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The Use of Cell Density to Estimate the Bacterial Promoter Strength

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The reporter gene *cat* can be used to determine promoter activation and to seek genes related to pathogenicity, where the promoter becomes tagged. The estimation of promoter expression in these cases can be evaluated by three approaches: *i*) determination of relative quantity of the mRNA; *ii*) quantification of protein (chloramphenicol acetyl-transferase); or *iii*) determination of specific enzymatic activity. However the ultimate result for *cat* expression in a cell is to endow resistance against chloramphenicol. In this work we use the cell density measure with chloramphenicol antibiotic as an analysis of the promoter regulation and strength during a study of *Pseudomonas syringae* pv. maculicola mutant screening. We found that the promoter expression level modifies accordingly the cell density in liquid media and also the colony size in solid media at defined times. We propose the determination of cell density in liquid media supplemented with a constant concentration of chloramphenicol to estimate not only the expression conditions of a promoter tagged with *cat*, but also the expression intensity in those conditions.

Key words: Promoter expression, *cat* gene, *Pseudomonas syringae* pv. maculicola M2, Tn*5-cat*1.

There are many strategies to record gene expression. It can be done by measuring promoter activity quantifying the mRNA levels or if possible, the encoded protein. The use of gene reporters fused to the promoter under study simplifies the estimation of its expression and allows us the use of standard measure methods and commercial kits giving reproducible and trustworthy results. The most used reporter gene has been *lacZ*[1] but recently *gfp* is widely used because of their interesting properties and simplicity[2,3,4].

A reporter gene must fulfill several basic criteria in order to be useful: 1) A simple cost-effective assay for quantifying the reporter protein or activity should be available, 2) The amount of protein must correspond to the amount of transcribed mRNA, 3) Endogenous protein or enzyme activity must be absent or minimal in target cells, 4) The assay should be sensitive to small changes in gene expression. *cat* reporter gene has been widely used to monitor gene expression in both bacteria[3,4,5] and plants[6], because of its positive selection and sensitivity. Most methods designed

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for measuring gene expression are difficult and time consuming, and in most cases very expensive due to the use of chemicals or equipment. Here, we report a cheap methodology to measure gene expression by using cell density of *P. syringae* pv. maculicola M2 as indicator. Results were compared with a P_{BAD}: *cat* fusion in order to prove that the cell density depends on the expression level of the reporter gene. We used a transposon tagged *Pseudomonas syringae* pv. maculicola M2 which was selected by its ability to grow in response to plant extract in M9 or KB media containing chloramphenicol. This bacterium contains a *hrpZ*::Tn*5cat*1 mutation (Fig. 1), so the expression of *cat* reporter gene is driven by *hrpAZB* operon native promoter. This strain can be used as a bioreporter to monitor chemicals or environmental conditions affecting the expression of the Type III Secretion System (TTSS), a crucial pathogenicity factor in Gram-negative bacteria[7,8].

MATERIALS AND METHODS

Pseudomonas syringae pv. maculicola strain M2 (Rif^R;PsmM2) was a kindly gift from Dr. Jeffrey L. Dangl[9]. *Pseudomonas syringae* pv maculicola *hrp Z*::*cat* (PsmMut8) was obtained in this work. *E. coli* S17-1 λpir (*thi pro hsd* R *hsd* MΔ *rec*A RP4-2*tra*Tc::Mu Km::Tn*7*;[10]) was obtained from Dr. Kate J. Wilson. *E. coli* TOP10 [*mcr*AD (*mrr-hsd*RMS-*mcr*BC) ϕ80 *lac*ZDM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 D(*ara-leu*) 7697 *gal* U *gal* K *rps*L (Str^R) *end* A1 *nup* G; (Invitrogen) was used for determination of *cat* expression experiments along with pBAD/His A (Invitrogen). *E. coli* HB101 (F-Δ *(mcr*C-*mrr) leu sup* E44 *hsd* S20 (rB-mB-) *ara*-14 *gal* K2 *lac*Y1 *pro*A2 *rps*L20 (Strr) *xyl*-5*mtl*-i *rec*A13;[11]) was used for cloning. Transposable element pTn*5cat*1 is derived from pTn*5cat*[12]. A synthetic DNA fragment with *Swa*I restriction site (CCCATTTAAATGCATGC) was added into the *Sma*I site in pUIRM504 to form the plasmid pMDC505. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Inc., or Invitrogen. SuperMix High Fidelity was purchased from Invitrogen. Enzymes were used following the supplier recommendations. King's B medium[13], minimal medium (M9)or M9 supplemented with casaminoacids (M9CA, Difco) were used to culture *P. syringae* pv. maculicola strains, or in the assays to determine the conditions for *cat* expression, with or without addition as it will be described below. LB medium was used to culture *E. coli* strains. Chloramphenicol (Cm), rifampicin (Rif) and kanamycin (Km) were purchased to Serva or Sigma-Aldrich Chemicals, all antibiotics were used at 50 µg/ml, except when it is mentioned.

Mutagenesis and mutant selection

Mutants of PsmM2 were generated using the transposable element pTn*5cat*1, according a published protocol[12]. *E. coli* S17-1 λpir(pMDC505) was used to mobilize pTn*5cat*1 to PsmM2 by conjugation and the bacteria were spread on M9 Rif_{50} Km_{50} plates. Mutants were screened to select those forming bigger colonies on M9 Cm_{50} with plant extract (2 ml per liter) than on KB Cm_{50} without additions. Plant extract was prepared from leaves of *Phaseolus vulgaris*. Leaves (10 g) were cut off, frozen in liquid nitrogen and ground in a cold mortar. Tissue debris were eliminated by centrifugation, the supernatant was collected, dispensed in microcentrifuge tubes and stored at –84°C until used, PsmMut8 was not able to produce HR in *Arabidopsis thaliana* assay (data not shown). After cloning and sequencing the mutated gene (GeneBank accession number AY325899) it was assessed that pTn*5cat*1 was inserted in *hrpZ* gene (Fig. 1).

Assay for promoter strength

pTn*5cat*1 containing *cat* as reporter gene[12] allowed to estimate the *hrpAZB* operon promoter strength. Promoter strength was evaluated as cell density after growing bacteria in media containing chloramphenicol. Assays were performed in 96-well polystyrene sterile plates by triplicate. 50 µl of a 0.04 OD_{620} culture of the mutant PsmMut8 in KB Km_{50} were added to wells containing 200 µl of M9, M9CA or KB media, supplemented or not with plant extract (2 µl/ml) or sucrose (5%); all media contained kanamycin (50 µg/ml) and chloramphenicol (150 µg/ml). Plates were incubated at 28°C and the cell density was measured at 0, 24 and 48 h using a Titertek Multiskan Plus (EFLAB, Jont Venture Company of Labsystem and Flow Laboratories) with a 492 nm filter.

Arabinose induction of P_{BAD}::*cat* expression

The promoterless *cat* gene was amplified from pTn*5cat*1 using SuperMix High Fidelity

(Invitrogen) and cloned in pCR4-TOPO (Invitrogen) to obtain pCR4*cat*. The *Eco*RI fragment containing *cat* was cloned in the *Eco*RI site of pBAD/His A (Invitrogen) obtaining pBAD*cat*. Time course experiments were performed in 96-well polystyrene sterile plates. 50 µl of a 0.04 OD_{620} culture of the *E. coli* TOP10 (pBAD*cat*) in LB Cb_{100} supplemented with arabinose 0.02 mM were added to wells containing 200 µl of LB Cb_{100} Cm_{75} supplemented with arabinose (0.0, 0.025, 0.25, 0.5, 0.4, 1.0, 2.0 and 4.0 mM). Plates were incubated at 37 °C and the cell density was measured during 24 h by triplicate, using a Titertek Multiskan Plus (EFLAB, Jont Venture Company of Labsystem and Flow Laboratories) with a 492 nm filter.

RESULTS AND DISCUSSION

Screening and selection of PsmMut8

An approach using a transposable promoter probe was performed to search for genes involved in the pathogenicity of PsmM2. pTn*5cat*1 was introduced by conjugation to PsmM2 from *E. coli* S17-1(pMDC505), and the colonies developed on M9 plates supplemented with kanamycin, rifampicin and plant extract of *Phaseolus vulgaris* were tested, comparing their development in the presence of chloramphenicol in the same medium against King's B medium (KB). The colonies that became bigger in M9-plant extract than in KB at the same incubation time showed higher resistance

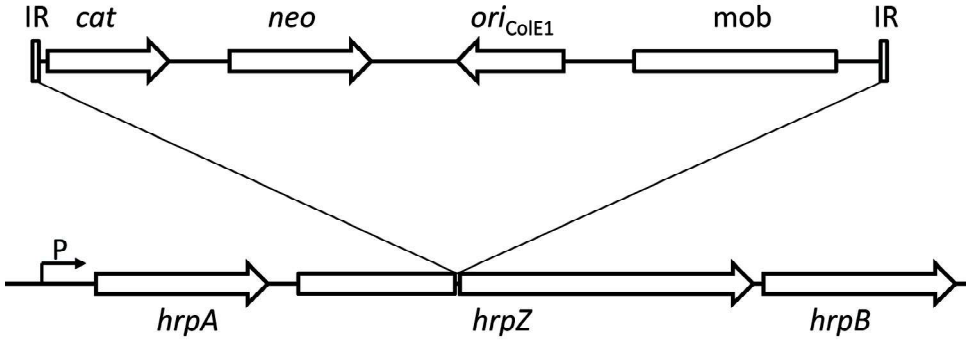

Fig. 1. pTn*5cat1* insertion in the *hrpAZB* operon of PsmM2, indicating the promoter (P) detected. The orientation of the transposon is shown, including the inverted repeats (IR), *cat* reporter gene, the genetic marker for kanamycin resistance (*neo*), replication origin (ori_{ColE1}) and mob region (mob).

to chloramphenicol, this was an indication that the promoters driving the expression of the reporter gene *cat* were induced in M9 by the plant extract, or they were repressed in KB. This approach allowed to obtain 14 mutants, among them PsmMut8. The mutants were proven to be pathogenic to *Arabidopsis thaliana* and only PsmMut8 was non-pathogenic. The transposon was inserted in *hrpZ,* which is included in the operon *hrpAZB*[14,15].

Analysis of the regulation in the tagged gene *hrpZ*

We used PsmMut8 (*hrpZ*::Tn*5cat*1) to analyze the expression of *hrpAZB* promoter in different media; all supplemented with chloramphenicol, and cell density was estimated by the determination of A_{492} at different incubation times. Fig. 2a shows the results after the incubation for 48 h at 28° C. It can be clearly observed that the expression of the *hrpAZB* promoter is negligible in rich medium (KB) and is induced by the minimal medium and the plant extract. Also the medium containing casaminoacids blocked the induction by plant extract.

Cell density as a measure of promotor strength

To assess that the results shown in figure 2a are indeed due to the different expression level of the *hrpAZB* promoter, an experiment using the promoter of operon *araBAD*, which is controllable modifying the arabinose concentration in the medium[16], driving the expression of *cat* gene was performed. Promoterless *cat* gene was cloned in the commercial plasmid pBAD to be expressed by the promoter P_{BAD} and controllable by AraC. Fig. 2b shows the results obtained when *E. coli* TOP10 harboring the plasmid pBAD-*cat* was cultured in LB supplemented with chloramphenicol and different concentrations of arabinose. As it can be shown, the variation in arabinose concentration

directly affects the bacterial growth. Key factor in expression analysis are those obtained through promoter strength determination. The use of reporter genes, such as those encoding for beta-galactosidase (*lacZ*), beta-glucoronidase (*uidA*), chloramphenicol acetyltransferase (*cat*) or the green fluorescent protein (*gfp*) facilitated the study of gene expression and led to the design and development of accurate techniques as a IVET technology or and commercial kits[4,10,17,18,19]. However, most methods are cumbersome, expensive or both. Almost every method, commercial or not, to determine the levels of gene expression, at least in bacteria, includes an initial step: the culturing. The approach depicted here to determine promoter strength is the easiest way to estimate *cat* expression. The assay is performed during the initial cultivation of the strain harboring a *cat* reporter gene fused to the promoter under study. The use of a transposable promoter probe (Tn*5cat*) to mutate PsmM2 and selecting on M9 versus KB (both media supplemented with chloramphenicol) shown mutants forming bigger colonies on the minimal medium than on the rich medium, the first evidence about biomass quantification could be a method to estimate gene expression.

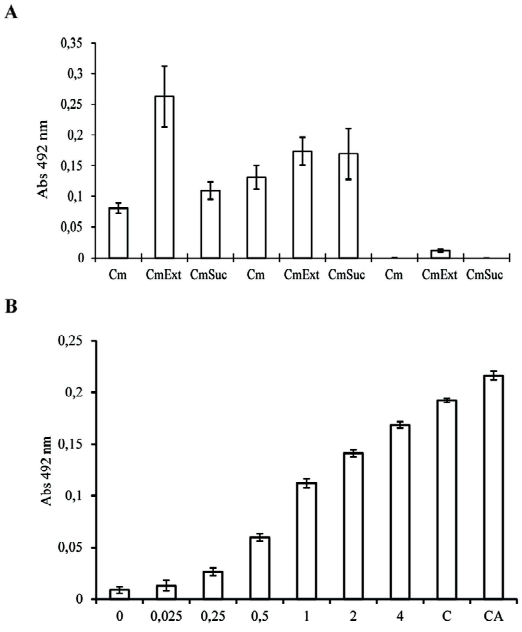

Fig. 2. Growth of PsmMut8 and *E. coli*pBAD::*cat* in different conditions. (**A**) PsmMut8 in minimum media (M9), M9 casamino acids (M9Ca) and King's B (KB), all the media was supplemented with chloramphenicol (Cm; 150 µg/ml). As inductor we used plant extract (Ext) or sacarose (Sac). (**B**) *E. coli* pBAD::cat under different concentrations of arabinose (mM), with chloramphenicol (150 µg/ml) C = control without chloramphenicol, CA = control without chloramphenicol

The use of transposable promoter probes to search for important genes is one of the easiest ways to identify genes involved in a particular process[9,12,20,21,22], such as the genes related to the pathogenicity or virulence of phytopathogenic bacteria. It is common sense to search for genes expressed under the conditions presented during infection[12,19] and *cat* reporter gene is convenient since it allows growth of mutants expressing it, leading to colony formation on solid media supplemented with chloramphenicol while the mutants lacking *cat* expression cannot growth at all or do it with a very low rate. pTn*5cat*1 was very useful in order to obtain mutants from PsmM2 where the *cat* reporter gene were fused to a promoter whose expression depends on plant metabolites and minimal medium. The cell concentration can be measured as optical density with a photocolourimeter or a 96-well plate reader. The method proposed here use only the first cultivation step and the bacterial cell density indicates directly the level of gene expression and the conditions for its expression. The comparison of different strains grown in the same media gives only an approximation of different promoter strength as their expression depends on different gene regulation.

Effect of M9 and plant extract on *hrpZ* gene expression

From a mutant collection of PsmM2, we chose mutant 8 (PsmMut8) because was not able to cause disease in *Arabidopsis thaliana*, and the *cat* reporter gene was expressed in M9 or M9 with plant extract, which indicates that this promoter was inducible and we could controlled their expression. To determine the effect of conditions where this promoter was induced, PsmMut8 was grown in M9 and KB added with chloramphenicol and supplemented with or without plant extract. Fig. 2 shows the optical density after 48 h. It can be seen that growth was induced by M9 and plant

extract. KB medium repressed or did not induce *cat* expression, because it was reported that aminoacids down regulated genes related to pathogenesis process and low pH and metabolites from the plant are able to stimulate genes related with phytopathogenesis process[14,23,24,25,26].

The cell density depends on the inductor

To show that the methodological approach indeed allows estimation of promoter strength, the promoter *cat* was cloned in pBAD/His, where the gene reporter was expressed by P_{BAD} and controlled by arabinose. The estimation of the *cat* expression was done by similar way as for PsmMut8. The result showed that P_{BAD} was controlled by arabinose and the measured biomass depends on the extent to which promoter is activated. This demonstrated that the technique presented here to measure promoter strength or gene expression is usable and sensitive for different concentration of the inductor or growth conditions.

This methodology is inexpensive, rapid and make possible to analyze large quantities of mutant easily with accuracy, also it could be used in an undergraduate course to show cloning and transcriptional control.

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