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NOTE

Measurement of Iron-dependence of *pupA* Promoter Activity by a *pup-lux* Bioreporter

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The promoter region of the *pupA* gene of *Pseudomonas putida* WCS358 was fused with the structural genes for bioluminescence (*luxCDABE*) from *Vibrio fischeri*, and the resulting fusion plasmid harbored by the WCS358 host. The *pup-lux* fusion gene was then used for quantitative analysis of the iron-dependence of *pupA* promoter activity. Factors affecting bioluminescence produced by the *pup-lux* bioreporter were found to be cell activity, iron-chelator concentrations, Fe(III) concentrations, and nutrient components. Light production rates of the *pup-lux* bioreporter were inversely dependent upon iron molecules when $FeCl_3$ concentrations were between 10^{-2} and 1 μM in nutrient-poor minimal media, and between 0.1 and 10 mM in nutrient-rich complex media.

Iron is an essential mineral nutrient for microorganisms but is often in limited supply in natural environments. Microorganisms have developed specific strategies to increase iron-uptake efficiency during starvation conditions (3), such as the synthesis of siderophores (iron chelators). Siderophores are excreted by the cell into the soil, where they bind tightly Fe(III) and are, in this form, resorbed by the microorganism. This process reduces the amount of iron that is available in the immediate vicinity of plant roots, causing an effect that induces iron starvation in pathogenic microorganisms and prevents their accumulation. Thus, siderophores are being examined to determine whether they can be used to create more effective biocontrol inoculants (12).

When grown under iron-limited conditions, fluorescent *Pseudomonas* species produce large quantities of yellow-green fluorescent siderophores and their receptor proteins (1, 7). In the *P. putida* WCS358 strain, ferric pseudobactin 358 is a siderophore with high affinity for Fe(III). Its transport is initiated by binding of this complex to a highly specific outer membrane protein, *pupA* (pseudobactin uptake protein A) (2). The *pupA* promoter region includes specific features that are recognized by a regulatory protein and induced in the absence of iron (5, 8). Although many other uptake proteins have been described, the *pupA* gene is specific for WCS358 and has been used as a marker to monitor wild-type *P. putida* WCS358 in natural environments (10). The control of *pupA* expression is a good model for mineral-regulated systems. For a better understanding of iron regulation in bacterial physiology, it would be useful to obtain more quantitative information about *pupA* promoter activity under iron-limited conditions. Bioluminescence may be of great use for this purpose because it provides a rapid and simple method of analyzing specific gene transcriptional activity without disturbing or destroying the cells. In this report, the *pupA* promoter region was fused with the structural *lux* genes of *Vibrio fischeri* in a promoter probe vector. The *pup-lux* fusion gene was then integrated into the chromosomal DNA of *P. putida* WCS358. This strain was used for analysis of the iron-dependence of *pupA* promoter activity.

The *Pseudomonas putida* WCS358 strain and the *pupA* gene in pAK21 were generously supplied by Dr. V. Venturi, and have been described previously (12). The *P. putida* WCS358 was grown at 30°C in modified King's medium B (KB) (4) containing (per liter): protease peptone #3 (Difco), 20 g; K_2HPO_4, 1.5 g; glycerol, 15 ml. Tetracycline (25 μg/ml) was used as a selection antibiotic. For the preparation of iron-limited KB medium, a non-metabolizable iron chelator, 2,2'-dipyridyl (DPR), was added to a final concentration of 1 mM. Minimal medium contained (per liter): NH_4Cl, 1 g; K_2HPO_4, 6 g; KH_2PO_4, 3 g; $MgSO_47H_2O$, 0.2 g; and 4 g of a carbon source (succinate, glucose, or glycerol).

As shown in Fig. 1, a 1.4-kb *SalI-EcoRI* fragment of pAK21 containing the *pupA* promoter region was fused with the promoterless *luxCDABE* genes of *Vibrio*

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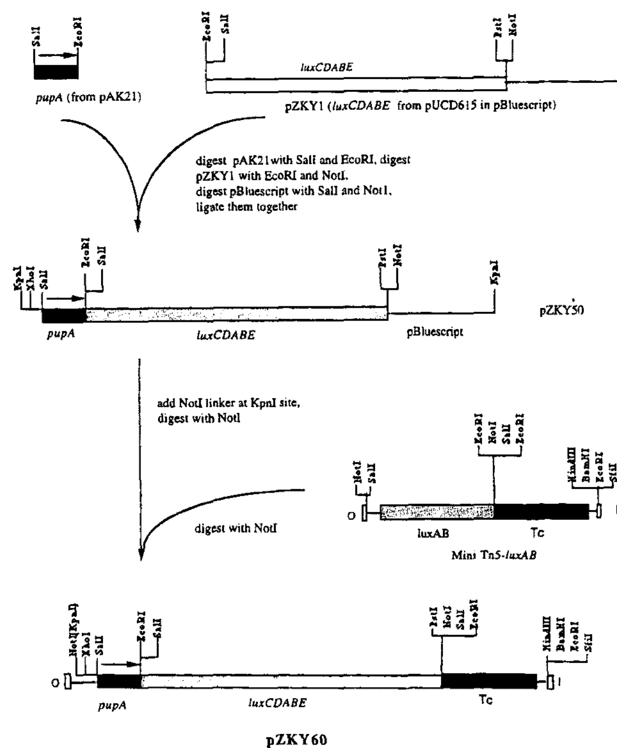

Fig. 1. Cloning scheme for the construction of a *pupA-lux* vector.

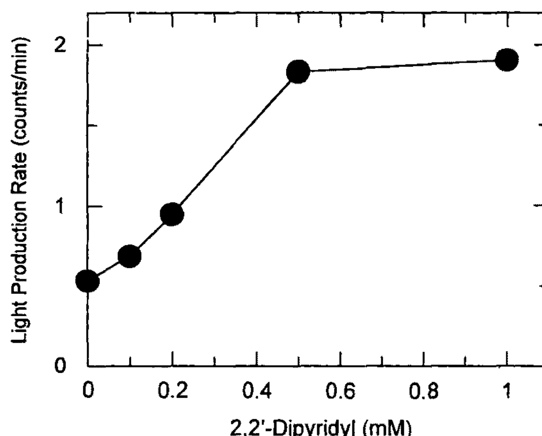

Fig. 2. The effect of 2,2'-dipyridyl in a complex KB medium on *pupA* promoter activity.

fischeri, derived from plasmid pUCD615 (11). The resulting plasmid (pZKY50) was linearized with *Kpn*I and the terminal were modified with *Not*I polylinker. A digestion with *Not*I was followed by isolation of the 9.7-kb fragment. This fragment was substituted for the *luxAB* genes of a mini-Tn5 promoter-probe transposon to eliminate the supply of aldehyde substrates and to facilitate introduction of the *pup-lux* fusion into the parent strain. This fusion transposon was named pZKY60 and transformed into *E. coli* SM10 (6). An aliquot of *E. coli* SM10 harboring pZKY60 was mixed with *P. putida* WCS358 in 10 mM $MgSO_4$ solution and mated as described previously (6). *P. putida* WCS358 clones that received pZKY60 and in which transposition events had occurred were selected with tetracycline (25 μg/ml) and renamed *P. putida* ZKY60.

In order to determine whether the *pup-lux* construction was responsive to induction by iron-starvation, *P. putida* ZKY60 was subjected to iron starvation conditions using a strong iron chelator, DPR. Cells were grown at 30°C in the modified KB medium with tetracycline selection until the mid-exponential range of growth (OD_{600nm}=0.8) was reached. Aliquots of the culture were exposed to concentrations of DPR ranging from 0 to 1 mM (final concentration), and incubated at 30°C. Bioluminescence was measured by use of a luminometer (Turner Design Inc., Model TD-20e, Mt. View, CA). Fig. 2 demonstrates that bioluminescence increased steadily as DPR concentrations increased up to 0.5 mM. A small increase in light production was observed even without the addition of DPR. This was probably caused by the use of KB medium, which is iron-poor. Clearly, the iron starvation conditions were exacerbated by the addition of DPR at any concentration. Light production increased slightly after 0.5 mM, indicating that the required maximum concentration of DPR was close to 1 mM.

It was assumed that the *pup-lux* gene expression level would vary with the growth rate of a batch culture. To determine whether this was true, *P. putida* ZKY60 was grown in KB medium at 30°C, and aliquots were taken at various time-points along the growth curve. These aliquots were amended with 1 mM DPR, and light readings were taken over the induction period. Optical density of the aliquots was measured at 600 nm. Fig. 3 shows the correlation of light production rate (LPR) and cell growth phase in a batch culture. The maximum LPR appeared in the mid-log growth phase, when the cell optical density was about 1.2 at 600 nm, indicating that cells were very responsive during exponential growth. To further refine our understanding of the induction of *pup-lux* fusion, we incubated the *P. putida* ZKY60 strain with DPR and added increasing amounts of iron (III) in the form of $FeCl_3$. It was anticipated that a threshold concentration of iron would be sufficient to saturate the DPR and to repress the *pup-lux* fusion, eliminating light production. Mid-exponential phase cells were grown in KB medium and then amended with DPR at 1 mM (final concentration). Various concentrations of $FeCl_3$ were then added, and incubation continued at 30°C with constant shaking. Monitoring of bioluminescence was performed throughout. Fig. 4 shows the effect of added $FeCl_3$ on light production rates. As expected, cultures

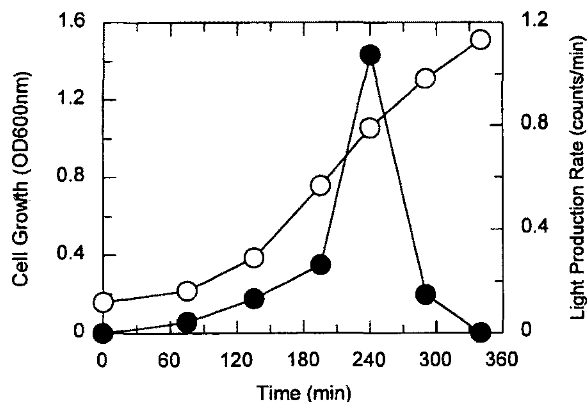

Fig. 3. Measurement of cell transcriptional activity during cell growth.

Cell growth (open circle) was measured at 600 nm and light production rate (closed circle) was determined from the initial slope of the light output. —○—, Cell; —●—, LPR.

without added iron (III) were induced to produce light, and a general pattern emerged in which the more iron (III) was added, the less bioluminescnce was observed over the course of the experiment. However, bioluminescence could be detected even in a very high concentration of $FeCl_3$ such as 6400 μM. The reason why LPR increased at lower concentrations is not known. It is possible that a confounding effect for this assay is the presence of some nutrient components in the modified KB medium that also stimulate lux gene expression under these conditions. To remove possible nutrient effects on the *pup-lux* bioreporter, it was necessary to develop a minimal medium that contained little iron.

A minimal defined medium based on the standard succinate medium (9) was tested to find a proper carbon source among succinate, glucose, and glycerol. To test the viability of cells growing in iron-limited conditions,

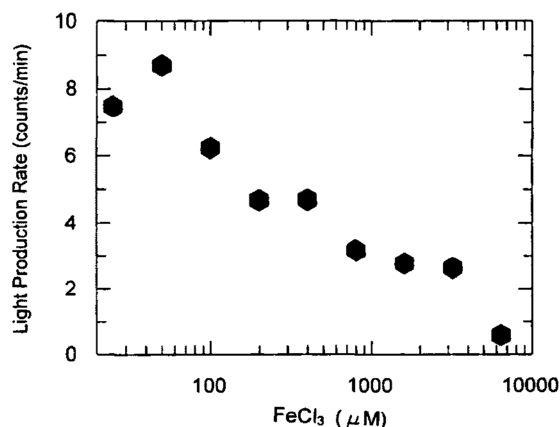

Fig. 4. Effect of $FeCl_3$ on light production rate in a complex KB medium containing 1 mM 2,2'-dipyridyl.

a colony of *P. putida* ZKY60 was directly inoculated in media containing one of three different carbon sources, and grown overnight at 30°C. The overnight cultures were transferred to fresh minimal medium. Cell growth and light output were monitored without the addition of DPR. As shown in Fig. 5a, cells growing in glycerol medium showed poor cell growth, while cell growth was rapid in either succinate- or glucose-containing medium. Light outputs were very poor when glycerol and succinate were used as the carbon sources, even after normalizing results for the lower growth rate (Fig. 5b). Light production on a cell basis was the highest when glucose was used, indicating that glucose may provide more metabolic energy to cells than the other two carbon sources. Consequently, glucose was selected as a proper carbon source in a minimal medium. Iron-dependence of the *pup-lux* bioreporter in the minimal medium was then measured in the absence of DPR with varying concentrations of $FeCl_3$. Fig. 6 shows that the Fe(III) concentration in this minimal medium was required to be at least 10^{-2} μM to inhibit *pupA* promoter activity. In lower concentrations than this, the differences among LPRs

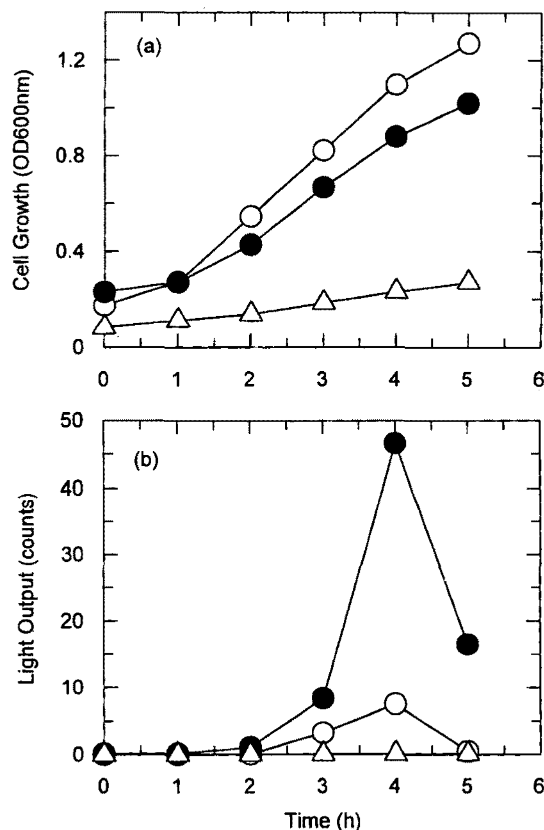

Fig. 5. Effect of carbon sources in minimal medium on (a) cell growth and (b) light output.

—○—, Succinate; —●—, Glucose; —△—, Glycerol.

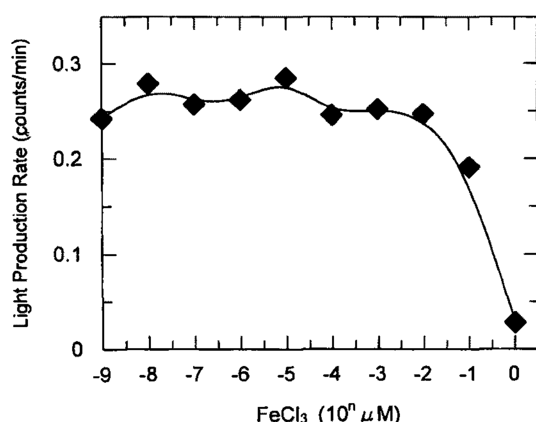

Fig. 6. Effect of $FeCl_3$ on the light production rate in a glucose-based minimal medium.

were not statistically significant (p=0.05). Bioluminescence in 10^{-1} μM $FeCl_3$ was first inhibited but increased later (data not shown). This may indicate that cells utilized all bioavailable irons in an hour, resulting in an iron-deficiency condition. Light output was completely inhibited by more than 1.0 μM $FeCl_3$.

In conclusion, our experiments show that light production rates of the *pup-lux* bioreporter were inversely dependent upon $FeCl_3$ concentrations between about 10^{-2} μM and 1 μM in minimal media, and between 0.1 mM and 10 mM in complex media. Requirement of higher ferric ions in complex media indicates that there is the possibility of another cellular process affecting light production.

The amount of Fe(III) ions in soil may provide a reliable indication of the microbial conditions in subsurface systems, which is essential to be known for a bioremediation of underground contaminants, because many microorganisms utilize Fe(III) as an electron acceptor that provides free energy during respiration. This *pup-lux* bioreporter therefore could be used as a tool for the simple and rapid detection of bioavailable Fe(III) ions in subsurface systems.

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REFERENCES

1. Ankenbauer, R. G. and H. N. Quan. 1994. FtpA, the Fe (III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. *J. Bacteriol.* **176**: 307-319.
2. Bitter, W. J. D. Marugg, L. A. de Weger, J. Tommassen, and P. J. Weisbeek. 1991. The ferric-pseudobactin receptor *pupA* of *Pseudomonas putida* WCS358: homology to *Ton*B-dependent *Escherichia coli* receptors and specificity of the protein. *Mol. Microbiol.* **5**: 647-655.
3. Guerinot, M. L. 1994. Microbial iron transport. *Annu. Rev. Microbiol.* **48**: 743-772.
4. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44**: 301-307.
5. Koster, M., J. van de Vossenberg, J. Leong, and P. J. Weisbeek. 1993. Identification and characterization of the *pupB* gene encoding an inducible ferric-pseudobactin receptor of *Pseudomonas putida* WCS358. *Mol. Microbiol.* **8**: 591-601.
6. De Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**: 6568-6572.
7. Marugg, J. D., H. B. Nielander, A. J. G. Horrevoets, I. van Mergen, I. van genderen, and P. J. Weisbeek. 1988. Genetic organization and transcriptional analysis of a major gene cluster involved in siderophore biosynthesis in *Pseudomonas putida* WCS358. *J. Bacteriol.* **170**: 1812-1819.
8. Marugg, J. D., L. A. de Weger, H. B. Nielander, M. Oorthuizen, K. Recourt, B. Lugtenberg, G. A. J. M. van der Hofstad, and P. J. Weisbeek. 1989. Cloning and characterization of a gene encoding an outer membrane protein required for siderophore uptake in *Pseudomonas putida* WCS358. *J. Bacteriol.* **171**: 2819-2826.
9. Meyer, J. M. and M. A. Abdallah. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J. Gen. Microbiol.* **107**: 319-328.
10. Raaijmakers J. M., W. Bitter, H. L. M. Punte, P. A. H. M. Bakker, P. J. Weisbeek, and B. Schippers. 1994. Siderophore receptor *pupA* as a marker to monitor wild-type *Pseudomonas putida* WCS358 in natural environments. *Appl. Environ. Microbiol.* **60**: 1184-1190.
11. Rogowsky, P. M., T. J. Close, J. A. Chimera, J. J. Shaw, and C. I. Kado. 1987. Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. *J. Bacteriol.* **169**: 5105-5112.
12. De Weger, L. A., J. J. C. M. van Arendonk, K. Recourt, G. A. J. M. van der Hofstad, P. J. Weisbeek, and B. Lugtenberg. 1988. Siderophore-mediated uptake of Fe^{3+} by the plant growth-stimulating *Pseudomonas putida* WCS358 and by other Rhizosphere microorganisms. *J. Bacteriol.* **170**: 4693-4698.

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