Functional Genomic Analysis of Global Regulator NolR in *Sinorhizobium meliloti*

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NolR is a regulator of nodulation genes present in species belonging to the genera *Rhizobium* and *Sinorhizobium*. The expression of the *nolR* gene in *Sinorhizobium meliloti* AK631 was investigated in relation to stage of growth, availability of nutrients, and different environmental stimuli using the *nolR::lacZ* fusion report system. It has been shown that the *nolR* gene is regulated in a population-density-dependent fashion and influenced by a number of environmental stimuli, including nutrients, pH, and oxygen. Exploration of the physiological functions of NolR under various laboratory conditions has shown that NolR is required for the optimal growth of the bacteria on solid media, optimal survival of the bacteria in carbon-starved minimal medium, and after heat shock challenge. NolR also is involved in recipient-induced conjugative transfer of a plasmid. Proteome analysis of strain AK631 and its Tn5-induced *nolR*-deficient mutant EK698 revealed that a functional NolR induced significant differences in the accumulation of 20 polypeptides in peptide mass fingerprinting early-log-phase cultures and 48 polypeptides in stationary-phase cultures. NolR acted mainly as a repressor in the early-log-phase cultures, whereas it acted as both repressor and activator in the stationary-phase cultures. The NolR protein and 59 NolR-associated proteins have been identified by peptide mass fingerprinting. The NolR protein was differentially expressed only in the NolR⁺ wild-type strain AK631 but not in its NolR⁻ derivative EK698, confirming that no functional NolR was produced in the mutant. The NolR-associated proteins have diverse functions in amino acid metabolism, carbohydrate metabolism, lipid metabolism, nucleotide metabolism, energy metabolism, metabolism of Co-factors, and cellular adaptation and transportation. These results further support our previous proposal that the NolR is a global regulatory protein which is required for the optimization of nodulation, bacterial growth and survival, and conjugative transfer of a plasmid.

Additional keywords: cellular functions, luteolin, protein networks, quorum sensing.

Sinorhizobium meliloti forms a nitrogen-fixing symbiosis in root nodules of a group of legumes generally referred to as “medics” which includes commercially significant species such as *Medicago sativa* (alfalfa or lucerne) and *M. truncatula* (Australian barrel medic).

During this plant–microbe interaction, bacterial nodulation (*nod*) gene expression is regulated both positively and negatively (Kondorosi et al. 1989a). 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 coordinately regulate the common nodulation genes (Kondorosi et al. 1989a and b).

Negative regulation is mediated by a repressor protein encoded by a chromosomal *nolR* gene (Cren et al. 1994; Kondorosi et al. 1991). This chromosomal gene encodes a 13-kDa protein which binds to the overlapping *nodD1* and *nodA* promoters at the RNA polymerase binding site (Cren et al. 1995). 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 because *nolR*⁻ *S. meliloti* strains are less efficient at nodule initiation and formation compared with the wild-type strain (Kondorosi et al. 1989b). It has been reported that the expression of *nod* genes of *S. meliloti* involved in core Nod factor synthesis is differentially downregulated by NolR which leads to the production of a low amount of fully decorated Nod factors (Cren et al. 1995). Therefore, fine tuning of Nod factor production appears to be required for optimal nodulation. These studies illustrate the interaction between the chromosomal and plasmid replicons and the finely tuned nature of the control of synthesis of the Nod factors of *Sinorhizobium* spp. (Cren et al. 1995).

A database search revealed that NolR was similar in size and structure to a group of small bacterial regulatory proteins (Kiss et al. 1998). The highest similarity of NolR was to a positive activator, HlyU, although it also was similar to a group of negative regulators. The possibility of NolR being both a positive and negative regulatory protein is further supported by proteomic studies on *S. meliloti* strains, with or without a functional NolR, which revealed that production of a number of proteins were up- or downregulated in the presence of a functional NolR protein (Chen et al. 2000b). Most identified NolR-associated proteins involved in various metabolic pathways and cellular functions, including the trichloroacetic acid (TCA) cycle, stress response, cell growth and maintenance, DNA transcription, and protein synthesis. It has been suggested that NolR is a global regulatory protein which responds to environmental factors to fine tune intracellular metabolism (Chen et al. 2000b).

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The NolR function also is present in species belonging to the genera *Rhizobium* and *Sinorhizobium* (Kiss et al. 1998). Recently, it has been shown that the alteration of *nolR* expression in *S. fredii* can affect the production of Nod factors, signal responsive (SR) proteins, and exopolysaccharide (EPS), which are three different bacterial symbiotic signals and also change its nodulation capacity on different hosts (Vinardell et al. 2004).

In *Bradyrhizobium japonicum*, negative regulation of the nodulation genes is mediated by Nola and NodD2. Nola activates expression of NodD2 which, in turn, leads to the repression of *nod* gene expression (Garcia et al. 1996). It has been demonstrated that the expression of *nola* and *nodD2* is population density-dependent which is mediated by quorum sensing (Loh et al. 2001). Quorum sensing is dependent on the synthesis, exchange, and perception of small signal molecules between bacteria. The population-density-dependent expression of *nola* and *nodD2* in *B. japonicum* was mediated by a novel quorum sensing signal molecule, designated bradyoxetin (Loh et al. 2002). Bradyoxetin is different from the acyl homoserine lactones (AHLs), the most widespread form of quorum sensing signal molecules found among gram-negative bacteria (Brelles-Marino and Bedmar 2001). Quorum sensing plays an important role in the symbiosis between alfalfa and its symbiont *S. meliloti*. *S. meliloti* has at least three quorum sensing systems, Sin, Mel, and Tra (González and Marketon 2003). The Sin quorum sensing system is responsible for the production of long-chain AHLs and controls production of the symbiotically active EPS II and low molecular weight succinoglycan (Hoang et al. 2004; Marketon et al. 2002). The Mel system is responsible for the production of short-chain AHLs (Marketon et al. 2002) and appears to be involved in nitrogen fixation (Hoang et al. 2004). The Tra system, which is present in strain Rm41 but not Rm1021, also is involved in the production of short-chain AHLs (Marketon et al. 2002) and controls conjugal plasmid transfer (González and Marketon 2003).

In this study, we have investigated i) the expression of the *nolR* in *S. meliloti* AK631, an *exoB* derivative of Rm41, in relation to the stage of growth, availability of nutrients, and different environmental stimuli using a *lacZ* reporter system; ii) physiological functions of NolR under various environmental conditions; and iii) proteins regulated by NolR using peptide mass fingerprinting (PMF). It has been shown that regulation of the *nolR* is population density dependent and influenced by a number of environmental stimuli. It also has been discovered that NolR is required for the optimal growth or survival of the bacteria grown on the solid media or under stress conditions and conjugative transfer of plasmids. Furthermore, at least 59 NolR-associated proteins have been identified.

RESULTS

Population-density-dependent regulation of the *nolR* expression.

The expression of the *nolR* gene was investigated by measuring the β -galactosidase activity of *S. meliloti* NolR⁺ strain AK631 carrying a translational *nolR::lacZ* fusion inserted into its chromosome (Cren et al. 1995). Bacterial growth measured by viable cell count was parallel with that by optical density at 600 nm (OD₆₀₀). An OD₆₀₀ unit corresponded to approximately 10⁹ cells ml⁻¹ (data not shown). A correlation between cell density and the *nolR* expression was observed by measuring β -galactosidase activity over the complete growth cycle of cells of AK631 carrying the *nolR::lacZ* fusion growing in TA medium (yeast tryptone broth; Chen et al. 2003) (Fig. 1). The β -galactosidase activity of the *nolR::lacZ* fusion was reduced continuously during the first 5 h of incubation time. When the cells grew to the early log-phase with a cell population of approximately 4.3 × 10⁸ ml⁻¹ after 7 h of incubation, the β -galactosidase activity was shown to increase steadily until the cell culture reached the stationary phase. The increase of the *nolR*

Fig. 1. Population-density-dependent expression of the *nolR::lacZ* fusion in *Sinorhizobium meliloti* AK631 grown in TA medium.

expression was correlated with the increase of cell population, indicating that the elevated *nolR* expression is population density dependent. A high level of approximately 4,000 Miller units of the *nolR* expression was sustained during the stationary phase. The population-density-dependent regulation of the *nolR* expression was tested further by the 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 1 h, it was found that the β -galactosidase activity decreased with an increase in culture dilution (data not shown). A similar pattern of population-density-dependent elevation of the *nolR* expression also was observed in the *NolR*⁺ *S. meliloti* EK698 background (data not shown).

The levels of the *nolR* expression were maintained nearly unchanged when the cells were grown into stationary phase, suggesting that cell growth is required for elevated *nolR* expression. The requirement of cell growth for elevation of *nolR* expression was tested by the exposure of the early log-phase cells to a nutrient-starved medium, the supernatant of stationary-phase cultures of AK631 grown in TA medium for 2 days. When the culture medium of the early log-phase TA culture of strain AK631 was replaced by the nutrient-starved medium, cellular growth stopped. Consequently, an unchanged *nolR* activity remained. However, once cellular growth resumed by the addition of the TA nutrients (tryptone and yeast extract) to the nutrient-starved culture, β -galactosidase activity of the *nolR*::*lacZ* fusion increased with the growing cells (data not shown).

Stimulation of *nolR* expression by a quorum factor.

Population-density-dependent regulation of the *nolR* expression suggested that quorum sensing may be involved in regulation of the *nolR* gene. The expression of the *nolR* was at the maximal level when the cells reached a high cell population in stationary phase; therefore, the supernatant of the stationary-phase cultures of AK631 grown in TA medium for 2 days was used to detect the presence of quorum factors. When the early log-phase cells of AK631 carrying the *nolR*::*lacZ* fusion ($OD_{600} = 0.2$) were exposed to fresh TA medium supplemented with or without the nutrient-starved supernatant, β -galactosidase activity of the *nolR*::*lacZ* fusion in the cultures containing the supernatant was 136% of that in the cultures without the supernatant (Table 1). The enhanced activity was statistically significant. However, the enhancement of the *nolR* expression by the supernatant was not significant when the middle log-phase cells ($OD_{600} = 1.1$) were used (i.e., only early log phase cells responded) (Table 1).

Regulation of the *nolR* by environmental factors.

When strain AK631 was grown in the minimal BIII medium, population-density-dependent regulation of the *nolR* expression

pattern again was detected. The highest level of β -galactosidase activity detected in the culture grown in BIII medium was approximately 1,000 Miller units, which was four times lower than the level detected in the culture grown in TA medium (Fig. 1).

To determine whether *nolR* expression was affected by the pH of the medium, the middle log-phase TA culture of AK632 carrying the *nolR*::*lacZ* fusion (pH 6.9) was adjusted to a pH of 5.7 and 8.3 (Fig. 2). Comparing this with the culture without pH shock, the cell growth in the acidified culture was slower whereas the cell growth in the alkalinized culture was faster. The expression of *nolR* was sustained at the initial level in both acidified and alkalinized cultures during the first 2 h of incubation. The β -galactosidase activity of the *nolR*::*lacZ* fusion in the acidified culture was still sustained at the initial level up to 6 h of incubation. In the alkalinized culture, however, β -galactosidase activity started to increase with a rate similar to the control culture when the pH of the alkalinized culture was dropped to 7.7 after 4 h of incubation.

The requirement of oxygen for *nolR* expression was tested by anaerobically incubating an early log-phase culture of strain AK631 carrying the *nolR*::*lacZ* fusion in a sealed flask filled with nitrogen gas. No bacterial growth or increase of *nolR* expression was observed under anaerobic conditions. However, *nolR* expression was able to increase quickly once the bacteria started growth by returning the growth condition of the treated culture to normal air condition (data not shown). This indicates that the *nolR* expression is elevated in growing cells.

Regulation of *NolR* by luteolin.

It was reported that *nolR* expression in both the *nolR*⁺ and *nolR*⁺ strains could be inhibited with the presence of luteolin at a low concentration of 5 μ M in GTS minimal medium (Cren et al. 1995). However, inhibition of *nolR* expression by luteolin was not found in either the *nolR*⁺ or *nolR*⁺ *S. meliloti* strains grown in the rich TA or minimal BIII medium. The effect of luteolin on *nolR* expression also was tested by the addition of different concentrations of luteolin (2.5, 5, or 7.5 μ M) to cultures at different stages of early growth phase. Still, *nolR* expression was not altered by luteolin (data not shown). When GTS minimal medium was used, luteolin had an inhibitory effect on *nolR* expression only when luteolin was added to a low-cell-density culture (Table 2). In fact, not only *nolR* expression but also bacterial growth was inhibited by luteolin. When luteolin was added to the middle log phase GTS cultures of strain AK631 carrying the *nolR*::*lacZ* fusion, it did not alter bacterial growth and did not inhibit *nolR* expression (Table 2). This indicates that luteolin did not directly regulate *nolR* expression.

Table 1. Expression of the *nolR*::*lacZ* fusion in *Sinorhizobium meliloti* AK631 in response to the population-density-dependent factor

Test medium	β -Galactosidase activity of the <i>nolR</i> :: <i>lacZ</i> fusion (units) ^a	
	Early	Middle
TA	851 $\pm$ 13	1,448 $\pm$ 132
TA + supernatant ^b	1,160 $\pm$ 60	1,612 $\pm$ 24

^a Early-log-phase (Early) (optical density at 600 nm [OD_{600}] = 0.2) and middle-log-phase (Middle) (OD_{600} = 1.0) TA cultures were harvested, washed with TA medium, and suspended into the test medium in the original volume. The cultures were incubated at 28°C for 1 h and tested for β -galactosidase activities. Values are the means four independent determinations.

^b Supernatant from stationary TA culture of *S. meliloti* AK631 (OD_{600} = 1.8).

Table 2. Effect of luteolin on cell growth and *nolR*::*lacZ* fusion activity^a

Luteolin added at	With luteolin treatment		Without luteolin treatment	
	OD_{600}	Miller units	OD_{600}	Miller units
Early log phase ^b	0.726	292 $\pm$ 32	1.723	716 $\pm$ 89
Late log phase ^c	1.527	367 $\pm$ 42	1.526	402 $\pm$ 66

^a OD_{600} = optical density at 600 nm.

^b Luteolin (5 μ M) was added into the early-log-phase GTS (Cren et al. 1995) culture of AK631 carrying the *nolR*::*lacZ* fusion (OD_{600} = 0.056). Cell growth and β -galactosidase activity were measured after 24 h of incubation.

^c Luteolin (5 mM) was added into the late-log-phase GTS culture of AK631 carrying the *nolR*::*lacZ* fusion (OD_{600} = 1.041). Cell growth and β -galactosidase activity were measured after 7 h of incubation.

Requirement of NolR for optimal survival under stress conditions.

Strains AK631 (*NolR*⁺) and EK698 (*NolR*⁻) were grown in the defined BIII medium and the nutrient-limited BIII medium with much reduced carbon and nitrogen nutrients for up to 16 days. Samples of these cultures were taken out every 2 days for viable count detection. When the cells reached stationary phase after 2 days of inoculation, the viability of cells of either strain AK631 or EK638 grown in the BIII medium was sustained in the prolonged stationary phase (Fig. 3). The cell number of both cultures grown in the nutrient-limited BIII medium reached the peak (10^9 CFU ml⁻¹) after 2 days of inoculation and then was shown to decrease steadily to a lower density (10^8 CFU ml⁻¹) up to 8 days of incubation, and then sustained this low density. Although there was no difference of cell viability between the *nolR*⁻ and *nolR*⁺ strains during a prolonged stationary phase when cells were

grown in the BIII medium, *nolR*⁺ cells did survive better than the *nolR*⁻ mutant in nutrient-limited BIII medium (Fig. 3). The difference between AK631 and EK698 viability becomes apparent after 8 days. From day 8 until day 16, AK631 maintained its viability at 1×10^8 CFU ml⁻¹, whereas the viability of EK698 further dropped to 4×10^7 from day 8 to 10 and then maintained this low viability. The half a log of difference was statistically significant. These results indicate that functional NolR is required for the optimization of stationary-phase survival of *S. meliloti* under nutrient-stressed conditions.

The physiological role of NolR also was investigated under heat-shock stress conditions. This was done by the incubation of stationary-phase cultures of strains AK631 and EK698 at 44 to 46°C for up to 6 h. Viability of the heat-shocked culture was detected every 2 h during the treatment. There was no significant difference in viability between

Fig. 2. Effect of pH shock on the *nolR::lacZ* expression in strain AK631. Middle-log-phase culture grown in TA medium (optical density at 600 nm [OD₆₀₀] = 0.8, pH 6.9) was adjusted to a pH 5.6 with 40 mM MES and to pH 8.3 with 1 M Tris base. **A**, β-Galactosidase activity, **B**, cell growth, and **C**, pH of the cultures under pH shock.

AK631 and EK698 upon 45°C heat-shock challenge (data not shown). However, at 46°C, a difference in viability between *NolR*⁺ and *NolR*⁻ cells was observed (Fig. 4). The viability of *NolR*⁺ cells was statistically significant half a log higher than *NolR*⁻ cells after 6 h of incubation at 46°C. A closer look at the viability of AK631 at the 46°C treatment reveals a tripha-

sic curve. This suggests that, between the 2-h and 6-h time course, there is a repairing system functioning within the cells of strain AK631 but not in strain EK698. Therefore, a functional *NolR* may play a role in the repairing mechanism to help maintain the viability of the cell under elevated temperatures.

Fig. 3. Viability of strains AK631 and EK698 in BIII medium and carbon-nitrogen-limited BIII medium in prolonged stationary phase.

Fig. 4. Effect of 46°C heat shock on the viability of strains AK631 and EK698 in BIII medium.

Requirement of *NolR* for colony development.

An observation on the colony size on agar plates during viable counting showed that the size of the colonies from the *nolR*⁺ cells initially was bigger compared with that of the colonies from the *nolR*⁻ cells. By measuring the size of colonies that appeared on the TA plates after 3 days of incubation, it was found that 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 were due to smaller cellular size or lower cell numbers in the colonies, the colonies were picked up randomly and used for viable count analysis. It was found that bigger colonies had more cells than 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 of 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 on solid medium. These results indicate that functional *NolR* is required for the optimization of *S. meliloti* to grow on solid media.

NolR regulates *Rhizobium leguminosarum* bv. *trifolii nodA* and affects conjugal plasmid transfer.

Kiss and associates (Kiss et al. 1998) indicated the conservation of the *nolR* in the genera *Sinorhizobium* and *Rhizobium* of the *Rhizobiaceae* family, including *Rhizobium leguminosarum* bv. *trifolii*. To detect the function of *NolR* of *S. meliloti* on regulation of *nod* genes of different *Rhizobium* spp., plasmid pRT311 (containing a transcriptional fusion between the *R. leguminosarum* bv. *trifolii nodA* promoter and the *lacZ* gene) was conjugally transferred into strains AK631 and EK698. It was found that there was a big difference in plasmid transfer frequency of pRT311 to the *NolR*⁺ strain AK631 compared with the *NolR*⁻ strain EK698. The transfer frequency of pRT311 to AK631 and EK698 was 1.2×10^{-2} and 2.3×10^{-4} , respectively. The plasmid transfer frequency in AK631 was 50 times higher than that in EK698, indicating that *NolR* plays a role in conjugal plasmid transfer. In the presence of luteolin, the β -galactosidase activity of *NolR*⁺ AK631 carrying plasmid

pRT311 was 60% of that shown by its *NolR*⁻ derivative EK698 carrying plasmid pRT311, indicating that *NolR* of *S. meliloti* is also functional on the expression of *R. leguminosarum* bv. *trifolii nodA*.

Effects of *NolR* on protein accumulation in wild-type *S. meliloti* AK631.

When two-dimensional (2D) maps of *NolR*⁺ AK631 and *NolR*⁻ EK698 were compared, one protein (spot 93) was found to exist only in AK631 but not in EK698 (Fig. 5; Table 3). The molecular weight (MW) and isoelectric point (pI) of spot 93 on the gel were 13.2 kDa and 6.4, respectively, which were close to the theoretical MW (13.3 kDa) and pI (6.1) of AK631 *NolR*. PMF analysis showed that this protein is highly homologous to *S. meliloti* *NolR* in the SwissProt database (Table 3). PMF of spot 93 also was matched to *NolR* in the *S. meliloti* 1021 genome database, although the confidence of the match is low (data not shown). This is due to a single insertional mutation in the C-terminal coding sequence of the *nolR* gene of 1021 which caused the open reading frame to be elongated by 50 amino acids, resulting in a 172-amino-acid-long, 18.8-kDa protein (Cren et al. 1995). No equivalent protein spot of *NolR* was found on the 2D gels used to analyze the *NolR*⁻ EK698, which demonstrates that no functional *NolR* has been produced in strain EK698. The *NolR* spots of the early exponential phase and stationary phase of strain AK631 were quantified and compared. It was found that the accumulation of *NolR* in stationary phase was 175% of that of in the early log phase (Table 3). The higher accumulation of *NolR* in the stationary phase corresponds to the higher expression of the *nolR* in the stationary phase (Fig. 1).

The accumulation of 59 protein spots was affected reproducibly and significantly by a functional *NolR* when the protein profiles of strains AK631 and EK698 grown in the TA medium at the stages of early log phase (OD₆₀₀ = 0.2) and stationary phase (OD₆₀₀ = 1.8) were compared on the 2D gels. The difference in the accumulation of the 59 protein spots in the *NolR*⁻ EK698 background is shown in Table 3. In early-log-phase cultures, 20 protein spots showed changes of at least 1.5- to 8-fold in accumulation. Among the 20 spots, 17 were increased and 3 were decreased in protein accumulation level. In stationary-phase cultures, however, 48 protein spots were altered in accumulation level at least 1.5- to 4-fold. Of the 48 spots, 25 were increased and 23 were decreased in protein accumulation level. Nine protein spots were altered in protein accumulation level in both early-log-phase and stationary-phase cultures,

Fig. 5. Enlarged views of the region containing *NolR* protein of *Sinorhizobium meliloti* (arrows show the position of the *NolR* spot). Left, AK631 with a functional *NolR*; right, EK698 without a functional *NolR*. SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

with six of the nine spots having increased or decreased protein levels in the both growth phase cultures and three having increased protein levels in the early-log-phase cultures but decreased protein levels in the stationary-phase cultures. The 59 NolR-associated polypeptides were identified by PMF and correspond to 54 proteins in the *S. meliloti* 1021 protein database (Table 3). These include 3 pSymA, 9 pSymB, and 47 chromosomal gene products.

Proteins associated with NolR, AHLs, and bacteroids.

Natera and associates reported that over 600 proteins of *S. meliloti* 1021 were up- or downregulated in the bacteroid form of the rhizobia compared with cultured cells (Natera et al. 2000). Also, proteomic studies of strain 1021 responding to the addition of AHL quorum sensing signals have identified a number of AHL-associated proteins (Chen et al. 2003, Teplitski et al. 2004). A possible overlap of protein networks regulated by NolR, AHLs, and in the bacteroid is shown in Table 4. In all, 12 NolR-associated proteins were found to be affected by

AHLs or in the bacteroid, and 4 of the 12 NolR-associated proteins also were expressed differentially by the addition of AHLs and in the bacteroid.

DISCUSSION

NolR expression during the growth cycle.

By examining the patterns of *nolR* expression throughout the complete growth cycle of *S. meliloti* carrying a *nolR-lacZ* fusion, evidence supporting a population-density-dependent regulation of *nolR* expression has been demonstrated. Once cell growth of strain AK631 carrying a *nolR::lacZ* fusion was stopped by the exposure of the bacterial culture to conditions which affect its cellular growth, such as in an anaerobic environment or when deprived of nutrients, the level of *nolR* expression remained unchanged unless oxygen or nutrients were provided. These findings indicate that the elevation of *nolR* activity only occurred in a growing cell. Because the growing cells change culture population density, the correlation be-

Table 3. Proteins differentially accumulated in early log phase and stationary phase cultures of NolR⁻ *Sinorhizobium meliloti* EK698

Spot no.	Accession ^d	Protein description	Protein accumulation response (%) ^a			Mascort search results ^b			MW/pI ^c	
			Early	Stationary	Rating ^e	Score	Peak	Seq. (%)	Exp.	Theor.
30	SMa1200	Hypothetical protein	65	...	3	55	5	37	17,754/5.08	17,202/4.84
43	SMa1227	fixJ, FixJ transcriptional activator	...	269	3	104	9	56	23,954/5.46	22,204/5.12
19	SMa1313	virB5, VirB5 type IV secretion protein	...	32	3	104	7	58	24,527/4.66	22,616/4.72
92	SMB20021	Transcriptional regulator	836	...	1	34	3	42	14,584/6.58	17,309/5.32
20	SMB20127	Hypothetical protein	...	56	3	99	7	35	33,080/4.67	34,545/4.88
22	SMB20127	Hypothetical protein	...	16	3	99	7	35	33,311/4.84	34,545/4.88
32	SMB20343	Aldehyde dehydrogenase subunit	...	70	1	38	3	36	16,542/5.11	16,109/4.96
73	SMB20703	Conserved hypothetical protein	...	65	3	51	3	40	31,954/6.50	18,698/6.19
72	SMB20751	(1.1.1.31) Putative 3-hydroxyisobutyrate dehydrogenase	358	...	3	79	6	45	29,841/6.41	30,268/5.89
14	SMB20895	chvE, Proline iminopeptidase	...	55	3	85	7	31	34,725/4.41	37,845/4.48
8	SMB21037	Putative oligopeptide/murein peptide ABC transporter periplasmic	...	70	3	99	8	24	43,022/5.81	57,267/6.54
116	SMB21566	groEL5, Putative heat shock protein groEL	...	50	3	165	12	34	56,941/5.50	57,847/5.23
46	SMc00042	ilvE2 (2.6.1.42) Aminotransferase	...	201	3	62	8	35	28,206/5.56	34,153/5.08
4	SMc00048	Conserved hypothetical protein	140	...	2	46	3	44	13,240/5.69	15,199/5.32
74	SMc00101	Oxidoreductase	...	214	3	162	10	60	30,022/6.81	30,346/6.72
111	SMc00154	gor (EC 1.6.4.2) Glutathione reductase	...	210	3	93	7	27	53,926/6.42	49,891/5.81
45	SMc00265	Periplasmic binding protein	...	26	3	85	7	25	28,899/5.50	36,903/6.26
113	SMc00335	rpsA, 30S ribosomal protein S1	...	150	3	218	15	40	60,841/5.32	62,602/5.12
90	SMc00347	mk, Regulator of nucleoside diphosphate kinase	...	39	3	77	5	62	13,676/6.66	14,310/6.11
10	SMc00361	Hypothetical protein	...	340	3	87	8	41	34,552/5.54	30,529/5.26
26	SMc00415	dnaN (EC 2.7.7.7) DNA polymerase III, beta chain	...	40	3	57	5	18	41,175/5.17	40,367/5.08
103	SMc00493	purQ (EC 6.3.5.3) Phosphoribosylformyl glycinamide synthase	...	150	3	90	6	43	25,530/6.75	23,477/6.08
80	SMc00863	moaB, Molybdenum cofactor biosynthesis protein B	...	40	1	37	3	27	20,828/6.35	20,000/5.84
104	SMc00912	groES1, 10-kDa chaperonin A	...	240	3	102	7	52	11,632/5.68	10,581/5.43
105	SMc00913	groEL1, 60-kDa chaperonin A	...	240	3	67	6	17	54,524/5.28	57,651/5.00
115	SMc00913	groEL1, 60-kDa chaperonin A	...	170	2	41	4	12	55,016/5.21	57,651/5.00
42	SMc01101	def1 (3.5.1.31) Polypeptide deformylase	...	274	1	39	4	16	21,222/5.38	19,881/4.99
29	SMc01218	Transcription elongation factor (transcript cleavage factor)	...	263	3	126	8	59	19,546/4.98	17,462/4.85
44	SMc01311	tufA, Elongation factor TU	...	302	3	156	13	48	40,881/5.54	42,722/5.30

(continued on next page)

^a Differential accumulation is based on statistical analysis of at least three gels per treatment. Numbers represent average percentage differences in spot volumes between NolR⁻ EK698 and NolR⁺ AK631, and each value is significant at the *P* = 0.05 level; Early = early log phase; Stationary = stationary phase; missing (Miss) and New refer to polypeptides not detectable in the mutant EK698 and wild-type AK631, respectively.

^b Peak = Number of peak matched and Seq. (%) = percent sequence coverage.

^c MW = molecular weight, pI = isoelectric point, Exp. = experimental, and Theor. = theoretical.

^d Genomic accession numbers. Smc, Sma, and Smb refer to products of open reading frames located on the chromosome and symbiotic plasmids A and B, respectively, in *S. meliloti* 1021 proteomic database. P28267 is a SwissProt accession number.

^e Confidence ratings: 3 = all criteria were met and with probability-based Mowse scores greater than 50; 2 = one criterion was not met with probability-based Mowse scores between 40 and 50; and 1 = at least one of the criteria met with probability-based Mowse score between 30 and 40.

tween *nolR* expression and the growing cell number further supports the idea that elevated *nolR::lacZ* fusion activities are population density dependent. However, this finding was not in line with earlier conclusions made by Cren and associates (1995), who stated that the *nolR* expression “did not depend on the growth phase of the bacteria, at least in the optical density (OD₆₀₀) range 0.1 – 1.0.” This may be due to the fact that the *nolR* expression activity was not measured throughout the complete bacterial growth cycle in Cren’s experiments. Actually, there was not much difference in the *nolR* expression levels if the cultures were tested with OD₆₀₀ between 0.1 and 1.0 (Fig. 1).

NolR expression and quorum sensing signals.

In bacteria, the regulation of gene expression in response to fluctuations in cell population density relies on a system of signaling between cells known as quorum sensing. Quorum sensing bacteria produce diffusible signal molecules that increase in concentration as a function of cell density. Population-density-dependent regulation of *nolR* suggests that quorum sensing may control NolR production in *S. meliloti* AK631.

This was supported by stimulation of *nolR* by quorum factors in the supernatants of the stationary-phase cultures. The significant stimulation of *nolR* expression by quorum factors occurred in the early-log-phase cultures but not in the middle-log-phase cultures. Presumably, amounts of quorum sensing signals produced in the middle-log-phase cultures are high enough for induction of *nolR*. AHLs are the most common quorum sensing signals among gram-negative bacteria. Strain AK631 produced several novel short- and long-chain AHLs in the defined medium (Teplitski et al. 2003). It will be interesting to see whether either of these AHLs is able to induce *nolR* expression, or if the induction is caused by another quorum sensing active component. Negative regulation of nodulation genes by quorum sensing has been reported in *B. japonicum* (Loh et al. 2001). A NolR-like repressor protein, NodD2, is required for repressing the nodulation genes (Garcia et al. 1996; Gillette and Elkan 1996). NodD2 is induced by bradyoxetin, a novel quorum sensing signal which is different from common AHLs detected in *Rhizobium* spp. (Loh et al. 2002), through a quorum sensing cascade system (González and Marketon 2003).

Table 3. (Continued from preceding page)

Spot no.	Accession ^d	Protein description	Protein accumulation response (%) ^a			Mascort search results ^b			MW/pI ^c	
			Early	Stationary	Rating ^e	Score	Peak	Seq. (%)	Exp.	Theor.
28	SMc01344	accB, Biotin carboxyl carrier protein of acetyl-CoA carboxylase	327	...	3	58	4	46	19,203/4.84	16,521/4.71
117	SMc01587	Conserved hypothetical protein	...	30	3	57	4	32	23,928/4.66	23,231/5.24
49	SMc01613	rpiB (5.3.1.6) ribose 5-phosphate isomerase B	203	...	3	102	6	67	15,680/5.60	15,181/5.33
50	SMc01613	rpiB (5.3.1.6) ribose 5-phosphate isomerase B	233	New	2	45	3	35	14,427/5.61	15,181/5.33
31	SMc01723	Transmembrane hypothetical/global homology	321	53	3	56	4	32	17,355/5.12	18,704/4.84
84	SMc01757	Conserved hypothetical protein	...	170	3	76	5	49	13,339/6.07	13,453/5.67
25	SMc01828	Transmembrane transport protein	...	57	3	136	9	42	40,165/5.02	41,062/4.99
118	SMc01874	ftsZ1, Cell division protein	50	50	3	154	12	28	64,716/5.34	62,935/5.06
107	SMc02100	tsf, Elongation factor TS	...	260	3	189	12	56	33,639/5.53	32,082/5.07
47	SMc02122	fpr (1.18.1.2) Ferredoxin—NADP reductase	192	361	3	169	10	65	26,318/5.64	30,319/5.30
5	SMc02123	Conserved hypothetical protein	67	...	3	65	5	37	18,437/5.82	18,124/5.39
102	SMc02171	ABC transporter periplasmic binding protein	...	63	3	134	9	43	37,205/4.57	35,239/4.62
91	SMc02351	Conserved hypothetical protein	550	258	3	74	5	54	14,479/6.53	17,115/6.08
23	SMc02365	degP1 (3.4.21.-) Protease precursor	...	222	3	142	10	35	60,979/4.86	53,003/4.80
58	SMc02499	atpA, ATP synthase alpha chain	241	...	3	95	8	27	46,137/6.69	54,647/6.30
59	SMc02499	atpA, ATP synthase alpha chain	320	...	3	80	10	23	52,692/6.69	54,647/6.30
109	SMc02509	sitA, ABC transporter iron-binding periplasmic protein	...	60	3	89	6	37	34,032/5.06	32,849/5.25
67	SMc02562	pckA (4.1.1.49) Phosphoenolpyruvate carboxykinase	215	50	3	153	11	34	52,692/5.92	58,083/5.56
99	SMc02761	trxA, Thioredoxin	184	294	2	41	4	38	10,809/5.04	11,383/4.88
101	SMc02857	dnaK, heat-shock protein 70 (hsp70) chaperone	...	191	3	135	10	28	67,379/5.03	68,907/4.91
100	SMc02857	dnaK, heat-shock protein 70 (hsp70) chaperone	...	227	3	147	11	29	67,321/4.99	68,907/4.91
27	SMc03019	fliG, flagellar motor switch protein	224	36	2	44	4	20	36,451/5.18	37,717/4.83
13	SMc03091	argI1 (EC 3.5.3.1) Arginase	...	420	2	50	6	35	41,508/4.39	32,841/5.34
21	SMc03131	ABC transporter amino acid-binding periplasmic protein	265	...	3	107	7	44	26,046/4.79	30,073/4.87
75	SMc03135	ABC transporter amino-acid transport ATP-binding protein	261	...	3	127	14	56	26,046/6.26	28,045/5.67
110	SMc03152	Transmembrane hypothetical	...	60	3	79	7	53	26,906/5.35	23,937/5.04
41	SMc03239	ppa (EC 3.6.1.1) Inorganic pyrophosphatase	...	385	1	39	3	24	19,781/5.28	20,036/5.67
9	SMc03786	brf, Bacterioferritin (cytochrome b-1, b-557)	...	35	3	83	5	56	18,269/4.92	18,337/4.75
89	SMc04040	ibpA, heat-shock protein	241	442	3	55	6	39	17,093/6.54	17,402/6.11
62	SMc04262	gnd (EC 1.1.1.4) 6-phosphogluconate dehydrogenase	...	70	3	126	9	33	41,359/6.49	50,772/5.92
93	P28267	Nodulation protein NolR (<i>S. meliloti</i>)	Miss	Miss	3	84	5	64	12,159/6.40	13,341/6.06

NolR as a regulator of cellular functions.

NolR has been identified as a repressor protein to control the production of the lipochitooligosaccharide Nod factor required for nodule organogenesis in *S. meliloti* AK631 and *R. leguminosarum* bv. *viciae* TOM (Kiss et al. 1998). Recently, it has been found in *S. fredii* that NolR controls production of three different symbiotic signals: Nod factors, EPS, and SR proteins secreted from the type III secretion system (Vinardell et al. 2004). Moreover, it has been shown that quorum sensing controls EPS production in *S. meliloti* (Marketon et al. 2003) and, recently, it has been reported that quorum sensing signals regulate type III secretion in the marine bacterium *Vibrio harveyi* (Henke and Bassler 2004). It is possible that production or secretion of symbiotic signals, such as Nod factors, EPS, and SR proteins in *S. meliloti*, are under the control of quorum sensing systems and NolR plays a coordinator in these quorum sensing cascade systems to control the secretion of symbiotic signals.

Luteolin addition and NolR regulation.

Luteolin, an inducer of the common *nod* genes, originally was reported by Cren and associates to have an inhibitory effect on *nolR* expression (Cren et al. 1995). In the presence of luteolin at the concentration of 5 μ M, the expression of *nolR* was lower both in *S. meliloti* NolR⁺ AK631 and the NolR⁻ mutant EK698 grown in the minimal GTS medium. It was suggested that luteolin indirectly controlled *nolR* regulation via activation of luteolin-inducible genes (Cren et al. 1995). Surprisingly, the inhibitory effect of luteolin on *nolR* expression could not be observed in the cells grown in rich TA or minimum BIII medium. In fact, the inhibition of *nolR* expression by luteolin occurred only when luteolin was added to the early-log-phase GTS cultures of strain AK631 carrying a *nolR::lacZ* fusion. However, luteolin inhibited not only *nolR* expression, but bacterial growth as well. Because the level of *nolR* expression is correlated to cell population density, the lower cell density in the luteolin-containing cultures would have a lower level of *nolR* expression. Therefore, the inhibitory effect of luteolin on *nolR* expression observed by Cren and associates (Cren et al. 1995) is the result of the reduction of cell population density of *S. meliloti* cultures by luteolin.

NolR and environmental stimuli and cellular growth.

The expression of the *nolR* gene also is related to nutrient availability and responsive to various environmental stresses, such as pH shock and low oxygen level. The expression level was higher in rich media than in minimal media and reduced by extremely acidic or alkaline pH and lack of oxygen. Nutrient-dependent gene expression has been reported in *S. meliloti*, in which the expression of the *pckA* gene was dependent on medium carbon sources, such as succinate and arabinose (Osteras et al. 1995). The fact that *nolR* is responsive to different environmental stimuli by changing its transcription level suggested that expression of the *nolR* gene is sensitive to environmental challenge and NolR could act as a regulator in some key metabolic steps, as proposed in previous proteomic studies (Chen et al. 2000b). 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 would influence the level of the global regulatory role of NolR.

Previous proteomic studies (Chen et al. 2000b) and the current proteomic studies of NolR-associated proteins in *S. meliloti* revealed that some of these proteins are heat-shock proteins. Heat-shock proteins are found in all organisms and presumably protect against thermal damage and accelerate recovery (Eriksson and Clarke 1996). 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 renaturing unfolded polypeptides (Parcell and Lindquist 1993). When *S. meliloti* was challenged with heat shock, cells with a functional NolR showed higher thermal tolerance. Those results revealed that NolR plays a role in optimal survival under heat shock, probably via heat shock proteins of a repairing system functioning directly or indirectly within the *nolR*⁺ cells to help maintain the cellular viability under elevated temperature.

The effect of NolR on *S. meliloti* growth and survival could not be detected when the cells were grown in rich or defined liquid media, even for as long as 3 weeks. However, when *S. meliloti* cells were grown in a defined liquid medium with much reduced carbon and nitrogen sources, the strain with a functional NolR was found to have a half of a logarithm cell number higher than that without a functional NolR.

Table 4. *Sinorhizobium meliloti* proteins respondent to NolR, acyl homoserine lactones (AHLs), and bacteroids

Protein ^b	Accession ^b	Differential expression in respond to			
		NolR ^a		AHLs	Bacteroids ^c
		Early-log phase	Stationary phase		
Putative heat shock protein groEL	SMb21566	...	Downregulated	Upregulated ^{d,e}	...
30S ribosomal protein	SMc00335	...	Downregulated	Upregulated ^e	...
Polypeptide deformylase	SMc01101	...	Downregulated	Upregulated ^d	...
Biotin carboxyl carrier protein of acetyl-CoA carboxylase	SMc01344	Downregulated	...	Downregulated ^f	Upregulated
Ribose 5-phosphate isomerase B	SMc01613	Downregulated	Downregulated	Upregulated ^d	...
Cell division protein	SMc01874	...	Upregulated	...	Downregulated
Ferredoxin--NADP reductase	SMc02122	Downregulated	Downregulated	Upregulated ^d	...
ABC transporter periplasmic binding protein	SMc02171	...	Upregulated	Upregulated ^d	...
Protease precursor	SMc02365	...	Downregulated	Upregulated ^{d,e}	Upregulated
Phosphoenolpyruvate carboxykinase	SMc02562	Downregulated	Upregulated	Upregulated ^{d,e}	Downregulated
Heat shock protein 70 (hsp70) chaperone	SMc02857	...	Downregulated	Upregulated ^g	Upregulated
Heat shock protein	SMc04040	Downregulated	Downregulated	...	Upregulated

^a Based on analysis of the proteins differentially accumulated in early log phase and stationary phase cultures of NolR⁻ *Sinorhizobium meliloti* EK698.

^b Genomic accession number. Smb, Sma, and Smb refer to products of open reading frames located on the chromosome and symbiotic plasmids A and B, respectively, in the *S. meliloti* 1021 genome database.

^c Proteins differentially expressed in bacteroids of *S. meliloti* 1021 (Natera et al. 2000).

^d Protein was upregulated by AHL 3-oxo-C16:1-HL in *S. meliloti* 1021 (Chen et al. 2003).

^e Protein was upregulated by crude AHLs produced by *S. meliloti* 1021 (Teplitski et al. 2004).

^f Protein was downregulated by AHL C14-HL in *S. meliloti* 1021 (Chen et al. 2003).

^g Protein was upregulated by unidentified AHLs (Chen unpublished).

after 1 week of entering the stationary phase. This difference in cell viability was sustained for up to 9 days. These results suggest that the functional NolR is able to sense nutrient stress during bacterial growth that leads to the induction of a cellular adaptation mechanism and improves stationary-phase survival of *S. meliloti*. Rhizobia are free-living organisms 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. In *R. leguminosarum* bv. *phaseoli*, cells survived under carbon-, nitrogen-, and phosphorus-starved conditions for at least 2 months (Thorne and Williams 1997). The starved cells had a low level of cell division and were cross-protected against some environmental shocks (Thorne and Williams 1999). There also is 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 *Escherichia coli*, the *rpoS* gene encoding the sigma S subunit is required for prolonged survival in the stationary phase in both nutrient-rich and minimal media (Lange and Henge-Aronis 1991) and nitrogen-starved minimal media (Kabir et al. 2004). Possible explanations of NolR's response to starvation stress resistance include i) NolR might detect the starvation stress signals, activated by the overlapping pathways related to population density (which often is mediated by sigma factors), and therefore is able to protect cell viability by directly or indirectly affecting the levels of some key macromolecular synthesis; and ii) NolR itself is responsive to starvation stress signals, which in turn activates related population density signals (for example, sigma factors), resulting in the regulation of essential genes to maintain viability during stationary-phase starvation and to generate cross-resistance to other environmental stresses, such as heat shock.

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. Cells with functional NolR were found to have a higher cell division rate than those without, indicating that these cells can recover much more quickly from starvation once the nutrients are provided. In nature, bacteria often must cope with hostile environmental conditions by developing sophisticated cooperative behavior and intricate communication capabilities, such as direct cell-to-cell physical interactions via extramembrane polymers, collective production of extracellular fluid for movements on hard surfaces, and long-range chemical signaling such as quorum sensing and chemotactic signaling (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 elevation 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, they have a higher rate of cell division and better adaptation to changed environments from liquid medium to agar plates and more nutrients. Actually, a cell-division protein encoded by *ftsZ1* gene (SMc01874) was found to be downregulated in the NolR⁻ *S. meliloti* AK698 in the current (Table 3) and previous studies (Chen et al. 2000b). It will be interesting to test whether the expression of *ftsZ1* is directly regulated by NolR and involved in colony development of *S. meliloti* AK631 on solid medium.

Interestingly, NolR was found to be involved in conjugative transfer of plasmids. The frequency of plasmid transfer was increased greatly by a recipient that carried a functional NolR.

This recipient-induced plasmid transfer has been reported in *Enterococcus faecalis* (Dunny et al. 1995) and *R. leguminosarum* bv. *viciae* (Danino et al. 2003). In *E. faecalis*, conjugative transfer of plasmids to a potential recipient is induced by the small peptides released from the recipient. In *R. leguminosarum* bv. *viciae*, recipient-induced plasmid transfer is dependent on the production of an AHL (3-OH-C_{14:1}-HSL) by the recipient. In addition to *R. leguminosarum* bv. *viciae*, the production of 3-OH-C_{14:1}-HSL has been detected in *R. etli* (Daniels et al. 2002) but not in the culture supernatants of *S. meliloti* (Marketon et al. 2002; Teplitski et al. 2003). It is possible that, as a global regulatory protein, NolR plays a role in the control of recipient cells excreting signal molecules into medium where they can diffuse to a potential donor cell and induce expression of conjugative transfer functions.

Proteomic analysis.

Proteomic analysis of *S. meliloti* revealed that at least 59 proteins were significantly altered in protein levels in a strain lacking a functional NolR grown at both the early-log and stationary phase. In the early-log-phase cultures, there were 20 proteins regulated by NolR. Among these 20 proteins, 17 proteins were downregulated by NolR and only 3 proteins were upregulated by NolR. In the stationary-phase cultures, there were 48 NolR-regulated proteins. Among these, 23 were upregulated and 25 were downregulated by NolR. These results indicate that NolR acts mainly as a repressor in cells grown at the early-log-phase cultures whereas, in the stationary-phase cultures, NolR acts as both repressor and activator. Using PMF analysis and the *S. meliloti* 1021 proteomic database search, 59 NolR-associated proteins were identified. The identified proteins involve various metabolic pathways and cellular functions, including amino acid metabolism, carbohydrate metabolism, lipid metabolism, nucleotide metabolism, energy metabolism, metabolism of Co-factors, adaptation, and transportation. These results further support the view that NolR is a global regulator (Chen et al. 2000b). In *S. meliloti*, NodD is a regulatory protein specific for induction of nodulation genes. However, NodD is a low-abundant protein and has not been detected on 2D gels from *S. meliloti* strains grown in various media supplemented with or without the *nodD* inducer luteolin (H. Chen, unpublished data). In contrast to NodD, NolR is an abundant protein which appears as a major spot on 2D gels; perhaps this high level is consistent with its global regulatory functions. The NolR protein was found only in the wild type, AK631, but not in its mutant, EK698, confirming that no functional NolR protein is produced in the Tn5-induced *nolR* mutant EK698.

An earlier proteome study of NolR-associated proteins in *S. meliloti* found that, from silver-stained 2D gels, over 100 protein spots were differentially accumulated in the stationary-phase cells of the NolR⁻ strain EK698 (Chen et al. 2000b). It was possible to isolate 52 spots from Coomassie-stained preparative 2D gels or polyvinylidene difluoride membranes blotted from preparative gels. Among the 52 spots, 38 were identified by N-terminal sequencing and PMF. However, only six spots were identified as *S. meliloti* proteins (Chen et al. 2000b). This was due to the unavailability of the *S. meliloti* 1021 genome database at that time. In the present study, PMF and the *S. meliloti* 1021 proteomic database search have been applied to identify NolR-associated *S. meliloti* proteins. This has allowed us to obtain *S. meliloti* protein identification of all 59 detected NolR-associated protein spots. There were 23 NolR-associated protein spots detected in both the previous and present studies. Among the 23 spots, 11 proteins were identified as NolR-associated in stationary phase cultures by N-terminal sequencing in the previous study and by PMF in the present study. These include elongation factor TS, ABC transporter iron-binding

periplasmic protein, inorganic pyrophosphatase, heat shock protein 70 chaperone, 10-kDa chaperonin A, 60-kDa chaperonin A (two isoforms), DNA polymerase III β chain, transmembrane hypothetical proteins, and two hypothetical proteins. Two proteins, putative heat-shock protein GroEL and a cell-division protein, were identified as NolR-associated in stationary-phase cultures by PMF in the previous and present study. Another 10 spots were detected as NolR-associated in the previous and present study. Through the search against the *S. meliloti* 1021 protein database, however, these 10 spots were identified by PMF in the present study but not identified by *N*-terminal sequences in the previous study (Chen et al. 2000b). Our results are in agreement with the suggestion made by Mathesius and associates that, in bacterial strains, PMF is more reliable for species-specific protein identification than *N*-terminal sequencing (Mathesius et al. 2002).

Because most of the NolR-associated proteins were differentially regulated during stationary-phase growth, expression of genes encoding these proteins might be population density dependent and mediated by quorum sensing systems. This possibility was supported by the results that the levels of 10 NolR-associated proteins also were found to be altered in *S. meliloti* 1021 by the addition of AHL quorum sensing signals (Chen et al. 2003, Teplitski et al. 2004). Strain AK631 is very similar to strain 1021 with regards to nodulation and nitrogen-fixation specificities on their host medic plants. It is possible that both NolR and AHL quorum sensing signals are involved in *S. meliloti* stationary-phase adaptation. The growth state of bacteroids can be regarded as similar to free-living rhizobia grown at stationary phase. Interestingly, six NolR-associated proteins also were differentially regulated in bacteroids of *S. meliloti* 1021 (Natera et al. 2000). Among these six proteins, four also were regulated in *S. meliloti* 1021 by the addition of AHLs. These results suggest that there is an overlap among gene networks that are regulated by NolR, quorum sensing, and bacteroids. 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. Because proteomic analysis can provide an overview 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.

Conclusion.

NolR is a global regulator in *S. meliloti*. The functional NolR is not an obligatory regulator protein, but it is important for the optimization of nodulation, bacterial growth on solid medium, survival under stress conditions, and conjugative transfer of plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

S. meliloti strains used were AK631 (Rm41 *exoB nolR*⁺) (Kondorosi et al. 1989b), EK698 (AK631 *nolR::Tn5*) (Kondorosi et al. 1991), AK631 carrying a *nolR::lacZ* fusion, and EK698 carrying a *nolR-lacZ* fusion (Cren et al. 1995). Plasmids used for conjugative transfer were pRT311 (a transcriptional fusion between the *R. leguminosarum* bv. *trifolii* *nodA* promoter and the *lacZ* gene (Innes et al. 1985) and pRK2013, a helper for plasmid transfer (Figurski and Helinski 1979). The following growth media were used: rich TA medium (Orosz et al. 1973), BIII minimal medium (Dazzo 1982), nutrient-limited BIII medium (mannitol at 0.2 g liter⁻¹ and glutamate at 0.5 g liter⁻¹ as carbon and nitrogen sources), modified

TM minimal medium (Innes et al. 1985), Luria-Bertani medium (Miller 1972), and GTS minimal medium (Kondorosi et al. 1984).

Microbiological techniques.

S. meliloti strains were grown at 28°C and *Escherichia coli* strains were grown at 37°C. Bacterial growth was monitored at 600 nm using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Units of β -galactosidase activity were measured using a modified Miller method (Miller 1972). The supernatants of TA cultures of AK631 (OD₆₀₀ > 1.8) were prepared by centrifugation of the culture at 6,000 $\times$ g for 10 min and filter sterilization (0.2- μ m filter) of the supernatant. Anaerobic incubation of the bacteria was carried out by flowing nitrogen gas at 10 kPa for 30 min into overnight TA cultures in flasks, after which the flasks were sealed with rubber stoppers. To resume the oxygen supply, the nitrogen-treated cultures were transferred to sterilized empty flasks. The heat-shock treatment was carried out by placing 1.5-ml Eppendorf tubes containing 0.1 ml of the 2-day-old TA culture into a rack in a 46°C water bath. After heat-shock treatment of up to 6 h, the surviving cells were detected by viable counts. Plasmid pRT311 was transferred to strains AK631 and EK698 with helper plasmid pRK2013 by a triparental patch-mating technique (Ditta et al. 1980). Bacterial matings were carried out on TA medium at 28°C. Transconjugants were counterselected on the modified TMR medium containing tetracycline at 5 μ g ml⁻¹.

Protein extraction, separation, quantification, and identification.

Proteins were extracted from cells grown in TA to early-log phase (at OD₆₀₀ of approximately 0.1 to 0.2) or to stationary phase (at OD₆₀₀ of approximately 1.8 to 1.9) as described (Chen et al. 2000a) and quantified based on a modified Bradford protein assay (Guerreiro et al. 1999). Protein concentrations were normalized and the samples were subjected to 2D gel separation as described (Chen et al. 2000a). Preparative gels were stained with Coomassie Brilliant Blue in a stepwise colloidal staining procedure (Neuhoff et al. 1988). Digitized images (600 dots per inch) of the stained gels were quantified using MELANIE 4 image analysis software (Bio-Rad, Hercules, CA, U.S.A.). Protein spot locations were compared with 10 landmark proteins and matched against a specialized proteomic database for *S. meliloti* 1021 (Weiller et al. 2001). OD of each spot over its area (volume) as a percentage of the relative OD of the gel image (percent volume) was used to quantify each spot. Digitized spot images were statistically analyzed using GenStat 4.2 software as described by Mathesius and associates (2003). A polypeptide was deemed differentially accumulated if χ^2 was less than 0.05.

Proteins were identified by tryptic digestion of the polypeptides isolated from the Coomassie-stained gels followed by PMF with MALDITOF mass spectrometry performed on a Micromass TofSpec 2E Time of Flight Mass Spectrometer (Bruker Daltonik GmbH, Leipzig, Germany). Peptide mass fingerprints were identified by comparison with the *S. meliloti* 1021 proteomic database using Mascot software (Micromass; Waters Corp., Milford, MA, U.S.A.) as described previously (Weiller et al. 2001). Scoring of PMF matches was based on the following criteria: i) a minimum three peptides matched within 100 ppm to the theoretical mass of the polypeptide without any protein modification, ii) good agreement between the actual and predicated molecular mass and *pI*, and iii) the absence of other polypeptides that match. A confidence rating of 3 was assigned if all criteria were met and if the probability-based Mowse scores were greater than 50; a rating of 2 was assigned if one criterion was not met and if the probability-based

Mowse scores was between 40 and 50; and a confidence rating of 1 meant that at least one of the criteria was met and the probability-based Mowse score was between 30 and 40.

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LITERATURE CITED

- Ben-Jacob, E., Cohen, I., and Levine, H. 2000. Cooperative self-organization of microorganisms. *Adv. Physics* 49:395-554.
- Brelles-Marino, G., and Bedmar, E. J. 2001. Detection, purification and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *J. Biotechnol.* 91:191-209.
- Chen, H., Higgins, J., Oresnik, I. J., Hynes, M. F., Natera, S., Djordjevic, M. A., Weinman, J. J., and Rolfe, B. G. 2000a. Proteome analysis demonstrates complex replicon and luteolin interactions in pSyma-cured derivatives of *Sinorhizobium meliloti* strain 2011. *Electrophoresis* 21:3833-3842.
- Chen, H. C., Higgins, J., Kondorosi, A., Kondorosi, E., Djordjevic, M. A., Weinman, J. J., and Rolfe, B. G. 2000b. Identification of *nolR*-regulated proteins in *Sinorhizobium meliloti* using proteome analysis. *Electrophoresis* 21:3823-3832.
- Chen, H., Teplitski, M., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to N-acyl homoserine lactone quorum-sensing signals and the transition to stationary phase. *J. Bacteriol.* 185:5029-5036.
- Cren, M., Kondorosi, E., and Kondorosi, A. 1994. An insertion point inactivates *NolR* repressor in *Rhizobium meliloti* 1021. *J. Bacteriol.* 176:518-519.
- Cren, M., Kondorosi, A., and Kondorosi, E. 1995. *NolR* controls expression of *R. meliloti* nodulation genes involved in the core Nod factor synthesis. *Mol. Microbiol.* 15:733-747.
- Daniels, R., De Vos, D. E., Desair, J., Raedschelders, G., Luyten, E., Rosemeyer, V., Verreth, C., Schoeters, E., Vanderleyden, J., and Michiels, J. 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. *J. Biol. Chem.* 277:462-468.
- Danino, V. E., Wilkinson, A., Edwards, A., and Downie, J. A. 2003. Recipient-induced transfer of the symbiotic plasmid pRL1J1 in *Rhizobium leguminosarum* bv. *viciae* is regulated by a quorum-sensing relay. *Mol. Microbiol.* 50:511-525.
- Dazzo, F. B. 1982. Leguminous root nodules. Pages 431-446 in: *Experimental Microbial Ecology*. R. G. Burns and J. L. Slater, ed. Blackwell Scientific, Oxford.
- Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. U.S.A.* 77:7347-7351.
- Dunny, G. M., Leonard, B. A., and Hedberg, P. J. 1995. Pheromone-inducible conjugation in *Enterococcus faecalis*: Interbacterial and host-parasite chemical communication. *J. Bacteriol.* 177:871-876.
- Eriksson, M., and Clarke, A. K. 1996. The heat shock protein ClpB mediates the development of thermotolerance in the *Cyanobacterium Synechococcus* sp. strain PCC 7942. *J. Bacteriol.* 178:4839-4846.
- Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. U.S.A.* 76:1648-1652.
- Garcia, M., Dunlap, J., Loh, J., and Stacey, G. 1996. Phenotypic characterization and regulation of the *nolA* gene of *Bradyrhizobium japonicum*. *Mol. Plant-Microbe Interact.* 9:625-636.
- Gillette, W. K., and Elkan, G. H. 1996. *Bradyrhizobium (Arachis)* sp. strain NC92 contains two *nodD* genes involved in the repression of *nodA* and a *nolA* gene required for the efficient nodulation of host plants. *J. Bacteriol.* 178:2757-2766.
- González, J. E., and Marketon, M. M. 2003. Quorum sensing in nitrogen-fixing rhizobia. *Microbiol. Mol. Biol. Rev.* 67:574-592.
- Guerreiro, N., Djordjevic, M. A., and Rolfe, B. G. 1999. Proteome analysis of the model microsymbiont *Sinorhizobium meliloti* isolation and characterisation of novel proteins. *Electrophoresis* 20:818-825.
- Henke, J. M., and Bassler, B. L. 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J. Bacteriol.* 186:3794-3805.
- Hoang, H. H., Becker, A., and Gonzalez, J. E. 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J. Bacteriol.* 186:5460-5472.
- Innes, R., Kuempel, P., Plazinski, J., Canter-Cremers, H., Rolfe, B., and Djordjevic, M. 1985. Plant factors induce expression of nodulation and host-range genes in *Rhizobium trifolii*. *Mol. Gen. Genet.* 201:426-432.
- Ishihama, A. 1997. Adaptation of gene expression in stationary phase bacteria. *Curr. Opin. Genet. Dev.* 7:582-588.
- Kabir, M. S., Sagara, T., Oshima, T., Kawagoe, Y., Mori, H., Tsunedomi, R., and Yamada, M. 2004. Effects of mutations in the *rpoS* gene on cell viability and global gene expression under nitrogen starvation in *Escherichia coli*. *Microbiol.* 150:2543-2553.
- Kiss, E., Mergaert, P., Olah, B., Kereszt, A., Staehelin, C., Davies, A. E., and Downie, J. A. 1998. Conservation of *nolR* in the *Sinorhizobium* and *Rhizobium* Genera of the *Rhizobiaceae* family. *Mol. Plant-Microbe Interact.* 11:1186-1195.
- Kondorosi, E., Banfalvi, Z., and Kondorosi, A. 1984. Physical and genetic analysis of a symbiotic region of *R. meliloti*: Identification of nodulation genes. *Mol. Gen. Genet.* 193:445-452.
- Kondorosi, A., Kondorosi, E., Györgypál, Z., Bánfalvi, Z., Gyuris, J., Putnok, P., Grosskopf, E., John, M., Schmidt, J., Ha, D. T. C., Lados, M., Horváth, B., Slaska-Kiss, K., and Schell, J. 1989a. Molecular genetic basis of *Rhizobium*-legume interactions. *Genome* 31:350-353.
- Kondorosi, E., Gyuris, J., Schmidt, J., John, M., Duda, E., Hoffman, B., Schell, J., and Kondorosi, A. 1989b. Positive and negative control of *nod* gene expression in *R. meliloti* is required for optimal nodulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1331-1340.
- Kondorosi, E., Pierre, M., Cren, M., Haumann, U., Hoffman, B., Schell, J., and Kondorosi, A. 1991. Identification of *NolR*, a negative transacting factor controlling the *nod* regulation in *Rhizobium meliloti*. *J. Mol. Biol.* 222:885-896.
- Lange, R., and Hengge-Aronis, R. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol.* 5:49-59.
- Li, B., Wang, J., Wang, B., Liu, W., and Wu, Z. 1995. Computer simulation of bacterial-colony formation. *Europhysics* 30:239-243.
- Loh, J. T., Yuen-Tsai, J. P.-Y., Stacey, M. G., Lohar, D., Welborn, A., and Stacey, G. 2001. Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. *Mol. Microbiol.* 42:37-46.
- Loh, J., Carlson, R. W., York, W. S., and Stacey, G. 2002. Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation. *Proc. Natl. Acad. Sci. U.S.A.* 99:14446-14451.
- Long, S. 1996. *Rhizobium* symbiosis: *nod* factors in perspective. *Plant Cell* 8:1885-1898.
- Marketon, M. M., Gronquist, M. R., Eberhard, A., and González, J. E. 2002. Characterization of the *Sinorhizobium meliloti* *sinR/sinI* locus and the production of novel N-acyl homoserine lactones. *J. Bacteriol.* 184:5686-5695.
- Marketon, M. M., Glenn, S. A., Eberhard, A., and González, J. E. 2003. Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. *J. Bacteriol.* 185:325-331.
- Mathesius, U., Imin, N., Chen, H., Djordjevic, M., Weinman, J., Natera, S., Morris, A., Kerim, T., Paul, S., Menzel, C., Weiller, G., and Rolfe, B. 2002. Evaluation of proteome reference maps for cross-species identification of proteins by peptide mass fingerprinting. *Proteomics* 2:1288-1303.
- Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B. G., and Bauer, W. D. 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc. Natl. Acad. Sci. U.S.A.* 100:1444-1449.
- Miller, J. H. 1972. Pages 352-355 in: *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Natera, S., Guerreiro, N., and Djordjevic, M. 2000. Proteome analysis of differentially displayed proteins as a tool for the investigation of symbiosis. *Mol. Plant-Microbe Interact.* 13:995-1009.
- Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9:255-262.
- Orosz, L., Svab, Z., Kondorosi, A., and Sik, T. 1973. Genetic studies on rhizobiophage 16-3. I. Genes and functions on the chromosome. *Mol. Gen. Genet.* 125:341-350.
- Osteras, M., Driscoll, B. T., and Finan, T. 1995. Molecular and expression analysis of the *Rhizobium meliloti* phosphoenolpyruvate carboxykinase (*pckA*) gene. *J. Bacteriol.* 177:1452-1460.
- Parcell, D. A., and Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27:437-496.
- Teplitski, M., Eberhard, A., Gronquist, M. R., Gao, M., Robinson, J. B., and Bauer, W. D. 2003. Chemical identification of N-acyl homoserine

- lactone quorum-sensing signals produced by *Sinorhizobium meliloti* strains in defined medium. Arch. Microbiol. 180:494-497.
- Teplitski, M., Chen, H., Rajamani, S., Gao, M., Merighi, M., Sayre, R. T., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2004. *Chlamydomonas reinhardtii* secretes compound that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. Plant Physiol. 134:137-146.
- Thorne, S., and Williams, H. 1997. Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. *phaseoli*: Analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. J. Bacteriol. 179:6894-6901.
- Thorne, S., and Williams, H. 1999. Cell-density dependent starvation survival of *Rhizobium leguminosarum* bv. *phaseoli*: Identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary phase survival. J. Bacteriol. 181:981-990.
- Vinardell, J. M., Ollero, F. J., Hidalgo, A., López-Baena, F. J., Medina, C., Ivanov-Vangelov, K., Parada, M., Madinabeitia, N., Espuny Mdel, R., Bellogín, R. A., Camacho, M., Rodríguez-Navarro, D. N., Soria-Díaz, M. E., Gil-Serrano, A. M., and Ruiz-Sainz, J. E. 2004. NolR regulates diverse symbiotic signals of *Sinorhizobium fredii* HH103. Mol. Plant-Microbe Interact. 17:676-685.
- Watson, S. P., Clements, M. O., and Foster, S. J. 1998. Characterization of the starvation-survival response of *Staphylococcus aureus*. J. Bacteriol. 180:1750-1758.
- Weiller, G. F., Djordjevic, M. J., Caraux, G., Chen, H., and Weinman, J. J. 2001. A specialised proteomic database for comparing matrix-assisted laser desorption/ionization-time of flight mass spectrometry data of tryptic peptides with corresponding sequence database segments. Proteomics 1:1489-1494.
- Zambrano, M. M., Siegle, D., Almiron, M., Tormo, A., and Kolter, R. 1993. Microbial competition—*Escherichia coli* mutants that take over stationary phase cultures. Science 259:1757-1760.