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The effect of phosphate deficiency on quorum sensing signaling pathway of *Sinorhizobium meliloti*

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Abstract

Introduction: Phosphorus is one of the most essential macroelements for bacterial cells. Since phosphate (PO_4^{-3}) limitation is frequently encountered in soils, bacteria developed some mechanisms in response to this sever condition. Phosphate transporter (PstS) and proteins involved in quorum sensing (QS) signaling pathway are affected by mediating PhoB, response regulator, following phosphate starvation. QS system of *Sinorhizobium meliloti* composed of at least three genes of *sinI* (autoinducer synthase), *sinR* and *expR* (autoinducer activated receptor) which involved in its free living and symbiotic functions.

Materials and methods: The optical density (OD_{600}) of different *S. meliloti* transformed strains carrying pLK004 (a *pstS* promoter- *egfp* fusion), pLK64 (a *sinI* promoter- *egfp* fusion), pLK65 (a *sinR* promoter- *egfp* fusion), pLK66 (an *expR* promoter- *egfp* fusion) and control (promoterless- *egfp* fusion) plasmids were read under different phosphate concentrations of 0.1 (phosphate deficiency), 0.5 and 2 mM (sufficient phosphate) at several time points of 16, 24 and 40h. The promoter activity of different genes of *pstS*, *sinI*, *sinR* and *expR* were measured as emitted fluorescence per bacterial cell density (OD_{600}) under different phosphate concentrations.

Results: By reducing phosphate concentration in the medium, the growth rate of transformed bacteria decreased, especially at 40h. The promoter activity of *pstS*, *sinI* and *sinR*, but not *expR*, genes was activated following phosphate starvation.

Discussion and conclusion: *S. meliloti* can upregulate PstS to partly compensate phosphate deficiency in the environment. The gene of *sinR* is also activated in a PhoB dependent manner as phosphate starvation is encountered. SinR is the activator of *sinI*, so the upregulation of QS pathway under phosphate deficiency may be facilitate free living and symbiotic bacterial functions.

Key words: Phosphate deficiency, Phosphate tansporter, Quorum sensing pathway, *Sinorhizobium meliloti*

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Introduction

The soil gram negative bacterium, *Sinorhizobium meliloti*, is a free living state or in symbiosis with some legume plants. Different molecules and signaling pathways are involved in bacterial lifestyles (1- 4). The best characterized rhizobial signal molecules, lipochito- oligosaccharidic Nod factors, are induced through plant signal molecules as flavonoids. These bacterial signal molecules are involved in the establishment of symbiosis with host plant (5 and 6). The Sin quorum sensing (QS) system is a signaling pathway that regulates multiple functions in both free living and symbiotic states of *S. meliloti*, as motility, root surface adhesion, biofilm formation, traveling into infection thread as well as other cellular processes (7- 13). QS pathway is a widespread phenomenon in bacteria which can regulate the expression of specific genes in response to population density (14- 18). It relies on the production and detection of signal molecules as auto-inducers via bacteria in a population. The best studied auto- inducer belongs to *N*-acyl hemoserin lactones (AHLs) that are produced by many gram negative bacteria (15 and 19- 25). The QS system of *S. meliloti* composed of at least three genes of *sinI*, *sinR* and *expR*. The gene of *sinI* encodes the enzyme catalyzing AHLs synthesis and its expression is controlled by at least two transcriptional regulators of SinR and ExpR (11 and 26- 28).

Apart from bacterial population density, environmental conditions particularly nutrition limitation also impact QS signaling pathway (29). Phosphorous was the only element recognized as an inducer of QS system (29 and 30). Phosphate (PO_4^{-3}) plays key roles in bacterial metabolism as a major structural element of phospholipids, nucleic acids and ATP molecules and regulates different cellular processes. Two-component regulatory system of PhoR-PhoB is involved in phosphate metabolism of many gram negative bacteria. Similar to *Escherichia coli*, it was estimated that under phosphate starvation, the sensor kinase of PhoR phosphorylates response regulator PhoB. Phosphorylated PhoB (P- PhoB) can regulate the expression of target genes by binding to a DNA sequence termed Pho box (31- 35). A microarray study revealed that the expression of QS gene of *sinR* and phosphate transporter *pstS* in *S. meliloti* strain 2011, increased about 3 times in a PhoB dependent manner under P starvation (30).

In the current report, the response of QS regulatory pathway to phosphate starvation is further characterized. We evaluated the expression of different genes of QS signaling system, *sinI*, *sinR* and *expR*, and phosphate transporter, *pstS*, by assaying the promoter activity of these genes at different times of 16, 24 and 40 hours (h) after incubation. The experiments have been conducted under different phosphate concentrations of 0.1 (phosphate starvation), 0.5 and 2 mM (sufficient concentration) in the medium.

Materials and methods

Bacterial strains and growth conditions

To study the expression of phosphate transporter (*pstS*) and QS genes, *S. meliloti* strain of Sm2B3001 (*sinI*$^+$, *sinR*$^+$ and *expR*$^+$) (36) carrying the plasmids of either pLK004 (37), a *pstS* promoter- *egfp* fusion, pLK64 (11), the fiusion of *sinI* promoter-enhanced green fluorescence protein (*egfp*), pLK65, the fusion of *sinR* promoter- *egfp*, or pLK66, *expR* promoter- *egfp* fusion (29) has been selected. *S. meliloti* strain of Sm2B3001 carrying pLK vector with a promoterless *egfp* was used as the background of fluorescence (control). Different bacterial transformed strains were cultured in the modified morpholine ethan sulfonic acid (MOPS) buffered minimal broth containing 48 mM MOPS (adjusted to pH 7.2 with KOH), 55 mM manitol, 21 mM sodium glutamate, 1mM $MgSO_4$, 250 µM $CaCl_2$, 37 µM $FeCl_3$, 48 µM H_3BO_3, 10 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.6 µM $NaMoO_4$, 0.3 µM $CoCl_2$, 4.09 µM biotin, and either 0.1, 0.5 or 2.0 mM K_2HPO_4 (38). The cultures were also supplemented with 10 µg ml^{-1} of both antibiotics of tetracycline and nalidixic acid (29).

Optical density (OD_{600}), EGFP fluorescence and promoter activity assay

To evaluate OD_{600} and promoter activity, *S. meliloti* strains were cultured in 15 ml test tubes containing 2 ml of modified MOPS medium at 30°C with shaking. The starting OD_{600} in the fresh broth was 0.002 OD_{600} and EGFP fluorescence (excitation at 485 nm and emission at 538 nm with a 97% scanning rate) were measured at different times of 16, 24 and 48 h after incubation. For that, 100 µl of bacterial cultures were transferred to 96- well microtitre plates (Greiner), and then OD and EGFP fluorescence were reported by using a Tecan Infinite M200 reader (Tecan Trading AG, Switzerland). Promoter activity was expressed as EGFP fluorescence per OD, F OD^{-1}.

The experiment was conducted in a factorial design with 4 replicates. Statistical analyses were performed through SPSS software (version 18). The data were analyzed by one- way analyses of variance (ANOVAs), and the means were separated using Duncan test.

Results

Bacterial density under different phosphate concentrations

The OD of different transformed *S. meliloti* strains at different times of 16, 24 and 40h after growing in MOPS medium supplemented with 0.1, 0.5 and 2 mM phosphate have been shown in Fig. 1. As the results of samples at 16h in Fig. 1a showed, bacterial OD of *S. melioti* with *sinI* promoter- *egfp* fusion increased significantly by decreasing P concentration from 0.5 to 0.1 mM. There was not any significant difference between 0.5 and 2 mM phosphate. At 24h, the results indicated that by decreasing phosphate concentration in MOPS medium from 2 to 0.5 mM, bacterial OD decreased significantly, but there was not any significant difference between bacterial OD at 0.1 and 2 mM phosphate supplemented medium (Fig. 1a). The results of measurements at 40h indicated that bacterial OD increased significantly as phosphate concentration increased in the medium from 0.1 to 0.5 and from 0.5 to 2 mM. As the results showed, the growth (OD) of *S. meliloti* with *sinI* promoter- *egfp* fusion under different phosphate

concentrations increased significantly by elapsing the time and the highest bacterial OD has been seen at 40h.

As the results of *S. meliloti* with *sinR* promoter- *egfp* fusion showed (Fig. 1b), bacterial OD increased significantly at 16h as phosphate concentration increased from 0.5 to 2 mM in MOPS medium and there was not any significant difference between bacteria grown under 0.1 and 2 mM phosphate. At 24h, OD increased significantly by increasing phosphate concentration in the medium from 0.5 to 2 mM, but there was no significant difference between 0.1 and 0.5 mM. At the last sampling time of 40h, OD increased significantly by increasing phosphate concentration in the medium from 0.1 to 0.5 and from 0.5 to 2 mM. As the results in Fig. 1b indicated, bacterial OD increased significantly under all phosphate concentrations of 0.1, 0.5 and 2 mM by elapsing the time of bacterial incubation from 16 to 24 or from 24 to 40h.

Bacterial OD of *S. meliloti* transformed with *expR* promoter- *egfp* fusion did not change significantly by growing after 16 h under different phosphate concentrations of 0.1, 0.5 and 2 mM (Fig. 1c). The results of measurements at 24 h showed, bacterial OD increased significantly by increasing phosphate concentration in the medium from 0.5 to 2 mM, but there was not any significant difference between 0.1 and 0.5 mM phosphate. As phosphate concentration increased from 0.1 to 0.5 or from 0.5 to 2 mM, *S. meliloti* OD increased significantly after 40 h incubation. The highest OD has been reported at 40h for all bacteria grown under different P concentrations (Fig. 1c).

The results of 16h incubation of *S. meliloti* containing *pstS* promoter- *egfp* fusion indicated that OD decreased by increasing phosphate concentration from 0.1 to 0.5 mM. There was not any significant difference between the bacterial OD at 0.1 and 2 mM or 0.5 and 2 mM phosphate supplemented medium (Fig. 1d). Bacterial OD did not change by increasing phosphate concentration from 0.1 to 0.5 at 24 h, but it was increased significantly at 2 mM phosphate. The results of measurements at 40h indicated that the growth of bacteria increased as phosphate concentration increased in the medium. By elapsing the time, bacterial OD increased under all phosphate concentrations (Fig. 1d).

After growing the control strain of *S. meliloti* with promoterless- *egfp* fusion under different phosphate concentrations (Fig. 1e), the results of 16h incubation showed that OD decreased significantly as phosphate concentration increased from 0.1 to 0.5 mM and there was not any significant difference between 0.1 and 2 mM phosphate. At 24 h after incubation in MOPS medium, OD increased significantly as phosphate concentration increased from 0.5 to 2 mM and it has not seen any difference between bacterial OD under 0.1 and 0.5 mM phosphate. The results of 40h incubation of the control strain (Fig. 1e), like the previous *S. meliloti* transformed strains (Fig. 1a, b, c and d), showed that bacterial OD increased significantly by increasing phosphate concentration in the medium. The results also indicated that the bacterial density of different *S. meliloti* strains increased by passing the time (Fig. 1e).

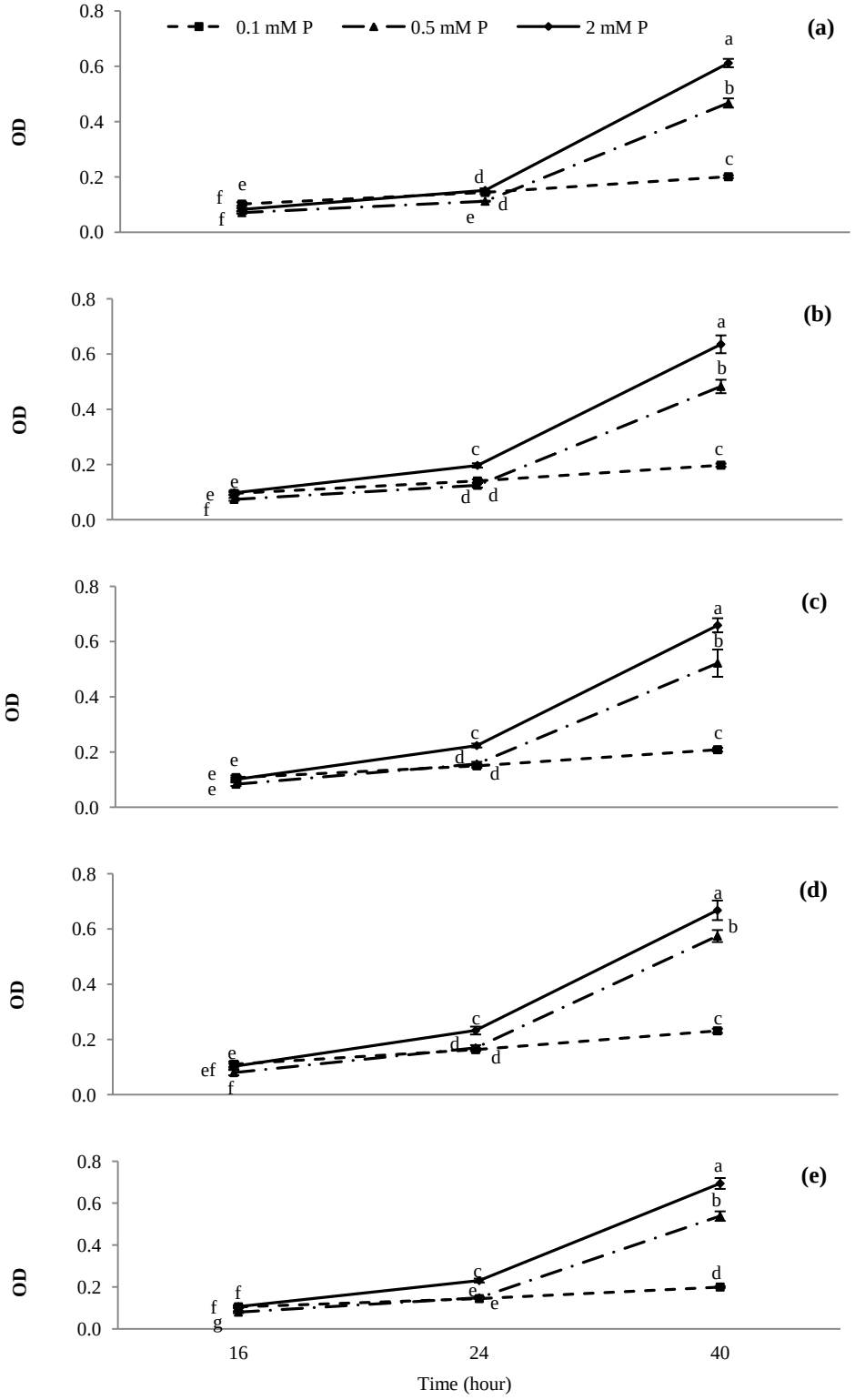

Fig. 1- Optical density (OD) of different strains of *Sinorhizobium meliloti* carrying plasmids of pLK64, *sinI* promoter- *egfp* fusion (a), pLK65, *sinR* promoter- *egfp* (b), pLK66, *expR* promoter- *egfp* (c), pLK004, *pstS* promoter- *egfp* (d), and pLK with a promoterless *egfp* as the control (e) under different phosphate (P) concentration. Variation for replicates (n = 4) is shown as errer bars. The letters above bars indicate significant defferences between means of OD (Duncan test, *P value* < 0.05).

Promoter activity of QS and phosphate transporter coding genes under different phosphate concentrations

Promoter activity (F OD^{-1}) of QS (*sinI*, *sinR* and *expR*) and phosphate transporter (*pstS*) coding genes along with the background fluorescence (F OD^{-1}) of the control strain of *S. meliloti* under different phosphate concentrations (0.1, 0.5 and 2 mM) was presented versus different sampling time in Fig. 2. As the results in Fig. 2a showed, promoter activity of *sinI* increased significantly by reducing phosphate concentration from 0.5 to 0.1 mM at all times of 16, 24 and 40h. By decreasing phosphate concentration from 2 to 0.5 mM, it did not change at 16 and 24h but increased significantly at 40h time point. Promoter activity of *sinI* increased significantly by the incubation time under all phosphate concentrations of 0.1, 0.5 and 2 mM (Fig. 2a).

Data in Fig. 2b indicated that *sinR* promoter activity decreased by increasing phosphate concentration of 0.1 to 0.5 mM at 16h after incubation. There was not any significant difference between promoter activity under 0.5 and 2 mM phosphate at this time point. At 24h after incubation, it was increased as phosphate concentration decreased from 2 to 0.5 mM or from 0.5 to 0.1 mM. *sinR* promoter activity did not change by increasing phosphate concentration from 0.1 to 0.5 mM, but decreased significantly at 2 mM phosphate supplemented MOPS medium at the last time point. The promoter activity of *sinR* was also increased by passing the time and the highest promoter activity of *sinR* was reported at 40h.

Promoter activity of *expR* increased significantly as phosphate concentration reduced from 2 or 0.5 to 0.1 mM at 16h (Fig. 2c). This response was reversed at 24h time and the expression of *expR* increased significantly by increasing phosphate concentration from 0.1 to 2 mM. There was not any difference between 0.1 and 0.5 or 0.5 and 2 mM phosphate at this time point. By passing the time to 40h, the promoter activity increased as phosphate concentration increased in the medium from 0.1 to 0.5 or from 0.5 to 2 mM. AS the results in Fig. 2c indicated, the time after incubation had positive effect on *expR* promoter activity and it was increased by elapsing the time.

Phosphate transporter gene of *pstS* in *S. meliloti* expressed more under phosphate starvation (0.1 mM) compare to 0.5 and 2 mM phosphate at 16 and 24h after incubation (Fig. 2d). Its expression did not differ significantly at 0.5 and 2 mM phosphate supplemented medium at these time points. At the last time of 40h, the promoter activity of *pstS* was reversed under 0.1 and 0.5 mM phosphate. As the promoter activity increased by increasing phosphate concentration from 0.1 to 0.5mM and there was not any significant difference between 0.1 and 2 mM phosphate. As the data of different sampling time showed, *pstS* promoter activity increased by passing the time and the highest activity has been reported at 40h after incubation.

The background fluorescence of *S. meliloti* was higher under 0.5 mM compare to 0.1 and 2 mM phosphate at all sampling times (Fig. 2e). It was also decreased by decreasing phosphate concentration in MOPS medium from 2 to 0.1 mM. The emission fluorescence increased in *S. meliloti* as the time elapsed after incubation.

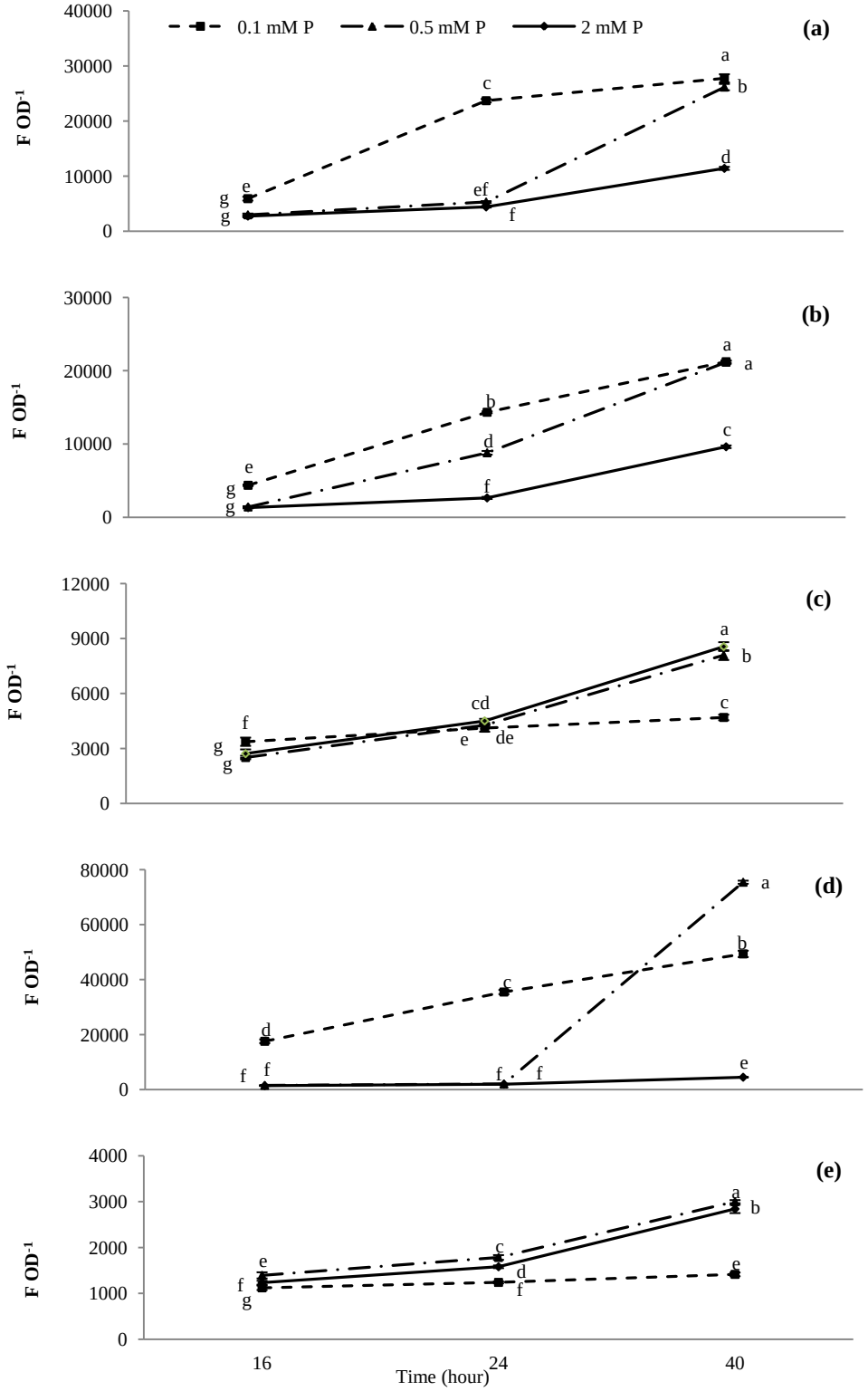

Fig. 2- Promoter activity (F OD^{-1}) of different genes of quorum sensing (QS) pathway, *sinI* (a), *sinR* (b), *expR* (c), and phostphate transporter coding gene of *pstS* (d) along with the background fluorscence of the control strain of *Sinorhizobium meliloti* (e). F OD^{-1} was measured at different times of 16, 24 and 40 h after bacterial growth in MOPS medium containing 0.1, 0.5 and 2 mM P. Variation for replicates (n = 4) is shown as error bars. Different letters above bars indicate significant differences between means of F OD^{-1} (Duncan test, *P value* < 0.05).

Discussion and conclusion

Phosphorous is an essential macroelement for all organisms and after nitrogen, it is the most limiting mineral nutrition for living cells (39 and 40). We demonstrated that the population density (OD_{600}) of all *S. meliloti* transformed strains containing pLK004, pLK64, pLK65, pLK66 and control plasmids decreased following phosphate deficiency especially at 40h.

Although the total amount of phosphate in lithosphere is quite high, it is frequently in inaccessible forms and its diffusion rate is also too slow. In addition, plants due to high rate absorption create some phosphate - depleted zones in soil. So, symbiotic and free living bacteria which normally encounter phosphate starvation developed some modified mechanisms to cope with these sever conditions (29, 30 and 40).

By measuring promoter activity in *S. meliloti* strain Sm2B3001, we showed that the expression of *pstS* increased following phosphate starvation. In fact, bacteria can respond to phosphate limited environment via increasing the expression of phosphate transporters. Our results in Fig. 2d also indicated that there is a threshold for increasing *pstS* expression and its promoter activity reversed at 40h for 0.5 and 0.1 mM phosphate. As phosphate concentration decreased from 0.5 to 0.1 mM in the medium, the expression of *patS* was also decreased about 53 percent.

QS signaling pathway is another bacterial volunteer to cope with phosphate limitation. In the current study, the responses of 2 genes *sinI* and *sinR* in QS signaling pathway to phosphate deficiency were more or less the same. The promoter activities of these genes were about 5- fold higher under 0.1 mM compared to 2 mM phosphate at 24h. A model was proposed for QS regulation in response to phosphate starvation (29). Based on this model, when deficiency in phosphate is encountered, the bacterial growth rate is slowed (Our data also indicated) and the production of AHLs in a population would be reduced, accordingly. In response to phosphate starvation, PhoB induces the expression of *sinR*. The increased level of SinR, as the transcriptional regulator, promotes *sinI* expression and thereby AHL production. This level of AHL which is bound to ExpR is sufficient for strong induction of *sinI*. So, QS pathway and AHL production would be upregulated under phosphate deficiency to facilitate bacterial functions involved in free living and symbiotic life styles. In this study, according to the described model (29), high SinR synthesis following phosphate limitation can promote *sinI* expression. The QS gene of *expR* did not respond to phosphate deficieny as two other genes of *sinI* and *sinR*. It might be because phosphate deficiency- activated *sinR* did not have any effect on *expR* promoter activity.

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تاثیر کمبود فسفر روی مسیر سگینالینگ *quorum sensing* در *Sinorhizobium meliloti*

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چکیده

مقدمه: فسفر یک از مهم‌ترین عناصر ضروری پرمصرف برای رشد باکتری‌هاست. به طور معمول باکتری‌ها در محیط با کمبود فسفر مواجه هستند و مکانیسم‌هایی در آن‌ها برای رویارویی با چنین شرایطی تکامل یافته است. ژن‌های ناقل فسفات (*pstS*) و مسیر انتقال سیگنال حس‌گر حد نصاب (Quorum sensing)، به واسطه تنظیم‌کننده PhoB، در پاسخ باکتری به این شرایط استرسی دخالت دارند. مسیر حس‌گر حد نصاب در باکتری *Sinorhizobium meliloti* حداقل از سه ژن *sinI*، *sinR* و *expR* تشکیل یافته است و در کنترل فعالیت‌های باکتری در شرایط آزاد-زی و همزیستی دخالت دارد.

مواد و روش‌ها: تراکم جمعیت در گونه‌های تراریخت باکتری *S. meliloti* حامل پلاسمیدهای pLK004 (ترکیب راه‌انداز *pstS* و ژن *egfp*)، pLK64 (ترکیب راه‌انداز *sinI* و ژن *egfp*)، pLK65 (ترکیب راه‌انداز *sinR* و ژن *egfp*)، pLK66 (ترکیب راه‌انداز *expR* و ژن *egfp*) و کنترل (ژن *egfp* بدون راه‌انداز) در غلظت‌های مختلف ۰/۱ (کمبود فسفات)، ۰/۵ و ۲ (غلظت کافی فسفات) میلی‌مولار فسفات در زمان‌های مختلف ۱۶، ۲۴ و ۴۰ ساعت اندازه‌گیری شد. همچنین، فعالیت پروموتر ژن‌های مختلف *pstS*، *sinI*، *sinR* و *expR* با تقسیم واحد فلورسنس ساطع شده بر تراکم جمعیت باکتری در غلظت‌ها مختلف فسفات محاسبه شد.

نتایج: سرعت رشد باکتری‌های تراریخت با کاهش غلظت فسفات در محیط کشت، به‌ویژه ۴۰ ساعت پس از کشت آن‌ها، کاهش یافت. فعالیت پروموتر ژن ناقل فسفات (*pstS*) و همچنین، برخی ژن‌های مسیر کروم‌سنسینگ (*sinI* و *sinR* و نه *expR*) به‌دنبال کاهش غلظت فسفات در محیط کشت، افزایش یافت.

بحث و نتیجه‌گیری: از جمله پاسخ‌های باکتری *S. meliloti* به کمبود فسفات می‌توان به افزایش بیان ژن ناقل فسفات (*pstS*) اشاره کرد که تاحدودی می‌تواند این کمبود در محیط را جبران نماید. بیان ژن *sinR* نیز در شرایط کمبود فسفات به کمک تنظیم کننده PhoB القا می‌شود. پروتئین SinR به‌عنوان فعال کننده ژن *sinI* عمل می‌کند و بنابراین، مسیر حس‌گر حد نصاب در شرایط کمبود فسفات فعال می‌شود که احتمالاً موجب تسهیل فعالیت‌های باکتری در شرایط آزاد- زی و همزیستی می‌شود.

واژه‌های کلیدی: کمبود فسفر، انتقال فسفر، مسیر حسگر حد نصاب، سینوریزوبیوم ملیلوتی

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