Characterisation of the regulatory gene *nolR* in *Sinorhizobium meliloti*

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Declaration of Original Work

The research in this thesis was conducted under guidance of Professor Barry G. Rolfe at the Research School of Biological Sciences. All of the work reported is my own, and has not been submitted towards a degree at this or any other institution or university.

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Abstract

Nitrogen-fixing symbioses between legumes and rhizobia are important for sustainable agriculture and contribute significantly to the global nitrogen cycle. The soil bacteria form symbiotic associations with the host plants, converting atmospheric nitrogen into an organic form which the plant can utilise. This process, termed symbiotic nitrogen fixation, is very significant in agriculture and in the recycling of nitrogen in the biosphere.

Sinorhizobium meliloti (previously known as *Rhizobium meliloti*) has been chosen by the international research community as the model microorganism to investigate nodulation, nitrogen fixation and the symbiotic interaction between plant and bacterium. *Sinorhizobium meliloti* strain 1021 is a free-living microsymbiont whose genome consists of three replicons. These replicons consist of a chromosome of 3,654,135 bp and two large plasmids containing genes involved in symbiosis that are thus labelled pSyma and pSymb of 1,354,226 bp and 1,683,333bp, respectively. With the completion of the genome database of *S. meliloti* strain Rm1021, focusing is now on the "functional" proteomics of the organism.

In *S. meliloti*, optimal nodulation requires both positive and negative control of *nod* gene expression. The expression of these nodulation genes can be activated in the presence of the *nodD* gene in conjunction with specific signals from the host plants. The negative regulation of the *nod* genes is controlled by the *nolR* gene, which has a single copy on the chromosome and was found highly conserved in the *Sinorhizobium* genus. Proteome analysis provides a sensitive tool to examine the functional organisation of the *S. meliloti* genome and the intracellular gene interactions between replicons. Subsequent analysis of proteins with altered mobility in 2D gels could identify targets of certain proteins, which will help to detect networks of co-ordinately regulated genes that may play cooperative roles in nodule formation or function. Recently, the *nolR* gene was found to have both negative and positive regulator functions, which made it an ideal candidate to study "functional" proteomics of *S. meliloti*.

In this thesis, by using the *nolR::lacZ* fusion reporter system, the regulation of *nolR* gene expression was found related to population density, nutrient availability, and responsive to various environmental challenges. In addition, the expression of *nolR* was stimulated by factor(s) accumulated with increasing population density. The physiological studies of the NolR protein revealed that NolR was essential for optimal survival in nutrient-limited stationary-phase grown cells or after a heat shock challenge, and it was also required for initial colony formation. Investigations of the intracellular regulation associated with the *nolR* gene expression was carried out using proteomic analysis that consisted of protein separation by 2-DE and protein identification by PMF analysis. Over 200 proteins were found to have detectable changes in levels in *nolR*$^-$ mutant on the silver-stained 2-DE gels. The number of NolR-associated proteins was greater in the stationary phase than in the early exponential phase. Among these NolR-associated proteins, 89 protein spots were isolated from the Coomassie-stained gels. PMF analysis shows that 74 of the 89 spots could be matched to the *S. meliloti* database.

In summary, concluded from these observations, the expression of the *nolR* gene is influenced by key stages of a cell cycle and its metabolism. On the other hand, the *nolR* gene encodes a fine-tuning global regulator, but not an obligatory protein or a lethal factor, although the NolR protein is essential under certain physiological situations. Proteomic analysis combined with genetic approaches also revealed that NolR is part of an integrated regulatory system, which influences some functions that the AHL signalling system affects.

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Characterisation of the regulatory gene *nolR* in *Sinorhizobium meliloti*

Chapter 1 General Introduction

1. *Rhizobium*-legume symbiosis

Members of the bacterial genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium* (collectively referred to as rhizobia) form symbiotic associations with the plant family Leguminosae, culminating in the conversion of atmospheric nitrogen into an organic form which the plant can utilise. This process, termed symbiotic nitrogen fixation, is very significant in agriculture and in the recycling of nitrogen in the biosphere.

In addition to the importance of symbiotic nitrogen fixation, the associations between legumes and nodule bacteria provide good experimental systems to investigate problems of great interest in plant science, such as (1) the specificity of recognition between plants and microbes; (2) the exchange of information between two symbiotic organisms to coordinate their development and functions, and (3) the induction of a major plant development switch, the organogenesis of a root nodule (Denarie & Roche, 1991).

1.1 The Rhizobia: a great variety of nodule bacteria

Micro-organisms forming nitrogen-fixing symbiotic associations with legumes have recently been classified into four genera, *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium* on the basis of their host specificity and of various taxonomical criteria (see Table 1.1). Systematic studies based on nucleic acid hybridisation have revealed that these four genera of Gram-negative bacteria are very distant phylogenetically (Young & Johnston, 1989). *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* are much closer to nonsymbiotic relatives than they are to each other. *Rhizobium* is closely related to *Agrobacterium* which is able to transform dicotyledonous plants and induce tumours, while *Rhodopseudomonas palestris*, a photosynthetic soil bacterium, is the closest known relative of *Bradyrhizobium*. *Azorhizobium* is most closely related to *Xanthobacter*. The reason why these distant genera

have been grouped in the same family, the Rhizobiaceae, is their unique property of establishing a nitrogen-fixing symbiosis with plants of the family Leguminosae.

Table 1.1 *Rhizobium* nodulation and host range (Fisher & Long, 1992)

Bacterial species	Host plant
Sinorhizobium meliloti	*Medicago* (alfalfa), *Melilotus alba* (sweetclover), *Trigonella*
Rhizobium leguminosarum	
biovar *trifolii*	*Trifolium* (clover)
biovar *viciae*	*Pisum* (pea), *Vicia* (vetch)
biovar *phaseoli*	*Phaseolus* (bean)
Rhizobium sp. NGR234	Broad host range; genera including *Vigna, Macroptilium, Lablab* and *Glycine;* Tropical legumes and *Parasponia*
Rhizobium fredii	*Glycine* (soybean), *Vigna* (cowpea), some tropical legumes
Rhizobium tropici	*Phaseolus* (bean), *Leuceana*
Mesorhizobium loti	*Lotus*
Bradyrhizobium japonicum	*Glycine* (soybean), *Vigna* (cowpea)
Azorhizobium caulinodans	*Sesbania* (root and stem nodules)

1.2 The Leguminosae

The Leguminosae is a very important family containing more than 15,000 species grouped in three subfamilies, the Caesalpinioideae, which is the most ancient group, and the more advanced groups Mimosoideae and Papilionoideae. Legumes are very diverse in morphology, habit and the ecology, ranging from arctic annual herbaceous plants to tropical trees (Gallon & Chaplin, 1987a).

The symbiosis between root-nodule bacteria and legumes is specific. However, the degree of specificity varies among rhizobia (Gallon & Chaplin, 1987a). Some strains have a narrow host range, for example, *R. leguminosarum* biovar *trifolii* strains elicit nitrogen-fixing nodules only on species of *Trifolium* (clover) and *Sinorhizobium meliloti* (originally called *Rhizobium meliloti*) strains only on species of the three genera *Medicago*, *Melilotus* and *Trigonella*. There are even strains that discriminate between genotypes within a legume species. Frequently, strains have been reported to form effective nodules on one plant species (or genus) and ineffective ones on another, showing that specificity is not limited to nodulation but also affect the late stages of nodule development and the establishment of a nitrogen-fixing symbiosis.

1.3 Infection and nodulation

Root infection and nodulation is a complex multi-step process involving a highly specific exchange of molecular signals between the plant and bacteria. Figure 1.1 illustrates the major stages of indeterminate nodule development.

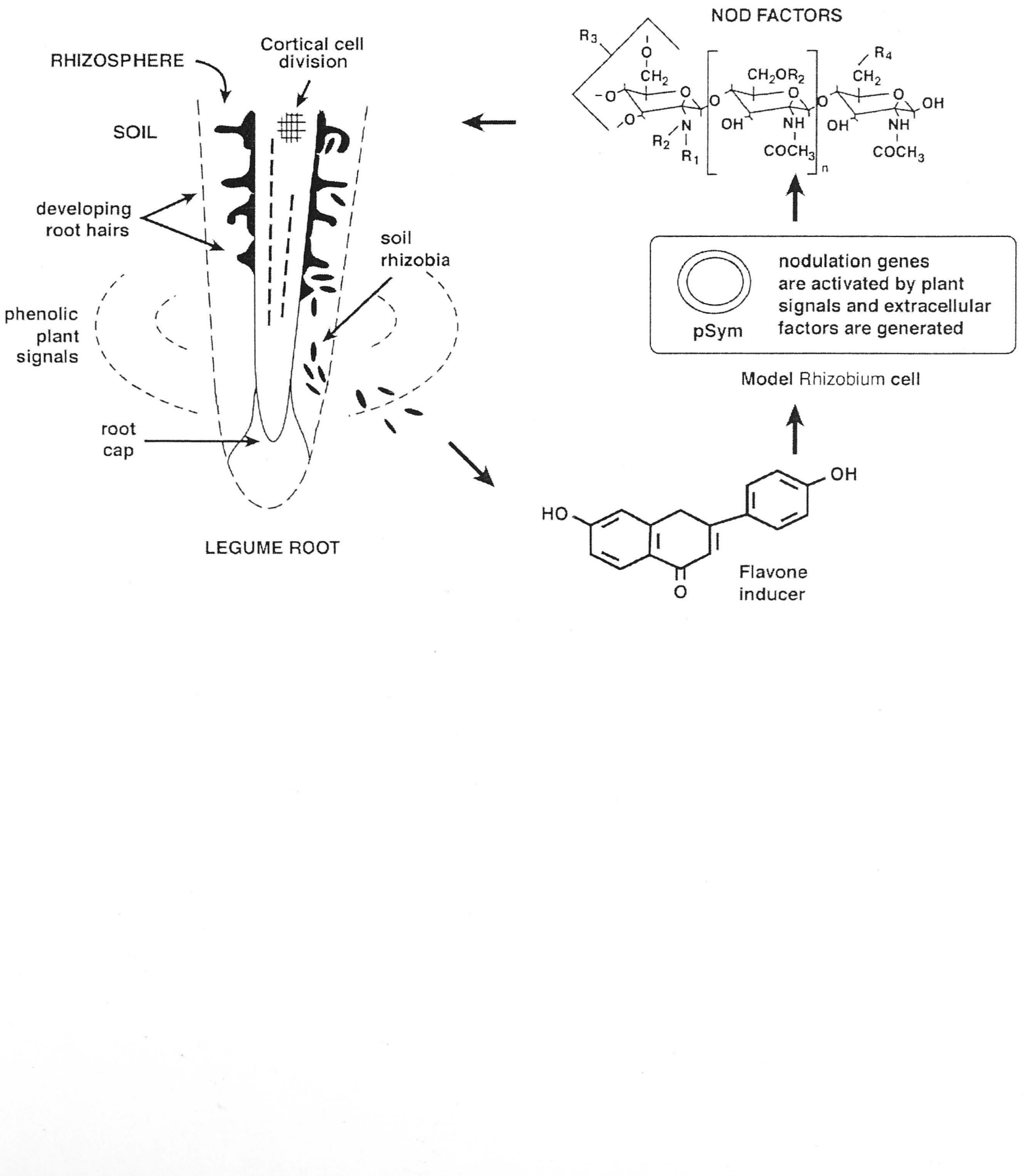

RHIZOSPHERE
Cortical cell division
SOIL
developing root hairs
soil rhizobia
phenolic plant signals
root cap
LEGUME ROOT
NOD FACTORS
pSym
nodulation genes are activated by plant signals and extracellular factors are generated
Model Rhizobium cell
HO
OH
O
Flavone inducer

Figure 1.1 Schematic representation of the signal exchange between the legume root and *Rhizobium* during the early stages of root infection and nodulation. Flavonoid compounds secreted by the legume root enter the rhizosphere and stimulate the expression of nodulation (*nod*) genes in the *Rhizobium* cells. These *nod* genes are involved in the synthesis and secretion of Nod factors, which are able to induce the critical steps leading to nodule formation including root hair curling and cortical cell division.

1.3.1 Recognition

The routes of infection are varied and include simple entry through cracks in the epidermis in peanut or formation of walled infection thread through root hairs in alfalfa, clover, pea and vetch (Rolfe & Gresshoff, 1988b). The mode of infection is a characteristic of the host since the same rhizobial strain can penetrate different host species by either root hair thread or epidermis crack entry, and a given species is infected by the same type whatever the strain. The infection process is initiated by pre-infection events in the rhizosphere, the environment surrounding the root. The developing legume excretes from its roots a variety of compounds which can support the growth of the rhizobia and stimulate the non-specific chemotactic migration of rhizobia to the rhizosphere. Subsequently, the rhizobia attach and multiply on the root surface and elicit a curling of the host's root hairs. The recognition system involves interchanges of a series of chemical signals between the host legume and rhizobia. Rhizobial LCOs (lipo-oligosacharides) play an important role in recognition and nodule initiation. The first visible sign that the infection process has started is the deformation of root hairs (Bauer, 1981).

1.3.2 Formation of infection thread

The next step is the formation of an infection thread, a tubular structure which carries the invading rhizobia cells from root surface into the cortex. The first visible sign is that the hair nucleus and cytoplasm become concentrate around the site of attachment. The site of initiation of an infection thread appears first as a local swelling and the region of cell wall is removed as the rhizobia gain entry into the plant tissue (Rolfe & Gresshoff, 1988a). The mechanism of host cell wall degradation is largely unknown, however it is thought to involve a two-step process (van Spronsen *et al.*, 1994). The first step involves the localised disruption of the cell wall by plant enzymes induces by rhizobial LCOs (lipo-oligosacharides) and then followed by the complete degradation of the cell wall by rhizobial hydrolytic enzymes (van Spronsen *et al.*, 1994).

1.3.3 Development of nodules

Nodules develop on the roots after the cortical cells have been infected by rhizobia. As the infection thread penetrates the outer layer of the root cortex, the cells of the inner cortex begin to divide in advance of the arrival of the infection thread. The infection thread advances towards these dividing cells and the rhizobia inside the thread continue to divide, filling the thread as it elongates. When the infection thread reaches the dividing cortical cells, rhizobia are released from its un-walled tip, into the cytoplasmic space of the primordial cells, surrounded by a plant membrane called the peribacteroid membrane. At this stage, the free-living motile rhizobia undergo morphological and biochemical changes and differentiate into their endosymbiotic form, termed bacteroids (Vasse *et al.*, 1990). The major effect of all these changes is that the bacteroids become capable of nitrogen fixation and the interchange of materials is facilitated between bacteroid and membrane envelope contents, and the cortical cell (Gallon & Chaplin, 1987b). Some bacterial mutants induce the formation of "empty nodules". For instance, *Agrobacterium* transconjugants carrying the *S. meliloti nod* genes or *S. meliloti* mutants defective in exopolysaccharide or β-1,2 glucan synthesis (ndv) (Finan *et al.*, 1985) (Stanfield *et al.*, 1988). These bacterial strains, which don't form infection threads and multiply at a reduced level within intercelluar spaces of the outer root cortex, elicit the formation of nodules.

2. Nodulation genes in rhizobia

The ability of rhizobia to invade legumes and stimulate the host to develop nodules depends upon a variety of genes, including those that control nodulation (*nod*, *noe* and *nol*), host specificity (*hsn*) and the nitrogen-fixation (*nif* and *fix*) genes.

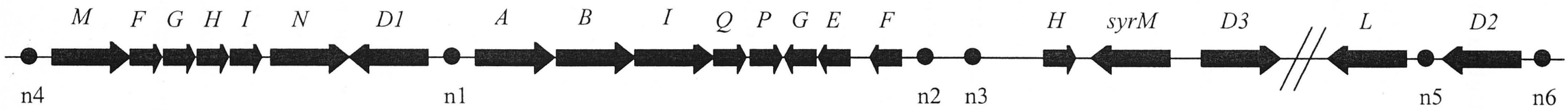
In *Rhizobium* and *Sinorhizobium*, these genes are located on a large symbiotic plasmid (Sym-plasmid or pSym), and on the chromosome in *Bradyrhizobium* and *Azorhizobium*. The *nod, noe* and *nol* genes are involved in Nod factor production and secretion and are required for infection, nodule formation and the control of host-specificity. The *Rhizobium nif* and *fix* genes are required to establish effective nodulation. The *nif* genes are not necessary for the development of the nodule structure, but they are required for effective, nitrogen-fixing nodulation.

Other *Rhizobium* genes and proteins, are required for effective symbiosis and influence nodule formation and nitrogen fixation. A summary of the genes that are necessarily required for effective symbiosis is given in Table 1.2.

2.1 Location and clustering of *nod* genes

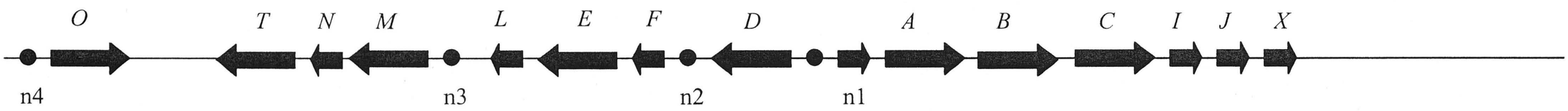
In the *Rhizobium* genus (the fast-growing rhizobia species), the *nod* genes are localised on large indigenous plasmid (Sym plasmids) in the vicinity of nitrogen fixation (*nif*) genes. In *Bradyrhizobium* (the slow-growing rhizobia species) and in *Azorhizobium* the symbiotic genes are located on the chromosome. In the three rhizobial genera the *nod* genes were found in one or in a few gene clusters and those genes clusters are located on a relatively short DNA segment. See Figure 1.2 for detailed location of the *nod* genes.

Sinorhizobium meliloti

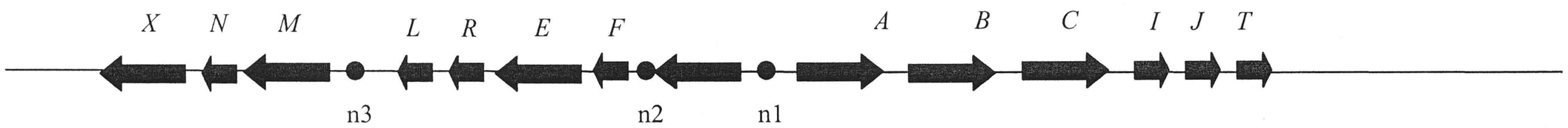


Rhizobium leguminosarum

biovar *viciae*



biovar *trifolli*



Bradyrhizobium japonicum

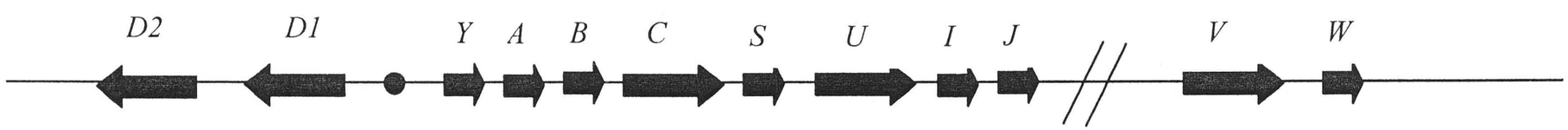


Figure 1.2

2.2 Common nodulation genes

The *nodABC* and *nodD* genes have been found in all rhizobial strains investigated so far. The *nodABC* genes are structurally and functionally conserved, therefore, they termed as common *nod* genes.

The functions of these genes have been determined. NodC is a chitin synthase which links N-acetyl glucosamine in β-1-4 linkages, NodB is a chitin deacetylase which removes the acetyl group from the non-reducing end of the extended chitin chain, and NodA is an acyl transferase which catalyses the addition of the acyl group to the deacetylated chitin oligomer (Djordjevic *et al.*, 1987). Mutation of these *nod* genes completely block the early interactions, root hair deformation and curling and consequently, nodulation. The NodD is a positive regulator of *nod* gene expression and it has common function in rhizobia, but its activation by the plant signal is specific, i.e., it induces *nod* gene expression in a host-dependent manner (Kondorosi, 1989a).

2.3 Nod factors

Flavonoid compounds secreted by the legume root enter the rhizosphere and stimulate the expression of nodulation (*nod*) genes in the *Rhizobium.* These *nod* genes are involved in the synthesis and secretion of bacterial signal molecules called Nod factors (Denarie *et al.*, 1996).

As outlined above *Rhizobium* Nod factors consist of a β-1,4-linked *N*-acetylglucosamine backbone, 4 to 5 residues in length, with the *N*-acetyl group of the terminal, non-reducing residue substituted by an acyl chain. This acyl chain can vary in length and number of unsaturated bonds, and a range of other modifications elsewhere on the Nod factor backbone have also been identified. Each *Rhizobium* strain can produce multiple Nod factor structures and the different modifications on the chitin oligomer backbone of the Nod factors secreted by a particular *Rhizobium* strain can influence the host range, activity and stability of the Nod factors (Denarie *et al.*, 1996).

2.4 Host specific genes

Host specificity is determined at several levels: variation in flavonoid plant signals; the interaction of flavonoids with *nodD* genes produces; and the chemical substituents on the Nod factor. Genes responsible for the host specific modification of the basic Nod factor structure are termed host specific genes. The gene products NodE, NodF and NodG are involved in the synthesis of the unsaturated fatty acids substitutions in the acyl group of the Nod factor: NodH and NodPQ account for the addition of sulphate to the reducing end of the Nod factors backbone at the C_6 position of *S. meliloti*; NodL and NodX are acetyl transferase which add acetate to the C_6 of the non-reducing and reducing ends of the chitin backbone respectively; *nodM* is considered to be a host specific gene, but it appears not to be directly involved in the production of Nod factor, instead it seems to be responsible for the synthesis of Nod factor precursors, by functioning as a glucosamine synthase (Denarie & Roche, 1991).

Studies show that these genes are species specific and cannot be interchanged with parallel functions. However, mutations in these genes do not prevent nodulation, it merely delays the nodulation process or alters the host specificity (Djordjevic *et al.*, 1987). For example, *nodH* and *nodPQ* genes are responsible for sulphonation of Nod factors in *S. meliloti*, but when *nodH* is mutated, the bacterium is unable to infect its usual host alfalfa but is able to infect vetch. In contrast, when the *nodPQ* genes are mutated, the bacterium is able to nodulate both hosts.

Table 1.2 Nodulation (*nod/nol/noe*) genes from *R. leguminosarum* and *S. meliloti* (Cohn *et al.*, 1998) (Denarie *et al.*, 1996)

Gene	Function of protein product	Cellular location	Species biovar*
nodA	*N*-acetyltransferase	Cytoplasm	Common
nodB	Chitin deacetylase	Cytoplasm	Common
nodC	*N*-acetylglucosaminyl transferase	Outer membrane	Common
nodD	DNA-binding protein, transcriptional regulator of nod gene expression	Cytoplasmic membrane	Common
nodE	β-ketoacyl synthase	Cytoplasmic membrane	Rt, Sm
nodF	Acyl carrier protein	Cytoplasm	Rt, Sm
nodG	Alcoholdehydrogenase, ß-ketoacyl reductase		Sm
nodH	Sulfo-transferase		Sm
nodI	Predicted ABC transporter ATP-binding protein, involved in Nod factor secretion	Cytoplasmic membrane	Rt; Sm
nodJ	Predicted ABC transporter permease, acts with *nodI* in Nod factor secretion	Cytoplasmic membrane	Rt; Sm
nodL	6-O-Acetyl-transferase	Cytoplasmic membrane	Rt, Sm
nodM	D-glucosamine synthase		Rt, Sm
nodN	Involved in the efficiency of Nod factor production		Rt, Sm
nodP	ATP-sulphurylase		Sm
nodQ	APS kinase		Sm
nodT	Transit sequences, positive role in cultivar specific nodulation of subterranean clover, Nod factor secretion	Outer membrane protein	Rt
nodX	Encode an O-acetyl transferase that transfers an acetyl group to the reducing terminal residue of LCOs		Rt
nolF, *nolG,* *nolH,* *nolI*	Transport		Sm
nolQ	Unknown		Sm
nolR	Repression of *nod* gene transcription		Sm
nolS	Unknown		Sm
nolZ	Unknown		Sm
noeA	Host-range gene along with *nodL*, *noeB* specifically required for nodulation of particular *Medicago* species		Sm
noeB	see *noeA*		Sm

* *Rhizobium* species and biovars: Rt: *R. leguminosarum* bv. *trifolii,* Sm: *S. meliloti*

3. Fine tuning of nodulation genes in *Sinorhizobium meliloti* by *nolR*

In *Sinorhizobium meliloti*, expression of the nodulation genes (*nod* and *nol* genes) is under both positive and negative controls (Kondorosi *et al.*, 1989b) (Kondorosi *et al.*, 1991). This

dual control provides a mechanism for fine tuning of the expression of nodulation genes, which enables a more successful interaction of the *S.meliloti* with the plant host.

3.1 The positive control of nodulation genes

The expression of these nodulation genes can be activated in the presence of the constitutive *nodD* gene in conjunction with specific signal molecules – flavonoid or isoflavonoid – exude from the roots of the host plants (Peters *et al.*, 1986) (Redmond *et al.*, 1986). The NodD proteins of different *Rhizobium* species interact with different flavonoids. The C-terminal part of NodD determines flavonoid-specificity as well as host-specificity (Savoure *et al.*, 1994) (Spaink *et al.*, 1989). The three copies of *nodD* (*nodD1*, *nodD2* and *nodD3*) have been detected in *S. meliloti* and shown to contribute differently to nodulation of distinct plant hosts. The region required for *nod* gene activation is known as the *nod* - box. In the presence of specific plant signals, various flavonoids, or isoflavones, the NodD protein interacts with the *nod* - box to regulate coordinately the common nodulation genes (Gottfert *et al.*, 1986).

3.2 The finding of negative control of nodulation genes - *nolR*

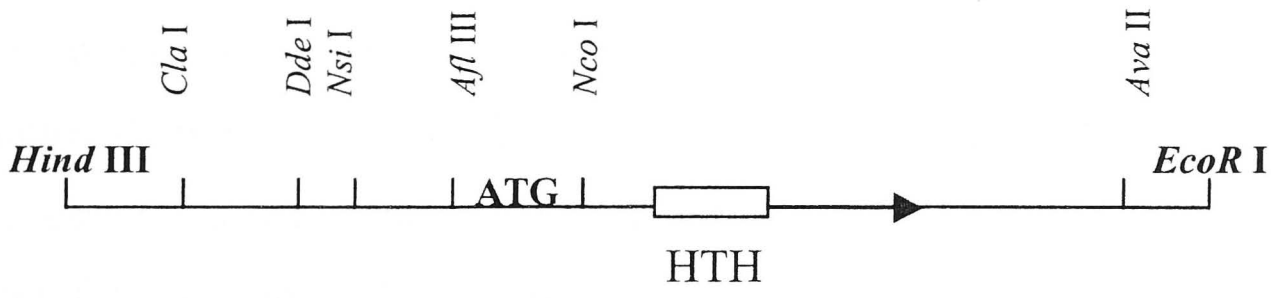
The first indication for the existence of negative regulation of the *nod* genes came from the observation that the induction level of the majority of *Sinorhizobium meliloti* strains was low. The levels of expression of *nod* genes were measured in the two widely used laboratory strains of *S. meliloti* strains AK631 and Rm1021 (Kondorosi *et al.*, 1989b). It was found that the induction of β-galactosidase activity of translational *nodABC-lacZ* fusions by *Medicago sativa* seed exudate or luteolin was low in both strains. However, this low induction of the *nod* genes was not unique, 80% *Sinorhizobium meliloti* strains tested by Kondorosi *et al* had low expression (Kondorosi *et al.*, 1991).

A plasmid containing a reporter region was isolated by using a gene library of strain AK631 (constructed in plasmid pLAFR1), and was mated to strain JM57. JM57 was a derivate of strain Rm1021 carrying *nodC-lacZ* on its pSyma plasmid. This region was later mapped on the chromosome of AK631 (Kondorosi *et al.*, 1989b). Gel retardation assays showed that the repressor binding site was located on a 33-bp fragment, which was downstream of the *nod*-

box overlapping the transcriptional initiation of *nodA* and *nodD1* genes. There was a conserved sequence of 21-bp in n1 and n6 of the *nod* – box region. Further investigation showed that the wild type strain AK631 had a low level of *nodD* gene expression, but in the absence of repressor, the NodD proteins were synthesised at a higher level (Kondorosi *et al.*, 1989b). The encoding gene of the repressor was called *nolR* (According to the nomenclature introduced at the 4th International Symposium on Molecular Genetics of Plant-Microbe Interaction, Acapulco, Mexico, May 15-20, 1988) (Kondorosi *et al.*, 1991).

Figure 1.3 The *nolR* promoter region: Restriction map

The *Cla*I-*Ava*II fragment is 822 base pairs, and the *Dde*I-*Afl*III fragment is 87 base pairs, which is essential for *nolR* expression (Cren *et al.*, 1995).



3.3 The role of the repressor - NolR in nodulation

Kondorosi *et al* compared the nodulation ability of AK631 on *Medicago sativa* in the presence and absence of the repressor. It was found that with the repressor, the percentage of nodulated plants between Day 5 and Day 20 was higher, and the average nodule numbers during the first days of nodulation was also higher (Kondorosi *et al.*, 1989b). The finding that the repressor-producing strain was more efficient in nodule induction was in line with earlier studies showing a low level of *nod* gene expression was sufficient for nodulation and over expressed *nodABC* genes inhibits nodulation (Mulligan & Long, 1985) (Knight *et al.*, 1986). Therefore, for optimal nodulation, a balance between positive and negative control of Nod factors production was required.

It was found that *nolR* was also expressed both at the early stage of infection in the infection thread and inside the bacteroids in the symbiotic zone in the bacteria. Other nodulation genes were found to have different expression levels inside the nodules. In the symbiotic zone the plasmid-borne *nodA-lacZ* fusion was induced in both strains AK631 and EK698 ($NolR^-$ derivate of AK631) while *nodC-lacZ* expression was detected only in EK698. In bacteroids, the fusions were not expressed in either strain, indicating that NolR had no direct role in switching off the *nod* gene in the bacteroid (Cren *et al.*, 1995).

3.4 Identification of NolR

By subcloning, deletion and Tn5 mutagenesis, a region of 594 bp on the chromosome was found to be necessary and sufficient for repressor production. Sequence analysis revealed that chromosomal *nolR* encodes a 13,349 Dalton protein of 122 amino acid residues. Purification of the NolR protein was achieved by affinity chromatography, using long synthetic DNA multimers of the 21 bp conserved repressor-binding sequence and the purified protein was detected corresponding well with the predicted size of NolR deducted from the nucleotide sequence. By using Biogel P30 to study the forms of NolR in the native protein exacts of AK631, it was found in NolR-DNA complex, NolR corresponded to a molecular weight of 22-24 kD, suggesting the active form of NolR bind to the cis-acting elements in a dimeric form. Studies of Cren et al (Cren *et al.*, 1995) found that NolR binds to the (A/T)TTAG-N(9)-A(T/A) target sequences present in its own promoters as well as in the promoters of n1, n4,

and n6 regions of *nod* - box. The binding motif was absent from n2, n3, and n5 regions of *nod* –box promoters, which controlled the expression of the host-specific *nod* genes *nodFEG*, *nodH* and *nodL*. Therefore, NolR differentially down-regulated the expression of the genes involved in core Nod factor synthesis resulting in low amounts which appeared to be optimal for nodulation.

Comparison of NolR to sequence database showed that NolR contained helix-turn-helix DNA binding motif homologous to that of the NodD proteins and other members of the bacterial activator LysR family as well as to the repressor XylR from *B.subtilis* (Kondorosi *et al.*, 1991). Further studies showed that NolR shared homology to and corresponded in both its size and structure to a group of small bacterial regulatory proteins. Most of the known members of this family were involved in the regulation of heavy metal resistance operons (Kiss *et al.*, 1998). The possible dual roles of *nolR* makes it an ideal subject to study the gene regulation in *S.meliloti*.

3.5 The *nolR* promoter and trans-acting factors

It has been proposed that expression of the *nolR* gene was regulated by proteins binding to the promoter region containing 87 bp sequence, providing full expression of *nolR* (Cren *et al.*, 1995). Gel retardation assays showed that *nolR* was regulated by its own products - NolR. Moreover, a group of proteins were also found to bind to the two distinct *nolR* promoter regions that differed from the NolR-binding site. These studies showed that NolR had at least two trans-acting factor roles, one as a factor able to down-regulated its own transcription and the other to regulate plasmid-located nodulation gene transcription.

3.6 Conservation of *nolR*

Conservation of *nolR* was studied by Southern blot analysis in different genera of legume symbionts and in *Agrobacterium* spp. The *nolR* probe of 285 bp fragment representing 80% of the *nolR* coding region, was hybridised to *EcoR*I-digested genomic DNA from strains of different genera of the *Rhizobiaceae* family (Kiss *et al.*, 1998). Based on the strength of hybridisation, conservation of the *nolR*-related sequences was higher in the *Sinorhizobium* genus than in the *Rhizobium* genus. In *Sinorhizobium* and *Rhizobium* Genera, a low level of

nod gene expression was observed, but not in *Bradyrhizobium*, *Azorhizobium* and *Agrobacterium* genera. Genomic DNA from *R. Leguminosarum* did not hybridise with the repressor probe, indicating that *nolR* is either missing or it is not highly conserved in these bacteria. However, the isolated *nolR* gene from *R. Leguminosarum* bv. *viciae* strain TOM showed similar functions to that of *S. meliloti* by repressing the expression of both the *nodABCIJ* and the *nodD* genes, resulting in decreased *Nod* factor production. It was also found that the presence of a functional *nolR* gene in *R. Leguminosarum* was correlated with an increased rate and extent of nodulation of pea than those that lacked the NolR repressor. The conserved structure, the location of the DNA-binding domain, and the similar size of NolR proteins, compared with a family of small bacteria regulatory proteins revealed that NolR belongs to this family (Kiss *et al.*, 1998).

3.7 *S.meliloti* strain 1021 has an inactivated NolR protein

Although *nolR* is highly conserved among rhizobia - not only the *nolR* gene but also the active NolR protein was detected in the majority of the in *S.meliloti* strains – however, the story is different with strain Rm1021, which is the most widely used laboratory strain. Rm1021 failed to produce the active repressor protein. Even though the $NolR^-$ mutant of strain Rm1021 nodulated alfalfa less efficiently than its isogenic counterpart containing the intact NolR protein (Kondorosi *et al.*, 1989b).

It was first found that protein extracts from strains AK631 and Rm1021 interacted differently with the 5' upstream region of the *nodABC* transcriptional unit (Kondorosi *et al.*, 1989b). Moreover, the expression of *nod* genes of strain 1021 was significantly higher than in strain AK631, indicating the lack of functional NolR in strain 1021 (Cren *et al.*, 1994). The nucleotide sequence carrying *nolR* fragment of strain 1021 was compared to that of strain AK631. It was found that at position 519, an insertion of an adenosine residue had occurred, resulting in a frame-shift in the coding region of the C-terminal part of protein. Because of this, the last 19 amino acids were different in strain 1021. The insertion resulted in the open reading frame being elongated by 50 amino acids and a protein of 172 amino acids' long and 18.8 kD of its molecular weight (Cren *et al.*, 1994).

A chimeric *nolR* gene was constructed containing the *nolR* promoter region encoding the N-terminal part from strain 1021 and the rest of *nolR* fragment from strain AK631. The gene

product of this molecule had an identical NolR to the repressor from strain AK631, indicating that the C-terminal part of the protein was essential for DNA binding (Cren *et al.*, 1994). The C-terminal of the repressor might be involved in the recognition of binding site, or the process of dimerization which is essential for binding, or both.

3.8 Proteomic analysis of *nolR* function

Chen *et al* (Chen *et al.*, 2000) applied proteomic analysis to investigate the intracellular regulation associated with *nolR* gene expression in *Sinorhizobium meliloti* strain AK631 and EK698, a *Tn5*-induced mutant of AK631. Total soluble proteins of strain AK631 and EK698 were compared and were shown to have 189 proteins altered significantly on silver stained 2-D gels. Of this group of proteins 52 *nolR*-responsive spots could be reproducibly detected and isolated from the *nolR* mutant on Coomassie stained 2D gels. N-terminal micro-sequencing or Mass Spectrometry of these proteins showed that they were involved in the TCA cycle, the stress response, protein synthesis and other cellular metabolic functions. The results indicated that the NolR function can act directly or indirectly as a positive regulator (Chen *et al.*, 2000). The proposed dual role of the NolR as a repressor and also as an enhancer involved in the broad spectrum of bacterial metabolic functions suggested that *nolR* is a global regulator.

3.9 Regulation of *nolR* by luteolin addition

Moreover, it was reported that the flavonoid – luteolin, which is an inducer during the symbiotic interaction, had an inhibitory effect on the expression of *nolR*. In the presence of luteolin, the transcription level was around two - fold lower in both wild type ($nolR^+$) and mutant ($nolR^-$) strains (Cren *et al.*, 1995). The proteomic analysis also revealed that the expression levels of 22 non-pSyma plasmid proteins were influenced by luteolin. Therefore, it was concluded that luteolin could have both positive and negative regulatory roles in *S.meliloti* (Chen *et al.*, 2000).

4. Symbiotic nitrogen fixation research in the postgenomics era

Nitrogen-fixing symbioses between legumes and rhizobia are important for sustainable agriculture and contribute significantly to the global nitrogen cycle. In the past three decades,

an enormous amount has been learned about rhizobial genes necessary for the symbiotic enterprise (Long, 2001). However, it has not been possible to extend nitrogen fixation to nonlegumes simply by modifying rhizobia. And plants require a core set of genes to recognize rhizobia and provide them access to the purpose-built environment of the nodule. The plant genes required for symbiotic nitrogen fixation have eluded largely molecular geneticists. Functional genomics brings together high throughput genetics with multiparallel analysis of gene transcripts, proteins and metabolites to uncover a truly holistic picture of life (Colebatch *et al.*, 2002b). Therefore, functional genomics approaches are likely to play a key role in identifying important gene and their precise roles in symbiosis as well (Colebatch *et al.*, 2002a).

4.1 Legume functional genomics

Classical forward genetics has led to the identification of many genetic loci that are essential for symbiotic nitrogen fixation in different legume species. Mutations in these genes lead to hypernodulation or supernodulation phenotypes. Most of the mutants were produced by chemical mutagens, so the indication is: map-based cloning currently represents a major bottleneck to gene discovery in legumes.

Several relatively recent developments promise to accelerate the isolation of important symbiotic genes in legumes via forward genetics. These developments will also provide a foundation for rapid genetics and functional genomics (Colebatch *et al.*, 2002a). The high throughput analysis of transcriptome, proteome, and metabolome of legumes allow the identification of genetic networks and sets of genes that work together during development or in nodule metabolism.

4.2 *Rhizobium* functional genomics

Functional genomics is set to provide new insights into rhizobia. In fact proteomics is valuable in identifying regulatory networks in rhizobia. Subsequent proteome analysis of proteins with altered mobility in 2D gels could identify targets of certain proteins (Guerreiro *et al.*, 1998) and in bacteroid state, (Natera *et al.*, 2000). Studies like these will help to

uncover networks of co-ordinately regulated genes that may play cooperative roles in nodule formation or function.

Therefore, combining genetic approaches with proteomic tools enables biochemical and reverse genetics experiments to be performed that will address the physiological functions of many proteins involved in symbiotic nitrogen fixation.

4.3 *Sinorhizobium meliloti* strain 1021 DNA sequencing - entering the post-genome era with *Rhizobium*

Proteome analysis provides a sensitive tool to examine the functional organisation of the *Sinorhizobium meliloti* genome and the intracellular gene interactions between replicons (Guerreiro *et al.*, 1998). Furthermore, proteome analysis provides a powerful analytical tool to complement the genome sequencing of the model micro-symbiont *Sinorhizobium meliloti* strain Rm1021. Hence, 2-dimensional gel electrophoresis of extractable cellular protein, coupled to post separation analysis, has been used to establish a proteome reference map for strain 1021 (Guerreiro *et al.*, 1999).

Sinorhizobium meliloti (previously known as *Rhizobium meliloti*) strain 1021 is a free-living microsymbiont whose genome consists of three replicons. These replicons consist of a chromosome of 3,654,135 bp and two large plasmids containing genes involved in symbiosis that are thus labelled pSyma and pSymb of 1,354,226 bp and 1,683,333bp, respectively.

4.3.1 Analysis of the chromosome sequence of strain 1021

The genome of this microsymbiont was sequenced by an international consortium of six laboratories in Europe and North America (Capela *et al.*, 1999). The project involved shotgun sequencing of 50 ordered recombinant bacterial artificial chromosome clones that cover the whole replicon. Sequence analysis and annotation were managed with the Integrated Annotation Tool web-based semi-automated annotation environment. Protein-coding ORFs were predicated and proteins were classified according to the Riley rules, modified to account for nodulation and nitrogen-fixation genes (Capela *et al.*, 2001) (Galibert *et al.*, 2001 Jul).

The chromosome sequence of *S. meliloti* has revealed that, besides the housekeeping genes (i.e., genes involved in nucleic acid and protein metabolism as well as universal biosynthetic pathways), this replicon also carries genetic information for mobility and chemotaxis, as well as stress responses such as adaptation to atypical conditions and oxidative stress protection (Capela *et al.*, 2001).

4.3.2 Analysis of the pSymA megaplasmid

Of the three replicons in *S. meloloti*, the megaplasmid pSymA is the smallest. The pSymA of Strain 1021 more closely resembles the plasmid of related bacteria than a true bacterial chromosome (Honeycutt *et al.*, 1993). In addition to the already known genes on pSymA (i.e. *nodD1*, *nodD2*, *nodD3*, *fix* and *nif* etc), which are necessary for nodulation and nitrogen fixation, the sequence analysis revealed that many pSymA genes involved in nitrogen metabolism have significant roles in providing versatility for dealing with nitrogen in many oxidation states and chemical combinations (Barnett *et al.*, 2001): one in twelve of the genes annotated encoded proteins related to nitrogen metabolism. A number of ORFs specified by pSymA may be involved in stress responses, including three for cold shock and one for heat shock. It is also important for a symbiotic soil bacterium to be able to withstand environmental oxidative stresses. Physiologically, low oxygen conditions characterize the nodule environment and may be encountered by rhizobia in soil. Two predicated haloperoxidases could be part of the mechanism of dealing with low oxygen condition.

The invaluable source of the pSymA DNA sequence will facilitate the studies of individual genes that can be viewed in the context of organism's potential and as the result of the action of a large set of possibilities (Barnett *et al.*, 2001).

4.3.3 Analysis of the pSymB megaplasmid

The 1683 kb megaplasmid of pSymB of *S. meliloti* is known to carry various gene clusters involved in exopolysaccharide (EPS) synthesis, C4-dicarboxylate transport and lactose metabolism (Finan *et al.*, 1986; Muller *et al.*, 1993).

The sequence analysis and annotation of pSymB revealed that this megaplasmid has a high gene density, similar to that of the bacterial chromosome (Finan *et al.*, 2001). Two major

observations from the analysis of the annotated pSymB sequence are: firstly, there are a large number of solute transport systems and genes involved in polysaccharide synthesis. Secondly, many genes that have potential catabolic activities such as alcohol dehydrogenases (Finan *et al.*, 2001). Thus, pSymB endows the bacteria with the ability to take up and oxidize many different compounds from the soil. Although pSymB doesn't play a direct role in nodulation and nitrogen fixation, it does play an important role in adaptation to the endosymbiotic lifestyle. In addition, pSymB encodes for several detoxification and antibiotic resistance functions. Therefore, pSymB is supposed to play a role in the survival of bacterium under the diverse nutritional living conditions in the soil and rhizosphere.

5. Stationary phase survival and quorum sensing

In the natural environment bacteria seldom encounter conditions that permit continuous balanced growth. When nutrients are plentiful, bacteria can sustain relatively fast growth. But under real conditions, bacteria are nutritionally starved most of the time. Still, these organisms can survive for extremely long periods in the absence of nutrients (Kolter *et al.*, 1993).

5.1 Stationary phase – a special phase in the life cycle of bacteria

Laboratory conditions do not exactly reflect the environment the bacteria find in nature. However, one can simulate periods of nutrient shortage and prolonged period of starvation by exposing bacteria in synthetic media. In most media, exponentially growing cells quickly use up the available nutrients and cease their exponential increase in biomass, thus enter a phase of the culture referred to as 'stationary phase' (Kolter *et al.*, 1993).

Bacteria in the stationary phase have attracted the attention from researchers in recent years, and with the application of modern genetic and biochemical approaches, more information has become available and understood regarding to the molecular mechanisms and defence systems that bacteria utilise to survive in the stationary phase.

5.1.1 Bacterial adaptation to stationary phase

For spore-forming bacteria such as *Bacillus subtilis*, an increase resistance is manifested by the formation of spores. In other gram-negative bacteria, such as *E.coli*, the molecular adaptation into the stationary phase can be viewed at several levels (Ishihama, 1997).

5.1.1.1 Morphological and physiological adaptation

In the stationary phase, cells can undergo a physical and morphological adaptation concomitantly acquiring a dormant state and a resistance against physical and chemical stresses. The stationary-phase bacteria achieve a multi-resistant state, becoming more thermo-tolerant and more resistant to oxidative acid and osmotic stresses in late exponential phase (Loewen & Hengge-Aronis, 1994).

5.1.1.2 The sigma factor

Proteins synthesised by starved cells during the entry into stationary phase are involved mainly in the maintaining viability during the prolonged starvation. It was found that the starvation-induced expression of many genes is controlled by an alternative 'sigma factor', known as δ^S. The δ^S, encoded by the *rpoS* gene is expressed maximally at the onset of the stationary stage. In *E.coli*, δ^S has seven subunits. The stationary phase related changes are mainly mediated by the modulation of the specificity of RNA polymerase by the replacement of the promoter recognition subunit δ^{70} with δ^S. Currently about 50 stationary specific genes have been identified, some of which have been known to be transcribed by the $E\delta^S$ holoenzyme (Loewen & Hengge-Aronis, 1994) (Merrick, 1993).

5.1.1.3 Multiple pathways for stationary adaptation

The highly significant difference in survival rates between *rpoS*$^+$ and *rpoS*$^-$ strains strongly indicated the role of δ^S in the stationary phase survival. However, the fact that some *E.coli* strains lacking δ^S can successfully adapt to the stationary phase, suggested that stationary phase survival has a complex modulation and there are multiple pathways for stationary phase adaptation (Ishihama, 1997).

5.2 Quorum sensing and entry into stationary phase

In recent years, it has become clear that many important behaviours in bacteria are regulated in a population density-dependent manner. Cell-to-cell signalling is termed 'quorum sensing' and has now been used to describe bacterial intercellular communication that involves small diffusible signal molecules, which leads to phenotypic changes in the cell population.

5.2.1 Signals in quorum sensing

The term 'quorum sensing' was coined to describe *N*-acyl homoserine lactones (AHL)-mediated signalling. Once considered exclusive to a few marine vibrios, AHL- mediated QS has now been demonstrated in diverse Gram-negative genera including *Agrobacterium, Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Enterobacter, Erwinia, Hafnia, Nitrosomonas, Obesumbacterium, Pantoea, Pseudomonas, Rahhnella, Ralstonia, Rhodobacter, Rhizobium, Serratia and Yersinia* (Lazazzera, 2000). The key protein components are the LuxI family of the AHL synthases and the LuxR family of transcriptional activators. A second family of AHL synthases, LuxM, lacks the homology with the LuxI family but appears to catalyse AHL formation from the same substrates as the LuxI proteins. There are also other quorum sensing signal molecules unrelated to AHLs (Lazazzera, 2000).

5.2.2 Regulating the production of AHLs by nutritional conditions and stationary phase

Williams et al (Thorne & Williams, 1997) reported that the nitrogen-fixing bacterium *Rhizobium leguminosarum* bv. *phaseoli* had general starvation response (i.e. the starved cells were cross-protected against pH, heat, osmotic and oxidate shock) similar to that found in other bacteria. Further studies have found that following starvation by exhaustion of carbon or nitrogen, but not of phosphorus, the survival of cultures was dependent in the cell density at entry into stationary phase. High-density cultures survived with little loss of viability in prolonged stationary phase. In contrast, low-density cultures lost viability rapidly. However, the decreased survival of cells at low-density could be rescued by the additions of an AHL, which was previously demonstrated to have a role in controlling cell density-dependent phenomena (Thorne & Williams, 1999).

In *E.coli*, the synthesis of AHLs arises as a natural response to starvation and stationary phase. In a screen for genes regulated by quorum sensing, it was found that a subset of these genes is co-regulated by the sigma factor. And it was found that in many Gram-negative bacteria, the transcription of the gene encoding the sigma factor is regulated by AHLs. Therefore, the AHL mediated quorum sensing is a part of the integral component of bacterial gene regulatory network that permits an individual bacterium within a community to facilitate adaptation to the chemical and physical status of the external environment (Withers *et al.*, 2001).

6. Scope of this thesis

Sinorhizobium meliloti has been chosen by the international research community as the model microorganism to investigate nodulation, nitrogen fixation and the symbiotic interaction between plant and bacterium. With the completion of the genome database of *S. meliloti* strain 1021, focusing is now on the "functional" proteomics of the organism. An important motivation for "functional" proteomics is to find ways of concentrating on those proteins/genes that are involved in a particular biological function of interest. The *nolR* gene of *S. meliloti*, which appears to have both negative and positive regulator functions, became an ideal candidate to study the "functional" proteomincs of *S. meliloti*.

The aim of this thesis is to identify factors involved in the regulation of *nolR* gene expression, proteins regulated by the NolR protein and physiological roles of the *nolR* gene. The objectives of this thesis are:

1. Utilising *S. meliloti* $nolR^+$ and $nolR^-$ strains carrying *nolR::lacZ* fusion to investigate the factors that affect the expression of *nolR*, including:
    - bacterial growth stages
    - bacterial growth medium
    - stress conditions such as heat-shock, low pH/ high pH and low oxygen
    - the *nod* gene inducer luteolin
    - quorum sensing signals

2. Exploring physiological roles of NolR in cellular survival under the following conditions:
    - in cell growth and survival in nutrient-starved media
    - in cell survival after heat-shock challenge
    - in bacterial colony development
    - in cell growth and survival under acidic conditions

3. Use proteomic analysis to examine the possible gene networks that might be influenced by a functional NolR protein by:
    - detecting number of proteins differentially altered in levels in the *nolR*- derivative
    - identification of proteins regulated by NolR
    - examining functions of NolR-regulated proteins in metabolic pathways and cellular functions
    - characteristics of the NolR protein in regulation of cellular protein synthesis

Finally, conclude from these experimental situations, features about the regulatory protein NolR and its role as a global cellular regulator.

Chapter 2 Materials and methods

1. Media

All media was prepared with Milli-RO water (reverse-osmosis purified water; Millipore Co., Bedford, MA) or distilled water, unless otherwise indicated. For solid media, DIFCO agar (Difco Laboratories, Detroit, Michigan) was added at 15 $g.L^{-1}$ media unless otherwise indicated.

Bergensen's modified media (Bergensen, 1961)

Chemical	Final concentration (per 1000 mL)
Macronutrients	
$CaCl_2.2H_2O$	40 mg
$FeCl_3$	3 mg
$MgSO_4.7H_2O$	80 mg
$Na_2HPO_4.12H_2O$	360 mg
Micronutrients[a]	
$CoCl.6H_2O$	0.25 mg
$CuSO_4.5H_2O$	0.25 mg
H_3BO_3	3 mg
$MnSO_4.4H_2O$	10 mg
$Na_2MoO_4.2H_2O$	0.25 mg
$ZnSO_4.7H_2O$	3 mg
Vitamins	
Biotin	0.2 mg
Thiamine-HCl	2 mg
Organic supplements	
Mannitol	3.0 g (solid media) 10.0 g (liquid media)
Monosodium glutamate	0.5 g
Yeast extract	0.5 g
pH (adjust to)	6.8

[a] Gamborg's trace elements (Gamborg & Eveleigh, 1968)

BIII media (Dazzo, 1982)

Chemical	Final concentration (per 1000 mL)
Macronutrients	
$CaCl_2.2H_2O$	7 mg
K_2HPO_4	230 mg
$MgSO_4.7H_2O$	100 mg
Micronutrients	
$CoCl.6H_2O$	0.059 mg
$CuSO_4.5H_2O$	0.005 mg
$FeSO_4.7H_2O$	0.125 mg
H_3BO_3	0.145 mg
$MnCl_2.4H_2O$	0.043 mg

$Na_2MoO_4.2H_2O$	0.174 mg
$ZnSO_4.7H_2O$	0.180 mg
Vitamins	
Biotin	0.02 mg
Calcium pantothenate	0.02 mg
Inositol	0.120 mg
p-amino benzoic acid	0.02 mg
Pyridoxine-HCl	0.02 mg
Nicotinic acid	0.02 mg
Riboflavin	0.02 mg
Thiamine-HCl	0.02 mg
Organic supplements	
Nitrilotriacetate[b]	0.007 g
Mannitol	10 g
Monosodium glutamate	1.10 g
Buffers	Na_2HPO_4 (pH 7.0)
(final concentration)	0.05 mM
pH (adjust to)	6.9

[b] Adjust to pH 5.0 before adding to prevent precipitation.

TA(YTB) media (Kondorosi *et al.*, 1984)

Chemical	Final concentration (per 1000 mL)
Tryptone	10g
Yeast extract	1g
NaCl	5g
$CaCl_2$	147mg
$MgCl_2$	203mg

TMR (Rogers modifications) media (Roger *et al.*, 1985)

Chemical	Final concentration (per 1000 mL)
TMR salts	50ml
MQ-water	940ml
$FeCl_3$	1ml
Sucrose	10g
$MgSO_4.7H_2O$(20%)	2ml
$CaCl_22H_2O$(25%)	0.2ml
TMR vitamins	2.5ml
Trace elements	1ml
Final pH will be 7.0 without adjust	

TMR salts (20 fold stock)

Chemical	Final concentration (per 1000 mL)
K_2HPO_4	20.8g
KH_2PO_4	8.8g
NaCl	2.0g
$(NH_4)_2SO_4$	10.0g

TMR vitamins

Chemical	Final concentration (per 100 mL)
Thiamine	40mg
Biotin	20mg
Nicotinic acid	40mg
Pyridoxine HCl	40mg

GTS media (Kondorosi, E.)

Chemical	Final concentration (per 1000 mL)
Glucose	2g
NH_4Cl	1g
KH_2PO_4	0.1g
NaCl	1g
Tris	3g
Sodium succinate	2.7g
MQ-H_2O	995ml
Adjust pH to 7.5 and autoclave, then add sterile stock solutions	

GTS Stock solutions

Chemical	Final concentration (per 1000 mL)
1M $MgSO_4$	1ml
0.1M $CaCl_2$	1ml
1mM $FeCl_3$	1ml
Gibson trace elements (1000 fold)	1ml
biotin	2mg

Gibson trace elements (1000 fold)

Chemical	Final concentration (per 1000 mL)
H_3BO_3	3g
$CuSO_4.5H_2O$	0.125g
$Na_2MoO_4.2H_2O$	0.12g
$CoCl_2.6H_2O$	0.119g
$ZnSO_4.7H_2O$	0.287g
$MnSO_4.H_2O$	2.23g

2. Bacterial strains and methods

2.1 Bacterial strains

Bacteria strains used in this study are:

***Sinorhizobium meliloti* strains**

Strains	Characterisation	References
AK631	wild type, Rm41, *exoB*, $NolR^+$	(Kondorosi *et al.*, 1989)
EK698	AK631, *nolR::Tn5*	(Kondorosi *et al.*, 1991)
ZB138	large *nod–nif* deletion of AK631	(Kondorosi *et al.*, 1984)

241	AK631 with *nolR::lacZ* on chromosome	(Cren *et al.*, 1994)
249	ZB138 with *nolR::lacZ* on chromosome	(Cren *et al.*, 1994)
273	ZB138 with *nolR::lacZ* on chromosome, *nolR::Tn5*	(Cren *et al.*, 1994)

***E.coli* strains**

Strains	**Characterisation**	**References**
HB101(pRT311)	*recA pro leu* Tc^r with the plasmid pRT311, which has *nodA::lacZ* - donor	(Innes *et al.*, 1985)
ANU1073	Km^r with - helper	

2.2 Storage of strains

Strains were grown on TA media plate containing the appropriate antibiotics (if required) at 28°C incubator. Stocks were maintained by collecting the freshly growing bacteria into 0.8ml sterile 20% (v/v) glycerol and 0.8ml BMM liquid media in 1.8 ml Nunc CryoTubes (Intermed, Denmark), After sealed with parafilm and labelled, the tubes are kept at -20°C and -80°C respectively.

2.3 Beta-galactosidase assay

Overnight cultures of *Sinorhizobium meliloti* strains carrying a translational *lacZ* fusion to the *nolR* promoter were grown at 28°C in TA medium and diluted to OD_{600} of 0.05 to initiate the experiments. Samples were removed to do beta-galactosidase assays by a modified Miller method (Miller, 1972) which was as follows:

to 400uL Z Buffer, 20uL 1% SDS, 10uL Chloroform and 150uL ONPG (4mg/ml), 400uL bacteria culture is added to start the reaction. The reaction was carried out in 37°C water bath. The reaction was stopped by adding 0.4 mL 1M Na_2CO_3 solution after sufficient yellow colour has developed. Stop watch to used to record the total reaction time. The Miller units are calculated using the following formula:

$$\text{Miller units} = (1000 \times OD_{420} - 1.75 \times OD_{550}) / (T \times V \times OD_{600})$$

OD_{420} and OD_{550} are read from the reaction mixture,

OD_{600} reflects the cell density just before assay,

T is the time of reaction in minutes,

V is the volume of bacterial culture used in the assay in ml.

(In most situations, the $1.75 \times OD_{550}$ is very small that it can be omitted.)

Beta-galactosidase assays also were done with extracted proteins from strains Rm1021, Rm1021(p*nolR-lacZ*), AK631 and strain 241. It was found that in terms of specific activity (Miller units/min/μg), the cell-free proteins had the same activity pattern as with the bacterial cells. After comparing the two procedures we concluded that the modified beta-galactosidase was reliable.

2.4 Medium effect experiment

Different media were used to test the medium effect on *nolR* expression.

Overnight cultures of strain 241 grown in TA medium were diluted to OD_{600} of 0.05 in same volume of fresh TA and TMR to initiate experiment.

Overnight cultures of strain 249 grown in BIII medium were re-inoculated into fresh BIII medium at the of optical density of OD_{600} of 0.2 to initiate the experiment.

2.5 The pH shock treatment

To the cultures of strain 241 grown in TA medium to mid-exponential phase (OD_{600} around 0.8-0.9), 40mM sterile Mes buffer was added to adjust the pH of the cultures down to pH 5.7, and 1M sterile Tris Base solution was added to adjust the pH of the cultures up to pH8.3 respectively. The culture without pH shock was control. The culture growth and *nolR* expression were measured at different time points.

2.6 The anaerobic condition experiment

The flasks containing the overnight cultures of strain 241 grown in TA medium were exposed to anaerobic growth conditions through the tube of a sealed rubber stopper connected to a nitrogen storage tank. Another tube was used as gas exhaust. The air pressure of nitrogen supply was maintained at 10kPa. To resume the oxygen supply, the nitrogen treated culture was transferred to a sterilised empty flask of the same size and was put back on the 28°C shaker to resume aerobic conditions.

2.7 The stationary phase survival experiment

Single colonies of *Sinorhizobium meliloti* strains AK631(pPH1JI), EK698 were transferred from TA plates to 5ml TA medium in McCartney bottles at 28°C shaker and grown to stationary phase. The culture then was diluted to 200ml freshly prepared BIII medium or 200ml modified BIII medium (with 0.6g/L sodium-glutamate, 0.3g/L mannitol. Other ingredients are same) in 500ml flasks to start the experiment.

A small volume of culture was removed every two days to do viable counts on TA plates. Isolated colonies at certain dilution level were randomly selected from TA plates. After size measurement under microscope, the colonies were cut out individually by surgical blades and put into 100ul 0.85% NaCl solution to do further serial dilution and plated out on TA plates to do viable count.

2.8 Temperature treatment experiment

Single colonies *Sinorhizobium meliloti* strains AK631, EK698 were transferred from TA plates to 5ml TA medium in McCartney bottles at 28°C shaker and grown to stationary phase. The culture then was diluted to 50ml freshly prepared BIII medium in 100ml flasks, and grow to the stationary phase. To start the experiment, a group of eppendorf tubes with 100ul of culture in each were put in to rack in 44°C, 46°C water bath respectively. 2 tubes were taken out every 2 hours to carry out viable count.

3. Two-dimensional gel electrophoresis

3.1 2-DE Solutions

All solutions were prepared with the highest available grade reagents. High purity MilliQ® water (Millipore Co., MA, USA) with resistance of greater than 18 MΩ.cm was used throughout. Gloves were worn at all times.

0.5 M Tris-HCl stock solution

15.25 g of Tris base was dissolved in 75 mL of MilliQ water and the pH adjusted to 8.8 using 1M HCl. The volume was made up to 250 mL and the solution filtered through a 0.45 μm Sterile Acrodisc® syringe filter (Gelman Sciences, MI, USA) before storing at 4°C.

Low-salt washing buffer

Chemical	Quantity	Final concentration
KCl	0.22 g	3.0 mM
KH_2PO_4	0.20 g	1.5 mM
NaCl	3.97 g	68 mM
NaH_2PO_4	1.24 g	9.0 mM

Made up to 1 L with MQ-water, filtered through 0.45 µm Sterile Acrodisc® filters and stored at 4°C.

Urea sample buffer

Chemical	Quantity	Final concentration
Urea	5.4 g	9 M
DTT	0.1 g	1% (w/v)
CHAPS	0.4 g	4% (w/v)
Bio-lyte® 3-10 ampholytes	200 µL (40% w/v)	0.8% (v/v)
Tris base	0.0424 g	35 mM
Final volume		10 mL

The solution was filtered through a 0.45 µm syringe filter and stored in small aliquots at -80°C for up to 2 months. Sample buffer thawed once should not be refrozen again.

Lysis buffer

Chemical	Quantity	Final concentration
Urea	5.4 g	9 M
DTT	0.1 g	1% (w/v)
CHAPS	0.4 g	4% (w/v)
Bio-lyte® 3-10 ampholytes	100 µL (40% w/v)	0.8% (v/v)
Tris base	0.0424 g	35 mM
PMSF (stock solution)	100 µL	1mM
EDTA (stock solution)	100 µL	5mM
Final volume		10 mL

The solution was filtered through a 0.2 µm syringe filter. The lysis buffer is prepared freshly each time.

100 mM Phenylmethanesulphonyl fluoride (PMSF) stock solution

87 mg of PMSF was made up to 5 mL with absolute ethanol and stored at 4°C.

Lasts for at least 9 months at 4°C.

0.5M EDTA (pH 8.0) stock solution

37.2 g of EDTA was dissolved in 150 mL MilliQ water with vigorous stirring. The pH was adjusted to pH 8.0 with NaOH and made up to 200 mL. The solution was sterilised by autoclaving and stored at 4°C. This solution is stable for months at 4°C.

Rehydration solution

Chemical	Quantity	Final concentration
Urea	7.2 g	8 M
CHAPS	0.075 g	0.5%
DTT	0.03 g	0.2%
Bio-®lyte 3-10 ampholytes	195 µL (40% w/v)	0.52%
Bromophenol blue	90 µL (1%)	0.6%
Final volume		15 mL

The solution was filtered through a 0.45 µm syringe filter prior to use. Only 5 mL was prepared if to be used for IPG strip rehydration in the reswelling tray.

Equilibration solution for 2 x 18 cm (or 24 cm) IPG strips

Chemical	Quantity	Final concentration
0.5 M Tris-HCl, pH 8.8	8 mL	50 mM
Urea	28.8 g	6 M
Glycerol	24 mL	40% (v/v)
SDS	1.6 g	2% (w/v)
Final volume		80 mL

The solution was divided into 2 x 40 mL. 0.8 g dithiothreitol (DTT, 2% w/v) was added to solution A. 1.6 g iodoacetamide (IAA, 4% w/v) and 200 µL of 1% (w/v) bromophenol blue was added to solution B.

3.2 Sample collection and protein extraction

Cultures of *R. meliloti* strains were grown to early exponential phase (optical density at OD_{600} of about 0.1-0.2) or to stationary phase (at OD_{600} of about 1.8-1.9). The bacterial cells were harvested by centrifugation at 7000xg for 15 minutes at 4°C on a GSA rotor. The collected cell pellet was resuspended in 20 mL of ice-cold sample washing buffer and centrifuged at 6000xg for 10 minutes at 4°C on a SS-34 rotor. The above washing step was repeated twice more.

The washed cell pellet was resuspended in five volumes of lysis buffer and vortexed vigorously. Cells were disrupted by sonification on ice (20 bursts of 15 second with 20 second intervals) using a MSE 100 ultrasonic disintegrator at maximum power. Insoluble materials and nucleic acids were pelleted by ultra-centrifugation at 200,000xg for one hour at 4-15°C using a SW 55-Ti rotor in Beckman TL-100 ultracentrifuge. The resulting supernatant was carefully collected. After the protein concentration was determined, small aliquots were stored at -80°C (100µg protein, 500µg protein and 1mg protein) for further use.

3.3 Bradford Protein Assay

Bacterial samples were assayed after extraction using the Bradford protein assay (Bio-Rad Laboratories, CA, USA). Bovine serum albumin (BSA) stock (3mg/mL) was used as a standard and dissolved in sample buffer over a range from 0, 0.15, 0.30, 0.45 and 0.60mg/mL, and the assay was carried out according to the manufacturers instructions. The dye concentrate was diluted 1 in 4 in distilled water and filtered by using 0.45μ filter. A standard assay was adapted for use in a microtitre plate by a proportional reduction in the volumes of sample and dye reagent. The samples were generally assayed as diluted 1 in 10, 1 in 50, and 1 in 100. 10 μL of sample or standard was added to each microtitre plate well (containing 200 μL diluted dye reagent) and thoroughly mixed using a pipette. Standard and sample dilutions were generally assayed in duplicate or triplicate. After a 5 min incubation at room temperature, optical density readings were taken using a microtitre plate reader (LabSystems Multiskan RC, Helsinki, Finland) with a 600nm filter. Standard curve was generated and sample concentrations were calculated according to the standard curve.

3.4 Isoelectric focusing

3.4.1 IPG strip rehydration

Precast, immobilised pH gradient (IPG) gel strips were purchased from Pharmacia-Biotechnology. 24 cm or 18 cm strips covering a pH ranges pH 4 to 7 (linear gradient) were used. IPG strips were rehydrated overnight in rehydration solution in either the Pharmacia Immobiline Dry Strip Reswelling cassette or tray, according to the manufacturers instructions.

3.4.2 First dimension set up

Following rehydration, the IPG strips were gently blotted on filter paper for a few seconds to remove any excess solution and to prevent formation of urea crystals on the gel surface during focusing. The rehydrated IPG strips were immediately aligned in Immobiline DryStrip Kit (Pharmacia-Biotechnology) which had been set up on a Multiphor II electrophoresis unit (Pharmacia-Biotechnology) according to the accompanying instructions. Kerosene was used between the bottom of the DryStrip Kit and the cooling plate which was used to keep the apparatus at a constant temperature of 20°C. The entire sept-up was covered with light paraffin oil (Ondina 18 oil, Shell, Australia). Exposure of the IPG gel strips to air as brief as possible.

3.4.3 Sample application

Protein samples were applied to the anode end of the IPG strips using plastic sample cups (Pharmacia-Biotechnology). Analytical gels which were to be silver stained had a protein loading of 100 to 200 µg of total cellular protein diluted with sample buffer to a volume of 100 µL on to a single IPG strip. Preparative gels required a protein loading up to 1 mg per strip. All samples which had been stored frozen were sonicated in a sonic bath and centrifuged for 5 mins in an Eppendorf centrifuge to remove any insoluble material which may clog on the surface of the application area. 2 µL of 1% bromophenol blue was added to each 100 µL of sample to aid in loading.

3.4.4 Isoelectric focusing protocols

The focusing protocols used depended upon parameters such as pH gradient, separation distance and sample volume. Focusing temperature was maintained at 20°C using a MultiTemp III thermostatic circulator (Pharmacia-Biotechnology), this was critical as any variation would lead toa change in protein pattern.

Multiphor power packs (EPS 3500XL, Pharmacia) were used to apply electrical current for isoelectric focusing. Focusing conditions and times given below varied with the length, pH and linearity of the IPG strips.

18 cm (or 24cm) IPG strips, pH 3-10 and pH 4-7

Step	Voltage (V)	Current (mA)	Power (W)	Volt hours (Vh)	Time (h)
1	150	1	5	18	0:15
2	150	1	5	75	0:30
3	300	1	5	1	0:01
4	300	1	5	1800	7:30
5	3500	1	5	9500	7
6	3500	1	5	189 kVh	54
Total				200 kVh	69:16

At the completion of focusing, the strips were either equilibrated for immediate use on the second dimension or individually wrapped in clean plastic wrap (Glad Wrap, NationalPak, NSW, Australia), labelled and stored at -80°C for up to a few months if required.

3.5 Second dimension - SDS PAGE

3.5.1 Equilibration of the IPG strips

The IPG strips were equilibrated for 10 minutes in Equilibration solution A followed by 10 minutes in Equilibration solution B. The strips were placed on their sides on filter paper for another 10 minutes to drain off excess equilibration solution, which may cause streaking in the second dimension.

3.5.2 Second dimension set up

Pre-cast ExcelGel SDS gels (T = 12 to 14% acrylamide) from Pharmacia were placed on the kerosene covered cooling plate of the Multiphor II unit. Care was taken not to trap any air bubbles beneath the plastic backing of the gel. The cover sheet of the gel was removed. SDS buffer strips (Pharmacia) were placed on the SDS gel according to the manufacturer's instructions. MilliQ-moistened forceps were used to stroke along the back of the buffer strips to remove any trapped air bubbles. The equilibrated IPG strips were placed gel side down about 1 mm from the anodic buffer strip. Forceps were used to gently stroke along the plastic backing of the IPG strips to remove any air bubbles and ensure good contact with the second dimension gel. Sample application pieces were placed under the ends of the plastic IPG backing to absorb any exuded moisture. Low molecular weight markers were pipetted onto extra sample application pieces placed at the cathode end of the IPG strips.

3.5.3 Second dimension running conditions

The gels were run at 6°C as given below:

SDS-PAGE running conditions

Step	Voltage (V)	Current (mA)	Power (W)	Time (h)
1	200	10	30	1:20 h
2	200	10	30	0:15 h
3	600	30	60	4:15 h*

* or until dye front reaches cathode buffer strip.

After the first phase, the run was paused and the IPG strips removed. The run was then continued in Phase 2 for another 15 mins to allow the proteins to stack at the interface of the stacking and

separating gels. The run was stopped and the anodic SDS buffer strip was moved to cover the region where the IPG strips had been. The run was then started for Phase 3 of protein separation and continued until the dye front reached the cathode buffer strip.

3.6 Silver staining

Analytical gels were stained using a long silver nitrate-tetrathionate protocol. Details of the method are given below in the table below and 500 mL of each solution was used per wash unless otherwise stated. Analytical grade chemicals were dedicated for 2-DE only and MilliQ water.All equipment used was thoroughly washed, rinsed in MilliQ water and handled only when wearing gloves. Stock formaldehyde was stored at room temperature and discarded 3 to 6 months after opening or when crystallisation was observed. The gels were stained in clean photographic trays with gentle shaking at room temperature. After completion of the staining and rinsing well, the gels were stored in sealed plastic bags containing a small amount of MilliQ water.

Silver nitrate-tetrathionate staining

Solution	Composition	Number and time of washes
Fixation	10% (v/v) acetic acid 40% (v/v) ethanol	3 x 30 mins
Sensitisation	30% (v/v) ethanol 2.5 g/L Potassium trihyrate 68 g/L sodium acetate trihydrate Make up to 990mL with MQ water and add 10mL 50% (v/v) glutaraldehyde in fume hood	18 hrs (Covered with foils)
Rinse	1L MQ water	6 x 20 mins
Silver solution[a]	2 g/L silver nitrate 0.623 g/L HEPES 700 µL formaldehyde (37%)	2 hrs (Covered with foils)
Rinse	1 L MQ water/gel	10 secs[b]
Developer[c]	30 g/L potassium carbonate anhyrous 12 mg/L sodium thiosulfate 250 µL formaldehyde	6 - 15 mins[d]
Stop	50 g/L Tris base 20 mL/L acetic acid	15-20 mins

[a] Prepare fresh, 30 to 45 mins before use and kept from light.
[b] This step is critical and must be carefully timed. Use stopwatch.
[c] Developer needs to be made fresh each time, 30 to 45 mins before use.
[d] Time varies. Add stopper when enough spots have developed.

3.7 Colloidal Coomassie staining for MALDI-TOF MS

Colloidal Coomassie staining was used for preparative gels (0.5 to 1 mg protein loaded in the first dimension) when proteins were to be analysed using matrix-assisted laser desorption ionisation

time of flight (MALDI-TOF) mass spectrometry. This quantitative staining method, which is modified from (Neuhoff, 1988 #55), uses Coomassie Brilliant Blue G-250. The compositions of the stain and rinsing solution are given below and used analytical grade chemicals and MilliQ water. The staining solution was freshly prepared each time in volumes of 100-250 mL per gel depending on the staining container and discarded after use.

Colloidal Coomassie stain solution 1

Chemical	Quantity	Final Concentration
Ammonium sulphate	170 g	17%
Phosphoric acid (85%)	36 mL	3%
Coomassie G-250	1 g	0.1%
Methanol	340 mL	34%
MilliQ water	make up to 1L	

To prepare the staining solution 1, the ammonium sulphate, phosphoric acid and 300 mL MQwater were combined in a Schotts bottle and dissolved by magnetic stirrer. In a separate bottle, 1g Coomassie G-250 was dissolved in the 5 mL methanol by magnetic stirrer. The two solutions were combined, add 335mL methanol and made up to 1L with MilliQ water. The solution should not be filtered in order to retain the colloidal dye particles.

Colloidal Coomassie staining solution 2 (Stock)

(Australian Proteome Analysis Facility)

Solutions	Chemical	Final Concentration
Stock staining solution A	Ammonium sulphate	10% (w/v)
	Phosphoric acid	2%
Stock staining solution B	Coomassie G-250	5%
Fixation solution	Ammonium sulphate	20%
Neutralisation solution	Tris-base (adjusted to pH6.5 with phosphoric acid)	0.1M

To prepare staining solution 2, 2 mL of stock staining solution B was mixed with 80 mL of stock solution A and then 20mL methanol was added. The solution should not be filtered in order to retain the colloidal dye particles.

Colloidal Coomassie staining process

Step	Solution	Quantity (per gel)	Treatment conditions
1.First stain	Staining solution 1	100 mL	24 hrs in sealed plastic bags, shake slowly
2.Neutralisation	Neutralisation buffer	250 mL	3 mins in staining tray, shake slowly
3.Rinse	Methanol (25%)	100-150 mL	10-20 secs in staining tray, shake slowly
4.Fixation	Fixation solution	200 mL	Overnight in sealed plastic bags, shake slowly
5.Second stain	Staining solution 2	100 mL	Overnight in sealed plastic bags, shake slowly
6.Neutralisation	Neutralisation buffer	250 mL	3 mins in staining tray, shake slowly
7.Rinse	Methanol (25%)	100-150 mL	10-20 secs in staining tray, shake slowly
8.Fixation	Fixation solution	200 mL	Overnight in sealed plastic bags, shake slowly

For further staining, repeat the steps from Step 5 through Step 8.

When the staining process was completed, the gels were stored in sealed plastic bags containing 5% methanol at 4°C.

3.8 Analysis of 2-DE images

3.8.1 Image processing

Silver stained analytical 2-DE gels were digitised at 600 dpi with a UMAX PS-2400X lamp scanner run under Photoshop 3.0 (Adobe, Mountain View, CA) using the transparent scanning mode. Auto adjust detection parameters were used and the images scanned at 30 bits and saved as TIFF files. The files for the image processing with Melanie were stored in the GIF or TIFF(8 or 16 bits). The resulting images were transferred into the UNIX environment of a SUNW, Ultra 5 station (Sun Microsystems, Mountain View, CA) for analysis.

Coomassie stained preparative gels were digitised at 600 dpi using a reflective scanning mode. For Coomassie stained gels, the gels were placed face-down on the scanner and a piece of white paper placed over the back to obtain an optimal reflective image.

3.8.2 Melanie 2.3

Gel analysis and feature detection was done using the Melanie 2.3 image analysis software package (Bio-Rad, Hercules, CA) run under the Unix operating system, OpenWindows 3.6. All gels to be compared were adjusted to the same colour levels and the features (spots) were detected using the same detection parameters to maintain as much consistency as possible. Protein spots (features) common to the compared gels were assigned as 'landmarks' to assist automatic alignment of the gel images before the remaining features were matched between the gels. In this way, features which were new or absent from a set of gels could be identified. On the basis of this gel pattern matching and subsequent quantification, proteins were tentatively assigned as constitutive or altered in expression. Quantification of the optical density (O.D.) of each feature over its area (vol) as a percentage of the total O.D. over the area of whole gel image (%vol) was used as a measure of changes in protein expression. Melanie 2.3 was also used to estimate apparent molecular weights and p*I*s using markers for reference points.

3.9 Protein characterisation by MALDI-TOF MS analysis

3.9.1 Preparation of samples for MALDI-TOF MS analysis

MALDI-TOF MS was used to determine the masses of the peptides generated following enzymatic digestion of an individual isolated protein. Spots were excised from preparatively loaded, colloidal Coomassie stained gels using sterile scalpel blades, cutting within the selective spot, or as close to it as possible. Each gel piece was placed in one of the 96 wells of a polypropylene microtitre plate (Medos, Langenselbold, Germany) which contained a small amount of 50% methanol. The methanol helped in getting the gel piece into the well and in preventing its loss by static repulsion. The plates were sealed with adhesive plate seals (Advanced Biotechnologies Ltd., Surrey, UK) and sent to the Australian Proteome Analysis Facility (A.P.A.F., Macquarie University, Sydney, Australia) for MALDI-TOF MS analysis.

3.9.2 Tryptic digestion and MALDI-TOF MS

The samples were subjected to a 16 hour, in-gel tryptic digest at 37°C and then were extracted from the gel with 50% (v/v) acetonitrile and 0.5% (v/v) TFA. A 1 μL aliquot was then spotted onto a sample plate along with 1 μL of matrix (α-cyano-4-hydroxcinnamic acid, 10 mg/mL in 70% v/v acetonitrile, 1% v/v TFA) and air-dried. A Micromass TofSpec 2E Time of Flight Mass Spectrometer (Waters Corporation, Milford, MA, USA) was used. The sample was irradiated with a 337nm nitrogen laser and spectra acquired in reflectron mode in the 600 to 4000 Da mass range. The obtained spectra was internally calibrated using two trypsin auto-digestion peaks at 842.51 Da and 2211.1 Da. Peptide mass fingerprints (PMFs) were taken as the monoisotopic peaks with a relative intensity of at least 5%.

3.9.3 Peptide mass fingerprint database searching

Peptide mass fingerprint peak lists were used as query masses to search protein and nucleic acid databases using the PeptIdent program available from ExPASy (http://www.expasy.ch/ch2d/2d-index.html). The documents of the web site are updated on a regular basis. This program compares the measured PMFs with computer-generated theoretical digests of the proteins in the databases to look for possible matches based on PMF homology. Searches of Swiss-Prot and TrEMBL bacterial

databases were done for proteins derived from *S. meliloti* grown in culture, all databases were searched for bacteroid proteins. Isoelectric point and molecular mass windows of ± 1.0 unit and ± 20% respectively were used. Initial searches were conducted using a mass accuracy of ± 0.1 Da but were increased to ± 0.5 Da if no matches were found. Confidence in the peptide mass fingerprint matches was based on multiple criteria, including the species of the matching peptide, the difference in the number of matching peptides between the first and second matches, the number of PMF matches, the number of missed cleavages, the percentage sequence coverage, and the compatibility of isoelectric and molecular mass of the analysed and database protein.

4. Chemical and reagent sources

General chemicals and reagents were obtained from Ajax Chemicals, Sydney, Australia. Chemicals listed below were of highest available purity unless otherwise indicated.

Chemical or Reagent	**Source**
Acrodisc® filters, sterile	Gelman Sciences, MI, USA
Agar	Difco Laboratories, Detroit, Michigan, USA
6-benzylamino purine, BAP	Sigma Chemical Co., St. Louis, MO, USA
Bio-lyte® ampholytes 3/10	BioRad Laboratories, CA, USA
Biotin	Sigma Chemical Co., St. Louis, MO, USA
Bovine serum albumin, BSA	Sigma Chemical Co., St. Louis, MO, USA
Bradford Dye Concentrate	BioRad Laboratories, CA, USA
Bromophenol blue	Sigma Chemical Co., St. Louis, MO, USA
CAPS	Sigma Chemical Co., St. Louis, MO, USA
CHAPS	Sigma Chemical Co., St. Louis, MO, USA
Coomassie Brilliant Blue R-250	BioRad Laboratories, CA, USA
Dihydroxy flavone, DHF	Indofine Chemicals, Somerville, NJ, USA
Dimethylsulfoxide, DMSO	Mallinckrot Inc., Kentucky, USA
EDTA	Ajax Chemicals, Sydney, Australia
Ethanol, 99.5%	BDH, Poole, UK
ExtraSep column, C_{18}	Activon Scientific Products, Thornless, NSW, Australia
Formaldehyde, 37% aqueous solution	Fluka, Neu-Ulm, Germany
Glutaraldehyde, 70% aqueous solution	Sigma Chemical Co., St. Louis, MO, USA
Inositol	Sigma Chemical Co., St. Louis, MO, USA
Iodoacetamide, IAA	Sigma Chemical Co., St. Louis, MO, USA
Methanol	BDH, Poole, UK
NAA	Sigma Chemical Co., St. Louis, MO, USA
NPA, N-(1-naphthyl)phthalamic acid	Gift from F.J. Katekar, CSIRO, Black Mountain, Canberra, Australia
Paraffin oil, light or Ondina 18 oil	Shell, Australia
Percoll	Sigma Chemical Co., St. Louis, MO, USA
Picloram (tordon or 4-amino-3,4,5	Sigma Chemical Co., St. Louis, MO, USA

-trichloropicolinic acid)	
PMSF	Sigma Chemical Co., St. Louis, MO, USA
Potassium tetrathionate	Fluka, Neu-Ulm, Germany
SDS	Amresco, Solon, OH, USA
Silver nitrate	ICN Biomedical Inc., Aurora, Ohio, USA
Sodium acetate trihydrate	BDH, Poole, UK
Sodium thiosulphate	BDH, Poole, UK
Streptomycin	Sigma Chemical Co., St. Louis, MO, USA
Thiourea	Fluka, Neu-Ulm, Germany
Tributylphosphine (TBP), 97%	Fluka, Neu-Ulm, Germany
Trichloroacetic acid	AnalaR
Tris base	Amresco, Solon, OH, USA
Urea	Amresco, Solon, OH, USA
Yeast extract	Difco Laboratories, Detroit, Michigan, USA

Chapter 3 Regulatory control of expression of the *nolR* gene

1. Introduction

Sinorhizobium meliloti forms nitrogen-fixing symbioses with Medic legumes and has been selected by the international research community as the model strain of *Rhizobium* species to investigate nodulation, nitrogen fixation and plant microbe symbiosis. In *Sinorhizobium*, expression of nodulation gene was regulated positively and negatively (Kondorosi *et al.*, 1989b). Positive regulation is controlled by three *nodD* gene products and other genes, e.g., *syrM*, located on the megaplasmid pSymA, in conjunction with signal molecules from the host plants (Long, 1996). The NodD protein is activated by specific plant signals and the activated NodD protein then interacts with a conserved *cis*-regulatory element (the *nod*-box) to co-ordinately regulate the common nodulation genes (Kondorosi, 1989a).

Negative regulation is mediated by a repressor protein encoded by a *nolR* gene (Kondorosi *et al.*, 1989b) (Cren *et al.*, 1995). This chromosomal gene encodes a 13 kDa protein, which binds to the overlapping *nod*D1 and *nod*A promoters at the RNA polymerase binding site (Kondorosi *et al.*, 1989b). The *nolR* gene is essential for the optimal nodulation of alfalfa (*Medicago sativa*) since the *nolR* mutants had a delayed nodulation phenotype, although all plants nodulated in time (Kondorosi *et al.*, 1989b). In addition, the expression of the inducible *nod* genes was differentially down-regulated in *S. meliloti* strains lacking the *nolR* repressor (Kondorosi, 1991) (Kiss *et al.*, 1998). These studies illustrate the interaction between the chromosomal and the plasmid replicons and the finely tuned nature of the control of synthesis of the Nod factors of the *Sinorhizobium* (Cren *et al.*, 1995).

Proteomic studies on *S. meliloti* strains with or without functional NolR have revealed that a number of proteins were significantly altered in protein production levels by the loss of the NolR function (Chen *et al.*, 2000). These proteins involve in various metabolic pathways and cellular functions including the TCA cycle, stress response, cell growth and maintenance, DNA transcription, and protein synthesis. It has been suggested that the NolR repressor is a global regulatory protein which responds to environmental factors to fine-tune intracellular metabolism (Chen *et al.*, 2000). However, little is known about the *nolR* gene expression and regulation at different growth stages and under different environmental conditions. Cren *et al* (1995) reported

that the *nolR* gene was highly expressed both in the free-living bacterium and in the bacteroid. Furthermore, Expression of *nolR* was negatively auto-regulated by NolR protein and affected by the *nod* gene inducer luteolin.

Many bacteria have been shown capable of responding to changes in population density and of coordinating the behaviour of individual cells in a local population through the exchange of extracellular signal molecules. This kind of regulation, called "quorum sensing", affects a diversity of bacterial behaviours (Whitehead *et al.*, 2001). Quorum sensing appears to be particularly important in coordinating gene expression within a local bacterial population during its interaction with a eukaryotic host (Winzer & Williams, 2001), and during adaptation to stress survival (Whitehead *et al.*, 2001). AHLs are the most common of signals used by Gram negative bacteria for quorum sensing regulation. Strains of *S.meliloti* have been reported to produce compound with AHL activity (Cha *et al.*, 1998) (Marketon & Gonzalalez, 2002). In addition, plants can secrete substances that mimic bacterial AHLs signal activities and affect population density-dependent behaviors in associated bacteria (Teplitski *et al.*, 2000).

In this chapter, I investigated the expression of *nolR* gene in relation to the stage of growth, availability of nutrients and different environmental stimuli. I showed that the regulation of *nolR* gene is influenced in a population density-dependent pattern and by a number of environmental stimuli. Moreover, I demonstrated that luteolin does not have a direct effect on *nolR* gene expression.

2. Results

2.1 The expression of *nolR* is population density-dependent

The expression of *nolR* gene was investigated by measuring the β–galactosidase activity of *S. meliloti* strain 241, a translational *nolR::lacZ* fusion inserted into the chromosome of the wild-type strain AK631 (Cren *et al.*, 1995). Bacterial growth was measured by viable-cell count and was parallel with the measured optical density at 600nm. An optical density unit at 600nm corresponded to approximately 10^9 cells ml^{-1} (Figure. 3.1). A correlation between cell density and *nolR* expression was observed by measuring β–galactosidase activity over the complete growth cycle of cells of strain 241 growing in TA medium, a tryptone-yeast-extract medium (Figure. 3.2). When

the overnight culture grown in TA medium was inoculated into TA medium, initially the β–galactosidase activity of the *nolR::lacZ* fusion was continuously reduced during the first 5 hours of incubation time. This was due to the rate of bacterial growth being greater than that of NolR protein production, so that the β–galactosidase activity per cell was diluted by the growing cells (Figure 3.3). In fact, after 3 hours of incubation the optical density of the culture was doubled with the cell population of about 1.2×10^8 ml^{-1} while the total β–galactosidase activity of the culture remained unchanged (Figure 3.1). From 3 to 5 hours after incubation, the optical density of the culture was increased about 75% with cell population of about 2.3×10^8 ml^{-1} while the total β–galactosidase activity of the culture was increased about 60%. When the cells grew to the early log-phase with cell population of about 4.3×10^8 ml^{-1} after 7 hours of incubation, the β–galactosidase activity was found to be increase steadily until the cell culture reached stationary phase (Figure 3.2, Figure 3.3). The increase of *nolR* expression was correlated with the increase of cell population, indicating that the expression of *nolR* is population density-dependent. The highest rate of *nolR* expression was observed during early stationary-phase rather than in log-phase, indicating that bacterial growth rate does not control *nolR* expression. A high level around 4000 Miller units of *nolR* expression was sustained during the stationary-phase.

The population density-dependent *nolR* expression was further tested by a dilution experiment. When the culture grown in TA medium to middle log or stationary phase was diluted with fresh TA medium and incubated for more than one hour, it was detected that the higher the initial dilution, the lower the optical density and the lower β–galactosidase activity as well (Figure 3.4).

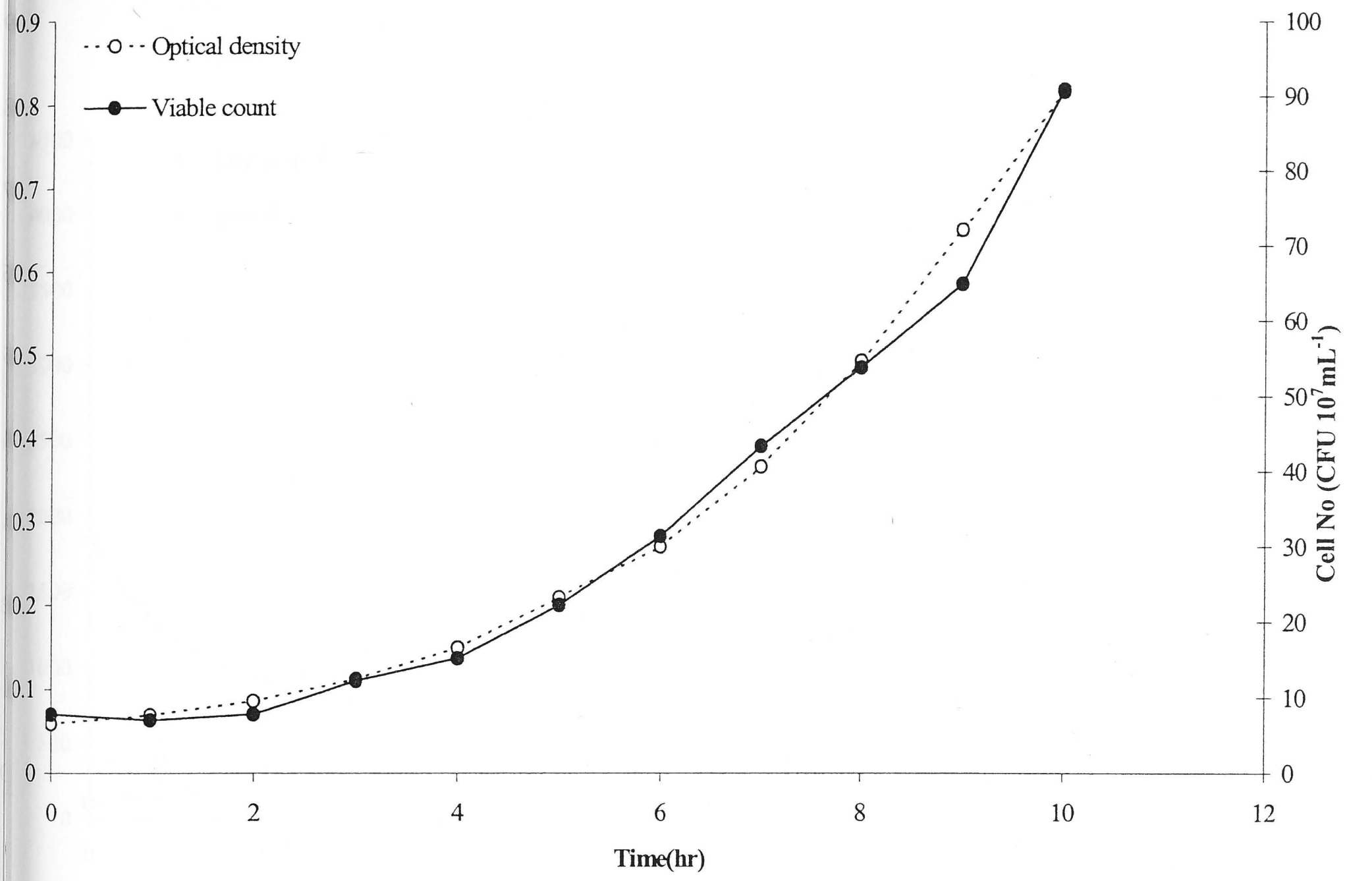


Figure 3.1 The comparison of viable count and optical density as two indications of growth.

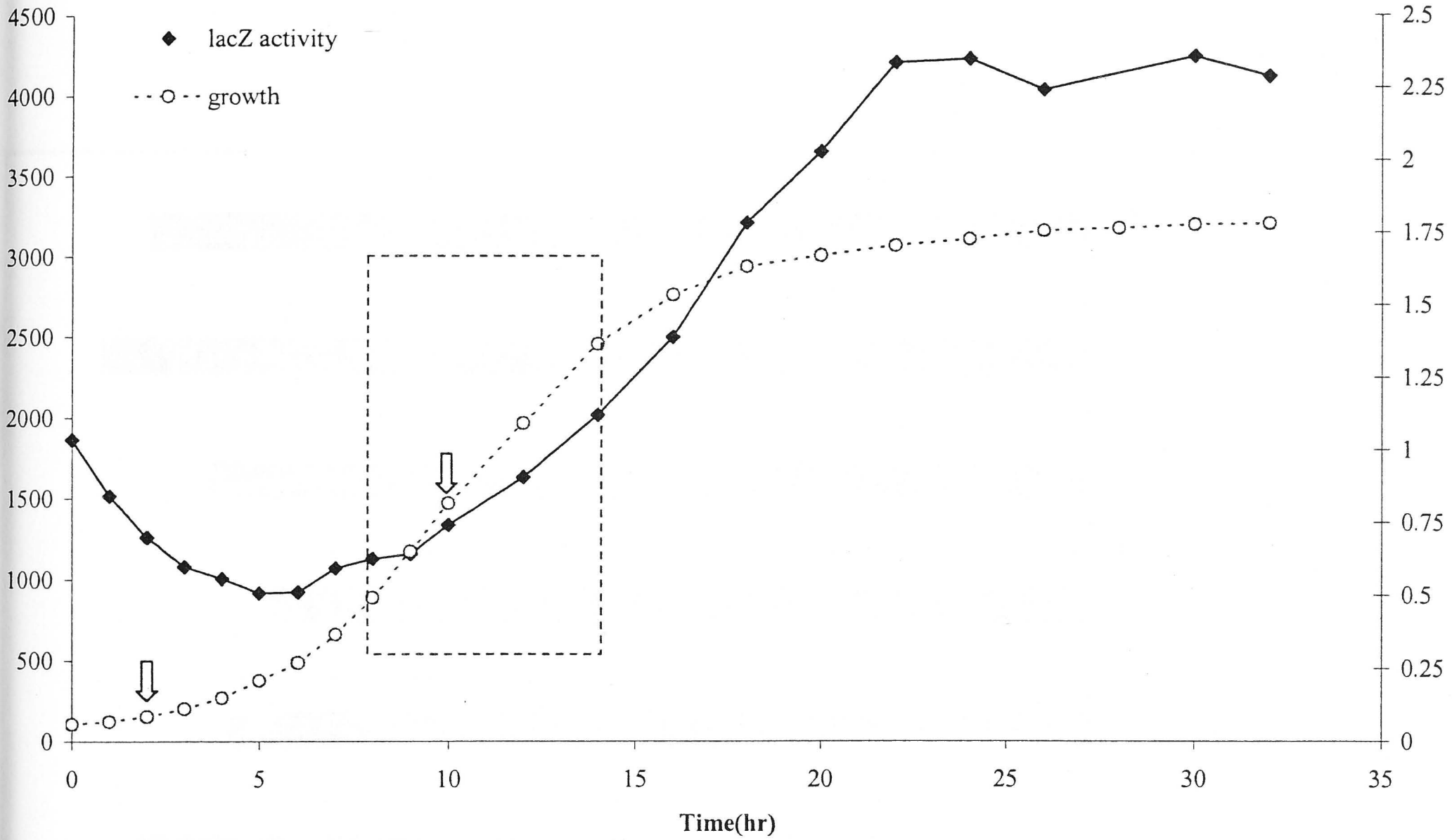


Figure 3.2 Expression of *nolR::lacZ* fusion in *S. meliloti* strain 241 grown in TA medium. Dashed rectangle indicates the period of mid exponential phase. (⇩) indicates the two different cell densities (OD_{600}0.1 and OD_{600}1.0) which have similar *nolR::lacZ* activities.

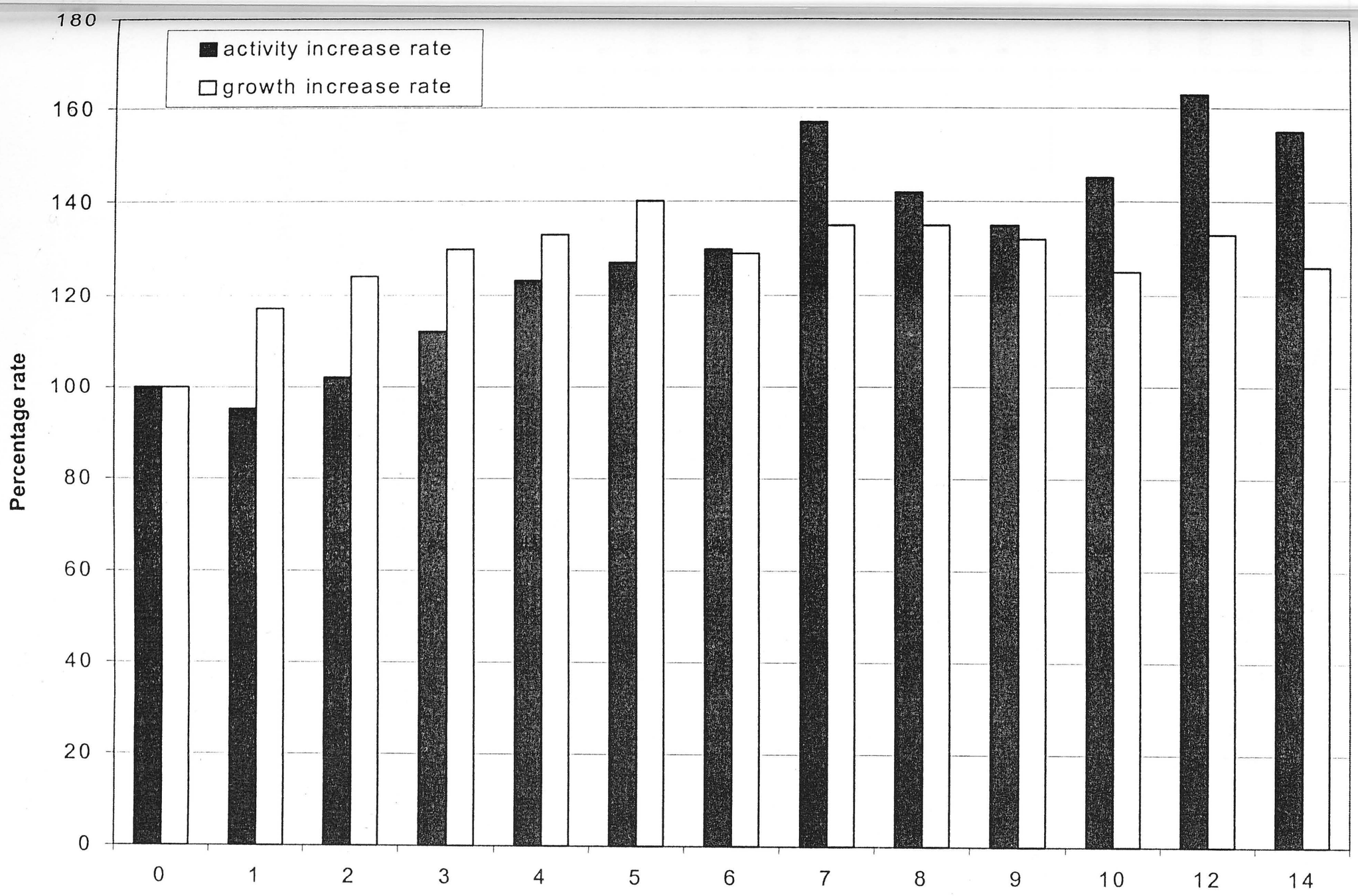


Figure 3.3 Rate changes in cellular growth and total *nolR::lacZ* activity during the first 14 hours after inoculation with *S.meliloti* strain 241. Rare changes were expressed as percentage activity and growth determined by comparing the total *nolR::lacZ* activity and optical density at a certain time point to the values of the previous time point respectively.

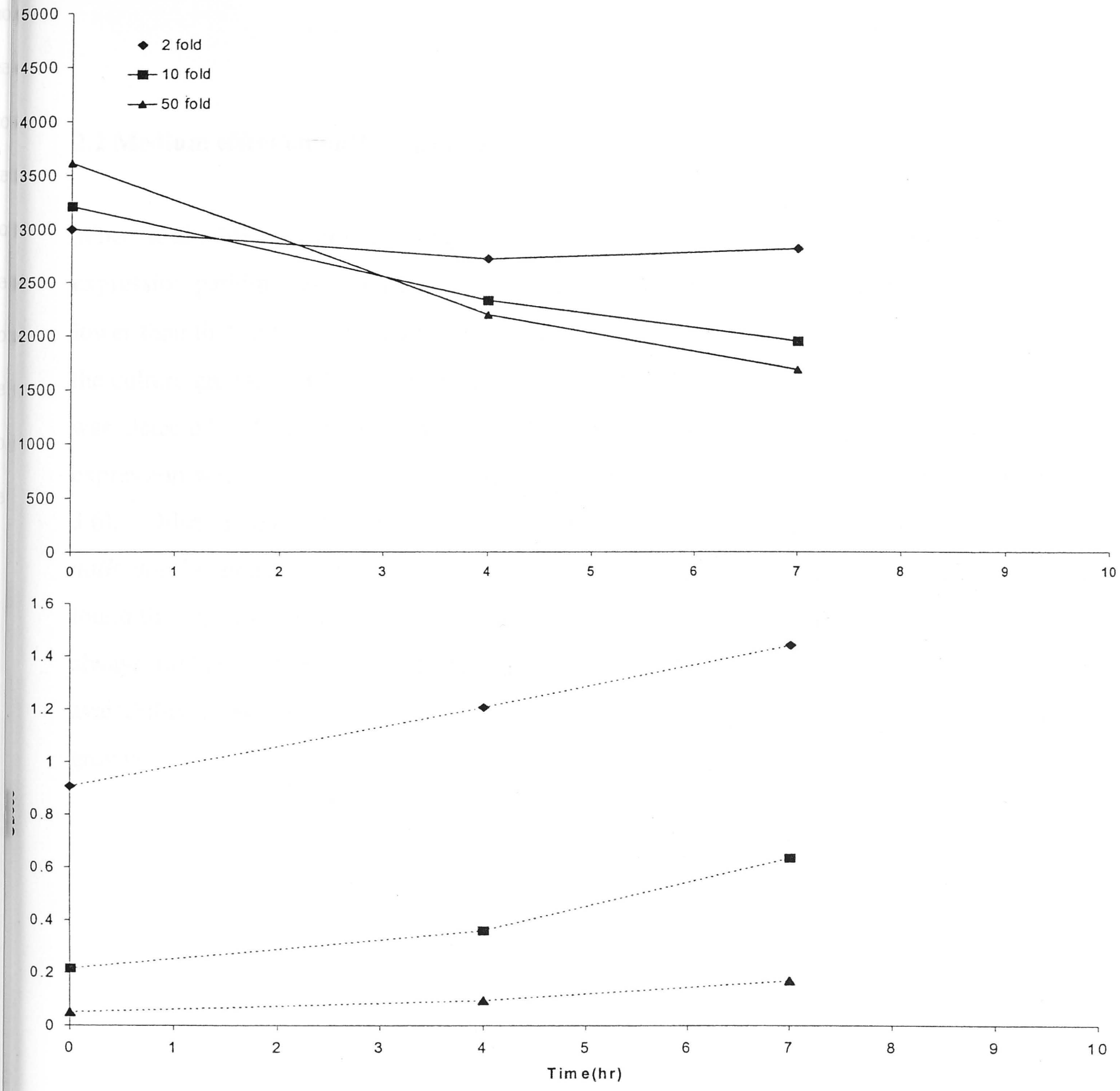


Figure 3.4. The effect of culture dilution on *nolR::lacZ* expression of strain 249 in TA medium. Early stationary phase cultures (at OD_{600} = 1.8) were diluted 2, 10, 50 fold to fresh TA medium to initiate the experiment. A) *nolR::lacZ* activity. B) Culture growth at OD600nm.

2.2 Medium effect on *nolR* expression

When strain 249 was grown in the minimal BIII medium, the population density-dependent *nolR* expression pattern was again detected. However, the level of *nolR* expression in BIII was much lower than that in the rich medium - TA. The highest level of β–galactosidase activity detected in the culture grown in BIII medium was around 1000 Miller units, which was 4 times lower than that was detected in the culture grown in TA medium (Figure 3.5). A similar low level of *nolR* expression was also observed in the other defined minimal media, such as TMR and GTS (Figure 3.6). Other *S. meliloti* strains, such as strain 241, 273 and ZB138, were also tested for the *nolR::lacZ* expression in different growth medium throughout the growth cycle, and it was also found that the *nolR* expression was related to the type of medium used: the rate of expression was always higher in the rich medium than in the defined minimal medium. Therefore, nutrient availability is an important factor that affects *nolR* expression presumably by influencing cell growth.

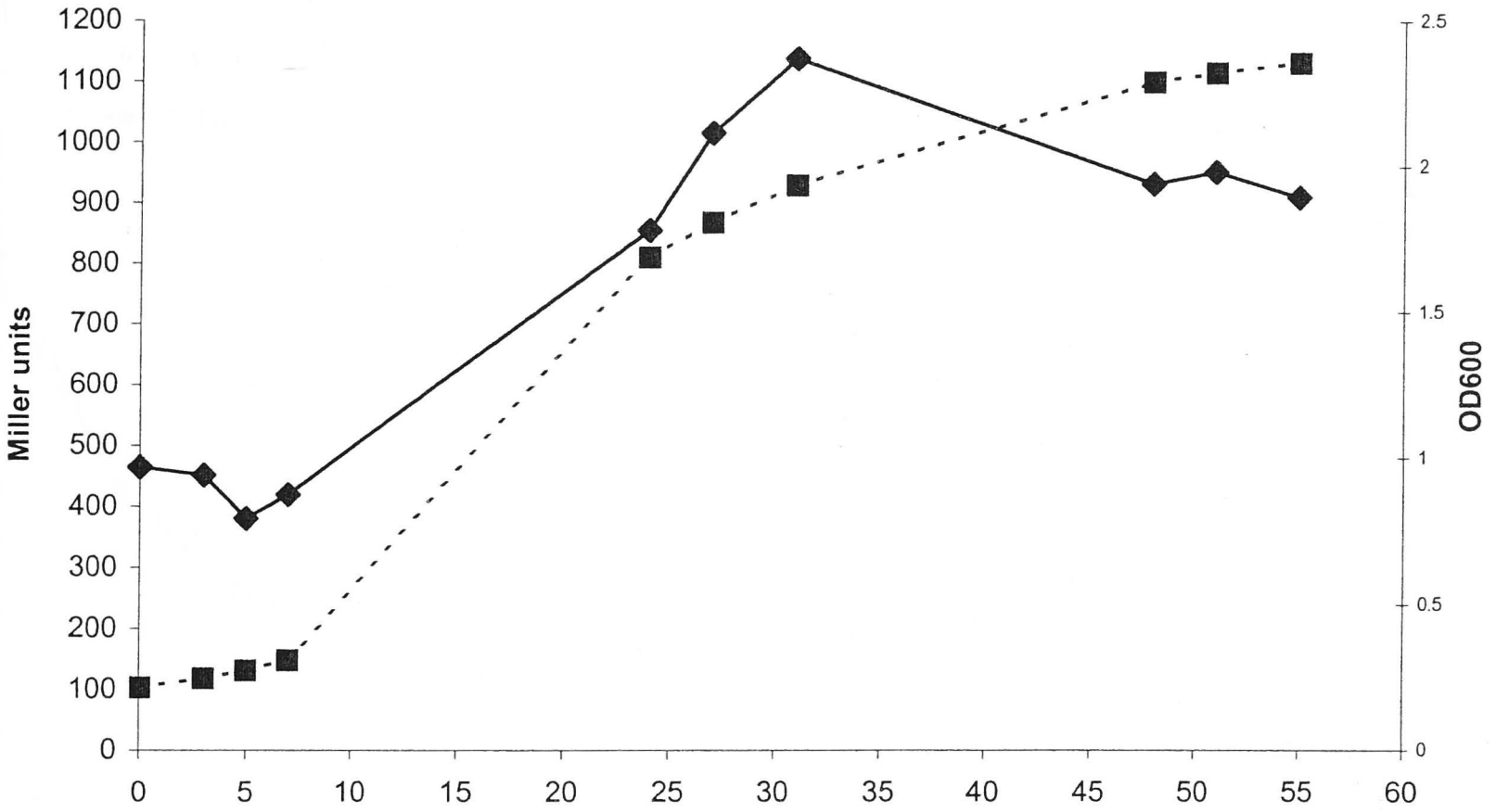


Figure 3.5 Expression of *nolR::lacZ* fusion in *S.meliloti* strain 249 in BIII medium. The overnight culture of strain 249 grown in BIII medium was re-inoculated into fresh BIII medium to initiate the experiment. Solid line represents β-galactosidase activity, dashed line represent cellular growth as optical density 600nm.

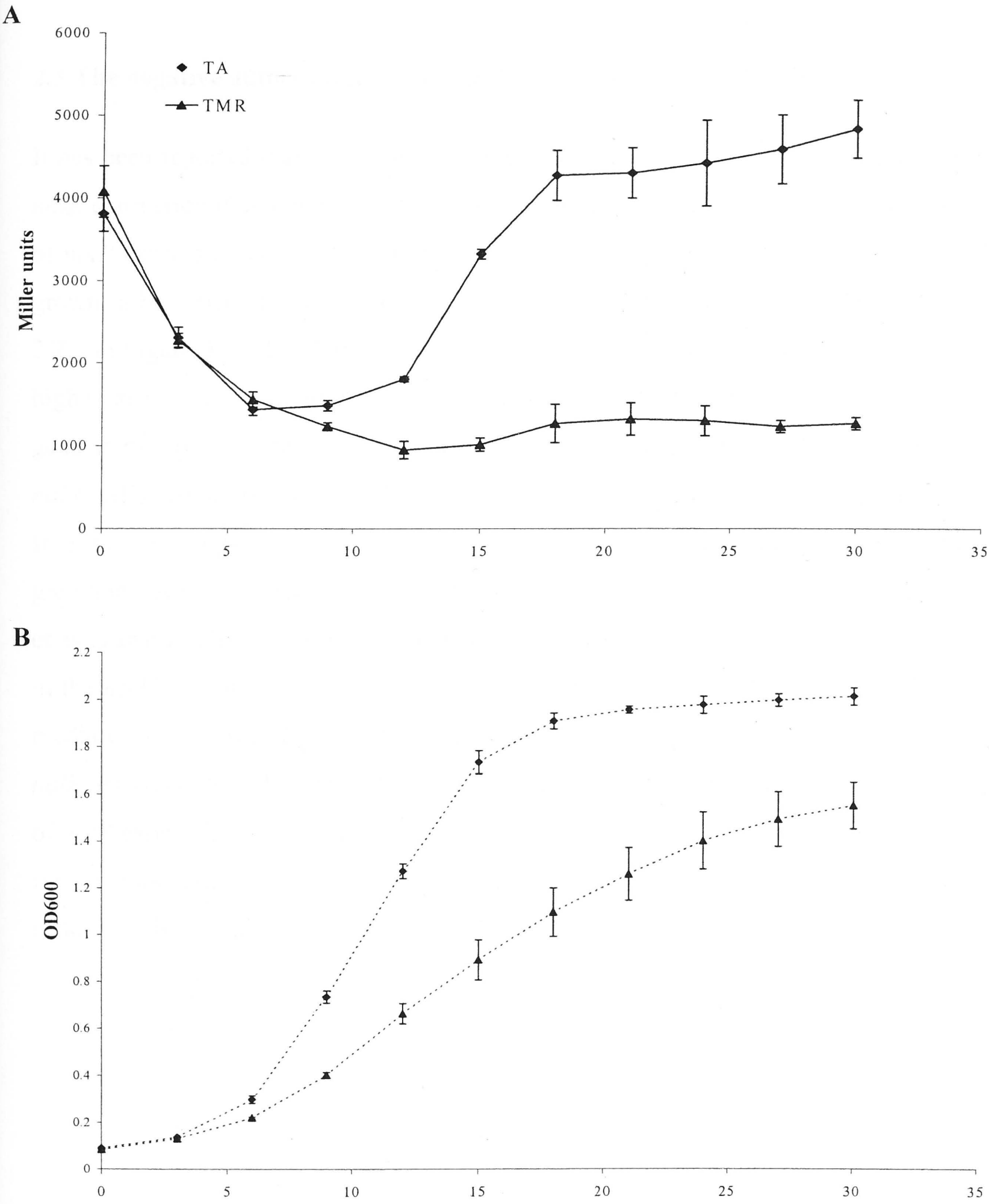


Figure 3.6. Comparison of the expression of *nolR::lacZ* in strain 241 grown in TA and TMR media. Overnight culture grown in TA medium was diluted around 50 fold (to OD_{600} = 0.08) with fresh TA and TMR respectively to initiate experiment. (A) *nolR::lacZ* activity. (B) Culture growth. Error bars represent standard deviations.

2.3 The negative autoregulation of *nolR* is more efficient in minimal media

It has been reported that the NolR protein binds to its own promoter and negatively auto-regulates *nolR* expression (Cren *et al.*, 1995). To determine whether the negative auto-regulation mechanism of *nolR* expression is correlated to the population density and medium, $nolR^+$ and $nolR^-$ cells were grown in both rich and minimal media and compared throughout a complete growth cycle (Figure 3.7 and Figure 3.8). The level of *nolR* expression in the $nolR^-$ mutant of strain AK631 was much higher than that in the wild-type strain, confirming that the NolR protein auto-regulates its own gene transcription. The population density-dependent *nolR* expression was also observed in the $nolR^-$ cells grown in rich and minimal media. However, the rate of *nolR* expression in $nolR^-$ cells from the early log-phase to the stationary-phase was much higher than that measured in $nolR^+$ cells grown in TA or BIII medium, suggesting the involvement of the negative auto-regulation of *nolR* in cells grown in different growth media. This medium-dependent *nolR* expression was also observed in the *nolR*⁻ mutant. In $nolR^-$ cells, the highest levels of *nolR* activity detected in the TA and BIII media were 13,500 and 5,671 Miller units, respectively. While in $nolR^+$ cells, the highest levels of *nolR* activity detected in the TA and BIII media were 4,700 and 1,017 Miller units. The inhibition of *nolR* expression in BIII medium grown cells was two fold for the $nolR^-$ mutant but was four fold for the $nolR^+$ strain. These results indicate that the level of the negative auto-regulation of *nolR* is influenced by cell nutrient.

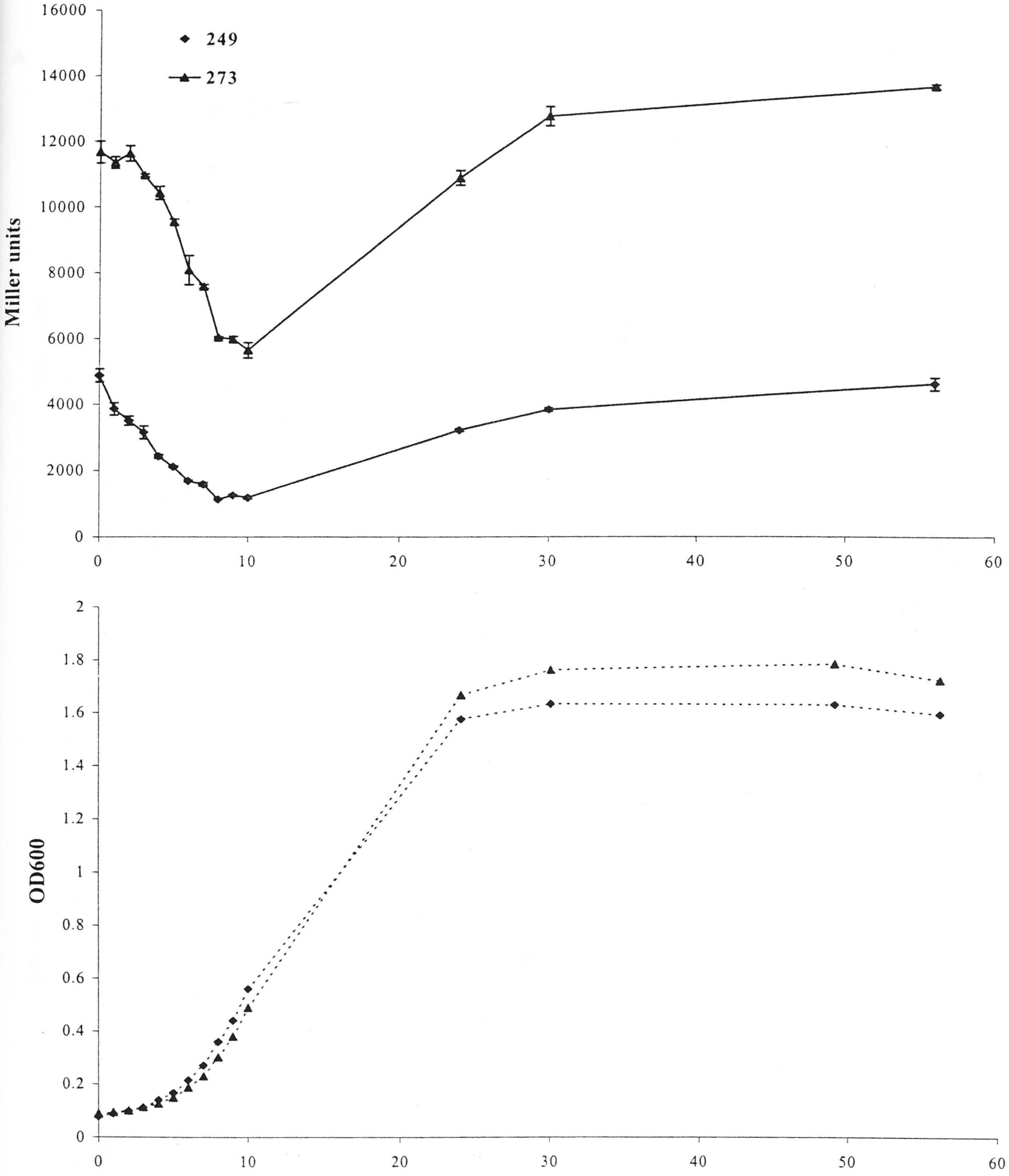
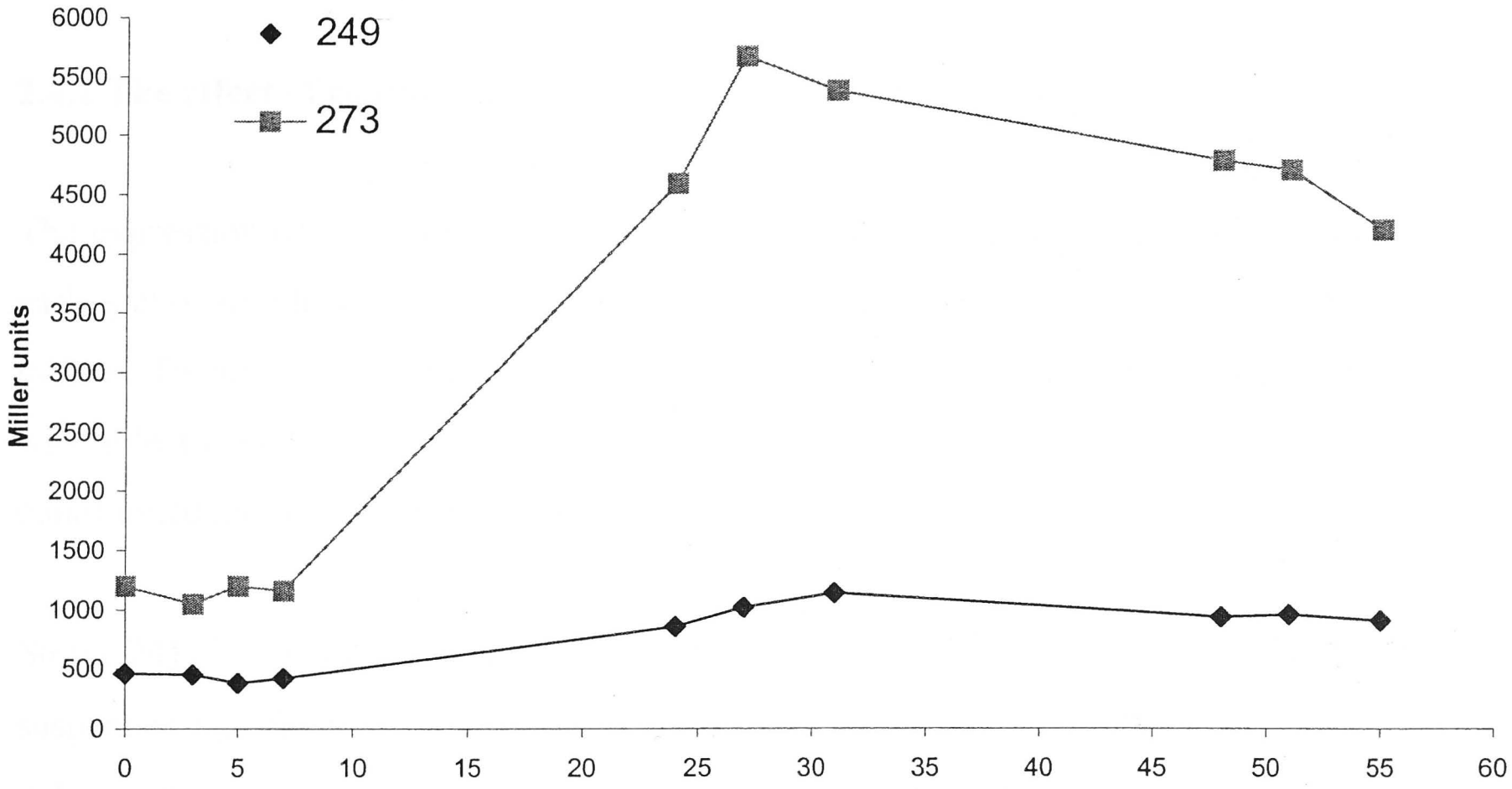
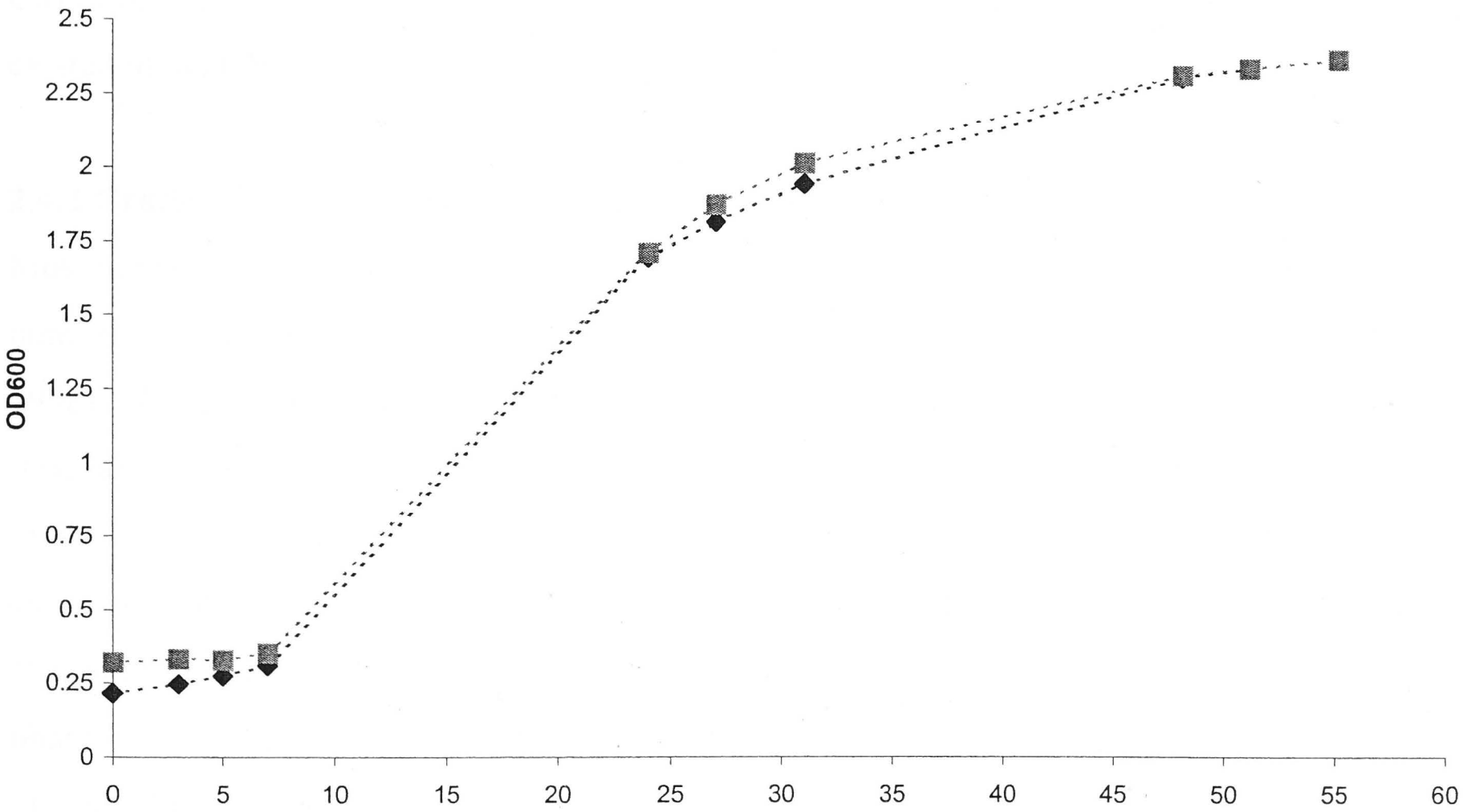


Figure 3.7 The of expression of *nolR::lacZ* in strains 249 (*nolR*$^+$) and 273 (*nolR*$^-$) in TA medium. Overnight cultures grown in TA medium were diluted to OD_{600} of 0.05 with fresh TA medium to initiate the experiment. A) *nolR::lacZ* activity. B) Culture growth. Error bars represent standard deviations.

A



B



Time (hrs)

Figure 3.8 The expression of *nolR::lacZ* in strains 249 (*nolR*$^+$) and 273(*nolR*$^-$) in BIII medium. Overnight cultures grown in BIII medium were diluted to OD_{600} of 0.2 with fresh BIII medium to initiate the experiment. A) beta-galactosidase activity. B) Culture growth.

2.4 Is *nolR* expression regulated by quorum sensing signals?

2.4.1 The effect of conditioned medium (CM) on *nolR* expression

The expression of *nolR* was at the maximal level when the cells reached a high cell population in early stationary phase. This suggested that *nolR* expression may be mediated by a quorum sensing system. To see whether there was any quorum factor presented in the stationary-phase culture that was able to induce the expression of *nolR*, the supernatant of stationary-phase cultures (called conditioned medium, CM) was used to test its effect on the *nolR::lacZ* fusion.

Strain 241 grown in TA medium to early log-phase or mid log-phase were centrifuged and re-suspended into fresh TA medium and TA supplemented with CM. After two hours incubation, β-galactosidase activities of the early log-phase and mid log-phase cells were increased by the added CM about 35% and 10%, respectively (Table 3.1), indicating that a cell population density factor existed in the CM.

2.4.2 Crude AHLs do not activate *nolR* expression

Most but not all of the quorum-sensing signals characterized to date are *N*-acylated derivatives of L-homoserine lactone (AHL). *S. meliloti* strain AK631 produces compounds with AHL activity (Cha, 1998). When *nolR*$^+$ wild-type strain AK631 was grown in TA and BIII media to stationary phase, AHL activity was detected in the supernatant of the culture by HPLC separation and electro-spray ionisation mass spectrometry (Teplitski *et al*, unpublished results). To determine whether the *nolR* expression was activated by AHL, crude AHLs were extracted from the supernatant of the stationary phase cultures. However, the addition of these non-purified AHLs into the early log-phase TA culture of strain 241 did not alter the β–galactosidase activity of the *nolR::lacZ* fusion (Table 3.1).

Table 3.1 Expression of *nolR::lac* Z fusion in response to the population-density-dependent factor

Test medium	b-galactosidase activity (Miller Uint)[a]	
	Early log-phase cells[a] in	Middle log-phase cells[b] in
TA	851 ± 13	1448 ± 132
CM[b]	1160 ± 60	1612 ± 24
TA (AHLs)[c]	876 ± 51	NT

a. Strain 241 was grown in TA medium. The early log-phase (OD_{600} = 0.2) or middle log-phase (OD_{600} = 1.0) cells were harvested, and washed with TA medium, and suspended into the original volume of the test medium. The cultures incubated at 28°C for 1 hr were used for b-galactosidase activities analysis. Values are the means with standard deviation of three independent determinations. NT = not tested

b. CM = conditioned medium from 241 culture (OD_{600} = 1.8)

c. TA medium supplemented with crude AHLs extracted from the conditioned medium.

2.5 The effect of pH shock on *nolR::lacZ* expression

In most conditions, cells grown in the TA medium started in a neutral pH (pH 6.9-7.0) environment. However, the pH of the cultures became basic as the cells grew into late exponential phase and stationary phase (pH 7.5-7.7). To determine whether *nolR* expression was affected by the pH of the medium, the middle log-phase TA culture of strain 241 (pH 6.9) was adjusted to a pH of 5.6 and 8.3, respectively (Figure 3.9). After 6 hours of incubation, the pH of the acidified culture was increased to 6.0 and the pH of the alkalified was decreased to 7.7. The pH of the culture without pH adjustment was 7.1. Compared with the culture without a pH shock, cell growth in the acidified culture was slower while cell growth in the alkalified culture was faster: after 2 hours of incubation, the growth in the acidified culture was 85% of the culture without a pH shock (control) while that of the alkalified culture was 119% of the control; after 6 hours, the growth of the acidified culture was still 86% of the control while that of the alkalified culture was 114% of the control (Figure 3.9). The expression of *nolR* was inhibited in both acidified and alkalified cultures. The inhibition of *nolR* expression in the acidified culture was greater than in the alkalified culture: the β–galactosidase activity of *nolR::lacZ* fusion in the acidified culture remained decreased up to 6 hours of incubation compared to that of the control (67% after 2 hours, 51% after 4 hours and 41% after 6 hours of incubation). In the alkalified culture, an increase of the β–galactosidase activity with a rate similar to the control culture was not observed until after 4 hours of incubation (69% after 2 hours, 69% after 4 hours and 85% after 6 hours). Therefore the acidic pH has a greater shock effect on *nolR* expression while its effect on cell growth is only moderate. However, the *nolR* expression of the pH shocked cultures and control culture were able to reach to a similar level after 24 hours of incubation (data not shown).

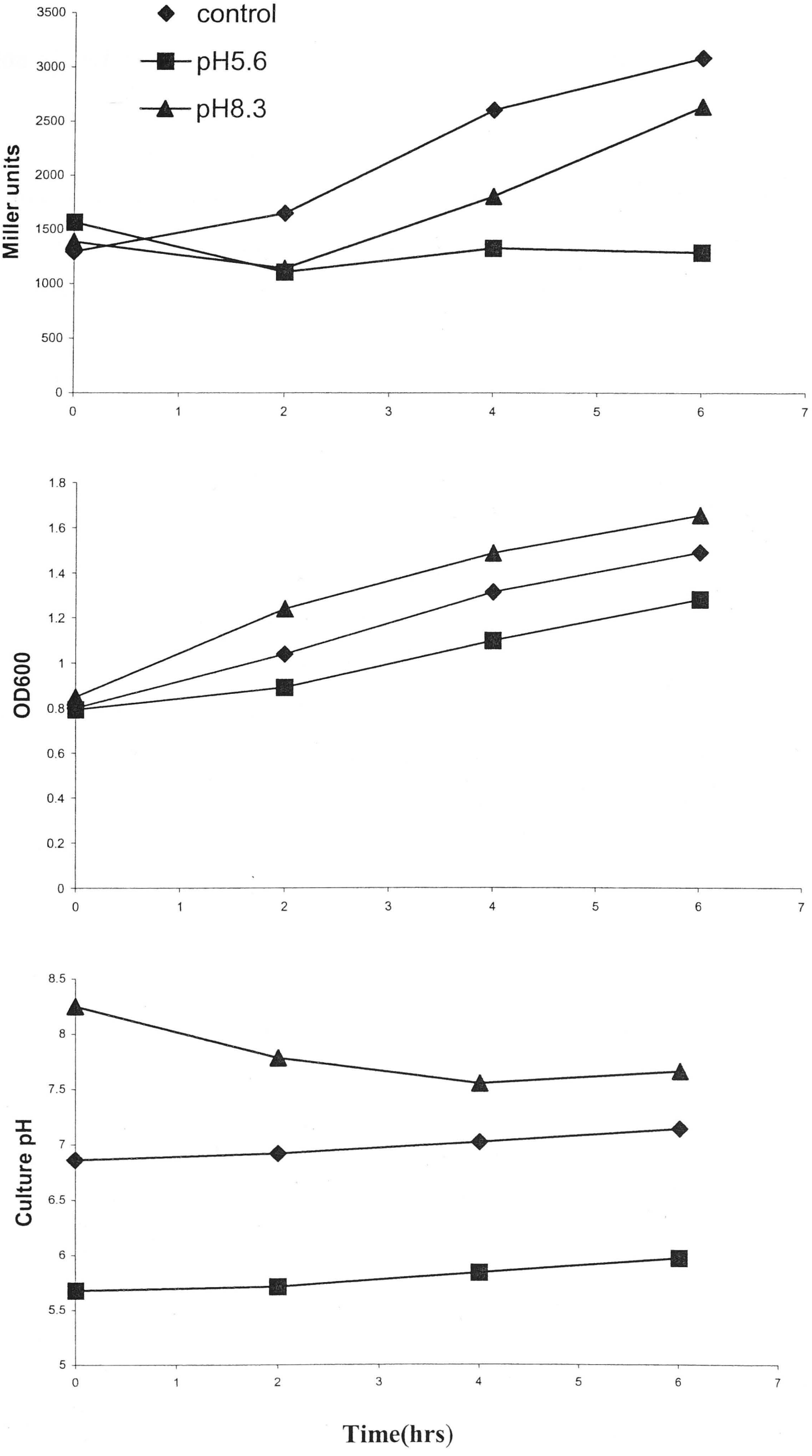


Fig 3.9 The effect of pH shock on *nolR::lacZ* expression. Middle log-phase culture of strain 241 grown in TA medium (pH6.9) was adjusted to a pH of 5.6 and 8.3, respectively. A) Beta-galactosidase activity. B) Culture growth. C) pH of the cultures under pH shock.

2.6 Expression of *nolR* under anaerobic conditions and in conditioned medium

To see whether oxygen was required for the *nolR* expression, an early log-phase culture of strain 241 was anaerobically incubated in a sealed flask filled with nitrogen gas. No bacterial growth or *nolR* expression was observed under anaerobic condition (Figure 3.10). However, the bacterial growth and *nolR* expression were able to recover quickly once the growth condition of the treated-culture had been returned to under normal air condition, suggesting that *nolR* is expressed in growing cells.

Expression of *nolR* in non-growing cells was also tested by changing the medium of the early log phase culture of strain 249 grown in TA medium with the conditioned medium (CM) prepared from the stationary phase culture of strain 249. When the growing culture was deprived of nutrient (treated with CM in this case), the cellular growth was stopped and the level of *nolR* expression remained unchanged (Figure 3.11). However, when the nutrients of TA medium (tryptone and yeast extract) were added to the CM culture, cellular growth and *nolR* expression remained at a higher and increasing pattern. Later on, when nutrients were added to the solely CM treated group, culture growth and *nolR* expression resumed (data not shown). Therefore, the expression of *nolR* is correlated with bacterial growth.

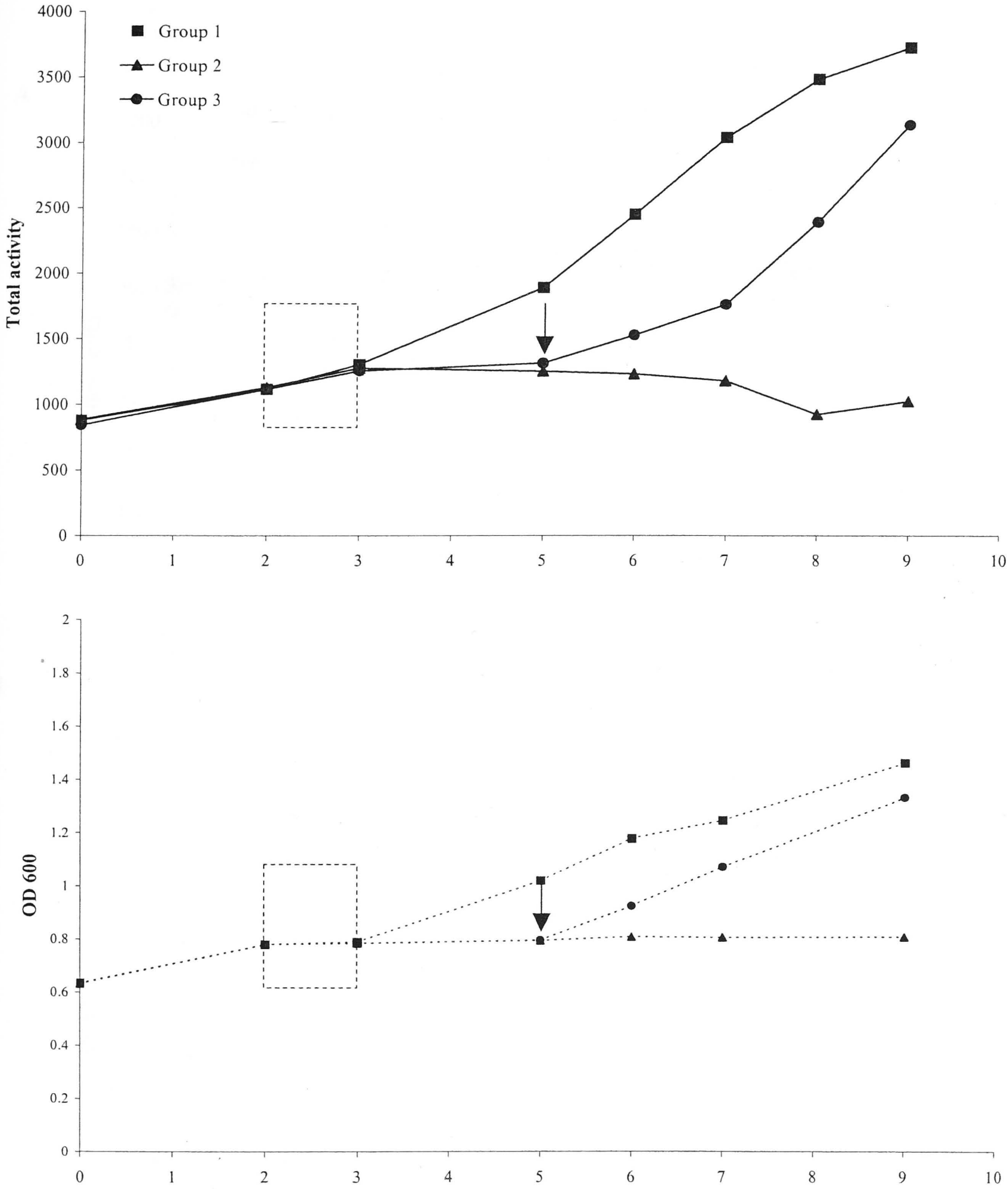
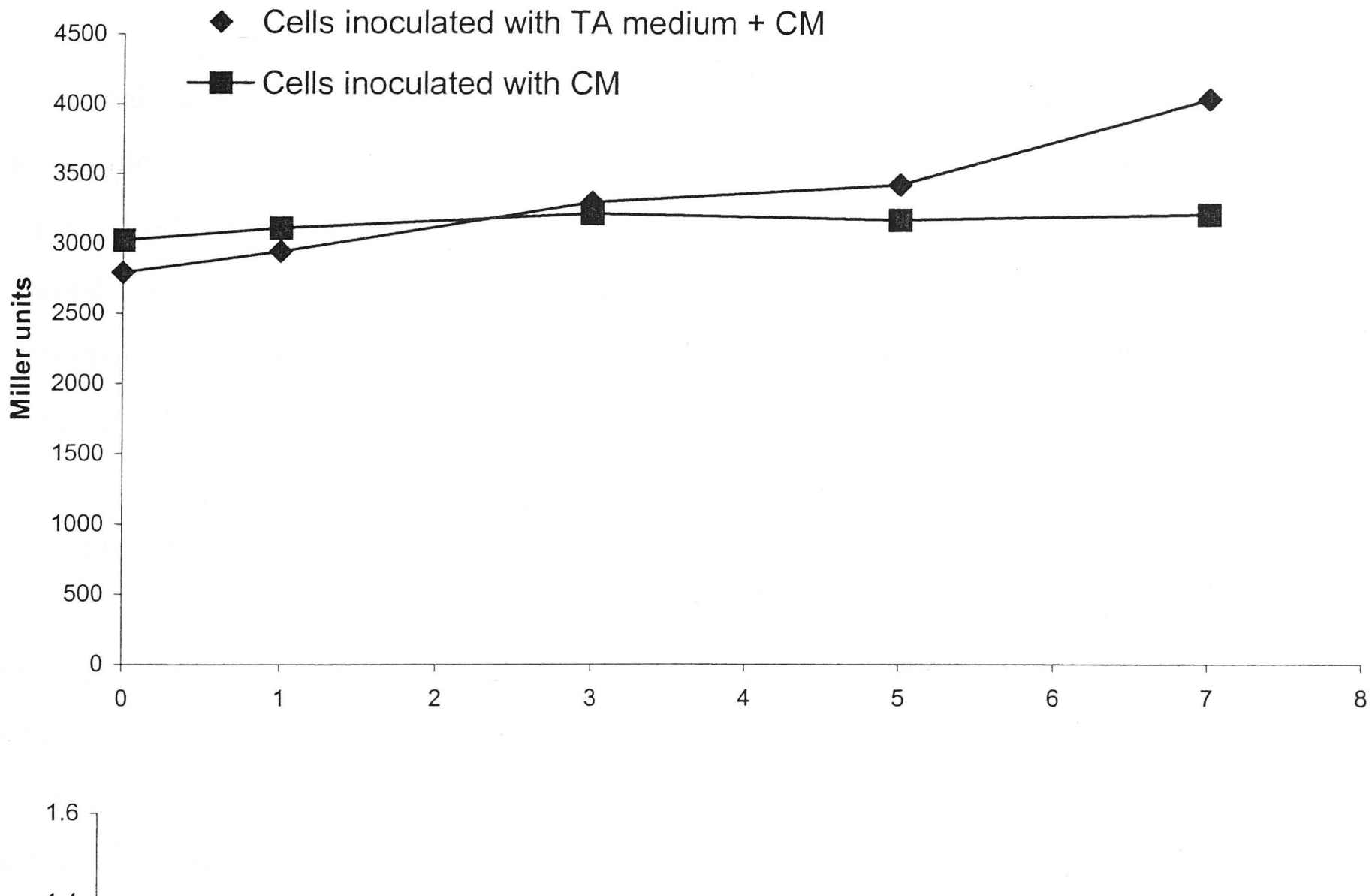


Figure 3.10. The effect of anaerobic conditions on *nolR::lacZ* expression in strain 241 grown in TA medium. The dashed rectangle indicated that the cultures of Group 2 and 3 were subjected to anaerobic treatment for 1 hour. The arrow (↓) indicates when Group 3 culture was re-exposed to the aerobic condition. The untreated culture (Group1) was the control. A) Total activity of *nolR::lacZ* expression. B) Culture growth.

A



B

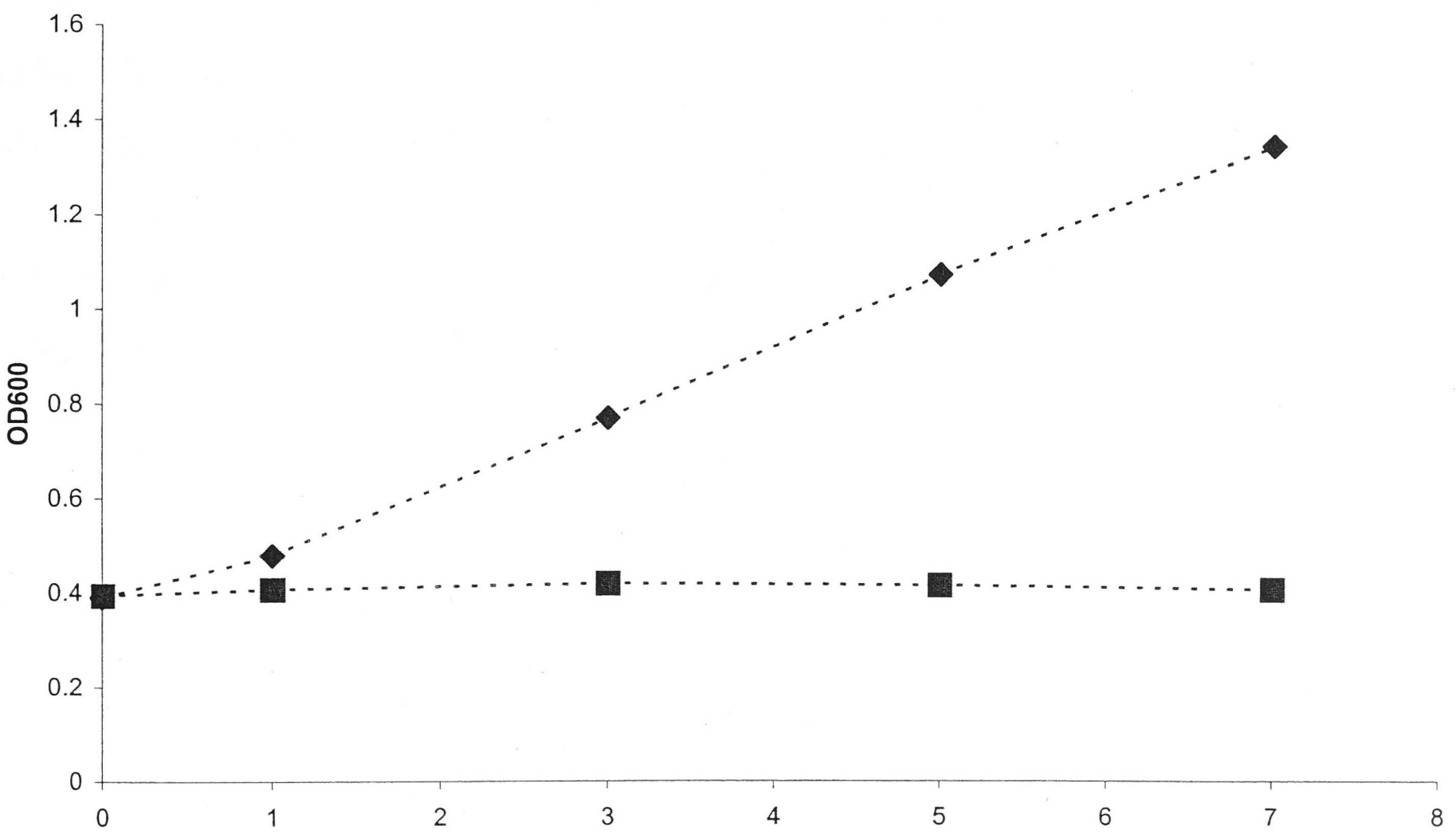


Figure 3.11 Growth related *nolR::lacZ* expression. Early log-phase culture of strain 249 grown in TA medium was centrifuged and cell pellets were resuspended into conditioned medium (CM) with TA medium supplemented with CM. CM was prepared from supernatant of stationary phase culture of strain 249. A) Beta-galactosidase activity. B) Culture growth.

2.7 Effect of luteolin on *nolR::lacZ* expression

It was reported that *nolR* expression in both *nolR*$^-$ and *nolR*$^+$ strains could be inhibited with the presence of luteolin at a low concentration of 5μM (Cren et al., 1995) in the GTS minimal medium (Cren *et al.*, 1995). However, the inhibition of the *nolR* expression by luteolin was not found in neither *nolR*$^-$ nor *nolR*$^+$ *S. meliloti* strains grown in TA or BIII medium. The effect of luteolin on *nolR* expression was also tested by the addition of different concentrations of luteolin (2.5μM, 5μM, 7.5μM). Still, *nolR* expression was not altered by added luteolin (Figure 3.12). Same results were obtained when luteolin was added to the culture grown in TA medium at different growth stages (data not shown).

When the GTS minimal medium was used, luteolin did have an inhibitory effect on *nolR* expression only when luteolin was added to a low cell density culture (OD600<0.2) (Figure 3.13 A). However, not only *nolR* expression but also bacterial growth was inhibited by added luteolin. When luteolin was added to the mid-log-phase (OD600 = 1.1) GTS culture of strain 241 (Figure 3.13 B), it did not alter the bacterial growth or *nolR* expression as well. Since the *nolR* expression is population density-dependent, the lower cell density in the luteolin-containing culture would have a lower level of *nolR* expression. Therefore, the inhibitory effect of luteolin on *nolR* expression was via its ability to reduce cell growth.

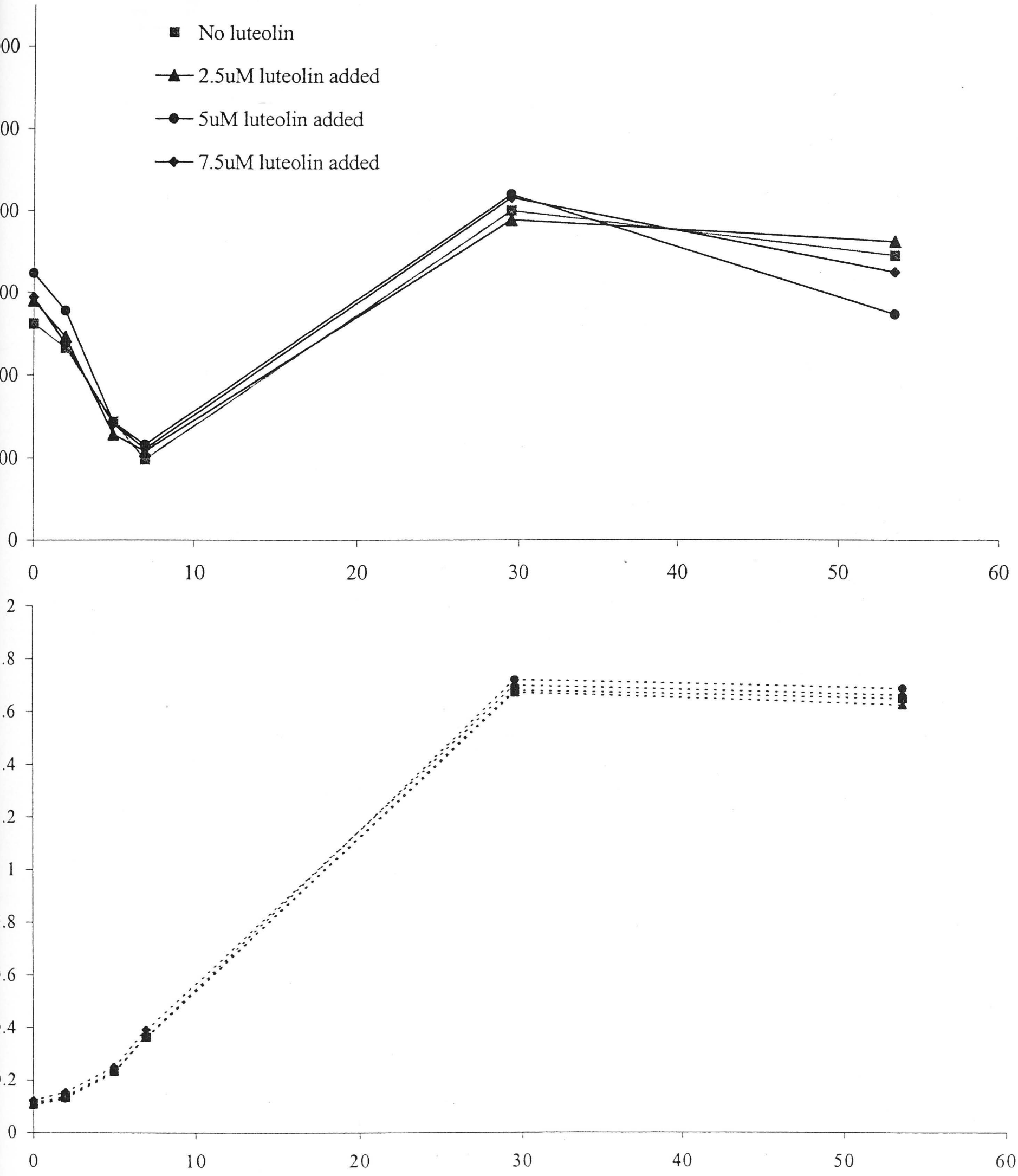
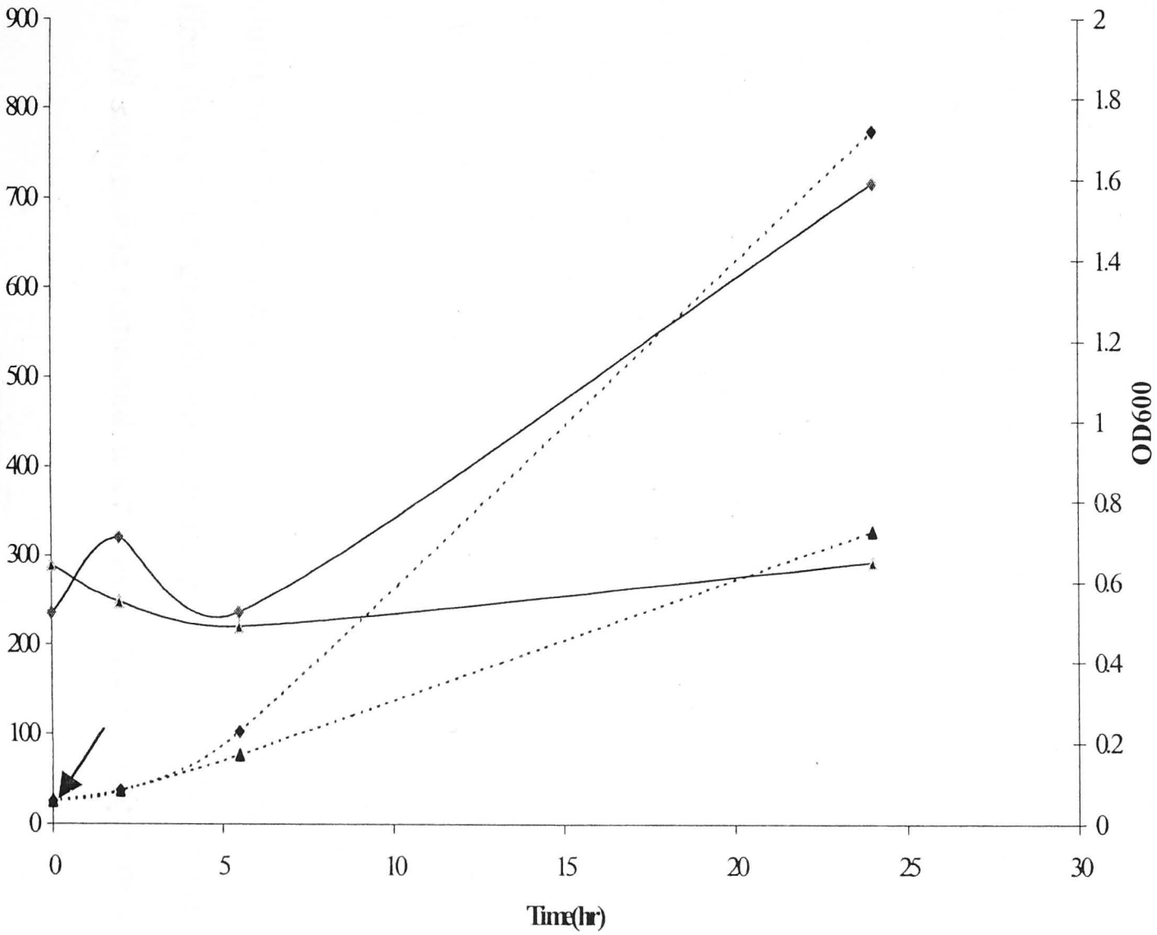


Figure 3.12. The effect of luteolin on *nolR::lacZ* expression in strain 249 grown in TA medium. Overnight culture of strain 249 was 50 fold diluted with fresh TA medium. Luteolin was added to the diluted culture at different concentrations (2.5uM,5uM and 7.5uM). A) β-galactosidase activity. B) Culture growth.



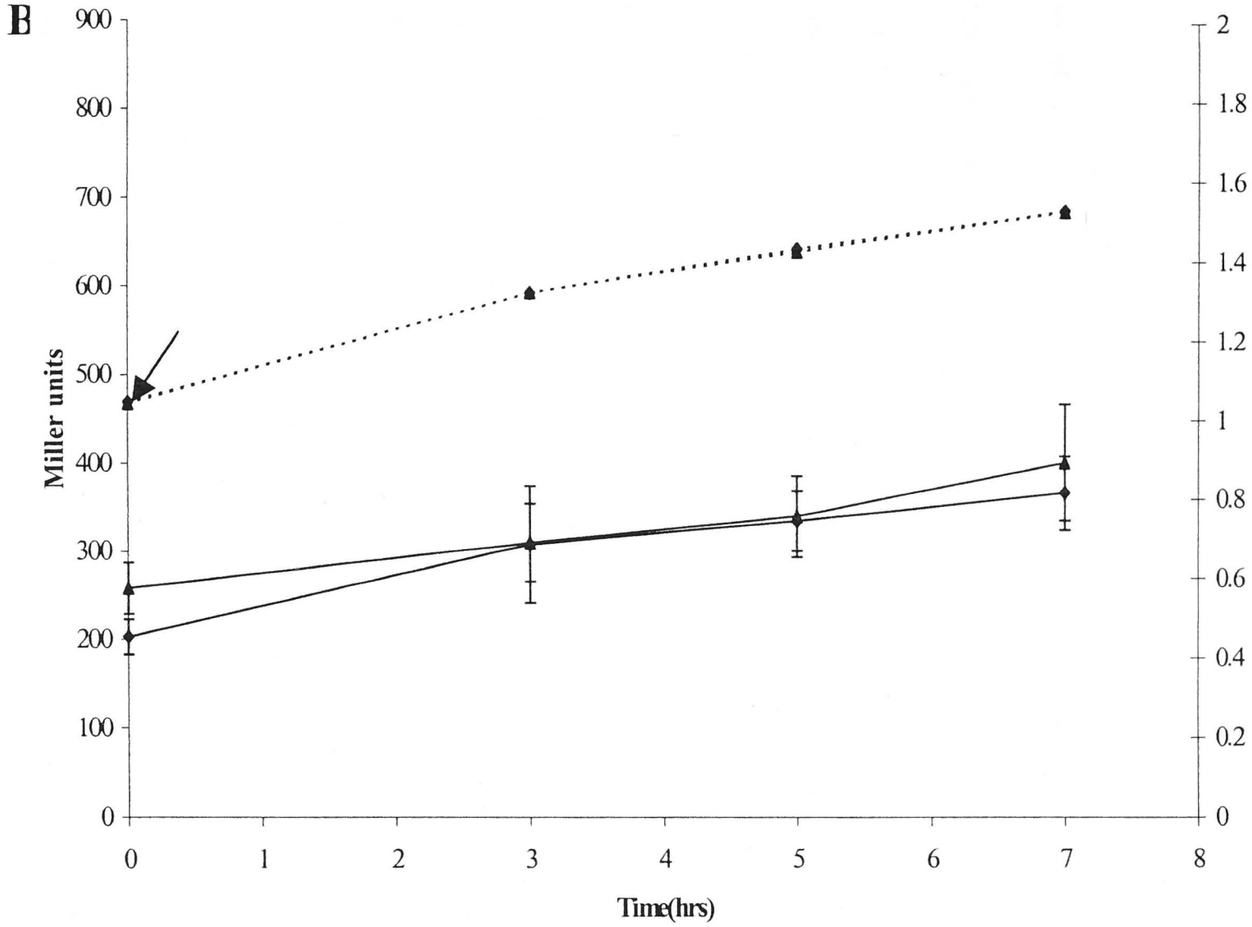


Figure 3.13. The effect of luteolin on *nolR::lacZ* expression in strain 241 grown in GTS minimal medium. Overnight culture of strain 241 grown in GTS was diluted 50 fold with GTS medium. A) Luteolin was added at inoculation time (OD_{600} of 0.05). B) Luteolin was added to the late log phase culture grown in GTS medium (OD_{600} of 1.1). Solid lines indicated beta-galactosidase activity while dashed lines indicated culture growth. The final concentration of added luteolin was 5uM. The arrow (↓) indicates when luteolin was added to the cultures.

(♦) The cultures without luteolin treatment. (▲) The luteolin-treated culture.

3. Discussion

3.1 Expression of *nolR* is population density-dependent

The *nolR* gene encodes an important regulator that negatively controls the nodulation genes in *S. meliloti* and it has a single copy on the chromosome (Cren *et al.*, 1995; Kondorosi *et al.*, 1989). The wild type strain, which has a functional NolR protein, is more efficient at nodule initiation and formation than the *nolR*$^-$ mutant strain (Kondorosi *et al.*, 1989). The functional NolR was part of a finely tuned system controlling the synthesis of the Nod factor molecules of *Sinorhizobium* that are necessary for the bacteria to optimally nodulate the host plant (Cren *et al.*, 1995). Besides controlling *nod* gene expression, NolR may act as a global regulator which responds to environmental factors to fine-tuned intracellular metabolism (Chen *et al.*, 2000). Studies of the regulatory control of *nolR* gene, therefore, could provide important clues to the hierarchical signal exchanges in the control of symbiosis and a series of key bacterial metabolic functions. By examining the patterns of *nolR* expression throughout the complete growth cycle of *S. meliloti* carrying a *nolR::lacZ* fusion, I have demonstrated that the expression of *nolR* was population density-dependent. Evidence supported this conclusion was obtained from (a) that *nolR* was not expressed during the early growth of a culture with cell population less than 10^8 CFU ml^{-1}; (b) that the level of *nolR* expression was increased with cell density of the culture and reached to its highest level when the culture was grown to early stationary-phase; (c) that the levels of *nolR* expression could be altered when the culture density was reduced by dilution. However, this finding was not in line with the conclusions made by Cren *et al.* (1995), who stated that the expression of *nolR* "did not depend on the growth phase of the bacteria grown in the minimal GTS medium, at least in the optical density (OD_{600}) range 0.1 – 1.0". The reason that the population density-dependent *nolR* expression was not observed by Cren *et al.* (1995) may be due to the fact that they did not measure the *nolR* expression activity throughout the complete bacterial growth cycle. Actually, there was not much difference in the levels of *nolR* expression when the cultures with and OD_{600} between 0.1 and 1.0 were tested. As showed in Figure 3.1 when the cultures were grown in TA medium, at OD_{600} between 0.1 to 1.0, the expression of *nolR* was at the similar levels in term of Miller units.

Once cellular growth of strain 241 was stopped by the exposure the bacterial culture to conditions which affect its cellular growth, such as in an anaerobic environment or deprived of nutrients, the level of *nolR* expression remained unchanged unless oxygen or nutrients were provided. These

results indicate that the elevation of the *nolR* activity can only occur in a growing cell. Because the growing cells change the culture population density, the correlation between *nolR* expression and growing cells supports that the expression of *nolR* is population density-dependent. That is, the change of the expression of *nolR* is correlated with the change of population density of the culture and when the cellular growth stops, the expression of *nolR* remains unchanged.

Many genes of the rhizobia family have been reported to have a population density-dependent expression pattern. For instance, the expression *nodC* gene in *Bradyrhizobium japonicum* was found to be related to culture age and the optimal gene expression occurred at very low cell density (Loh *et al.*, 2001). The *pckA* gene of *Rhizobium meliloti* was found to be another example of population density-dependent expression: in LB media, the gene expression was at a constant level during log phase, while at the onset of the stationary phase, when the cell population reached a higher level, the expression was 10 fold higher (Osteras *et al.*, 1995). However, in our studies, the expression of *nolR* was found not to be of those patterns and must occur in growing cells.

3.2 The population density-dependent *nolR* expression and quorum sensing

The cell density-dependent gene expression has been observed extensively in a range of bacteria. For instance, Liu *et al* (Liu *et al.*, 2000) reported that in *E.coli*, cell density could influence the level of the protein RNA polymerase - RpoS, which acted as a global regulator to modulate stationary phase gene expression and general resistance properties of a cell culture. The population density-dependent gene expression is often described as a control of gene expression by the quorum sensing system (Withers *et al.*, 2001). Most of quorum sensing signal molecules so far identified are AHLs. Large amounts of AHLs are excreted from cells into culture medium when bacterial cultures reach the stationary-phase. In our experiments, when the medium of the middle log-phase TA cultures of strain 241 was replaced with fresh TA medium, the unit galactosidase activity of *nolR::lacZ* fusion was reduced about 20% compared to that without a growth medium change. However, the medium change did not affect *nolR* expression when the early log-phase TA cultures were used. It is possible that the *nolR* activity was stimulated by a quorum factor that accumulated with increasing population density. When a crude preparation of AHLs produced from *S. meliloti* strain 1021 was added into the early log-phase culture of *S. meliloti* strain 241, no significant effect on *nolR* expression was observed, which suggested that either the cell population effect is not an AHL-type molecule or that the partially purified preparation did not retain the relevant molecules. A non-

AHL-type quorum sensing signal has also been found in *Bradyrhizobium japonicum* (Loh *et al.*, 2001). Although the expression of *nolA* in *Bradyrhizobium japonicum* was population density-dependent, the quorum signal molecule which mediated *nolA* expression isolated from the bacterial culture was not the typical AHL-type molecule. Instead, a two-component regulator system exists in controlling nodulation genes, which senses the quorum sensing molecule and acts as an interface between the quorum signal and its target genes (Loh *et al.*, 2002).

3.3 The expression of *nolR* was related to nutrient sources

The expression of *nolR* was found to be affected by the media in which the bacteria grew. The expression level was higher in rich medium than that in minimal medium, although the minimum medium did not affect the bacterial growth rate. In minimal medium, with the defined ingredients of carbon source, such as mannitol, sucrose, succinate and nitrogen source such as glutamate, $(NH_4)_2SO_4$ etc, the expression level of *nolR* was always lower than cultures in tryptone-yeast extract TA medium. Thus, nutrient availability is an important factor that affects *nolR* expression. The nutrient related gene expression has also been reported in *S. meliloti*. In *Rhizobium meliloti*, for instance, the expression of *pckA* gene was dependent on medium carbon sources (Osteras *et al.*, 1995). The *pckA* gene was expressed when the defined media containing succinate or arabinose as carbon sources but wasn't expressed when the defined media containing glucose, sucrose or glycerol as carbon sources (Osteras *et al.*, 1995). Presumably, the level of NolR in a culture reflects the sensitivity of this function to the cellular environment, growth medium status and which metabolic pathways are required by the dividing bacterial cells. These factors influence the level of the global regulatory role of NolR.

3.4 Auto-regulation of *nolR* transcription

By comparing the expression of *nolR::lacZ* in a strain which has a functional NolR protein and its *nolR::Tn5* mutant counterpart (which lacks a NolR protein), the expression level was found to be much higher in mutant strain in both rich medium (TA) and defined minimal medium (TMR) throughout the growth cycle. This further confirmed that NolR protein negatively auto-regulates its own gene expression (Cren *et al.*, 1995). The negative auto-regulation system of NolR also plays a role in the population density-dependent expression of *nolR*, since the rate of *nolR* expression in the $nolR^-$ background was higher compared with that in the $nolR^+$ background. Besides, the auto-

regulation mechanism plays an important role in the control of transcription of *nolR* in response to nutrient availability. The auto-regulation was found to be much more efficient in nutrient limited minimal medium than in rich medium since the level of *nolR* expression in *nolR*$^-$ *S. meliloti* strains grown in the BIII minimal medium was only reduced two times compared with those in the TA rich medium while the *nolR* expression level in *nolR*$^+$ *S. meliloti* strains grown in BIII minimal medium was reduced four times compared with those in the TA rich medium. It is possible that in minimal medium, the *nolR* inducers produced are much reduced or do not produce at all under nutrient limited condition so that the negative auto-regulation system of NolR becomes more efficient controling *nolR* expression when the cells are grown BIII. These results indicate that the auto-regulation mechanism of NolR is crucial for *S. meliloti* to maintain an appropriate level of NolR regulatory protein to respond to the changing growth conditions of the individual cell, its culture population and growth medium status.

3.5 *nolR* is responsive to environmental changes

When rapidly dividing cells of mid-exponential phase were subjected to more stressful conditions and environmental changes, such as pH shock, limited nutrients or anaerobic conditions, *nolR* was found to be responsive to these stimuli and its expression was greatly reduced. However, once the cells were rescued back into a free-living condition with neutral pH and sufficient oxygen its transcription level was restored. The lower transcription level of *nolR* under environmental challenges might due to be the lower level of availability of suitable nutrients, low pH or lack of oxygen in the growth medium.

The fact that *nolR* is responsive to different environmental stimuli by changing its transcription level suggested that expression of *nolR* is sensitive to environmental challenges and could act as a regulator in some key metabolic steps as proposed in the previous proteomic analysis studies (Chen *et al.*, 2000). Thus, different metabolism states have impact on the level of the product of *nolR*, which in turn modulate behaviours of other cellular functions through a gene network of interations.

3.6 Effect of luteolin on *nolR* expression is the result of effect of luteolin on bacterial growth

Luteolin, an inducer of common *nod* genes, was originally reported by Cren, *et al.* (1995) to have an inhibitory effect on *nolR* expression. In the presence of luteolin at the concentration of 5μM, the

expression of *nolR* was lower both in *S. meliloti* strains 241(NolR$^+$ wild type) and 249 (NolR$^-$ mutant) grown in the GTS minimal medium. It was suggested that luteolin indirectly controlled the *nolR* regulation via activation of luteolin-inducible genes (Cren *et al.*, 1995). Surprisingly, the inhibitory effect of luteolin on the expression of *nolR* could not be observed in my experiments, using strain 249 grown in TA or BIII medium. No inhibitory effect of luteolin on the *nolR* expression was detected even different concentrations of luteolin were added to the TA cultures at different growth stages in early log-phase. The inhibitory effect of luteolin on *nolR* expression was only able to be repeated in *S. meliloti* strains grown in GTS minimal medium supplemented with luteolin at very early growth stages. In fact, luteolin inhibited not only the expression of *nolR*, but the bacterial growth as well. Because the expression of *nolR* is correlated with population density of culture, the lower *nolR* expression activity in luteolin containing culture resulted from the lower cell density of the culture. This was confirmed by observations that no inhibition on *nolR* expression by luteolin could be detected when cellular growth was not affected by luteolin. For instance, luteolin did not change the growth of strain 241 grown in TA and BIII media and consequently did not alter the *nolR* expression. Furthermore, luteolin was unable to inhibit growth of *S. meliloti* strains grown in the GTS medium when it was added to the cultures at the middle log-phase. As a result of bacterial growth not affected by luteolin, the inhibitory effect of luteolin on *nolR* expression in *S. meliloti* strains grown in the GTS medium was shown. Thus, the inhibitory effect of luteolin on *nolR* expression observed by Cren *et al.* (1995) is in fact the result of the alteration of population density of *S. meliloti* cultures by luteolin.

Chapter 4 Physiological Role of NolR Under Different Stress Environment

1. Introduction

In rhizobia, the only well-characterized nodulation gene repressor is NolR from *Sinorhizobium meliloti* (Kondorosi, 1991; Kondorosi *et al.*, 1989). The functional NolR protein is in the dimeric form which negatively controls not only *nod* gene expression but also its own gene expression (Cren *et al.*, 1995). The functional NolR protein is required for the optimal nodulation since *nolR*$^-$ *S. meliloti* strains are less efficient at nodule initiation and formation compared with the wild-type strain (Kondorosi *et al.*, 1989). Cren *et al* (1995) has reported that the expression of *nod* genes of *S. meliloti* involved in core Nod factor synthesis are differentially down-regulated by NolR which leads to a production of low amounts of fully decorated Nod factors. Therefore, the fine-tuning of Nod factor production appears to be required for optimal nodulation.

NolR may also have a positive regulatory function since a database search revealed that NolR is most similar to HlyU, a transcriptional activator of the hemolysin *hlyA* gene from *Vibrio cholerae* (Kiss *et al.*, 1998). This possibility is further supported by proteomic studies on *nolR*-regulated proteins in *S. meliloti* which show that production of a number of proteins are up-regulated in the presence of a functional NolR protein (Chen *et al.*, 2000). The proteomic studies revealed that in the *nolR*$^-$ mutant of *S. meliloti*, 189 proteins were significantly altered in their levels with 101 protein spots up-regulated and 88 down-regulated. Most identified proteins involve in various cellular metabolism and functions which lead to the proposal that NolR is a global regulatory protein which responds to environmental factors to fine-tune intracellular metabolism. In the previous chapter, studies on the expression of *nolR* have shown that *nolR* expression is sensitive to various environmental stimuli including nutrition, pH, and oxygen. Therefore, it will be very interesting to discover other NolR functions besides its role of optimal nodulation via the fine control of *nod* gene expression.

The common habitat for rhizobia is the soil. In the soil, these organisms usually find themselves in unfavourable environmental stress conditions, including nutrient starvation, acidic soils, low oxygen, elevated temperature or a cold environment. All of these have a major impact on the survival of these organisms. Since there is no obvious physiological change in *S. meliloti* strains which lack a functional NolR protein when grown in normal laboratory conditions, I have grown

strains and exposed the cells to different growth and stress conditions to study its growth under different conditions and its survival strategy under different stress conditions.

In this chapter, I have investigated the functions of the *nolR* gene in terms of cell growth and maintenance under different environmental conditions. I have found that NolR is required for the optimal colony development and the optimal survival in nutrient-starved stationary-phase or after a heat shock challenge.

2. Results

2.1 NolR$^+$ cells are better survivors in nutrient-starved stationary-phase

To test whether the NolR protein plays a role in bacterial survival in stationary-phase cells, strains AK631 and EK698 were grown in BIII and modified BIII media with different mannitol and glutamate concentrations. BIII medium has 10 g L^{-1} mannitol and 1.1 g L^{-1} glutamate whereas the modified BIII medium (as the nutrient limited medium) has only 0.3 g L^{-1} mannitol and 0.6 g L^{-1} glutamate. Samples of these cultures were taken out every 2 days for viable count detection.

As shown in Figure 4.1, when the cells reached stationary phase two days after inoculation, the viability of cells of either strain AK631or EK638 grown in BIII medium sustained in the prolonged stationary phase (16 days). The cell number of both cultures grown in the nutrient limited BIII medium reached the peak (10^9 CFU ml^{-1}) two days after inoculation and then was found to decrease steadily until after 8 days of incubation. After 8 days incubation, a lower viability (10^8 CFU ml^{-1}) in nutrient limited conditions was maintained up to 16 days.

Although there was no difference of cell viability with the *nolR*$^-$ and *nolR*$^+$ strains during a prolonged stationary-phase when cells were grown in ordinary BIII medium, *nolR*$^+$ cells did survive better than *nolR*$^-$ mutant in the nutrient-limited BIII medium (Figure 4.1). The difference between AK631 and EK698 viability becomes apparent after 8 days. From day 8 until day 16, AK631 maintains its viability at 1×10^8 CFU ml^{-1} whereas that of EK698 drops to 4×10^7 and then is maintained at this lower cell viability. The half a logarithmic unit of difference, although small, was significant, which indicates that a functional NolR is required for optimal survival of *S. meliloti* under nutrient-limited growth conditions.

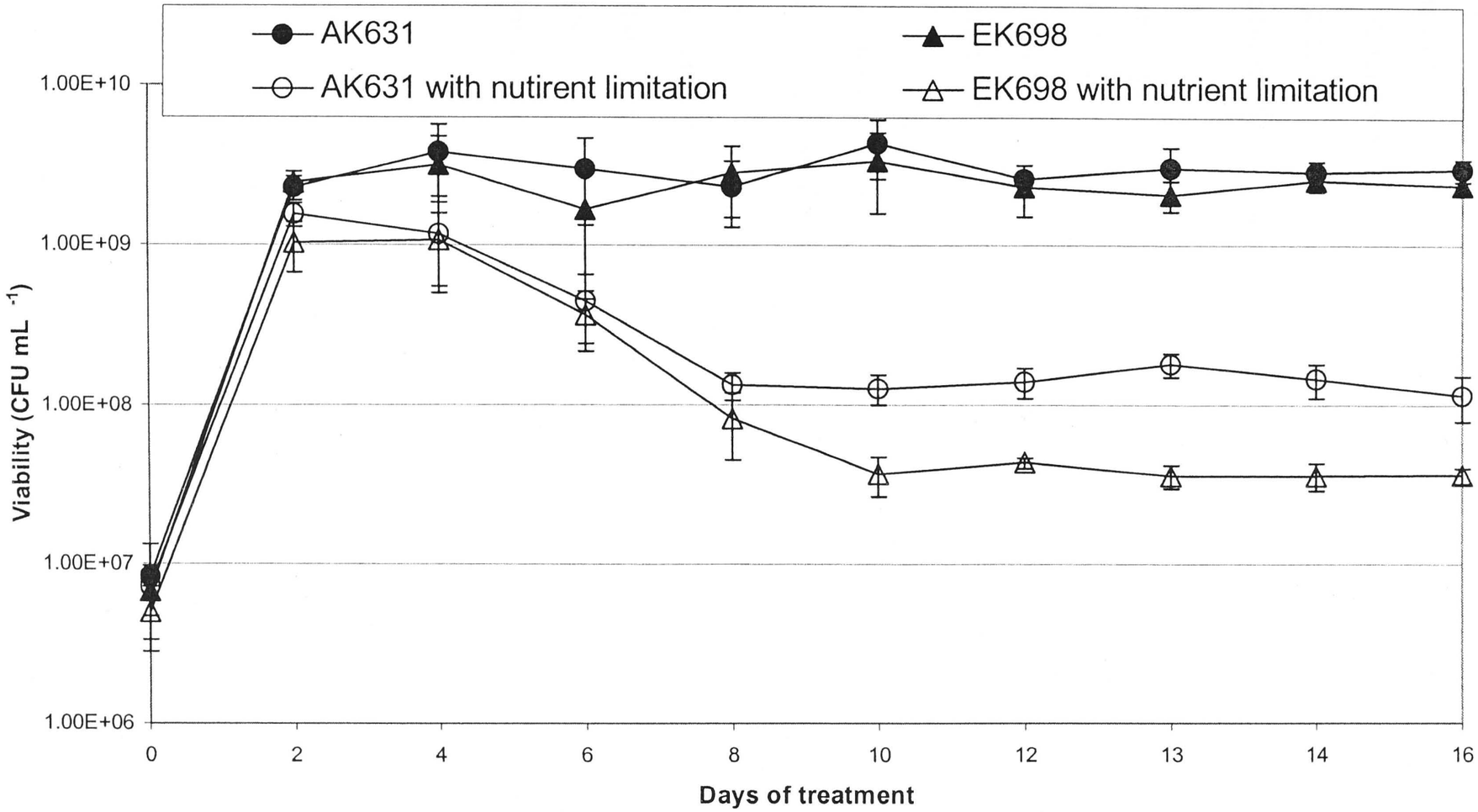


Figure 4.1 The viability of *S.meliloti* cells of strain AK631 and EK698 in BIII medium and refined BIII medium (with reduced carbon and nitrogen sources) in prolonged stationary phase. Cell viability is indicated with CFU ml^{-1}. Error bars represent standard deviations.

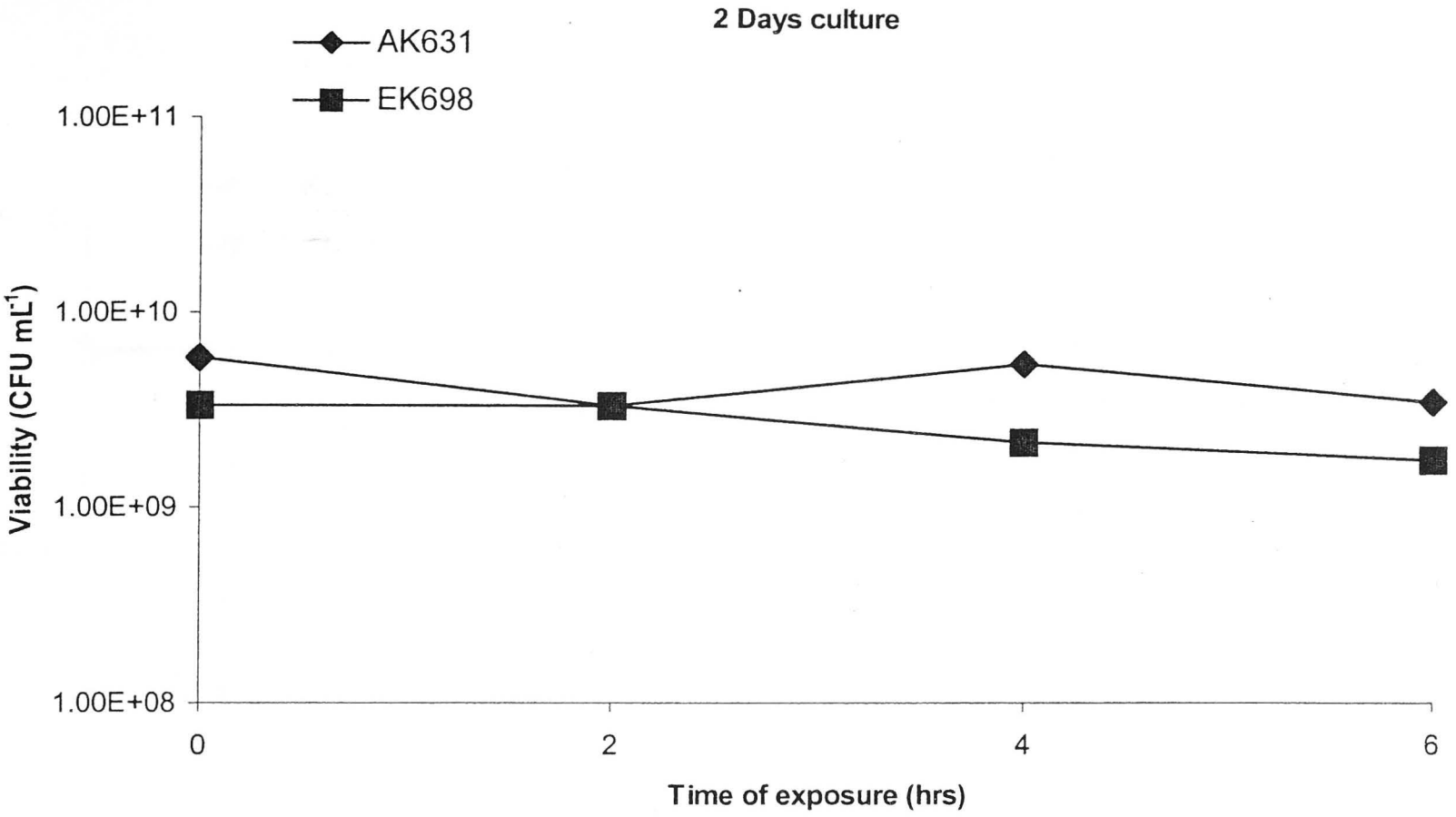
2.2 NolR$^+$ cells are better survivors after heat shock

Chen *et al.* (2000) reported that the level of a number of heat-shock proteins were altered in NolR$^-$ *S. meliloti* strain EK698. To investigate the physiological role of a functional NolR under the conditions of heat-shock stress, the NolR$^+$ AK631 and the NolR$^-$ EK698 strains were grown in BIII medium. After two days, nine days and thirteen days of the incubation, the cells were subjected to heat shock at 44°C for up to 6 hours. The viability of the heat-shocked culture was detected at every two hours during the treatment.

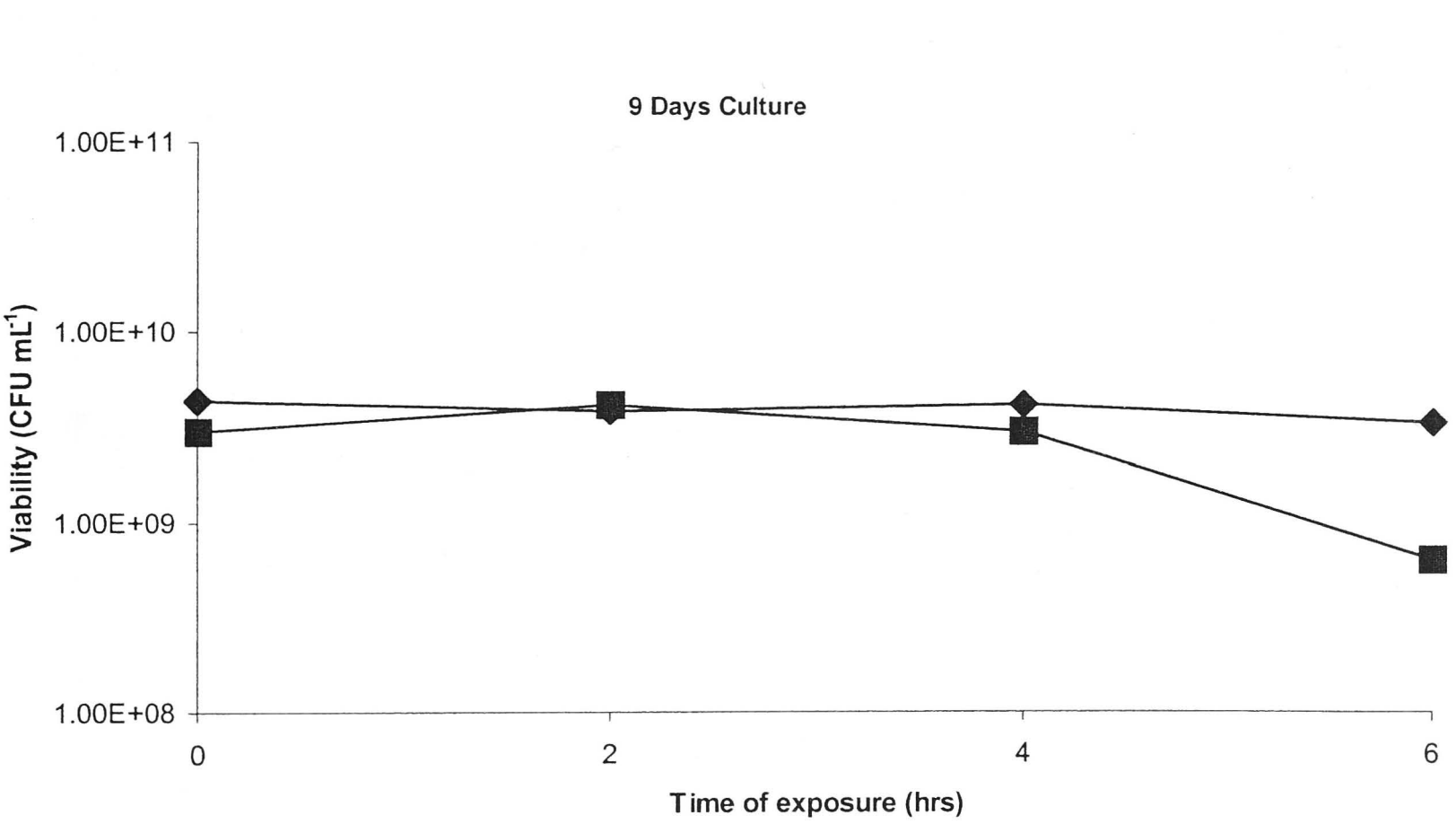
There is no significant difference in viability between AK631 and EK698 in 2-day old cultures upon 44°C heat-shock challenge (Figure 4.2 A). However, the difference in viability between the two strains become apparent in the 9-day old and 13-day old cultures (Figure 4.2 B & C). The half a log of difference in the 9 and 13 day-old cultures was significant.

The 2 days old BIII cultures of AK631 and EK698 were further subjected to elevated temperatures. Both strains had a similar viability after 45°C treatment (Figure 4.3 A). However, at 46°C, the difference in viability between the NolR$^+$ and NolR$^-$ cells was observed (Figure 4.3 B). A closer look at the viability of AK631 at the 46°C treatment is actually a biphasic curve. This suggests that between the 2-hour to 4-hour time course, there is a repairing system functioning within the cells of strain AK631, but not in the EK698 cells. Therefore, Functional NolR plays a role in the repairing mechanism to help maintain the viability of the cell under the elevated temperature.

A



B



C

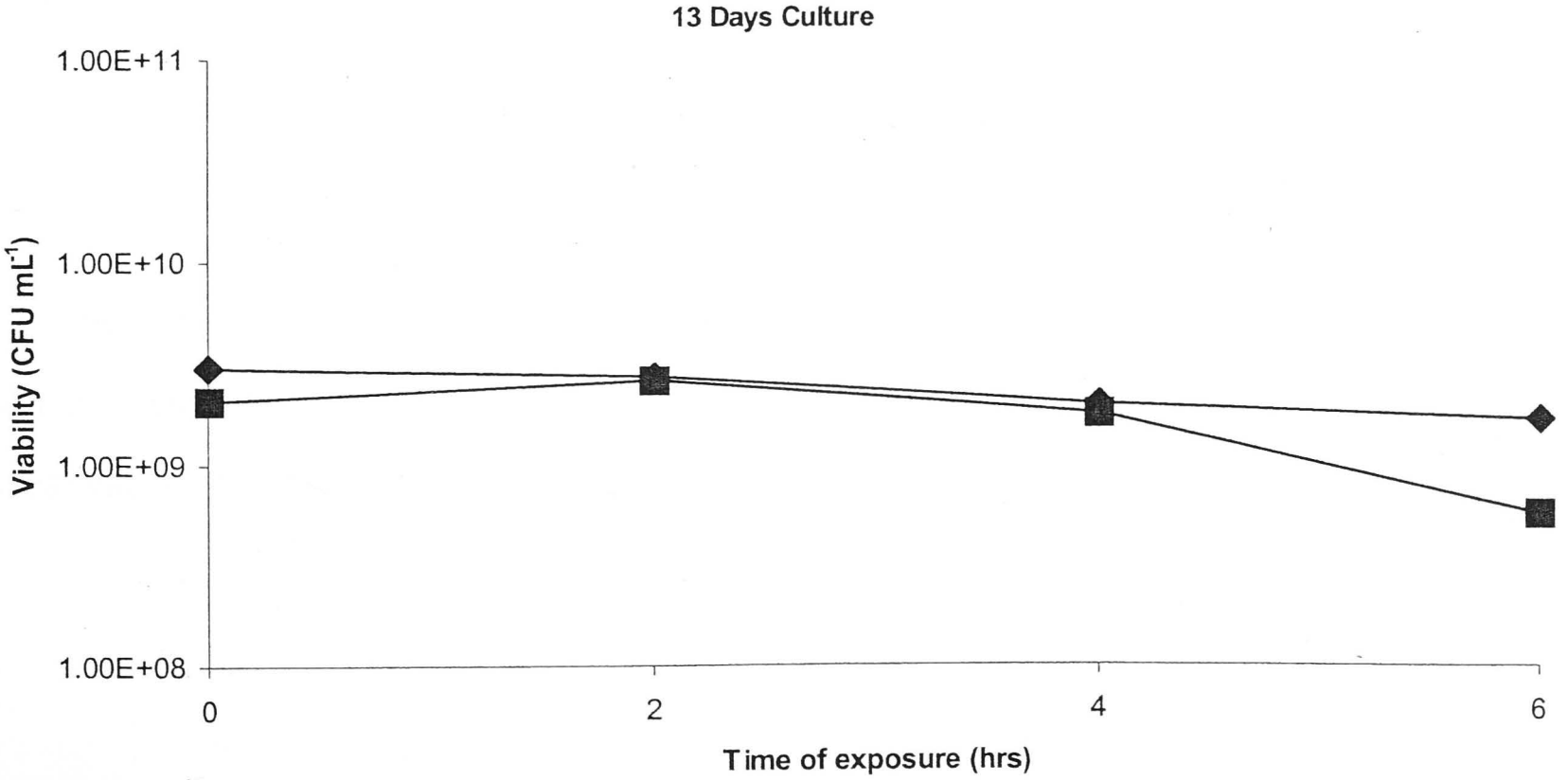


Figure 4.2 The effect of 44°C heat shock on BIII-grown *S.meliloti* strains AK631 and EK698 viability. The cultures were 2 days (A), 9 days(B) and 13 days old (C).

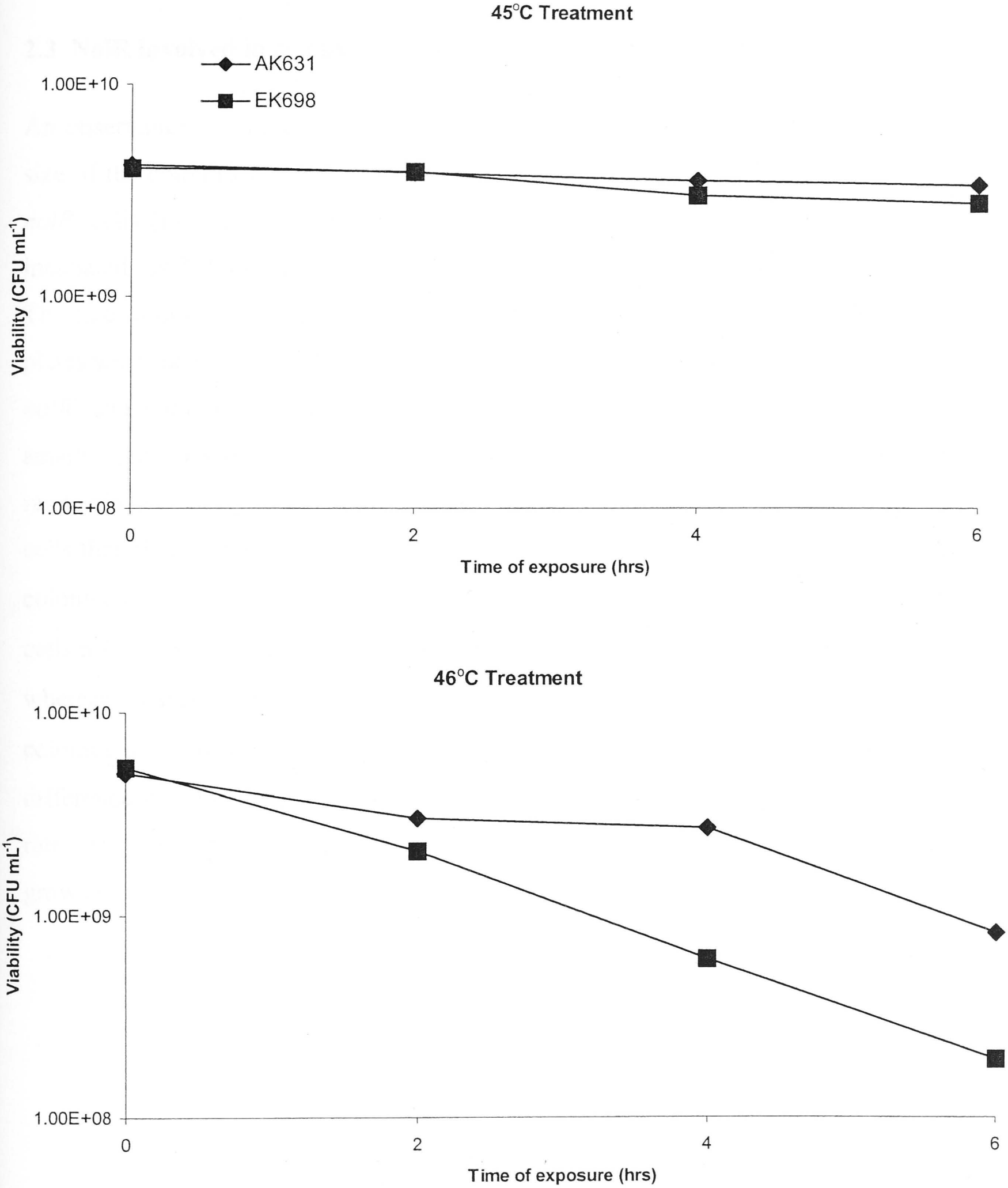


Figure 4.3 The effect of 45°C (A) and 46°C (B) heat shock on BIII-grown *S.meiloti* strains AK631(♦) and EK698 ( ) viability. The optical density at 600nm measurement of the 2 days old cultures was 2.153 and 1.959 for AK631 and EK698 respectively.

2.3 NolR involved in colony development

An observation on the colony size on the agar plates during the viable counting showed that the size of the colonies from *nolR*$^+$ cells was initially bigger compared with that of the colonies from *nolR*$^-$ cells (Figure 4.4). By measuring the size of colonies appeared on the TA plates that were incubated for 3 days, the average size of colonies of AK631 was 30% bigger than that of EK698. The size of colonies of AK631 was still slightly bigger (6% bigger) than that of EK698 when the plates were incubated for 4 days. After 5 days of incubation, difference in colony size between the *nolR*$^-$ and *nolR*$^+$ cells disappeared. To see whether the smaller NolR$^-$ colonies was due to the smaller cellular size or the less cell numbers in the colonies themselves, the colonies were picked randomly and assayed for viable count analysis. It was found that the bigger colonies had more cells than that of the small colonies. After 3 days of incubation on agar plates, an average AK631 colony consisted of 1.52×10^8 cells mL^{-1}, whereas an average EK698 colony consisted of 7.76×10^7 cells mL^{-1}. After 4 days of incubation, an average AK631 colony consisted 5.18×10^8 cells mL^{-1}, whereas an average EK698 colony consisted of 4.83×10^8 cells mL^{-1}. After 5 days on agar plates, colonies of AK631 and EK698 had similar cell number (1.2×10^9 cells mL^{-1}). Therefore, the difference in colony size was due to different cell division rate rather than different cell expansion rate. These results indicate that the functional NolR is required for the optimisation of *S. meliloti* to grow and divide on agar media.

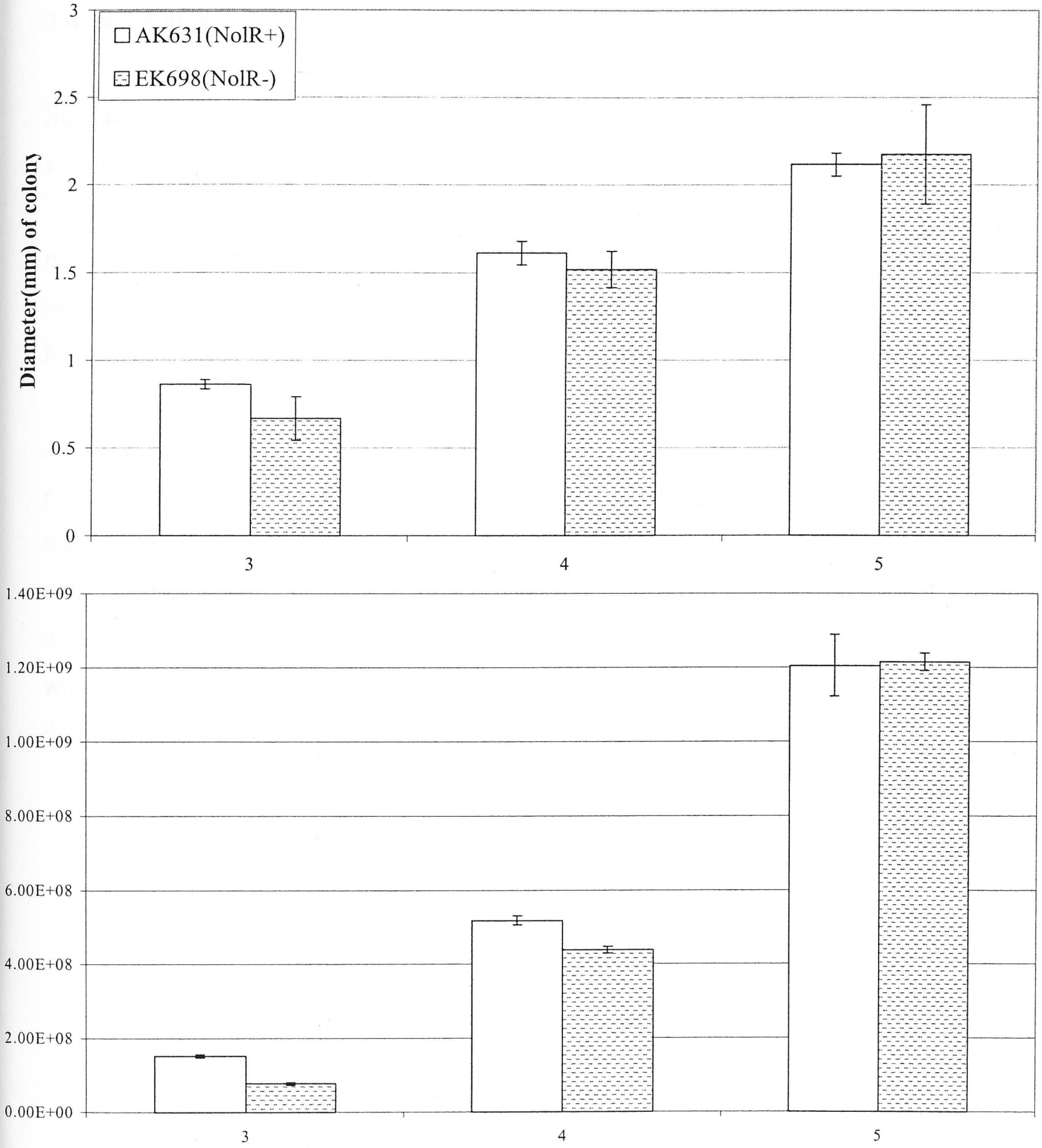


Figure 4.4 Colony development of strain AK631 ($NolR^+$) and EK698 ($NolR^-$) on TA plates. A) The average size of colonies appeared on TA plates were measured after 3,4 and 5 days. B) The average number of cells of colonies of A) were detected. Error bars represent standard deviations.

2.4 NolR does not affect bacterial growth in an acidic environment

Soil acidity is a major factor limiting legume growth and nitrogen fixation because of its adverse effects on the growth of the host plant, its root nodule bacteria and symbiotic development (Munns, 1986). To test the physiological role of functional NolR in the growth and maintenance of *S. meliloti* in an acidic condition, strains AK631 and EK698 grown in BIII medium for 7 days were inoculated into the defined JMM medium with a pH range adjusted from 5.5 to 5.8. No difference in acid-tolerance between the NolR$^-$ EK698 and the NolR$^+$ AK631 could be observed (Figure 4.5).

As shown above, the physiological role of NolR in bacterial survival in nutrient-starved stationary-phase appears only when the cultures are at a very late stage of stationary-phase. However, when the aged cultures grown in BIII and the modified nutrient-limited BIII media for one or two weeks were used to test their abilities to grow in the low pH JMM medium (pH 5.5), no significant difference in bacterial growth measured by viable count between the NolR$^-$ and the NolR$^+$ cultures was detected. In addition, no difference in colony development on JMM agar plates could be detected between strains AK631 and EK698 (data not shown).

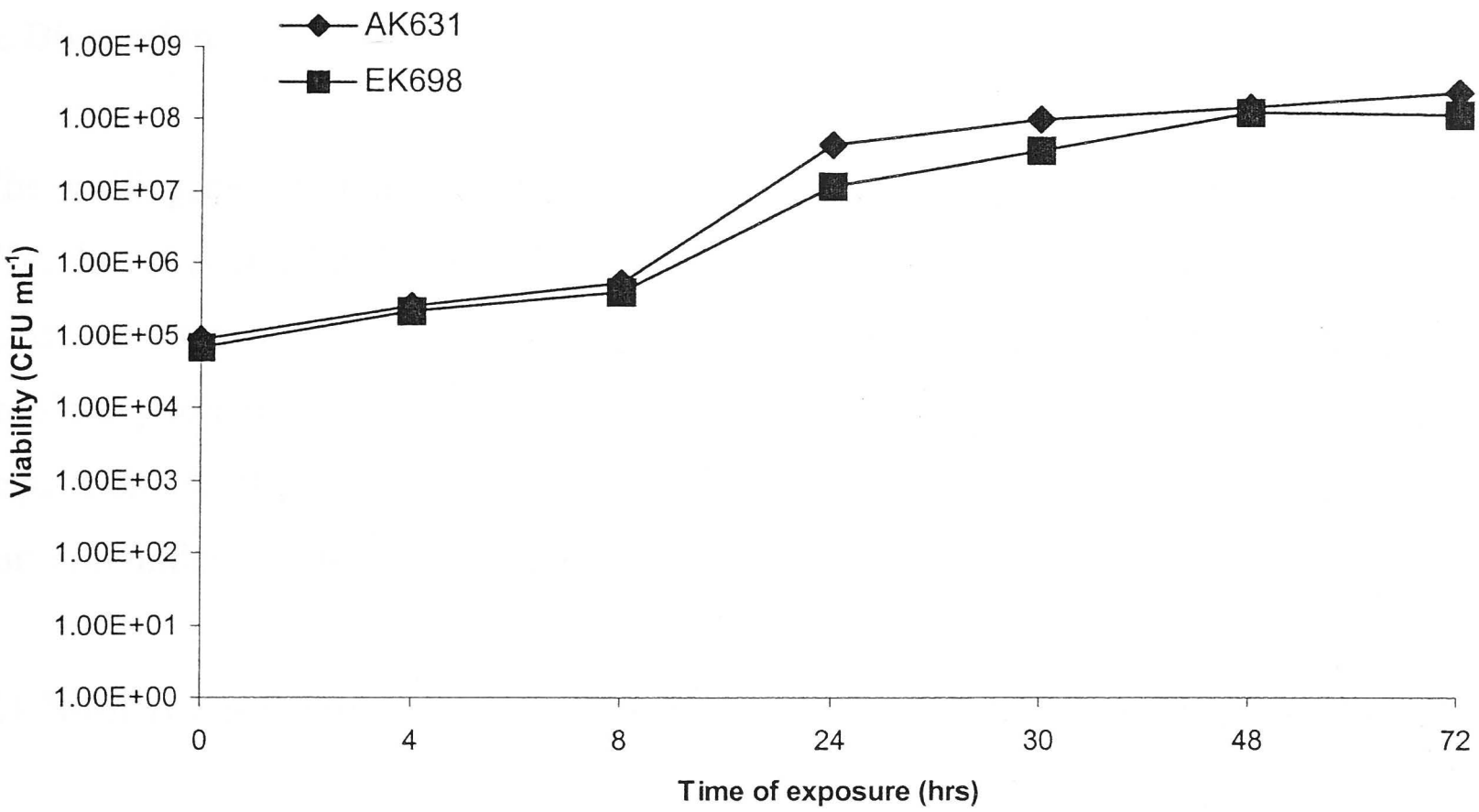


Figure 4.5 The effect of NolR on the growth of *S.meliloti* strains AK631 and EK698 in an acidic environment. The 7-day old BIII cultures of AK631 (OD600 = 1.811) and EK698 (OD600 = 2.165) were inoculated into JMM medium with pH adjusted to 5.5. Cell growth was measured by viable count.

3. Discussion

The *nolR* gene has been reported as a negative controller for optimal nodulation in *S. meliloti* (Kondorosi *et al.*, 1984). In addition, proteomic studies have showed that NolR can be a positive modulator for many genes and therefore a global regulator as well (Chen *et al.*, 2000). The regulatory role of *nolR* on nodulation genes and other genes makes it an ideal subject to study gene regulation. In this chapter, I studied the physiological functions of NolR in terms of optimal survival under different environmental stresses.

3.1 NolR is important for population density-dependent optimal starvation survival

3.1.1 Strain with a functional *nolR* gene has higher viability in nutrient-rich medium than in poor medium

A *S. meliloti* strain with a functional NolR protein was found to have higher transcription level in rich medium than in minimal medium even though cells grew well in both nutrient status (Chapter 3). Furthermore, the viability experiments showed that the strain with a functional NolR has at least one and a half logarithms higher viability during prolonged stationary phase in nutrient-rich medium than in defined minimal medium. The higher transcription and viability of the *nolR* strain in rich medium might be due to the type and availability of the carbon and nitrogen sources in rich medium.

3.1.2 NolR is important for optimal survival of *S. meliloti*

In the natural environment, bacteria seldom encounter conditions that permit continuous balanced growth - thus they are in the stationary survival phase most of the time, and can survive for extremely long periods in the absence of nutrients. Many bacteria have developed stress-survival strategies. The *nolR* gene expression was found to be responsive to some environmental changes such as the pH shock, medium shift and lack of oxygen (Chapter 3), thus could the *nolR* gene product also play a role in the stationary phase adaptation to survival conditions?

NolR was found to be essential for prolonged stationary phase survival in a nutrient-limited environment. By studying cell viability during a prolonged stationary phase of *S. meliloti*, it was

found that cells survived better when there was abundant carbon and nitrogen sources in nutrient-rich than in defined minimal medium. However, the strain with functional NolR had no higher viability under this condition than that without a functional NolR, at least for the first two weeks entering stationary phase. But in the nutrient-limited conditions, the strain with functional NolR protein was found to have a viability one third of a logarithm higher than that without a functional NolR protein after more than one week of entering stationary phase. This difference of cell viability was sustained over the rest of the prolonged stationary phase.

S.meliloti is a free living organism in the soil when not in symbiotic association with legumes. In the soil, with little available nutrients, the bacteria cease to increase their biomass during stationary phase. The mechanism which enables bacterial cells to adapt changes in their stationary phase has always attracted attentions of investigators. Thorne *et al.* (1997) reported that in *Rhizobium leguminosarum* bv. phaseoli, cells were able to starve carbon, nitrogen and phosphorus shortage for as long as two months with little loss of viability. The starved cells had low level of cell division and were cross-protected against some environmental shocks (Thorne & Williams, 1997). There is also considerable evidence to suggest that starved cultures are not static populations (Zambrano *et al.*, 1993), and continued protein synthesis was found to be essential for starvation survival in *Staphylococcus aureus* (Watson *et al.*, 1998). In this chapter, NolR was found to be essential for the cell viability during prolonged nutrient-starved stationary phase. Moreover, the transcription level of NolR is related to cell population density signal(s) (Chapter 3). Two pathways have been characterized in *E.coli* and other bacteria that respond to extra-cytoplasmic stress, one pathway is under the control of the alternative sigma factor, encoded by the *rpoE* gene (Connonlly *et al.*, 1997) (Kenyon *et al.*, 2002).

Thorne *et al.* (1999) has reported population density-dependent survival in *Rhizobium leguminosarum* bv. Phaseoli and the role of an *N*-acyl homoserine lactone to the adaptation to stationary phase survival. The size of the population on entry into stationary phase was essential for cell survival: under certain thresholds, cells could not survive. In our studies, the cells of higher density in nutrient-rich medium were found also to have better survival chances during stationary phase starvation. It is only when cells were grown in defined minimal medium that the role of NolR in cell survival was observed. Possible explanations of NolR's response to starvation stress resistance are: (a) NolR might detect the starvation stress signals, activated by the overlapping pathways related to population density (which is often mediated by sigma factors), and therefore is able to protect cell viability by directly or indirectly affecting the levels of some key

macromolecular synthesis; (b) NolR itself is responsive to starvation stress signals, which in turn activates related population density signal(s), for example, sigma factors resulting in the regulation of essential genes to maintain viability during stationary-phase starvation and also generate cross-resistance to other environmental stresses, such as heat shock (as discussed below) and extreme pH.

3.2 NolR plays a role in aged culture survival under heat shock

The proteomic studies *S. meliloti* revealed that among the proteins altered in the *nolR*$^-$ mutant are some heat shock proteins, which respond to elevated temperatures (Chen *et al.*, 2000). Heat shock proteins are found in all organisms and presumably protect against thermal damage and accelerate recovery (Eriksson & Clarke, 1996). Some multiple small heat shock proteins have been found in rhizobia (Munchbach *et al.*, 1999). Many heat shock proteins are molecular chaperones or components of various energy-dependent pathways, which can protect cells during thermal stress by stabilizing protein structure and by renaturing unfolded polypetides (Parcell & Lindquist, 1993).

In our research, cultures of different of age were subjected to 44°C, 45°C and 46°C heat shock challenges. It was found that at lower temperature (44°C) it is only in older cultures (9 or 13 days) that NolR functioned in heat resistance. While in higher temperature (46°C), 2-days old cultures showed higher heat shock resistance due to the presence of NolR. Those results revealed that NolR plays a role in optimal survival under heat shock, probably via (a) heat shock proteins of a repairing system functioning directly or indirectly within the *nolR*$^+$ cells to help maintain the cellular viability under elevated temperature; (b) in aged cultures, NolR activates the synthesis of σ^E, which cross-protects cell viability in the thermal stress.

3.3 NolR is important for colony development

Further studies of shifting stationary phase cells from nutrient limited medium onto plates of rich medium saw the adjustment of NolR to a "step up" medium situation. Cells with a functional NolR were found to have higher cell division rate than those without the NolR function, indicating these cells can recover much quicker from starvation once the nutrients are provided. In nature, bacteria must often cope with hostile environmental conditions by developing sophisticated cooperative behaviour and intricate communication capabilities, such as direct cell-cell physical interactions via extra-membrane polymers, collective production of extracellular fluid for movements on hard

surfaces, long range chemical signalling such as quorum sensing and chemotactic signalling (Ben-Jacob *et al.*, 2000). The growth of a bacterial colony depends on two local conditions: multiplication rate of bacteria in the surface and occupation rates of the sites of the cluster inside a small circle around the potential growth site, representing the nutrient concentration (Li *et al.*, 1995). The expression of *nolR* was found to be population density and medium-dependent, which means cells with functional NolR have better communication capabilities and can quickly turn on the synthesis of macromolecules. Hence NolR containing cells have higher rates of cell division and better adaptation to the changed environment from liquid medium to agar plates and more nutrient.

Under ideal laboratory conditions, when sufficient nutrients and optimal growth environment are provided for *S. meliloti* cells, there is no difference in terms of growth between the cells with a functional NolR and the cells without it. For example, both types of strains have similar growth curves and generation times. They can both form nodules with legumes, except that cells without functional NolR have lower nodulation efficiency. Therefore, it is only under certain conditions that the fine-tuning functions of NolR can be observed in these two types of strains. In this chapter, I have found that NolR is important for optimal survival in some stressful conditions, such as in starved and prolonged stationary phase cells, or aged cultures exposed to elevated temperatures or efficient colony development on agar plates, though NolR does not affect bacterial growth in an acidic environment. Hence, from these observations, it can be concluded that a functional NolR is not an obligatory regulatory protein, but it is important for the optimisation of survival under stress conditions at a very late stage of stationary phase. Whether other fine-tuning regulator(s) are also involved physiologically is still unknown.

Chapter 5 Functional analysis of the *nolR* gene

1. Introduction

Sinorhizobium meliloti has been a focus of research because of both its role in symbiosis and its close relation to bacterial plant and animal pathogens including *Agrobacterium* and *Brucella*. The knowledge and extensive genetic, biochemical and metabolic research of *S. meliloti* provides a solid foundation for genomic experimentation. With the annotation of the genome sequence of *S. meliloti* model strain Rm1021, more and more will be revealed in understanding the dynamics and integrated regulation of gene functions and symbiosis.

However, with the expanding genome database, the functions of the genome are predicted only. Proof of functions awaits functional tests (Barnett *et al.*, 2001). Recently, the concept of proteomics has been expanded to cover functional analysis of gene products or 'functional genomics' on a large-scale. The term "proteome" has been coined to describe the proteins expressed by an organism's genome. It was first used by the Wilkins group to reflect the concept of the analysis of the PROTein complement expressed by a genOME (Williams *et al.*, 1996). Traditionally, proteomics has been associated with the separation and display of a large number of proteins using two-dimensional electrophoresis (2-DE). The proteins separated by 2-DE can range from those in a protein complex or subcellular fraction to a particular cell line or whole organism.

The technique of two-dimensional gel electrophoresis (2-DE) was originally introduced by O'Farrell and independently, Klose, in 1975. This technique exploits two independent properties of proteins to separate complex mixtures of proteins in two steps. The first-dimension step of isoelectric focusing (IEF) separates proteins according to their isoelectric points (p*I*). The second-dimension step separates proteins according to their molecular masses using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Exploiting these two properties of p*I* and molecular mass can allow the separation of a mixture of thousands of proteins to individual protein spots on a single two-dimensional array. 2-DE also provides a great deal of information about a separated protein including not only p*I* and molecular mass but also relative quantity, solubility and post-translational modification that cannot necessarily be predicted from the genome sequence (Lopez, 2000).

Proteome analysis has been applied to identify NolR-associated proteins in *S. meliloti* (Chen *et al.*, 2000). Although 189 protein spots were detected to be significantly altered in levels in the *S. meliloti nolR*$^-$ strain, only 38 proteins were identified using N-terminal microsequencing. This was

due to (a) that the more efficient and economic peptide-mass-finger-printing (PMF) analysis could not be used to identify proteins since the complete genome database of *S. meliloti* was not available at that time; (b) that the N-terminal sequences of many *S. meliloti* protein spots did not have a confident match to the databases of other organisms.

In this chapter, I have investigated the intracellular regulation associated with the *nolR* gene expression using proteomic analysis that consists of protein separation by 2-DE and protein identification using PMF analysis. Proteins extracted from *S. meliloti* strains AK631 and EK698 (a *Tn*5-induced *nolR*$^-$deficient mutant of AK631) grown to different growth stages, were separated by two-dimensional gel electrophoresis and compared. I was able to detect over 200 proteins that have detectable changes in levels in *nolR* mutant on the silver-stained 2-DE gels. The number of NolR-associated proteins was greater in the stationary phase than in the early log-phase. Among these NolR - associated proteins, 89 protein spots were isolated from the Coomassie-stained gels. PMF analysis shows that 74 of the 89 spots could be matched to the *S. meliloti* database.

2. Results

2.1 Two-DE reference maps for the *nolR*$^+$ and *nolR*$^-$ strains

Over 2000 protein spots were visualised on the silver-stained 2-DE gels when the total soluble proteins from *S. meliloti* strains AK631 and EK698 were compared using two-dimensional gel electrophoresis. Similar spot profiles were reproducible with different batches of cells grown under the same conditions. The protein spots of the parent strain AK631 *nolR*$^+$ and the EK698 *nolR*$^-$ strain grown in TA medium at the stages of early log-phase (OD_{600} = 0.2) and stationary-phase (OD_{600} = 1.8) were detected and compared. Comparison of the silver-stained gels of the *nolR*$^+$ and *nolR*$^-$ strains showed that the stationary-phase cultures of the *nolR*$^-$ mutant had 313 changes (Figure 5.1) and the early log-phase cultures had 219 changes.

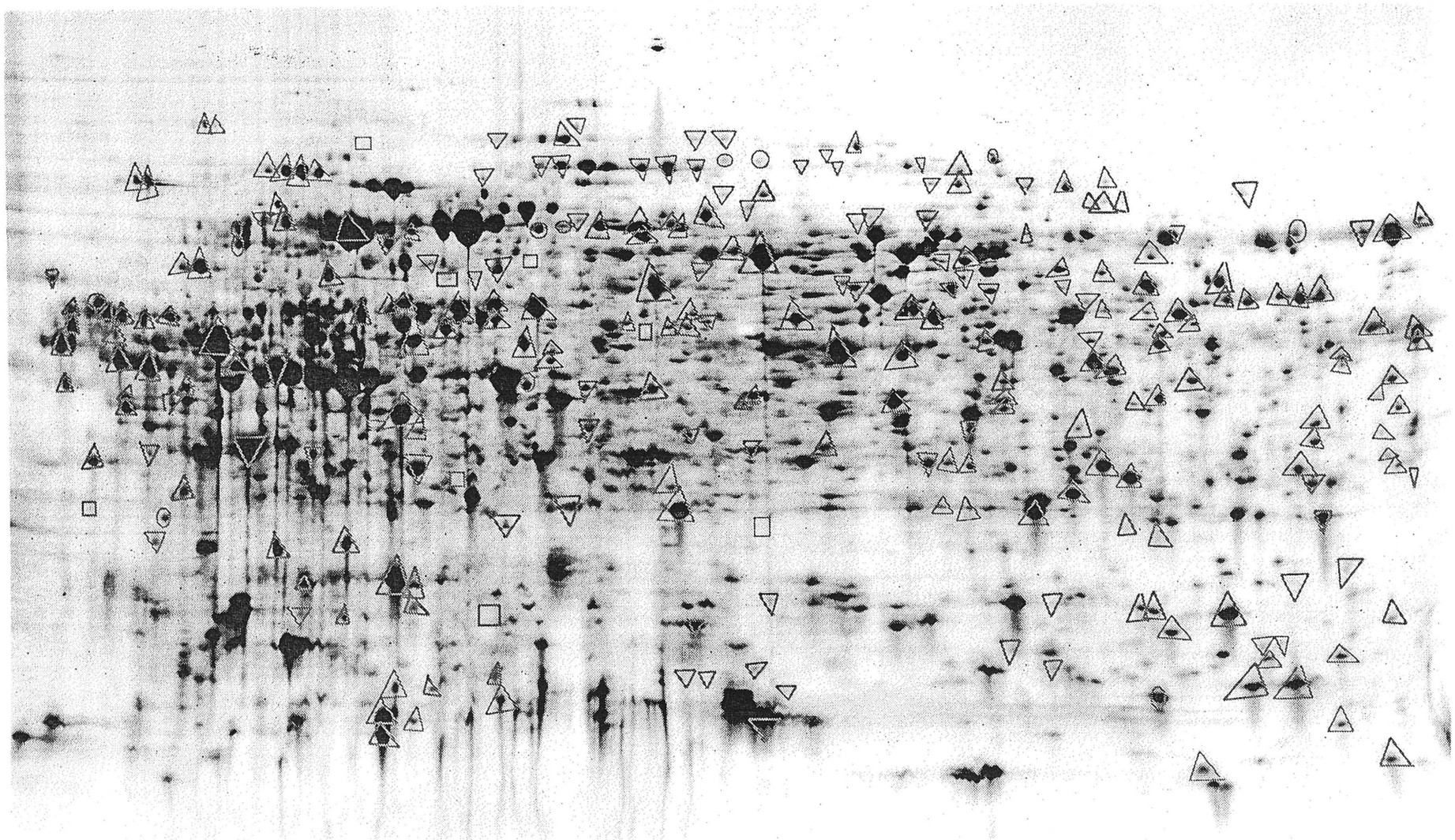


Figure 5.1 2-DE map of protein synthesised in *S.meliloti nolR*+ strain AK631 after stationary phase growth and compared with its *nolR*- mutant strain EK698. 100ug proteins were separated on 24cm 2-DE gels, pH range 4-7 and silver stained. The simbols used were (△)Up-regulated; (▽) Down-regulated; (O)New; (□)Missing.

2.2 Classification of NolR-regulated proteins in *S. meliloti*

Since protein spots from the Coomassie-stained gels are most suitable for peptide mass fingerprinting analysis, the total proteins of AK631 and EK698 were separated on the preparative gels and visualised by Coomassie staining. A minimum of 89 differentially expressed protein spots could be detected on the Coomassie-stained gels. The 89 protein spots were classified into nine different groups based on the alteration of their protein levels in the early log-phase and stationary-phase cells of *nolR*$^-$ EK698. Table 5.1 shows the group characteristics in the *nolR*$^-$ background.

Group A (36 proteins): the accumulation levels of these proteins were increased in both early log-phase and stationary-phase cultures;

Group B (8 proteins): levels of these proteins were decreased in both early log-phase and stationary-phase cultures;

Group C (2 proteins): levels of these proteins were decreased in early log-phase culture but increased in stationary-phase cultures;

Group D (5 proteins): levels of these proteins were increased in early log-phase culture but decreased in stationary-phase culture;

Group E (6 proteins): levels of these proteins were increased in early log-phase culture but not changed in stationary-phase culture;

Group F (1 protein): the level of this protein was decreased in early log-phase culture but not changed in stationary-phase culture;

Group G (8 proteins): levels of these proteins were decreased in stationary-phase culture but not changed in early log-phase culture;

Group H (22 proteins): levels of these proteins were increased in stationary-phase culture but not changed in early log-phase culture;

Group I (1 protein): this protein was undetectable in both early log-phase and stationary-phase cells.

The characteristics of NolR on regulation of cellular protein synthesis can be summarised as follows:

- The effect of NolR on regulation of protein synthesis is more efficient in stationary-phase than in early log-phase since the stationary-phase cells had 81 protein spots altered as a result of the *nolR* mutation while the early log-phase cells had 58 protein spots changed due to the *nolR* mutation;

- Most of NolR-regulated proteins are growth phase-related proteins. Among the 89 proteins, 41 had higher expression levels in stationary-phase and 17 had higher expression levels in early log-phase;
- The function of NolR is more efficient in negative regulation of protein production than in positive regulation of protein production. The number of proteins down-regulated as a result of NolR activity was 58 in stationary-phase cells and 47 in early log-phase cells while the number of proteins up-regulated by NolR was 24 in stationary-phase cells and 11 in early log-phase cells.

Table 5.1 Quantification and classification of NolR - assiciated proteins in *S.meliloti*

Group	Spot ID	Protein levels[a] (% of *nolR*+ control) EL	SP	Growth phase-related protein[b]
A (36 spots)	G73	462%	584%	N
	G45	184%	268%	SP
	G90	196%	212%	SP
	G85	146%	326%	SP
	G86	643%	169%	SP
	G81	148%	186%	SP
	G48	252%	200%	SP
	G31	522%	157%	SP
	G51	206%	257%	SP
	G05	529%	198%	SP
	G15	467%	182%	EL
	G61	201%	190%	N
	G59	1600%	354%	SP
	G58	271%	267%	N
	G76	345%	202%	N
	G66	188%	252%	SP
	G55	327%	274%	EL
	G64	813%	537%	SP
	G07	269%	906%	SP
	G16	338%	524%	N
	G62	152%	2185%	EL
	G56	478%	1756%	N
	G30	218%	767%	SP
	G02	260%	534%	N
	G54	493%	185%	SP
	G22	192%	237%	SP
	G70	2393%	179%	EL
	G32	2453%	225%	SP
	G14	245%	164%	SP
	G71	205%	174%	EL
	G63	832%	1057%	N
	G06	314%	200%	N
	G13	168%	153%	N
	G34	289%	158%	SP

	G57	210%	323%	EL
	G80	161%	314%	SP
B (8 spots)	G10	61%	59%	SP
	G83	48%	29%	N
	G91	32%	21%	SP
	G75	58%	30%	SP
	G43	62%	61%	SP
	G01	52%	31%	EL
	G41	31%	22%	SP
	G92	14%	14%	N
C (2 spots)	G19	45%	1084%	SP
	G72	48%	1659%	SP
D (5 spots)	G46	203%	49%	EL
	G89	1023%	4%	N
	G88	198%	46%	EL
	G29	331%	65%	N
	G40	243%	55%	EL
E (6 spots)	G35	170%	86%	SP
	G37	246%	118%	N
	G17	186%	99%	N
	G03	231%	84%	N
	G24	148%	106%	N
	G60	499%	104%	EL
F(1 spot)	G28	45%	104%	EL
G (8 spots)	G11	103%	53%	SP
	G77	90%	36%	EL
	G42	97%	52%	EL
	H91	104%	63%	N
	G36	81%	57%	N
	H90	87%	48%	N
	G12	93%	23%	EL
	G52	131%	57%	SP
H (22 spots)	G79	91%	155%	N
	G04	121%	146%	EL

	G65	135%	460%	SP
	G87	94%	148%	N
	G69	89%	424%	SP
	G82	117%	183%	N
	G96	nt	9%	UNKNOWN
	G49	89%	157%	SP
	G25	76%	161%	SP
	G53	102%	180%	SP
	G47	93%	215%	SP
	G23	113%	146%	EL
	G67	91%	528%	SP
	G18	135%	155%	SP
	G08	103%	166%	EL
	G21	83%	165%	SP
	G09	92%	308%	SP
	G20	72%	205%	SP
	G27	120%	293%	SP
	G68	130%	258%	N
	G38	nt	24%	UNKNOWN
	G95	nt	9%	UNKNOWN
I(1 spot)	G93	new[c]	new[c]	SP

[a]Protein levels: % spot density in *nolR* mutant of the wild type strain AK631

EL: early log phase SP:stationary phase

[b]In wild type strain AK631,

EL=protein level expressed was related to early log phase,

SP=protein level was related to stationary phase,

N=protein level was not growth phase related,

UNKNOWN=no data.

[c]Only appeared in wild type strain AK631.

2.3 Identification of proteins by peptide mass fingerprinting

The 89 NolR-associated proteins were isolated from the Coomassie-stained gels that were used for the PMF analysis (Table 5.2). Database searches show that PMFs of 74 proteins had a confident match to a *S. meliloti* strain 1021 PMF database. For most proteins with PMF match identities, the experimental molecular mass and p*I* were in good agreement with the theoretical mass and p*I* of the matched proteins, further supporting the validity of this protein identification approach. No confident match could be obtained for 15 proteins due to either the heterogeneous database of *S. meliloti* Rm1021 used or the poor signals of the generated peptides. Among the unidentified protein spots, no data could be generated for 1 protein spot due to low abundant protein level.

For the 74 proteins, there were 55 proteins encoded by genes located on the chromosome of *S. meliloti*, 6 proteins encoded by genes located on the pSyma mega-plasmid and 13 proteins encoded by genes located on the pSymb mega-plasmid. Therefore, most of the proteins altered in response to NolR were encoded by the chromosomally located genes.

It was found that the proteins altered due to the *nolR* mutation involved extensive metabolic pathways and cellular functions and some proteins had multiple functions in different metabolisms (Table 5.3). Those proteins had vital functions in amino acid metabolism (21 proteins), carbohydrate metabolism (12 proteins), energy metabolism (10 proteins), lipid metabolism (4 proteins) and nucleotide metabolism (3 proteins). Other aspects of cellular functions, such as small or large molecular transportation and binding, cell adaptation and biosynthesis of secondary metabolites were also affected. In addition, there were a large number of proteins (20 proteins) with unknown functions due to their matches to hypothetical proteins in the database.

Table 5.2 PMF matching of putative *S.meliloti* proteins affected by *nolR* mutatation

Spot ID	*Mr / p* I[a]	Mowse score	Likehood	No.of peptide matched	% Coverage	*Mm / p* I[b]	*S.meliloti* Gene ID[c]	protein
G73	32.0/6.5	4.38E+02	4.38E+02	7	30.65	35.3/6.8	SMc00035	Conserved hypothetical protein
G46	28.2/5.7	3.78E+04	3.78E+04	11	51.45	34.2/5.1	SMc00042	ilvE2, Aminotransferase (EC 2.6.1.42)
G79	21.9/6.2	1.06E+04	1.06E+04	5	59.5	22.4/6.2	SMc00043	Superoxide dismutase FE (EC 1.15.1.1)
G04	13.2/5.7	4.26E+02	4.26E+02	3	44.29	15.2/5.5	SMc00048	Conserved hypothetical protein
G74	30.0/6.8	6.00E+04	6.00E+04	13	62.55	30.4/7.2	SMc00101	Oxidoreductase
G45	28.9/5.5	1.98E+02	1.98E+02	9	32.74	36.9/6.6	SMc00265	Periplasmic binding protein
G65	40.3/5.8	1.31E+05	1.31E+05	10	39.41	43.0/5.7	SMc00327	fabB, 3-oxoacyl-acyl-carrier-protein synthase I (EC 2.3.1.41)
G90	13.7/6.7	2.33E+03	2.33E+03	8	73.33	14.3/6.5	SMc00347	rnk, Regulator of nucleoside diphosphate kinase
G10	34.6/5.5	1.37E+04	1.37E+04	9	41.79	30.5/5.4	SMc00361	Hypothetical protein
G35	10.9/5.1	2.61E+01	2.61E+01	3	36.54	11.6/4.8	SMc00496	Hypothetical protein
G85	16.6/6.4	8.52E+02	8.52E+02	6	54.84	16.6/6.2	SMc00524	bcp, Bacterioferritin comigratory protein
G86	16.5/6.4	8.38E+02	8.38E+02	8	58.06	16.6/6.2	SMc00524	bcp, Bacterioferritin comigratory protein
G37	11.4/4.4	1.64E+03	1.64E+03	6	81.98	12.1/4.4	SMc00538	Conserved hypothetical protein
G87	15.8/6.5	1.79E+02	1.79E+02	5	43.24	15.9/6.2	SMc00777	Conserved hypothetical protein
G81	24.3/6.6	1.22E+03	1.22E+03	7	53.77	20.7/6.6	SMc00943	wrbA1, TRP Repressor binding protein homologue
G11	16.4/6.0	5.80E+03	5.80E+03	8	72.73	15.9/5.6	SMc01106	Small heat shock protein
G48	16.9/5.7	2.50E+10	2.50E+10	8	58.06	19.3/8.8	SMc01137	Transmembrane hypothetical
G69	34.7/5.7	3.97E+02	3.97E+02	8	35.89	34.3/5.9	SMc01242	Signal peptide hypothetical/partial homology
G82	20.3/6.6	3.01E+03	3.01E+03	8	69.32	19.9/6.5	SMc01322	nusG, Transcription antitermination protein
G28	19.2/4.8	8.24E+02	8.24E+02	4	46.84	16.5/4.7	SMc01344	accB, Biotin carboxyl carrier protein of acetyl-coa carboxylase (bccp)
G96	9.0/6.5	3.90E+01	3.90E+01	3	24.19	13.3/4.8	SMc01418	Signal peptide hypothetical/global homology
G83	22.6/6.8	7.23E+03	7.23E+03	6	62.29	19.2/6.3	SMc01609	ribH2, 6,7-Dimethyl-8-ribityllumazine synthase (EC 2.5.1.9)
G49	15.7/5.6	1.08E+04	1.08E+04	8	82.76	15.2/5.4	SMc01613	rpiB, Ribose 5-phosphate isomerase B (EC 5.3.1.6)
G77	41.3/6.8	1.79E+03	1.79E+03	10	31.09	46.7/7.1	SMc01770	glyA1, Serine hydroxymethyltransferase (EC 2.1.2.1)
G25	40.2/5.0	5.08E+06	5.08E+06	14	55.95	41.1/5.0	SMc01828	Transmembrane transport protein
G53	11.3/5.4	6.06E+02	6.06E+02	6	85.71	10.7/5.4	SMc01833	Conserved hypothetical protein

G31	17.4/5.1	2.70E+03	2.70E+03	7	43.53	24.7/11.1	SMc02039	Oxidoreductase small molecule metabolism (EC 1.-.-.-)
G51	11.2/5.7	6.15E+01	6.15E+01	6	60.68	12.7/5.5	SMc02111	Conserved hypothetical protein
G47	26.3/5.6	7.03E+05	7.03E+05	13	69.26	30.3/5.4	SMc02122	fpr, Ferredoxin--NADP reductase (EC 1.18.1.2)
G05	18.4/5.8	1.94E+01	1.94E+01	4	27.11	18.1/5.5	SMc02123	Conserved hypothetical protein
G15	31.1/4.4	1.30E+03	1.30E+03	4	23.08	27.7/5.0	SMc02259	Abc transporter periplasmic binding protein
G93	12.2/6.4	6.25E+02	6.25E+02	6	40.12	18.9/10.3	SMc02330	nolR, Nodulation protein NolR
G91	14.5/6.5	9.70E+06	9.70E+06	8	74.19	17.1/6.5	SMc02351	Conserved hypothetical protein
G23	61.0/4.9	5.04E+04	5.04E+04	14	39.68	53.0/4.8	SMc02365	Protease precursor (EC 3.4.21.-)
G61	54.0/6.8	1.20E+03	1.20E+03	6	24.15	48.8/6.6	SMc02487	lpdA2, Transmembrane dihydrolipoamide dehydrogenase (E3 component of 2-oxoglutarate dehydrogenase complex (EC 1.8.1.4)
G58	46.1/6.7	4.30E+03	4.30E+03	9	29.27	54.7/6.7	SMc02499	atpA, Atp synthase alpha chain (EC 3.6.1.34)
G59	52.7/6.7	3.75E+07	3.75E+07	9	30.06	54.7/6.7	SMc02499	atpA, Atp synthase alpha chain (EC 3.6.1.34)
G67	52.7/5.9	1.65E+09	1.65E+09	21	57.09	58.1/5.8	SMc02562	pckA, Phosphoenolpyruvate carboxykinase (EC 4.1.1.49)
G18	31.1/4.7	6.67E+01	6.67E+01	5	24.5	33.4/6.0	SMc02710	fic, Cell filamentation protein
G42	21.2/5.4	1.11E+01	1.11E+01	3	20.51	21.6/5.6	SMc02720	clpP2, CLP protease proteolytic subunit (EC 3.4.21.-)
G17	29.1/4.6	3.75E+03	3.75E+03	7	42.45	34.0/4.6	SMc02737	opuC, ABC transporter glycine betaine-binding protein
G08	34.5/5.6	5.00E+02	5.00E+02	8	38.44	32.9/5.5	SMc03091	argI1, Arginase (EC 3.5.3.1)
G21	26.0/4.8	1.89E+04	1.89E+04	10	49.45	30.1/4.9	SMc03131	ABC transporter amino acid-binding periplasmic protein
G75	26.0/6.3	7.00E+06	7.00E+06	15	56.97	28.1/5.9	SMc03135	ABC transporter amino-acid transport system ATP-binding protein
G76	41.2/6.7	1.39E+06	1.39E+06	13	46.34	45.5/6.6	SMc03201	bkdAa, 2-Oxoisovalerate dehydrogenase alpha subunit (EC 1.2.4.4)
G09	18.3/4.8	5.22E+03	5.22E+03	5	56.52	18.3/4.8	SMc03786	bfr, Bacterioferritin (bfr) (cytochrome b-1) (cytochrome b-557)
G66	38.3/5.9	2.13E+04	2.13E+04	10	38.74	39.7/5.8	SMc03950	pntAa, Proton-translocating nicotinamide nucleotide transhydrogenase subunit (EC 1.6.1.1)
G55	67.9/6.2	6.63E+08	6.63E+08	9	22.42	71.3/6.1	SMc03978	tkt2, Transketolase (EC 2.2.1.1)
G64	36.2/6.8	4.65E+02	4.65E+02	6	32.44	36.1/7.4	SMc03979	gap, Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)
G89	17.1/6.5	1.09E+02	1.09E+02	6	62.75	17.4/6.5	SMc04040	ibpA, Heat shock protein
G07	62.2/6.2	2.91E+02	2.91E+02	11	27.61	65.5/5.9	SMc04045	ilvD2, Dihydroxy-acid dehydratase (EC 4.2.1.9)
G03	31.4/5.8	1.51E+02	1.51E+02	7	41.81	25.9/5.5	SMc04094	Conserved hypothetical protein
G16	26.3/4.5	4.35E+01	4.35E+01	4	18.22	28.3/5.3	SMc04112	Signal peptide pilus assembly protein
G62	41.4/6.5	2.95E+07	2.95E+07	15	57.14	50.8/6.2	SMc04262	gnd, 6-phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44)

H91	40.7/6.6	3.43E+05	3.43E+05	11	40.24	44.4/6.4	SMc04386	aatB, Aspartate aminotransferase B (EC 2.6.1.1)
G56	61.0/5.9	4.03E+05	4.03E+05	16	38.18	64.6/5.8	SMa0235	Putative dihydroxy-acid dehydratase (EC 4.2.1.9)
G30	17.8/5.1	4.55E+02	4.55E+02	5	37.74	17.2/4.9	SMa1200	Hypothetical protein
G43	24.0/5.5	2.14E+04	2.14E+04	9	57.35	22.2/5.2	SMa1227	fixJ, FixJ transcriptional activator
G02	38.7/6.1	9.76E+04	9.76E+04	13	59.41	36.2/5.9	SMa1296	adhA1, ADHA1 alcohol dehydrogenase (EC 1.1.1.1)
G19	24.5/4.7	4.71E+03	4.71E+03	8	61.46	22.6/4.7	SMa1313	virB5, Virb5 type iv secretion protein
G54	73.5/6.0	1.45E+03	1.45E+03	8	17.33	80.3/5.8	SMa2379	Catalase/peroxidase (EC 1.11.1.6)
G20	33.1/4.7	2.23E+04	2.23E+04	10	46.32	34.6/4.9	SMb20127	Hypothetical protein
G22	33.3/4.8	3.79E+03	3.79E+03	9	39.88	34.6/4.9	SMb20127	Hypothetical protein
G70	34.5/6.1	5.32E+01	5.32E+01	4	22.4	35.0/5.7	SMb20138	Conserved hypothetical protein
G32	16.5/5.1	1.07E+05	1.07E+05	4	36.84	16.1/5.0	SMb20343	Aldehyde dehydrogenase subunit
G27	36.5/5.2	1.76E+03	1.76E+03	7	23.58	61.6/5.1	SMb20585	ggt, Putative gamma-glutamyltranspeptidase (EC 2.3.2.2)
G72	29.8/6.4	5.60E+03	5.60E+03	7	43.62	30.3/6.2	SMb20751	Putative 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)
G88	17.4/6.5	9.00E+02	9.00E+02	8	42.51	18.4/6.5	SMb20874	Conserved hypothetical protein
G14	34.7/4.4	9.83E+01	9.83E+01	6	27.04	37.9/4.5	SMb20895	chvE, Probable sugar uptake abc transporter periplasmic solute-binding protein precursor
G24	41.6/5.0	5.05E+02	5.05E+02	7	31.12	45.9/5.2	SMb20945	exoF1, Outer membrane protein, similar to wza, oma family
G71	31.7/6.2	1.16E+06	1.16E+06	11	69.44	32.6/6.0	SMb20960	Udpglucose pyrophosphorylase (EC 2.7.7.9)
G68	43.0/5.8	3.26E+03	3.26E+03	11	27.31	57.3/7.0	SMb21037	Putative oligopeptide/murein peptide abc transporter periplasmic solute-binding protein precursor
G63	40.9/6.5	1.97E+04	1.97E+04	15	53.57	43.7/6.4	SMb21321	expA4, Putative membrane-anchored protein
G01	44.6/6.1	9.46E+04	9.46E+04	14	43.1	45.5/5.8	SMb21630	Conserved hypothetical protein
G06	17.4/5.8						ngm[d]	
G12	11.2/4.5						ngm	
G13	41.5/4.4						ngm	
G29	19.5/5.0						ngm	
G34	16.5/5.0						ngm	
G38	11.3/4.6						ngm	
G40	11.2/4.7						ngm	
G41	19.8/5.3						ngm	

G52	12.3/5.4	ngm
G57	66.4/6.5	ngm
G60	53.7/6.8	ngm
G80	20.8/6.4	ngm
G92	14.6/6.6	ngm
G95	9.3/6.5	ngm
G36	12.5/4.9	No data[e]

[a]Experimental molecular weight (*Mr*) and isoelectric point (*p* I) obtained from the 2-DE spot mobility by Melanie analysis using known standards markers.

[b]Theoretical molecular mass (*Mm*) and *p* I calculated for the predicted amino acid sequence of the protein. The units for molecular mass are kDa.

[c]Database accession number: SMc - chromosomal gene, SMb - gene on pSymA plamid, SMa - gene on pSymB plasmid

[d]ngm = No good match.

[e]No PMF data.

Table 5.3 Functional classification of proteins altered in *nolR*⁻ mutatant of *S.meliloti*

Amino acid metabolism (21 proteins)
- alanine and aspartatemetabolism
- arginine and proline metabolism
- cyanoamino acids metabolism
- cycteine metabolism
- D-arginine and D-ornithine metabolism
- glutamate metabolism
- glutathione metabolism
- glycine,serine,and threonine metabolism
- lysine degradation
- methane metabolism
- phenylalanine,tyrosine and tryptophan biosynthesis
- selenoamino acids metabolism
- taurine and hypotaurine metabolism
- tryptophan metabolism
- urea cycle and matabolism of animo groups
- valine,leucine and isoleucine biosynthesis/ degradation
- ABC transporter glycine betaine-binding protein
- degradation of proteins, peptides, glycopeptides

Carbohydrate metabolism (12 proteins)
- Aldehyde dehydrogenase subunit
- citrate cycle (TCA cycle)
- glycolysis/gluconeogenesis
- pentose and glucuronate interconversions
- pyruvate matebolism
- sucrose/nucleotide sugars metabolism
- A1 surface polysaccharides/antigens
- Probable sugar uptake abc transporter

Adaptation (4 proteins)
- Small heat shock protein
- Cell filamentation protein (cell division)
- adaptation
- Heat shock protein

Energy metabolism (10 proteins)
- photosynthesis
- ATP synthesis
- carbon fixation
- Nodulation protein
- Oxidative phosphorylation
- Oxidoreductase(small molecule metabolism)
- acceptor of superoxide radicals

Lipid metabolism (4 proteins)
- fatty acid biosynthesis (path 1)
- prostaglandin and luteokotriene metabolism
- fatty acid/glycerolipid metabolism
- bile acid biosynthesis

Metabolism of co-factors and vitamins (5 proteins)
- prophyrin and chlorophyll metabolism
- pantothenate and CoA biosynthesis
- riboflavin metabolism
- nicotinate and nicotinamide metabolism

Nucleotide metabolism (3 proteins)
- transcriptional activator
- nucleotide interconversions
- Transcription antitermination protein

Transport of molecules (8 proteins)
- transport and binding proteins
- transport of large molecues
- transport of small molecules
- surface structrues

Biosynthesis of secondary metabolites (1 protein)
- alkaloid biosynthesis I

Hypothetical proteins (20 proteins)

2.4 Discovery of the NolR protein

On the Coomassie stained 2-DE gels, there was only one spot (Spot ID G93, Group I) that was found expressed only in the wild type strain during both early exponential phase and stationary phase. This protein matched the NolR protein encoded by *nolR* gene of *S. meliloti* Rm1021 (Table 5.2). The Accession Number of spot G93 is SMc02330, indicating the gene encodes G93 is on chromosome. G93's experimental molecular weight and isoelectric point are 12.2 kDa and 6.4 respectively, compared with its theoretically calculated values of 18.9 kDa and 10.3. In order to confirm the spot is the NolR protein, ExPASy database search was conducted (ExPASy home page: http://tw.expasy.org). We also used the sequence analysis tool PeptIdent, which is a tool that allows the identifications of proteins using p*I*, molecular weight and peptide mass fingerprinting data. Experimentally measured peptide masses were compared with the theoretical peptides calculated for all proteins in the SWISS-PROT/TrEMBL databases. It was found that this protein (G93) had a very good match to the NolR protein encoded by *nolR* gene of *S. meliloti* strain AK631. The experimental molecular mass and p*I* of the spot G93 were 13.2 kDa and 6.4 (Table 5.2), which were close to the theoretical molecular mass (13.3 kDa) and p*I* (6.1) of the NolR of strain AK631. In addition, 5 matching peptides were found, which had no-miss cleavage and no-modification, covering 65% of the sequence of spot G93. The match score was 0.12, well above the standard of a good match.

As expected, no equivalent protein spot of NolR was found on the gels used to analyse mutant strain EK698. This was the first time that the NolR protein was observed on 2-DE gels, which provides concrete evidence for the researches on the *nolR* gene concerning the genetic modifications of the strains and the expression levels in different growth phases.

Further quantification studies of the NolR proteins were carried out using Melanie 2.3 software. The NolR spots of early exponential phase and stationary phase of strain AK631 were quantified and compared. It was found that the protein accumulated level of NolR in stationary phase was more than 175% higher than that of the level of early exponential phase. This confirms that high expression of *nolR* in stationary-phase observed in the chapter 3 was real.

2.5 Comparison of proteins altered by NolR with proteins regulated by AHLs

Proteomic analysis of *S. meliloti* proteins affected by AHL quorum sensing signals revealed that addition of AHLs to early log phase cultures of the wild-type strain 1021 caused significant differences in accumulation of over 100 polypeptides, 57 of which have been identified by PMF analysis (Chen *et al.* unpublished results). Comparing the NolR-associated proteins with the AHL-associated proteins, it was found that there were 15 proteins affected by both NolR and AHLs (Table 5.4). Interestingly, among these 15 proteins, ten were stationary phase-related proteins and one was an early log-phase-related protein.

Table 5.4 *S.meliloti* proteins affected by NolR, AHLs

Spot ID	*S. meliliti* Accession No.	Gene / protein name
G79	SMc00043	Superoxide dismutase FE
G35	SMc00496	Hypothetical protein
G37	SMc00538	Conserved hypothetical protein
G55	SMc03978	tkt2, Transketolase
G90	SMc00347	rnk, Regulator of nucleoside diphosphate kinase
G81	SMc00943	wrbA1, TRP Repressor binding protein homologue
G49	SMc01613	rpiB, Ribose 5-phosphate isomerase B
G53	SMc01833	Conserved hypothetical protein
G51	SMc02111	Conserved hypothetical protein
G47	SMc02122	fpr, Ferredoxin--NADP reductase
G67	SMc02562	pckA, Phosphoenolpyruvate carboxykinase
G17	SMc02737	opuC, ABC transporter glycine betaine-binding protein
H90	SMc02761	trxA, probale thioredoxin protein
G64	SMc03979	gap, Glyceraldehyde 3-phosphate dehydrogenase
G14	SMb20895	chvE, Probable sugar uptake abc transporter periplasmic solute-binding protein precursor

3. Discussion

Proteomic analysis is a post genomic high throughput study of the proteins compliment of cells and organisms. New developments in mass spectrometry enable identification of single protein spots from 2-DE protein gels. It is the combination of genomics and proteomics that forms the basis of a functional genomics approach to biological research (Sperling, 2001). Functional genomics will help determine when, where and how gene networks are expressed as an orchestrated system (Banerjee & Zhang, 2002). In this chapter, proteomic analysis has been successfully applied to investigate the intracellular regulation and gene networks associated with the *nolR* gene. Using peptide mass fingerprinting analysis (PMF) and *S. meliloti* genome database search, 74 of 89 NolR-associated proteins isolated from Coomassie-stained 2D gels were identified. The protein identification ratio is 83%. It is impossible to achieve such high ratio of protein identification without the availability of the *S. meliloti* genome database. These results indicate that with the availability of genome database, PMF analysis can be a quick, efficient and economical tool of high throughput proteomic analysis to investigate a gene expression network in an organism.

3.1 NolR is a global regulator

The *nolR* gene encodes a regulator that negatively controls the nodulation genes in *S. meliloti* (Kondorosi *et al.*, 1989b) (Cren *et al.*, 1995). In addition, NolR was shown to be similar in size and structure to a group of small bacterial regulatory proteins, with the highest similarity to a positive activator of HlyU (Kiss *et al.*, 1998). Proteomic analysis of NolR-regulated proteins of *S. meliloti* revealed that at least 52 proteins involved in the TCA cycle, stress response, cell growth and protein synthesis were up- and down-regulated by the *nolR* gene product suggesting that it is a global regulator (Chen *et al.*, 2000). In this chapter, at least 89 proteins on the Coomassie-stained gels and 200 proteins on the silver-stained gels were differentially expressed in the *S. meliloti* mutant lacking of a functional NolR protein. The identified proteins involve various metabolic pathways and cellular functions including amino acid metabolism, carbohydrate metabolism, lipid metabolism, nucleotide metabolism, energy metabolism, metabolism Co-factors, adaptation and transportation. These results provide further supporting evidence that NolR is a global regulator.

A large number of proteins that were up- and down-regulated in *S. meliloti* strain lacking of a functional NolR indicate that NolR may have a dual regulatory role, as both a repressor and an activator. Quantification of the NolR-associated spots revealed that number of proteins that were down-regulated by NolR was two times more than of proteins that were up-regulated by NolR.

This suggests that the repressor function is NolR's major role. The activator function of NolR could have resulted from an indirect regulation leading to the activation of other secondary functions.

NolR is necessary for the optimal survival of *S. meliloti* strains in the prolonged stationary phase when the bacteria were cultured in the nutrient-limited medium (Chapter 4). Interestingly, about half of NolR-regulated proteins are stationary phase-related proteins that had higher expression levels in stationary-phase. Bacterial stationary phase survival is a complex adaptation process (Lazazzera, 2000). The stationary-phase adaptation of *E. coli* is accompanied by marked changes in the pattern of global gene expression. Approximately 1000 genes highly expressed in the exponentially growing cells are mostly turned off or markedly repressed in the stationary phase cells and instead a set of 50 - 100 genes that are repressed in the growing cells begin to be expressed upon entry into the stationary phase (Ishihama, 1997). The stationary phase-related proteins that were regulated by NolR might be part of the adaptation mechanism. Therefore, it is possible that NolR is a global regulator involved in *S. meliloti* stationary phase adaptation that is required for the optimal starvation survival of *S. meliloti*.

3.2 Discovery of the NolR protein

Previous studies on the *nolR* gene were mainly focused on its gene expression and how it regulated nodulation genes on gene level. We demonstrated that on the Coomassie stained 2-DE gels the protein expressed only in wild type strain AK631 during both early exponential phase and stationary phase is the NolR protein. NolR protein was first discovered by searching the *S. meliloti* Rm1021 database. Because *nolR* in strain 1021 was spontaneously mutated that caused a frame shift in the C-terminal region and produced 50 more amino acids at the C-terminal of NolR (Cren *et al.*, 1994). The experimental iso-electro point (p*I*) and molecular weight (MW) of the NolR protein of *S. meliloti* strain AK631 were quite different from the theoretical p*I* and MW of NolR protein deduced from *nolR* gene of *S. meliloti* Rm1021. However, ExPAsy database search showed that the PMF profiles of the NolR protein of AK631 were perfectly matched to that of the NolR protein encoded by *nolR* gene of AK631. The experimental p*I* and MW of the NolR were very close to the theoretical p*I* and MW of the NolR deduced from *nolR* gene of AK631.

This was the first time that the NolR protein was observed on 2-DE gels, which provides concrete evidence for research on the *nolR* gene concerning the genetic modifications of the strains and the expression levels in different growth phases. The protein product encoded by the *nolR* gene was only found in AK631, but not in its mutant EK698, confirming that no NolR protein, at least no

functional NolR protein is produced in the *Tn*5-induced *nolR* mutant EK698. In addition, the expression level of *nolR* was much higher during stationary phase than in early log phase (Chapter 3). This was confirmed by further quantification studies of the NolR spots of early exponential phase and stationary phase of strain AK631. Protein level of NolR in stationary phase was 175% higher than that of early exponential phase, confirming the high expression of *nolR* in stationary-phase as observed earlier.

3.3 Proteins affected by NolR and AHLs

Because most of NolR-associated proteins were growth phase-related proteins, especially stationary phase-related proteins, expression of genes encoding these proteins might be population density-dependent and mediated by a quorum-sensing system. This possibility was supported by the results that synthesis of 15 NolR-associated proteins were also regulated by AHL quorum sensing signals and 10 of the 15 proteins were stationary phase-related proteins. It is possible that both NolR and AHL quorum sensing signals are involved in *S. meliloti* stationary phase adaptation.

Bacterial adaptation into stationary phase is a complex process involving global regulation of gene expression (Ishihama, 1997). This adaptation process should be regulated by multiple regulators. Since proteomic analysis can provide a large-scale view of the possible functions of an organism at the protein level, it can be applied to uncover networks of coordinately regulated genes that may play cooperative roles in many bacterial physiological situations, such as stationary phase adaptation.

Chapter 6 General Discussion

The *Rhizobium*/legume symbiosis is the most important nitrogen-fixing association in nature. *Sinorhizobium meliloti* has been chosen by the international research community as the model microorganism to investigate nodulation, nitrogen fixation and the symbiotic interaction between plant and bacterium. With the completion of the genome database of *S. meliloti* strain Rm1021, focusing is now on the "functional" proteomics of the organism. The *nolR* gene of *S. meliloti*, which appears to have both negative and positive regulator functions, became an ideal candidate to study "functional" proteomics of *S. meliloti*.

In this thesis, I have identified factors involved in the regulation of *nolR* gene expression, proteins regulated by the NolR protein and the physiological roles associated with its activity. The main observations and results in this investigation were as follows:

- Expression of the *nolR* gene is population density-related.
- The expression of *nolR* is related to nutrient availability.
- The expression of *nolR* is responsive to various environmental stresses, such as heat-shock, low pH/ high pH and low oxygen
- *nolR* is auto-regulated, but luteolin addition has no direct inhibitory or regulatory effect on *nolR* expression.
- The expression of *nolR* is stimulated by factor(s) accumulated with increasing population density.
- NolR is essential for optimal survival in nutrient-limited stationary-phase grown cells or after a heat shock challenge.
- NolR is required for initial optimal colony development.
- NolR is not essential for cell growth and survival under acidic conditions.
- Proteomic analysis showed that the NolR protein was a global regulator: there are numbers of proteins positively or negative regulated by the activity of the *nolR* gene. These genes were involved in various metabolic pathways and cellular functions.
- Furthermore, NolR was found to be part of an integrated regulatory system, which influences some functions that the AHL signalling system also affects.

The regulation of *nolR* gene expression, and functions regulated by the NolR protein and physiological roles of the *nolR* gene can be summarised as in Figure 6.1.

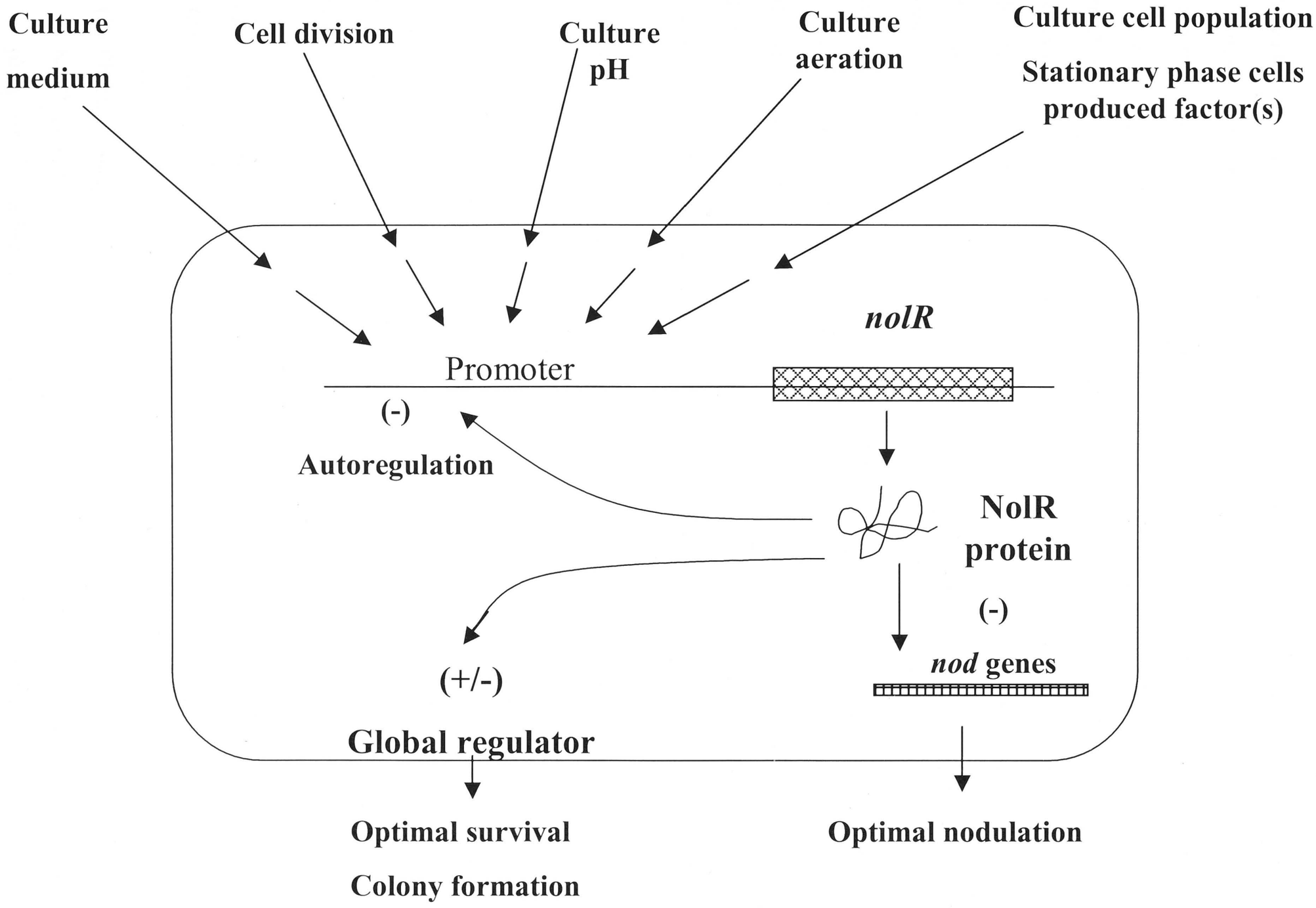

Culture
medium
Cell division
Culture
pH
Culture
aeration
Culture cell population
Stationary phase cells
produced factor(s)
nolR
Promoter
(-)
Autoregulation
NolR
protein
(-)
nod genes
(+/-)
Global regulator
Optimal survival
Colony formation
Optimal nodulation

Figure 6.1

1. The *nolR* gene encodes a global regulator

In *Sinorhizobium meliloti*, there exists a fine tuning mechanism of nodulation genes (*nod* and *nol* genes). Expression of the nodulation genes is under both positive and negative controls (Kondorosi *et al.*, 1989b) (Kondorosi *et al.*, 1991). This dual control provides a mechanism for fine tuning of the expression of nodulation genes, which enables a more successful interaction of the *S.meliloti* with the plant host. The expression of these nodulation genes can be activated in the presence of the constitutive *nodD* gene in conjunction with specific signal molecules exude from the roots of the host plants (Peters *et al.*, 1986) (Redmond *et al.*, 1986). The negative regulation of the *nod* genes was controlled by the *nolR* gene (Kondorosi *et al.*, 1989b). The low induction of the *nod* genes was found in 80% *Sinorhizobium meliloti* strains tested (Kondorosi *et al.*, 1991). In addition, strains with the NolR repressor were found more efficient in nodule induction, which confirmed the earlier investigation that a low level of *nod* gene expression was sufficient for nodulation and over expressed *nodABC* genes inhibits nodulation (Mulligan & Long, 1985) (Knight *et al.*, 1986). Therefore, for optimal nodulation, a balance between positive and negative control of Nod factors production was required.

The *nolR* gene has a single copy on the chromosome and was found highly conserved in the *Sinorhizobium* genus (Kondorosi *et al.*, 1989b) (Kiss *et al.*, 1998). Recent proteomic analysis work revealed that a number of proteins appear to be regulated by *nolR* gene in *S. meliloti* (Chen *et al.*, 2000). These included proteins involved in the TCA cycle, stress response, cell growth and protein synthesis. In addition, the NolR protein was shown to be similar in size and structure to a group of small bacterial proteins, with the highest similarity to a positive activator HlyU (Kiss *et al.*, 1998).

In this thesis, the NolR protein was proposed as a global regulator based on the following:

(a) Earlier studies have showed that NolR is important for optimal nodulation as a repressor of *nod* genes (Kondorosi *et al.*, 1989b) (Cren *et al.*, 1995).

(b) Physiological functions of the NolR protein were investigated and it was found that NolR is essential for optimal survival in cells grown in nutrient-limited stationary phase or after a heat shock challenge. In addition, NolR is required for initial optimal colony formation. Under ideal experimental conditions, when sufficient nutrients and optimal growth environment are provided, the phenotypes of cells, which have a

functional NolR and the cells without it show no difference in terms of growth pattern or nodule formation ability although the cells without functional NolR have lower nodulation efficiency. Therefore, it is only under certain conditions, in this case, cells grown in starved and prolonged stationary phase, or aged cultures exposed to elevated temperatures or colony development on agar plates, that the fine-tuning functions of NolR can be observed.

(c) Numbers of proteins regulated by the activity of the *nolR* gene involved in various cellular functions. At least 89 proteins on the Coomassie-stained gels and 200 proteins on the silver-stained gels were differentially expressed in the mutant strain lacking of a functional NolR protein. These identified proteins involve various metabolic pathways and cellular functions such as amino acid metabolism, carbohydrate metabolism, lipid metabolism, nucleotide metabolism, energy metabolism, metabolism Co-factors, adaptation and transportation.

(d) A large number of proteins that were up- and down-regulated in *S. meliloti* strain lacking of a functional NolR, indicating NolR may have a dual regulatory role, as both a repressor and an activator. Quantification of the NolR-associated spots revealed that number of proteins that were down-regulated by NolR was two times more than of proteins that were up-regulated by NolR. This suggests that the repressor function is NolR's major role. The activator function of NolR could have resulted from an indirect regulation leading to the activation of other secondary functions.

Hence, these evidence and observations revealed that the NolR protein is a fine tuning global regulator. However, NolR is not an obligatory regulatory protein or a lethal factor, although it is essential under certain physiological circumstances. Whether there are other regulator(s) involved physiologically is still to be investigated.

2. Key stages of a cell cycle and its metabolism influenced the level of expression of the *nolR* gene

In *S. meliloti*, the *nolR* gene encodes an important regulator that negatively controls the nodulation genes (Cren *et al.*, 1995). The wild type strain, which has a functional NolR protein, is more efficient at nodule initiation and formation (Kondorosi *et al.*, 1989b). The functional NolR was part of a finely tuned system controlling the synthesis of the Nod signal

molecules of *Sinorhizobium* that may be necessary for the bacteria to optimally nodulate the host plant (Cren *et al.*, 1995). Besides controlling *nod* gene expression, NolR acts as a global regulator which responds to environmental factors to fine-tuned intracellular metabolism. Studies of regulatory control of *nolR* gene, therefore, could provide important clues to investigate the hierarchical signal exchanges in the control of symbiosis and a series of key bacterial metabolic functions. Expression of the *nolR* gene is regulated by proteins binding to its promoter region contained within 87 bp sequence, providing full expression of *nolR* (Cren *et al.*, 1995). Cren *et al* (1995) had showed that *nolR* was also negatively regulated by its own products. Furthermore, a group of proteins were found to bind to two distinct *nolR* promoter regions that differ from NolR binding sites. Therefore, the level of *nolR* was regulated by its own products as well as other trans-acting factors binding to its promoter region.

In this thesis, I found that the expression of the *nolR* gene is population density-related. The cell density-dependent gene expression has been observed extensively in a range of bacteria. In the rhizobia family, many genes of have been reported to have the population density-dependent expression pattern and can be of different forms (Loh *et al.*, 2001) (Osteras *et al.*, 1995). However, the expression of *nolR* was found to be of none of those patterns reported. The population density-dependent gene expression is often described as control of gene expression by the quorum sensing system mediated by AHLs signal molecules (Withers *et al.*, 2001), but our studies suggest the quorum factor accumulated in age cultures is not an AHL-type molecule.

The expression of the *nolR* gene is also nutrient availability-related and responsive to various environmental stresses, such as heat shock, pH shock and low oxygen level. The lower transcription level of *nolR* might be due to the lower level of availability of suitable nutrients, unsuitable pH conditions or lack of oxygen in the growth medium, suggesting the *nolR* gene is sensitive to environmental challenges and its product can serve as a regulator in some key metabolic steps as indicated by the previous proteomic analysis studies (Chen *et al.*, 2000).

Thus, multiple factors and different metabolism states have impacts on the level of the product of *nolR*, which in turn modulate behaviours of other cellular functions, confirming the role of NolR as a global regulator.

3. The NolR protein

Coupling the expression of a gene with an easily assayable reporter gene provides a simple way for studying the regulation of gene expression. In this thesis, *nolR::lacZ* reporter system was used to investigate the expression of *nolR*. The lactose operon represents one of the most worked-out systems of gene expression and regulation. The advantages of the *lacZ* reporter system include: (a) Simple enzyme assay for β-galactosidase, which is stable and can be assayed for many hours. (b) The enzyme can be detected by chromogenic non-inducing substrates and the colour development can be watched during reaction. (c) The produce of β-galactosidase can be induced by gratuitous inducer, for instance, IPTG. (d) There are many useful lactose analogues for the selection and screen of various mutants (Bassford *et al.*, 1978). Previous studies on the *nolR* gene were mainly focused on its gene expression and how it regulated nodulation genes on gene level by using the *nolR::lacZ* fusion reporter system. However, can this specific reporter system truly reflect the *nolR* gene expression? Since no *nolR* gene product was assayed but only the β-galactosidase activity to represent the level of the NolR protein.

In this thesis, we observed for the first time on 2-DE gels the NolR protein, which was encoded only by the *nolR* gene of wild type strain AK631, but not in its mutant EK698, confirming that no NolR protein, at least no functional NolR protein is produced in the *Tn*5-induced *nolR* mutant EK698. This specific NolR protein spot was expressed in strain AK631 during both early exponential phase and stationary phase, and the protein level was 175% during stationary phase than that of early log phase by quantification studies, thus confirming the high expression of *nolR* in stationary-phase as observed by using the *nolR::lacZ* reporter system in earlier studies.

This proteomic analysis provides powerful evidence on the *nolR* gene concerning the genetic modifications of the strains and the gene products due to different growth phases. With this analytical tool, combining with genetic studies, confidence level of the research is greatly elevated and data become more reliable.

4. NolR is part of an integrated regulatory system

Proteome analysis is a powerful tool to examine cellular global regulation. It is particularly valuable in identifying sets of differentially regulated gene products that are expressed due to physiological growth conditions or genetic mutations. With the completion of genomic sequence project of model symbiont *S.meliloti* Rm1021, it is now becoming possible to address questions about the global structure of gene regulatory network. By searching the available genome data and putative functions of certain sets of genes, one can detect connectivity of the corresponding regulatory network by a given regulatory protein, or proteins, to get an overview of an integrated regulatory system.

In this thesis, most of the NolR-associated proteins were stationary phase-related, while the expression of the *nolR* gene was population density-dependent related and mediated by a quorum-sensing system. Thus expression of genes encoding these NolR-related proteins might be population density-dependent and mediated by a quorum-sensing system. When the large amount of proteomic data were compared and those obtained through the annotated DNA sequence, we found that there was overlapping of the proteomes of the two regulatory networks in *S. meliloti*. We found that 15 NolR-associated proteins were also regulated by AHL quorum sensing signals and 10 of the 15 proteins were stationary phase-related proteins. Therefore it is possible that both NolR and AHL quorum sensing signals are involved in *S. meliloti* stationary phase adaptation.

NolR has been indicated to be a global regulator, which controls essential cellular functions and metabolic pathways. However, how NolR is related to other regulatory systems is still unknown. Traditionally, biology has focus in studying individual genes, proteins and cells in isolation. Hood (2001) first proposed the concept of Systems Biology, which studies biological systems by systematically perturbing them (biologically, genetically, or chemically); monitoring gene, proteins, and information pathways responses; integrating these data and ultimately, formulating mathematical models that describe the structure of the system and its response to individual perturbations (Hood, 2001) (Ideker *et al.*, 2001).

Our research has invested and compared several members of regulatory networks of the model rhizobia strain by using genetic tool, the *nolR* promoter, and proteomic technology.

The findings suggest that the regulatory network of the NolR protein was neither simple nor isolated, but a part of an integrated regulatory system, which involves nodulation, various metabolisms, stationary phase survival, and other essential cellular functions.

5. Future work

Future investigations will be carried out in the following directions:

- Study the quorum sensing system mediating the *nolR* gene expression. Identify and characterise the signal factor(s) involved.
- Amplify and label the 87-bp promoter region of the *nolR* gene, and isolate the specific proteins (*trans*-acting factors) binding to this region by affinity chromatography.
- Further proteome analysis of the *trans*-acting factors by 2-DE electrophoresis, peptide mass fingerprints using the established *S.meliloti* genome database.
- Investigate the NolR protein of model strain Rm1021, which has a 50 more animo acids at C-terminal.
- Choose one of the bio-pathways affected by NolR and investigate NolR's regulatory function in it.
- Study other regulatory systems in *S.meliloti* and investigate the possible connection with the NolR regulatory system.

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