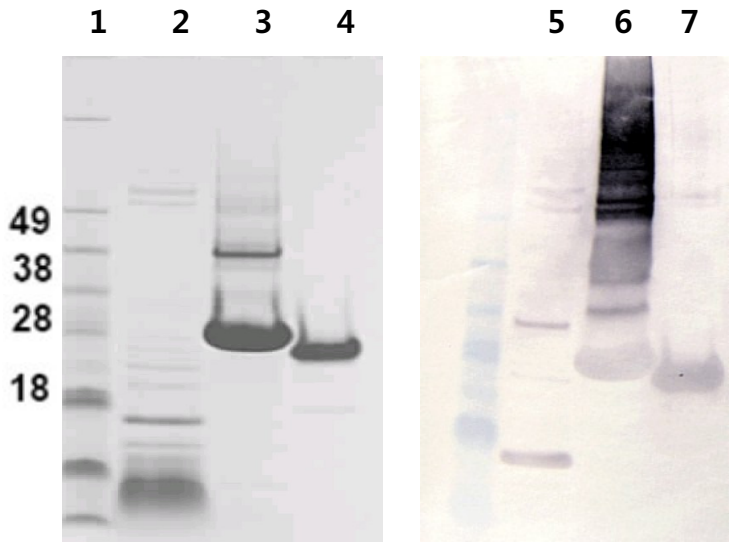


Figure 3.32 Extracellular accumulation of recombinant ZMO0994 and ZMO1034. Lane 1, Protein marker; Lane 2, 3 and 4 represent the SDS PAGE of ZMO1147, 1034 and 0994, respectively, from cloned *E. coli* culture medium (ZMO1147, presumably 29kDa, was –

Figure 3.31 *Continued*

already digested in the culture medium). A Western-blot of an SDS PAGE gel of purified recombinant proteins shows that ZMO0994 (lane 7) and ZMO1034 (lane 6) can be obtained successfully from *E. coli* culture medium, but not ZMO1147 (lane 5).

Considering that ZMO0994 and ZMO1034 were found in the *E. coli* extracellular medium, whilst ZMO1147 was not, would argue against these former proteins being released due simply to some of the cells bursting.

Chapter 4 Discussion

4.1 Quorum sensing in *Z. mobilis* and its applications

Z. mobilis is an obligately fermentative bacterium of industrial interest for ethanol production (Novak, 1981). The organism ferments glucose to ethanol and carbon dioxide at a level of up to 98% of the theoretical yield (two ethanol molecules per glucose molecule), achieving high concentrations of ethanol that are higher than those achieved by *Saccharomyces cerevisiae* (Osman and Ingram, 1987b) (98% in *Z. mobilis* and 86% in *S. cerevisiae* from 25% glucose). This level of metabolic activity is made possible by high-level expression of the glycolytic and ethanologenic enzymes, which account for approximately 50% of the soluble proteins (Algar and Scopes, 1985). For these reasons, this bacterium has been studied for ethanol fermentation using molecular genetic and biochemical methods.

Z. mobilis overproduced pyruvate decarboxylase (ZMO1360) and enolase (ZMO1608) in the presence of AI-2 quorum sensing molecules. Pyruvate decarboxylase, which has a limited occurrence in bacteria, is regarded as a key enzyme in ethanol formation and enolase is the most abundant of the glycolytic enzymes in *Z. mobilis*, as determined by two-dimensional gel electrophoresis (An *et al*., 1991) and biochemical assay (Osman and Ingram, 1987a). It has been suggested that enolase plays a role in metabolic flux control, since the intracellular concentrations of the triose phosphates and phosphoglycerates are relatively high (Algar and Scopes, 1985; Barrow et al., 1984). Considering that these are sequential enzymes in the ED pathway: our findings led us to hypothesize that AI-2 might stimulate ethanol production in *Z. mobilis*. Consistent with this hypothesis, we found, using a chromate assay, that ethanol was

over-produced by cells treated with AI-2. Under routine aerobic growth conditions, the maximal level of ethanol production increased by 58%, from 9% to 14.2% (v/v), in AI-2 treated cells. Although the absolute concentration of the AI-2 applied to the cells was not determined, the average luminescence of *in vitro* synthesized AI-2 was 1.0 x 10^6, which compared with the luminescence of 2.45 x 10^7, for AI-2 produced naturally by *E. coli* cells (20ml of culture, with *E. coli* grown to an OD_{600} of 1.0). There was approximately a 10-fold difference (Figure 3.12, 13 and 18). Thus, 5 Fold AI-2 could be regarded as a maximal threshold concentration for ethanol production by *Z. mobilis*, because ethanol contents induced by 5 and 10 Fold AI-2 concentrations were pretty much the same as each other. Furthermore, our AI-2 uptake experiments indicated that the added AI-2 concentration progressively decreased and was almost entirely consumed within 8 hours (Figure 3.12). Moreover, *Z. mobilis* could also consume the added glucose in 24 hours. Therefore, in *Z. mobilis* AI-2 might not only have a major role in determining the final ethanol production in short-term (e.g. 24hours) but also might affect the ethanol tolerance, osmotic tolerance and ethanol productivity (ethanol concentrations/hour) in much higher glucose-conditioned medium in the long-term (e.g. >300g/L).

Pyruvate decarboxylase (PDC) is the key enzyme for all homo-fermentative ethanol pathways. This enzyme catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde using Mg^{2+} and thiamine pyrophosphate (TPP) as co factors. Acetaldehyde is reduced to ethanol by alcohol dehydrogenase (AHD) during NADH oxidation. AHD enzymes are widely distributed throughout nature (Reid and Fewson, 1994). In contrast, PDC is common to only plants, yeasts and fungi; it is absent from animals and rare in prokaryotes (Konig, 1998). Recombinant *Z. mobilis* PDC has been used for the metabolic engineering of

ethanol pathways in many other organisms (Deng and Coleman, 1999; Kaczowka *et al*., 2005; Raj *et al*., 2002; Tao *et al*., 2001). Enolase has been studied extensively in eukaryotes organisms but has received less attention in bacteria (Burnett *et al*., 1992). The suggested function of enolase is to control metabolic flux, especially when ethanol is present at high concentration. An earlier glycolytic enzyme, glucose-6-phosphate dehydrogenase has been found to be inhibited by phosphoenolpyruvate (PEP) (0.5mM) (Scopes, 1997), and PEP is generated by enolase. Thus, it has been suggested that enolase regulates metabolic flux (Burnett, 1992, Uldis, 2006). According to our observation that AI-2 induced two sequential glycolytic enzymes, enolase (ZMO1608) and pyruvate decarboxylase (ZMO1360), the latter must be responsible for ethanol production and the former might control (presumably inhibit) this rapid metabolic process. Because glucose enters the cell by facilitated diffusion (Di Marco and Romano, 1985), it can accumulate intracellularly to quite high concentrations. Thus, in our study intracellular glucose rather than extracelluar glucose may be consumed at high rate by sequential overproduced enzymes within 24hours. It is known that *Z. mobilis* can complete sugar (e.g. >10%) conversion to ethanol within 40 hours (Doelle and Greenfield, 1985; McGhee *et al*., 1982; Osman and Ingram, 1987a).

It is generally accepted that most of bacteria can produce signal molecules, such as small peptide, AHLs and AI-2: and that these bacteria possess operons consisting mainly of genes for signal production, secretion and recognition (Bassler, 2002; Vendeville *et al*., 2005). However, having looked for and failed to find the *luxS* gene in the *Z. mobilis* genome, suggests that it uses the SAH dehydrolase pathway. Thus, *Z. mobilis* is one of a few examples in nature that responds to AI-2 but do not produce it. There is only one other example, *P. aeruginosa*, isolated from the hospital (Winstanley and Fothergill, 2009). An interesting

difference is that *P. aeruginosa* possesses the receptor gene for the signal (*lsrR*) but *Z. mobilis* does not. Although *Z. mobilis* could take up and respond to AI-2, to alter the gene expression of the bacterium, currently we do not have any knowledge of how AI-2 regulates the transcriptional processes. According to the results (Figure 3.13), the *Z. mobilis* AI-2 quorum sensing system seems to resemble those found in *Salmonella typhimurium* (Taga *et al*., 2003) and *E. coli* (Xavier and Bassler, 2005b), both of which produce transporters to take-up AI-2, which is then phosphorylated before interacting with a transcription factor. In contrast, *Vibrio* species utilize a two-component signal transduction system, in which a membrane bound histidine-kinase sensor, detects the AI-2 at the membrane (Miller *et al*., 2002). This difference (section 1.2.3.1) in behavior allows *E. coli* to interfere with AI-2 signaling by *Vibrio* species, by consuming the AI-2 produced by the *Vibrio* cells (Xavier and Bassler, 2005b). Recent studies suggest that some enteric bacteria respond to the presence of competitor bacteria by sequestering and destroying AI-2, thereby eliminating the competitor's intercellular communication capabilities (Xavier *et al*., 2007). For the purpose of determining the putative fate of AI-2 taken-up by *Z. mobilis*, I screened the *Z. mobilis* genome with the Lsr and Lux protein sequences to detect if it possessed homologues of these proteins, but apparently none were present. This suggests that *Z. mobilis* utilizes a novel transporter to take up AI-2 (section 4.3.1) and possesses a unique quorum sensing system.

As mentioned previously, *Z. mobilis* is an obligately fermentative bacterium that produces ethanol and carbon dioxide as principal products. This organism has been reported to be capable of sugar conversion rates several fold greater (Cook and Russell, 1994), and has a higher ethanol tolerance than *S. cerevisiae* (Panesar *et al*., 2006). One problem associated with large-scale ethanol production is the decrease in the rate of substrate conversion

observed during ethanol accumulation in the medium. Besides, to maintain vitality, specificity and fermentation activity depends on the ability of bacteria to survive under ethanol-induced stress conditions (Shakirova *et al*., 2008). Many studies have hypothesized that this inhibition by ethanol could be due to direct action of ethanol on key enzymes of glycolysis and ethanol production could involve feedback inhibition or alcohol dehydrogenase II inactivation due to reduction of NADH (Diefenbach and Duggleby, 1991; Osman and Ingram, 1985; Scopes 1997), resulting in use of the ATP production pathway (TCA cycle) rather than ethanol production. Previous studies with a variety of systems have shown that ethanol increases membrane leakage in a number of bacteria (Ingram, 1989; Micheal and Straka, 1986; Osman and Ingram, 1985). There are three basic ways in which ethanol would be expected to decrease the effectiveness of the cell membrane as a hydrophobic barrier: 1) by altering the colligative properties of the environment, 2) by directly interacting with the membrane, and 3) by altering the dielectric properties of the environment. A variety of enzymes in the ED pathway require metal ions as co-factors, such as magnesium for enolase and calcium for pyruvate decarboxylase activation. Thus, the inhibition of fermentation by ethanol could result from the leakage of the hydrophobic membrane barrier, leading to a loss of cofactors, coenzymes and intermediates of glycolysis.

Calcium ions are known to regulate a variety of regulatory processes in bacteria, such mobility, chemotaxis, cell division and differentiation (Smith, 1995). Intracellular Ca^{2+} levels are about a 1000 times lower than those found outside the cell. In eukaryotic cells, the cytoplasmic free Ca^{2+} concentration is tightly controlled by the action of specific pumps and channels in the membrane and subcellular organelles. The response of eukaryotic cells to increased cytoplasmic free Ca^{2+} concentration is mediated by either calcium-sensitive

enzymes or a protein transducer, such as calmodulin (Swan *et al*., 1987). Although more feature of eukaryotic cells, calmodulin-like proteins have been found in several bacteria, including Gram negative species (Gravarek, 2006). These proteins, with an average Mr of 20kDa, contain an EF-hand, Ca^{2+}-binding domain (Michiels *et al*., 2002; Rigden *et al*., 2003). It has been shown that there is an optimal calcium ion concentration for increased ethanol tolerance in yeast, allowing for the rapid production of an higher concentration of ethanol (Nabais *et al*., 1998). Calcium ions could increase the stability of the membrane, leading to an increase in the ethanol tolerance of fermenting yeast (van Uden, 1985). Interestingly, I found that *Z. mobilis* produces and excretes a 26kDa putative calcium binding protein (ZMO1043), which might play a role in conferring ethanol tolerance on *Z. mobilis*.

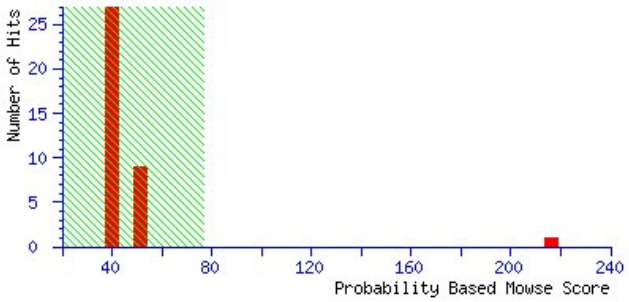
In addition *Z. mobilis* secretes the ZMO1147, putative chaperone. ZMO1147 is clearly transcriptionally linked to ZMO1148, a homologue of *yaeT*, being directly downstream of ZMO1148, so that they share the same promoter. This chaperone works in conjugation with the outer-membrane protein (OMP) YaeT, which has been shown to be a key component of a complex that is responsible for the insertion of proteins into the outer-membrane (Wu *et al*., 2005). Consequently, it is possible that both ZMO1147 and ZMO1148 are induced by AI-2. Considering that members of the YaeT family have homology with the OMP component of type II secretion system, suggests that ZMO1148 is utilized for the secretion of ZMO1147 and the other proteins induced by AI-2.

Considering the membrane leakage by ethanol, the finding that the ZMO1147 chaperone is secreted might suggest an additional role in maintaining the integrity of outer-membrane proteins or other secreted proteins; whilst the ZMO1034 Ca^{2+}-binding protein might have a

role in sequestering Ca^{2+} released from cells due to ethanol induced leakage of the membranes. Another secreted protein, ZMO0994, has homology to members of group-3 of the LEA (Late Embryogenesis Abundant) protein family that are associated with tolerance to dehydration. One proposal is that these proteins act as chaperones for water stress that suppress desiccation-induced protein aggregation (Goyal *et al*., 2005); a role that can be potentiated by interacting with non-reducing sugars, such as trehalose and sucrose (Browne *et al*., 2002). These sugars can also protect lipid membranes from dehydration which causes solute leakage and membrane protein aggregation (Hoekstra *et al*., 2001). The protective effect is due to the sugars forming a glassy matrix that prevents mechanical disruption and denaturation of membrane proteins. It has been shown that sucrose glasses are stabilized *in vitro* by interaction with LEA proteins (Browne *et al*., 2002).

In *Z. mobilis* both glucose and fructose are taken up by a low-affinity, high-velocity facilitated diffusion system encoded by *glf* (Di Marco and Romano, 1985; Parker *et al*., 1995), and another trait of *Z. mobilis* as a typical saccarophilic bacterium is that it can thrive on exceptionally high concentrations of sugar (Sprenger, 1996). After a long lag phase, *Z. mobilis* is able to grow in up to 40% glucose medium (Swing and De Ley, 1977). Early reports suggested that the ability to grow on high concentration of glucose was due to a rapid equilibrium of the internal and external glucose concentrations (Di Marco and Romano, 1985). Subsequently, studies of glucose transport confirmed that *Z. mobilis* has a facilitated diffusion system which enables a rapid equilibration between internal and external glucose concentrations. Studies using the non-metabolisable sugars maltose (impermeable) and xylose (permeable) revealed that these sugars were able to alter the osmotic pressure on the cytoplasmic membrane resulting in volume changes (Struch *et al*., 1991). However, nuclear

magnetic resonance spectrometry studies, using ^{13}C-labelled sugars, showed that the internal concentration of glucose in growing cells was comparatively low (Loos *et al*., 1994), prompting the suggestion that the ability of *Z. mobilis* to counteract detrimental osmotic stresses, due to growth on glucose or fructose, is attributable to the formation of sorbitol as a result of the activity of glucose-fructose oxidoreductase. Since the function of another screened protein ZMO0689 (Figure 4.1) from *Z. mobilis* is to produce the non-reducing sugar sorbitol; this might point towards a role for ZMO0994 and ZMO0689 in producing a protective sorbitol glass to counteract the dehydrative effect of increasing ethanol concentrations.



gi|61679830 Mass: 43188 Score: 216 Expect: 7.8e-16 Queries matched: 25
Chain D, Crystal Structure Of The Shifted Form Of The Glucose- Fructose Oxidoreductase From Zymomonas Mobilis
gi|56551585 Mass: 47388 Score: 209 Expect: 3.9e-15 Queries matched: 25
glucose-fructose oxidoreductase chain A [Zymomonas mobilis subsp. mobilis ZM4]
gi|17942860 Mass: 47486 Score: 207 Expect: 6.2e-15 Queries matched: 25
Chain B, Reduced Precursor Form Of Glucose-Fructose Oxidoreductase From Zymomonas Mobilis
gi|2098328 Mass: 42365 Score: 202 Expect: 2e-14 Queries matched: 25
Chain F, Glucose-Fructose Oxidoreductase
gi|12084748 Mass: 39420 Score: 200 Expect: 3.1e-14 Queries matched: 25
Chain D, Crystal Structure Of Glucose-Fructose Oxidoreductase (Gfor) Delta1-22 S64d
gi|155588 Mass: 48056 Score: 168 Expect: 4.9e-11 Queries matched: 22
glucose-fructose oxidoreductase
gi|1177393 Mass: 14719 Score: 42 Expect: 2e+02 Queries matched: 6
myophilin [Echinococcus multilocularis]

Figure 4.1 MASCOT search results from the MS-MS data generated for ZMO0689, glucose-fructose oxidoreductase.

(http://www.matrixscience.com/cgi/master_results.pl?file=../data/20051212/FsGcOGcn.dat)

The discovery that the metabolic pathway leading to ethanol production is regulated by AI-2 is of considerable biotechnological importance because it will provide a basis for further engineering of strains for more efficient ethanol production (Figure 4.2). Indeed, to engineer *Z. mobilis* by introducing the genes that encode Pfs and LuxS proteins to produce AI-2 would be a means to stimulate increased ethanol production. The benefit of using AI-2 to enhance ethanol production is that it is likely to promote the expression of the metabolic enzymes in a co-ordinated manner, for efficiency of metabolic flux through the pathway that leads to ethanol production.

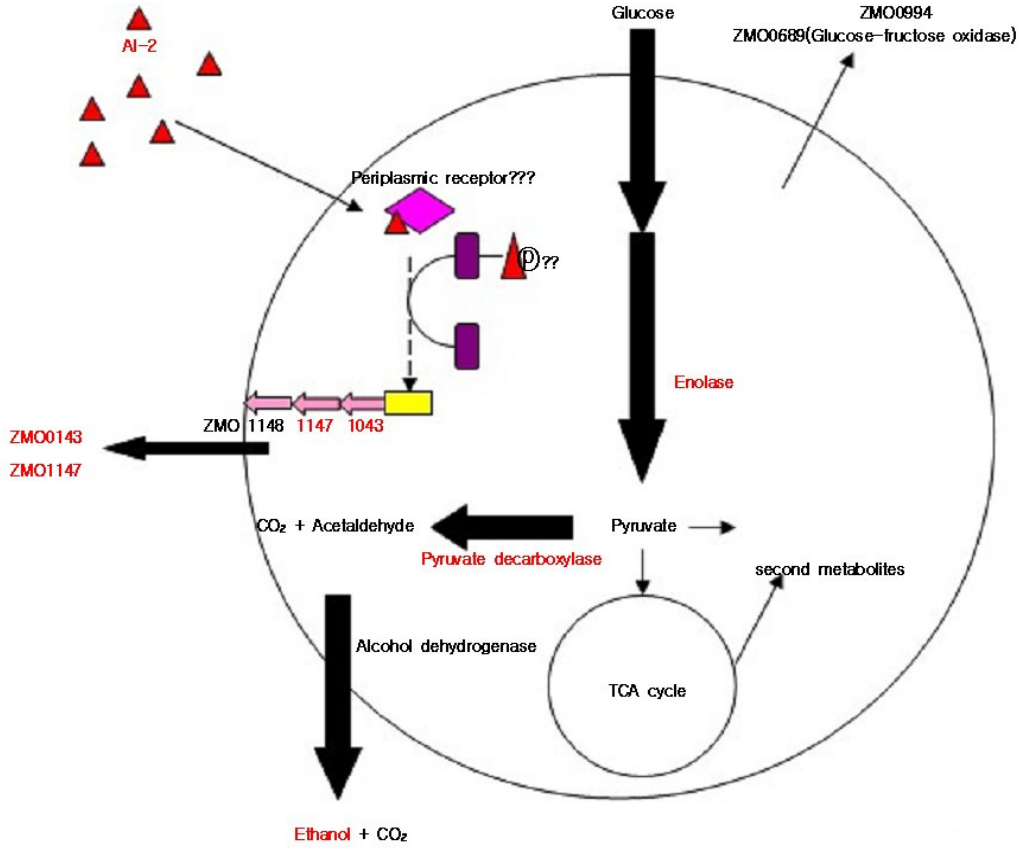


Figure 4.2 Proposed quorum sensing regulation in *Z. mobilis*. For the AI-2-stimulated production of ethanol, the following steps would be necessary: 1) AI-2 uptake, 2) internalization of AI-2 by an as yet unknown regulatory system, 3) (glucose uptake), induction of enzymes and extracellular proteins, 4) ethanol production.

Figure 4.2 *Continued*

Red letters indicate overexpressed extracellular proteins or overproduced substances (except AI-2), and the black arrows represent the metabolic pathway of glucose catabolism. The proposed relative flux through the different pathways is shown as the boldness of the arrows.

Considering that ethanol production is regulated by AI-2, it may be advantageous to introduce genes for hydrolytic enzymes, which degrade cellulose, tagged with the N-terminal sequence of the secreted proteins that would also be under the control of AI-2 responsive promoter to co-ordinate this process with sugar uptake and ethanol biosynthesis. Transcriptomic and proteomic studies will be instrumental in identify additional AI-2 regulated genes, to provide a better understanding of the biosynthetic process. Interestingly, it was found that both the enzymes involved in ethanol biosynthesis and those secreted are regulated by AI-2, suggesting they have an interrelated function. It is a notable feature of the secreted proteins that they all appear to play a protective role, which might be necessary to overcome the detrimental effects of ethanol. Increasing ethanol would be expected to increase the fluidity of the membrane, causing them to leak, while increasing its dehydration, which would inactivate membrane proteins. Further biochemical experimentation will be require to determine if the *Z. mobilis* secretome is associated directly in conferring tolerance to ethanol production. The discovery of a connection between AI-2 induced proteins secretion and increased ethanol production provides a lead in this direction. Understanding these processes will be a key to engineering *Z. mobilis* strains for increased ethanol production and tolerance.

4.2 An ecological and evolutionary perspective on quorum sensing in *Z. mobilis*

The findings that *Z. mobilis* overproduces ethanol in responding to AI-2 provokes the following ideas: since in many bacterial species AI-2 controls the expression of pathogenicity determinants, *Z. mobilis* may have developed the ability to consume AI-2 as a protective response that attenuates the virulence of bacterial species with which it co-exists. Indeed, *Z. mobilis* is known to have antibacterial activity, and has found use as a probiotic, which might in part be attributable to its ability to consume the quorum sensing molecules, attenuating the virulence of these bacterial pathogens. The first step for ethanol production in *Z. mobilis* is the uptake of the glucose, which is then shunted through the ED pathway. Thus, it probably utilizes the glucose more rapidly than the AI-2 producing strains (Presumably AI-2 induced). Because *Z. mobilis* may gain the benefit without the cost of producing AI-2, this could result in the outgrowth of *Z. mobilis* as it out-competes the other bacterial strains for resources, such as glucose. At the same time, enhanced ethanol production by *Z. mobilis* in the natural environment, in which bacterial mixtures exist, could result in the destruction of neighboring bacterial populations. *Z. mobilis* is known to have antibacterial effects against a wide range of Gram negative and Gram positive bacteria, as well as certain protozoa, yeasts and filamentous fungi (Swing and De Ley, 1977). The biochemical nature of these antagonisms is unknown. However, it is clear that by-products or possibly peptides would be toxins to outcompete neighbouring cells. There are several reports of bacteriocin production being linked to *luxS* gene regulation. The oral pathogen *Streptococcus mutans* employs a variety of mechanisms to maintain a competitive advantage over many other oral bacteria that occupy the same ecological niche (Riley and Gordon, 1999). Production of the bacteriocin, mutacin I,

is one such mechanism with *luxS* deletion mutants failing to produce of bacteriocins (Merritt *et al*, 2005).

To test if AI-2 induced killing effect of *Z. mobilis* on two different *E. coli* mutants (AI-2 producer and non-producer), well-diffusion assay and co-culture experiment (wild type and KX1186) were performed. In the well-diffusion assay after *Z. mobilis* was spotted onto the well, two *E. coli* strains were overlaid onto RM medium. In the co-culture experiment the CFU of *Z. mobilis* and each *E. coil* strain were counted in the mixture of *Z. mobilis* and *E. coli* (*Z. mobilis*+AI-2 producer, *Z. mobilis*+non-producer). We expected that *Z. mobilis* would kill AI-2 producer *E. coli* strain much more effectively than the non-producer, because of AI-2 induced ethanol overproduction in *Z. mobilis*. However, it was difficult to obtain solid evidence from experiments such as the growth rate of *E. coli* and *Z. mobilis* with/without AI-2. Presumably, these results may due to 1) unknown antibacterial substances from *Z. mobilis* that kill the two *E. coli* (AI-2 producer and non-producer) regardless of a critical ethanol concentration, and 2) the differences in the physiological nature of *Z. mobilis* and *E. coli* strain (e.g. growth difference in RM medium). Alternatively, the failure to find growth superiority in *Z. mobilis* may be explained by the counterbalance of the cost of gene expression for AI-2 production in *E. coli* and the cost for AI-2 response in *Z. mobilis*. In other words, *E. coli* strains may pay costs for (presumably) their growth, while *Z. mobilis* may spend costs (extra-energies) for excessive metabolism and growth to respond to AI-2.

In mixed-species consortia, production and detection of AI-2 by enteric bacteria should have reciprocal effects on biological functions in other bacterial species that communicate using

AI-2. When enteric bacteria provide AI-2 to a nearby species, it could be used as information for counting enteric cells in a mixed-species community or activating the expression of quorum-sensing-regulated genes. However, when enteric bacteria remove AI-2, a neighbouring species could underestimate the cell number or metabolic potentials and fail to initiate or incorrectly terminate quorum sensing. Thus, consuming AI-2 could allow enteric cells to interfere with AI-2-mediated communication in other bacteria so that they gain the public benefit for themselves (Bassler *et al*., 1997; Xavier and Bassler, 2005a).

Zymomonas might have the opportunity to utilize the free AI-2 from neighbouring cells sharing the same ecological niche, resulting in overproduction of antibacterial substances such as ethanol. Indeed, Lukas *et al.* (2008) suggested that commensal intestinal bacteria such as *Bacteroides vulgates*, *Clostridium proteoclasticum*, *Escherichia coli*, *Eubacterium rectal*, *Lachnospira multipara*, *Pseudobytyrivibrio rumins*, *Roseburia intestinals*, *Ruminococcus albus* and *Ruminococcus flavefaciens* carry the *luxS* gene for production of AI-2 (e.g. detected with the *Vibrio harveyi* luminescence assay of their culture fluids and PCR amplification of the *luxS* gene). However, it has not yet been proved whether AI-2 is used for bacterial communication (Lukas *et al*., 2008). It is widely accepted that the mammalian gastrointestinal tract harbors a complex population of bacteria that comprises of several hundred bacterial species at high cell density (10^{11} cells per g). The existence of this microbial complex in the intestine suggests that quorum sensing may be involved in cell-cell or host-cell communication (Kaper and sperando. 2005).

Z. mobilis is presumed to use the one-step SAH dehydrolase pathway for detoxification of

SAH because it does not possess a *luxS* gene and cannot produce AI-2. However, it does respond to AI-2, which regulates the metabolic pathway leading to ethanol production. It is conceivable that the ethanol itself may act as a toxic chemical to other bacteria. In fact, in the cell-free culture fluid of *Z. mobilis*, the Gram-negative and Gram-positive bacteria of *E. coli* and *S. epidermidis,* respectively could not survive, but yeast which has a greater ethanol tolerance could survive. The benefit of using AI-2 may be that *Z. mobilis* is far superior to competitors in obtaining the energy sources, because it is likely to promote the expression of glucose metabolic enzymes in a co-ordinate manner, for efficiency of metabolic flux through the pathway that leads to ethanol production. It has been suggested that AI-2 might be taken back by the bacteria to be metabolized as a carbon source (Taga *et al*., 2001). Thus, the therapeutic use of drinking a beverage of *Z. mobilis* may originate from the advantage that *Z. mobilis* consume AI-2 molecules as signals or carbon source and produce toxic metabolites or bacteriocins. The ability to consume AI-2 might interfere with the communication of enteric pathogens, which is necessary to maintain their ecological niche in the environment.

The signalling cheaters (limited in AI-2 signal) may respond to AI-2 produced by other bacteria species, and interfere with the communication of neighboring AI-2-producing bacteria, resulting in the malfunction of gene-regulation of the neighboring bacteria. While the LuxS enzyme is necessary for AI-2 production, it is not required for AI-2 signal transduction. Theoretically, whilst a bacterium may not be able to produce AI-2, it could have the ability to detect the presence of coexisting or competing bacterial species by sensing the environmental concentration of AI-2. From an evolutionary interests, there is a limitation to our ability to prove that the AI-2 signaler or non-signaler really behave like producer or cheater over short/long term periods. Indeed, one of these difficulties is that the cell culture

condition in the lab ignores the natural condition of the cell's environment. One example is that of diffusion sensing suggested by Redfield in 2002 which explain that autoinducer secretion monitors the diffusive properties of the local environment, with benefits that are directly realized by individual cells rather than populations.

Basically conversion of acetaldehyde-ethanol is catalyzed by alcohol dehydrogenase (Adh). *Z. mobilis* possesses two alcohol dehydrogenase, named AdhI and AdhII which catalyse the reaction in both directions; acetaldehyde-to-ethanol and ethanol-to-acetaldehyde. Reversely converted acetaldehyde by AdhII finally forms acetyl-CoA, which is then used in the TCA cycle for energy production especially in the presence of oxygen. This energy is used by *Z. mobilis* for biosynthesis, including for cell division, membrane synthesis and enzyme production. Thus, when the nutrients are depleted, *Z. mobilis* may have more advantage over its competitors, because internal ethanol may be consumed by the AdhII of *Z. mobilis* (Piskur *et al*., 2006), maintaining the *Z. mobilis* populations survival for a short-term.

From the study of phylogenic distribution of SAH detoxification pathways, Eukarya and Archaea use exclusively the one-step pathway to degrade SAH, while bacteria use either the one-step or the two-step pathway (Figure 1.13). The two-step Pfs/LuxS pathway is consistently present in all Firmicutes and absent in Actinobacteria (Figure 4.3). However, *Bifidobacterium longum* is the only species of Actinobacteria having the *luxS* and at the same time has SAH dehydrolase (Figure 4.3). *Bifidobacterium longum*, being a commensal of the healthy human gut, shares the same habitat with other bacteria. Consequently, there would be ample opportunities for *B. longum* to acquire *luxS* by horizontal gene transfer from intestinal bacterium such as *Lactobacillus* (Sun *et al*., 2004). The Lactobacillus branch also contains a

small part of *luxS* from *Borrelia burgdorferi*, a Spirochete, which is most similar to *luxS* from *Clostridium acetobutylicum* (Sun *et al*., 2004). Interestingly, most of the well-known enteric bacteria, including the above examples, *helicobacter pylori* and *E. coli* strains have *luxS* homologues. *Z. mobilis* belongs to the alpha-subclass of the class Proteobacteria and has no LuxS/Pfs pathway. Therefore, one might suggest that long-term exposure of *Z. mobilis* to AI-2 would induce *Z. mobilis* to become susceptibility to AI-2, because it is from the same phylogenic tree and can be found in the same niche.

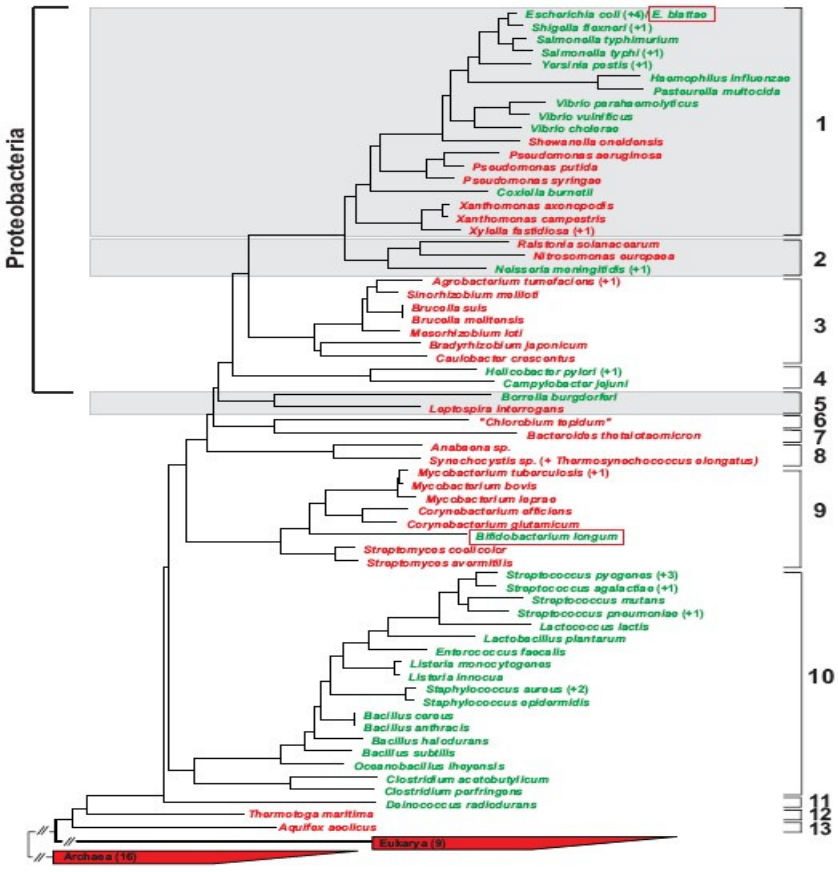


Figure 4.3 Distribution of one-step (red) and two-step (green) detoxification pathway of S-adenosylhomocystein within the three domains of life. Phyla are numbered from 1 to 13. 1,Gammaproteobacteria; 2,Betaproteobacteria; 3,Alphaproteobacteria; 4,Epsilonproteobacteria; 5,Spirochaetes; 6,Chlorobia; 7,Bacteroidetes; 8,Cyanobacteria; 9,Actinobacteria; 10,Firmicutes; 11,Deinococcus-Thermus; 12,Thermotogae; 13,Aquificae. (Adapted from Sun *et al*., 2004)

There are lots of excellent studies of knock-out mutants to investigate modifications of infectious phenotypes in some of the currently sequenced pathogens. In *V. cholerae* (Miller *et al*., 2002; Zhu *et al*. 2002), *Streptococcus pyogenes* (Lyon *et al*., 2001; Marouni *et al*.,2003), *Streptococcus pneumonia* (Stroeher *et al*., 2003), *Neisseria meningiltidis* (Winzer *et al*., 2002), *Clostridium perfringens* (Ohtani *et al*., 2002), *Borrelia burgdorferi* (Hubner *et al*., 2003), *Porphyromonas gingivalis* (Burgess *et al*., 2002) and *Shigella flexneri* (Day *et al*., 2001), *luxS* mutants showed changes in the expression of virulence factors, ranging from subtle to severe defects in virulence.

Cheaters can collect the benefit of the cooperating population without contributing "public goods" or costly resources that benefit the community as a whole. As previously mentioned, the discovery that AI-2 regulates the metabolic pathway leading to ethanol production in *Z. mobilis* is very similar to cheating in the *P. aeruginosa luxS* mutant strain (Section 1.2.4.3). It is likely that this involves AI-2 being used as 'cue' by *Z. mobilis* as a guide to future action. Bacteria are able to modify their properties according to their nutrient supply and adapt to a limited nutrient. It was difficult to determine the exact cellular mechanism of AI-2 regulation resulting in ethanol over-production and ethanol tolerance mechanism in *Z. mobilis*, but it is likely that *Z. mobilis* compete with *E. coli* in acquisition and utilization of nutrients by monitoring the local cell density of *E. coli* without investing effort into the dissemination of AI-2. In addition, *Z. mobilis* likely deploys ethanol as an antibacterial chemical. Yun (1996) reported that fructooligosaccharides stimulate the growth of bifidobacteria and discourage the growth of potentially pathogenic microorganisms. Fructooligosaccharides are produced by levansucrase in *Z. mobilis* and the productivity of levan is roughly direct proportional to ethanol productivity (Calazans *et al*., 1997).

Considering together the ethanol production and tolerance of *Z. mobilis*, along with its osmotic tolerance in the presence of AI-2; these features would be expected to confer a considerable advantage on *Z. mobilis* for its survival and growth in the environment.

4.3 Further studies

4.3.1 Characterisation of the AI-2 recognition mechanism

The first question is 'how is AI-2 recognized by *Z. mobilis*'? One simple test would be to determine unequivocally that *Z. mobilis* takes-up AI-2, because if it does, this would suggest that it has a similar system to *S. typhimurium,* rather than *V. cholerae,* which utilizes a two-component signal transduction system (TCS) to detect AI-2 at the membrane. To initially distinguish between these possibilities an AI-2 producing strain of *E. coli* was cultured alone and in the presence of *Z. mobilis*, separated within a dialysis bag; and, using the bioluminescence assay with *V. harveyi* BB170 (Bassler *et al*., 1997), the culture medium for AI-2 content. To avoid the potential complication of the *E.coli* taking-up the AI-2 a *luxK*-deletion strain which does not have this capacity (Xavier and Bassler, 2005a) was used. It was found that there was less AI-2 in the medium for *E. coli* co-cultured with *Z. mobilis*. Similarly, It was found that when AI-2, produced *in vitr*o, was added to the *Z. mobilis*, the AI-2 content of the medium progressively decreased with time. These findings indicating that *Z. mobilis* takes-up AI-2 and consequently a priority would be to determine if it has a quorum sensing system similar to that found in *S. typhimurium* and *E. coli*.

Further test should be performed to determine whether disrupted cells release enzymes that

can catalyse the phosphorylation of AI-2. Any AI-2 that is radio-labelled by incubation with [γ-^{32}P]-ATP, could be recovered using immobilized YdeW from *E. coli*. The YdeW is inhibitory transcriptional regulator which combines to phosphorylated AI-2 (Xavier and Bassler, 2005b). An alternative approach would be to incubate the cell extract with cold ATP, boil and sieve the extract through a 10 kDa membrane, to allow the AI-2 and any phosphorylated product to be separated from the proteins. To detect free AI-2, it could be incubated with YdeV and [α-^{32}P]-ATP, since any unphosphorylated AI-2 would be available for phosphorylation by YdeV, leading to a decrease in [α-^{32}P]-ATP as it is converted to [α-^{32}P]-ADP. The radio-labelled ATP and ADP could be separated by TLC and quantified using a phosphoimager. If these experiments indicate that AI-2 is phosphorylated by *Z. mobilis* cytoplasmic proteins then [γ-^{32}P]-AI-2, produced enzymatically with [γ-^{32}P]-ATP and YdeV could be utilized to screen a native-gel for proteins that bind AI-2-Pi. The *E. coli* proteins YdeW and YdeV would be preferable over the corresponding *S. typhimurium* proteins LsrR and LsrK, because of their ease of purification. Failure to identify phosphorylated AI-2 would raise the possibility that it is sensed at the membrane, probably by a two-component signal transduction system (TCS). *Z. mobilis* appears to have 12 TCS histidine-kinases of unknown function. So further experiment would delete these genes using suicide vectors (A PCR-amplified tetracycline resistance cassette) (Senthilkumar *et al.* 2004)

4.3.2 Identification of AI-2-responsive transcription factors

The finding that AI-2 treatment of *Z. mobilis* causes the genes for enolase and pyruvate decarboxylase to be overexpressed suggests that their expression is regulated by an AI-2-responsive transcription factor. Thus, this hypothesis could be tested using the 5' untranslated

region (UTR) of these genes to control the expression of reporter genes in *Z. mobilis*: the plasmids pAEG1 and pHS119, with *gfp* and *lacZ* reporters respectively, which have previously been used for detecting promoter function in *Z. mobilis* (Christogianni *et al*., 2005) could be used. Similarly, another test that could be performed is whether the 5' UTR of the genes for the secreted proteins can drive the AI-2-induced expression of reporter genes. It is unknown if AI-2 induces the expression of these genes, leading to their secretion, or if they are already available for secretion upon the AI-2-induced expression of the secretion machinery.

4.3.3 Transcriptomic and proteomic approaches to identify novel AI-2 and ethanol responsive genes

Both transcriptomic and proteomic approaches could be adopted to identify genes whose expression is induced by AI-2 and in response to ethanol. There have been few microarray studies of the effects of AI-2 on gene expression; probably because to date all those strains found to be responsive to AI-2, also produce it, complicating analyses. One study used a *luxS*-strain of *E. coli* and demonstrated a significant increase in the expression of YdeV, which was later confirmed as the transcription factor controlling expression of the AI-2 response machinery (De Lisa *et al*., 2001). For these experiments it would be necessary to design oligonucleotides that cover the 1,998 ORFs that are apparent in the *Z. mobilis* genome (Seo *et al*., 2005) for fabrication of a microarray.

Our 1D-gel analyses successfully identified a number of proteins that are up-regulated or secreted in response to AI-2 and ethanol. To identify additional proteins that are of

differential abundance following AI-2 and ethanol treatment two-dimensional differential in-gel electrophoresis (2D-DIGE) could be used. This would give us a better appreciation of the global effects of AI-2 and ethanol on the cellular response of *Z. mobilis*.

4.3.4 Identification of the molecular basis of ethanol tolerance in *Z. mobilis*

To test the hypothesis that the secreted protein products of the *ZMO0689*, *ZMO0994*, *ZMO1034* and *ZMO1147* genes confer ethanol tolerance, deletion mutation of these *Z. mobilis* genes using suicide vectors (Senthikumar *et al*., 2004) could be undertaken. Several studies suggest that ethanol tolerance is a phenotype controlled by several genes (Gonzalez *et al*., 2003) requiring global genomic screening procedures. One approach that has been applied to engineer an increase in ethanol tolerance of *E. coli* is global transcriptional machinery engineering (gTME), which is based upon making a random-mutagenesis library of the *rpoD* gene (Alper and Stephanopoulos, 2007). The *rpoD* gene encodes the σ^{70} protein that binds to RNA polymerase, so that mutations in the *rpoD* gene cause an alteration in the promoter preferences of the polymerase affecting transcription levels and thus modulate the transcriptome at the global level. We could use the same approach, to make a random-mutagenesis library of the *Z. mobilis rpoD* gene *(ZMO1623)*, and screen the library transformed into *Z. mobilis* for mutants may have increased ethanol tolerance.

Previous studies have established that plasmids carrying the transposable element mini Mu (e.g. pULB113 [RP4::miniMu3A]) can be transferred from *E. coli*, by conjugation, into *Z. mobilis* for transposon mutagenesis (Pappas *et al*., 1997). An alternative approach would be to create a transposon mutagenesis library for *Z. mobilis* that can be screened for genes that

confer ethanol tolerance - e.g. isolating colonies that have lost their ability to survive in 10% ethanol. Subsequently, this library could be used to screen for novel components of the AI-2 response, by transforming the library with a plasmid that carries a reporter gene under the control of an AI-2-responsive promoter and screening for colonies that do not produce the reporter gene product.

Such future studies will be important in elucidating, what must be a novel quorum sensing system and have biotechnological application in improving *Z. mobilis* strains for improved bioethanol production. It is to be noted that although *S. cerevisiae* is currently the organism of choice for bioethanol production, DuPont are developing their bioethanol production based upon the use of *Z. mobilis*.

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