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Establishment of an arabinose-inducible system in *Stenotrophomonas maltophilia*

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Abstract A pBBad22T-derived conditioned arabinose (Ara)-inducible expression system was evaluated in *Stenotrophomonas maltophilia* (an opportunistic pathogen and has gained increasing attention as a cause of healthcare-associated infection). *S. maltophilia* cannot grow well when Ara is the sole available carbon source. The induction kinetic study, optimal inducer concentration determination, and depletion experiment were performed by using a *xylE* gene fusion construct, pBxylE, to monitor the expression of pBBad22T in *S. maltophilia*. For induction survey, the expression of catechol 2,3-dioxygenase (C23O), encoded by *xylE* gene, continuously increases during an 8-h induced course and can be modulated by different inducer concentrations. The applied induction condition of pBBad22T in *S. maltophilia* is the inducer concentration ranging from 0.1% to 0.5% for an induction time of 4 h. For repression evaluation, the C23O expression is rapidly turned off within 30 min after the removal of Ara. Accordingly, the established Ara-inducible system can provide a convenient tool for the study of *S. maltophilia*.

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Abbreviations

Ap	Ampicilin
Ara	Arabinose
BHR	Broad host range
C23O	Catechol 2,3-dioxygenase (EC 1.13.11.2)
Km	Kanamycin
LB	Luria–Bertani
MCS	Multiple cloning site(s)
R	Resistance/resistant
Tet	Tetracycline
xylE	Gene encoding C230

It is often necessary to express a cloned gene from an inducible promoter and assess the influence of the gene expression in many bacterial studies. Accordingly, it is highly desirable to establish a controlled system that can quantitatively turn on or shut off the expression of the cloned gene. Many of such systems have been developed (Tabor and Richardson 1985; Elvin et al. 1990; Guzman et al. 1995; Lutz and Bujard 1997). Among them, the *araC*–

P_{BAD} system is the typical one that can tightly regulate the inducible expression of the cloned genes (Guzman et al. 1995).

In the field of bacteriology, a conditioned mutant for some essential genes is necessary for the study of gene functions and its involved complex regulation or physiological properties. The essential genes are indispensable for bacterial survival. Hence, the null mutation of essential genes is unavailable for further study. An alternative strategy is to develop a tight regulated induction implement to determine the essentiality and function of these essential genes. The *araC–*P_{BAD}-derived plasmid is such a design that can meet the requirement. The viability and phenotype of the mutants depends on the presence of arabinose (Ara) to induce the expression of the studied genes. The depletion experiment, in which the expression of the studied genes is shut off by shifting the bacterial culture from an Ara-containing medium to a no Ara-containing medium, allows us to examine the null mutant phenotype.

In *Escherichia coli*, the P_{BAD} promoter is induced in the presence of L-Ara and in the absence of glucose. AraC is a cAMP-catabolite repressor protein that can bind to the operator sequence upstream P_{BAD} and repress the expression of *araBAD* genes (Hahn et al. 1984). Guzman et al. have developed an *araC–*P_{BAD} controlled expression vector that is tightly regulated in enteric bacteria (Guzman et al. 1995). Some broad host range (BHR) *araC–*P_{BAD}-derived systems have been reported, including the plasmids of pCF430, pJN105, pBBad18-series, and pBBad22-series (Newman and Fuqua 1999; Sukchawalit et al. 1999). Among them, the pCF430 and pJN105 have been used in the plant pathogen *Agrobacterium tumefaciens* (Newman and Fuqua 1999). The application of pBBad18- and pBBad22-series plasmids in *Pseudomonas aeruginosa* and *Xanthomonas campestris* pv. *phaseoli* has also been characterized (Sukchawalit et al. 1999).

Stenotrophomonas maltophilia is an aerobic, ubiquitous Gram-negative bacillus and has gained increasing attention as a cause of nosocomial infection (Nyč and Matějková 2010). A compatible inducible expression system for *S. maltophilia* is of great urgency. In this study, we described the application of the BHR pBBad22T vector and established the induction parameters for the expression of cloned gene in the *S. maltophilia* system.

Materials and methods

Strains and culture conditions *S. maltophilia* KJ (Hu et al. 2008), a clinical isolate, and *E. coli* S17-1 were grown aerobically in Luria–Bertani (LB) medium. Tetracycline (Tet) was added, if needed, at a concentration of 30 mg/L. The XOL medium (Yang et al. 2002) contained basal salts (grams per liter): K_2HPO_4, 0.7 g/L; KH_2PO_4, 0.2 g/L; $(NH_4)_2SO_4$, 1.0 g/L; $MgCl_2{\cdot}6H_2O$ 0.1 g/L; $FeSO_4{\cdot}7H_2O$, 0.01 g/L; and $MnCl_2$, 0.001 g/L, at pH 7.15. Carbon sources were autoclaved separately and added before use.

Growth conditions To study the utilization of Ara by *E. coli* and *S. maltophilia*, the growth characteristics were evaluated of *S. maltophilia* KJ and *E. coli* S17-1 in the XOL medium containing 0.4% Ara or glucose. The bacterial growth was monitored by recording the A_{450} (for *S. maltophilia*) or A_{600} (for *E. coli*) in 1-h intervals.

Construction of the araC–P_{BAD}*::xylE fusioned plasmid, pBxylE* The *xylE* gene of *Pseudomonas putida* was obtained from pX1918G (Schweizer and Hoang 1995) by PCR using the paired primers XylE-F: 5′-GAA TTC GCG GCC GCG ATC AAG GAC TAC TAC CAT T-3′ and XylE-R: 5′-GCG GCA AGT CGT ACC GGA CCT TCA G-3′, and then cloned into yT&A vector (Yeastern Biotech, Taiwan), yielding the recombinant plasmid pTXylE. The 1.3-kb *xylE* gene was subcloned from pTXylE into the *Hind*III/*Eco*RI-pretreated plasmid pBBad22T. Plasmid pBBad22T is an L-Ara-inducible BHR expression vector with the BAD promoter and *araC* gene of *E. coli* (Sukchawalit et al. 1999). The resultant recombinant plasmid pBxylE (Fig. 1) was sequenced to confirm the correct orientation of *xylE*, under the driving of promoter P_{BAD}. The recombinant plasmid was transported into the assayed *S. maltophilia* strain via conjugation as described

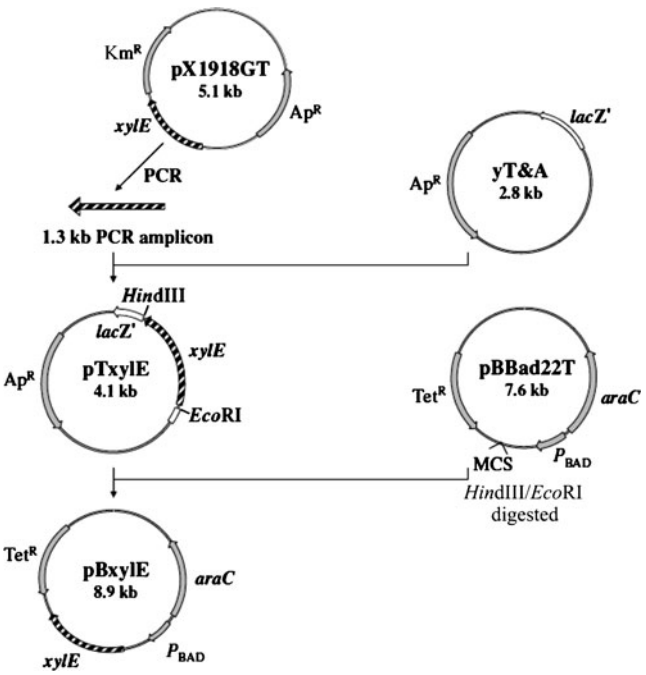

Fig. 1 Construction of pBxylE. The 1.3-kb *xylE* gene from pX1918GT was PCR amplified and ligated to yT&A vector, yielding pTxylE. For construction of pBxylE, a1.3-kb *Hin*dIII–*Eco*RI fragment from pTxylE was gel-purified and ligated to *Hin*dIII/*Eco*RI-treated pBBad22T

previously (Lin et al. 2009). Acquisition of the appropriate plasmids was confirmed by a colony-PCR method (Lin et al. 2008) using the paired primers XylE-F and XylE-R.

Induction kinetics studies Twenty milliliters of fresh LB broth was inoculated at a turbidity of 0.15 A_{450} with the assayed bacteria grown overnight at 37°C. The culture was further incubated for 0.5 h, and then Ara was added to the culture (final concentration of 0.02%); control group without Ara was simultaneously assayed. Samples were taken in 1-h intervals, and the catechol 2,3-dioxygenase (C23O) activity was determined. Meanwhile, the growth of bacteria was also monitored by recording the A_{600} for *E. coli* and the A_{450} for *S. maltophilia*.

Determination of the optimal inducer concentration An overnight culture was used as a starting inoculum to give the absorbance A_{450}=0.15. The culture was further grown for 0.5 h. Then, the distinct amounts of Ara were added, and incubation was continued for 4 h. For investigation of Ara-induction potency, the C23O activity was determined.

C23O activity C23O activity was measured in intact cells according to Lin et al. (2009). Activity assays were performed in buffered mixture (50 mmol/L sodium phosphate buffer, 10% acetone and 0.1 mol/L pyrocatechol as the substrate). Hydrolysis of pyrocatechol was examined spectrophotometrically with readings in 10-s intervals for 3 min at the wavelength of 375 nm. The rate of hydrolysis was calculated by using 44,000 mol^{-1} cm^{-1} as molar extinction coefficient. One unit of enzyme activity was defined as the amount of enzyme that converts 1 nmol substrate per min. The specific activity of the enzyme was defined in terms of units per 3.6×10^8 cells (assuming that an A_{450} of 1 corresponds to 3.6×10^8 cells per milliliter).

Depletion experiment S. maltophilia (pBxylE) was cultured in the LB medium supplemented with 0.2% Ara at 37°C. Overnight cultures were washed by a fresh LB medium once and subsequently diluted to an A_{450} of 0.15 in the fresh LB medium containing 0.2% Ara and in non-supplemented medium, respectively. The C23O activity of *S. maltophilia* (pBxylE) was monitored before cell washing, immediately after washing the cells, and during the period of further 1.5-h culturing with intervals of 0.5 h.

Results

The Ara utilization in *E. coli* and *S. maltophilia*

The Ara utilization by *S. maltophilia* was evaluated so as to establish the Ara-induction system in *S. maltophilia*. In the meantime, the Ara utilization by *E. coli* was also monitored. Cultures of *E. coli* S17-1, *S. maltophilia* KJ, *E. coli* S17-1 (pBxylE), and *S. maltophilia* KJ (pBxylE) were grown in the minimal medium supplemented with 0.4% Ara and glucose, respectively. The growth of the assayed strains was observed up to 9 h because the residual nutrition inside cells can support their growth (Fig. 2). The growth of *E. coli* in 0.4% Ara continued with prolonged culture, indicating that *E. coli* can metabolize Ara and use it as a sole carbon source. In contrast, the growth of *S. maltophilia* in 0.4% Ara was suppressed after 9 h, demonstrating that Ara is not an autotrophic carbon source for *S. maltophilia*. The presence of plasmid pBxylE did not interfere with the growth of *E. coli* and *S. maltophilia*.

Induction time courses of *E. coli* (pBxylE) and *S. maltophilia* (pBxylE)

The induction time course was performed to monitor the C23O activity of *E. coli* (pBxylE) and *S. maltophilia* (pBxylE). Taking the optimal Ara inducing conditions proposed by Sukchawalit's group as the reference, the induction kinetics were firstly investigated by the induction of 0.02% Ara; meanwhile, an additive-free control was also

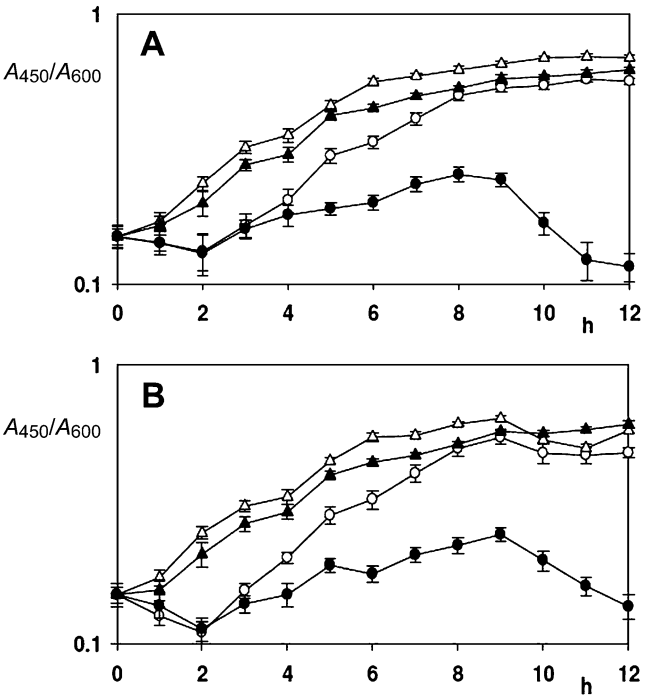

Fig. 2 Growth curves of *E. coli* and *S. maltophilia*; *circles*, *S. maltophilia*; *triangles*, *E. coli*, *open symbols*, 0.4% glucose; *closed symbols*, 0.4% arabinose. Cells from overnight cultures were separately harvested and then inoculated into XOL containing glucose or Ara at final concentration of 0.4%. Cell growth was measured by monitoring A_{600} for *E. coli* and A_{450} for *S. maltophilia*. *Errors bars* indicate standard deviations (n=3). **a** Strains of *E. coli* and *S. maltophilia*. **b** Strains of *E. coli* (pBxylE) and *S. maltophilia* (pBxylE)

carried out. Without the addition of Ara, the C23O activity of *E. coli* (pBxylE) was below the detection limit. A maximum C23O activity for *E. coli* (pBxylE) was obtained at 2 h after the addition of Ara, and thereafter, the activity decreased monotonously (Fig. 3). However, the non-induced C23O activity of *S. maltophilia* (pBxylE) was low but detectable after 4-h induction, indicating that the promoter of pBBad22T is not tightly repressed in the *S. maltophilia* system. For the induced *S. maltophilia* (pBxylE), the C23O activity was detectable for the first sampling, and an obvious increase in the C23O activity was observed when the induction time was extended to 8 h.

Establishment of optimum induction parameters of pBBad22T in *S. maltophilia*

Based on the results of growth-curve and induction time course, the fourth hour after addition of the inducer was considered to be the appropriate time point for the assay of optimum inducing concentrations. The C23O activity displayed an increase as the Ara concentration exceeded 0.002%. Moreover, the C23O activity changed insignificantly when more than 0.2% Ara was added (Table 1). In conclusion, the applied induction condition of pBBad22T for *S. maltophilia* was the inducer concentration ranging from 0.01 to 0.5% for induction time of 4 h.

Table 1 also shows that the induced C23O activity at 0.2% concentration was twofold higher than that in 0.02% condition. It is, therefore, feasible to modulate the expressed amounts of the cloned gene in pBBad22T by the inducer concentrations.

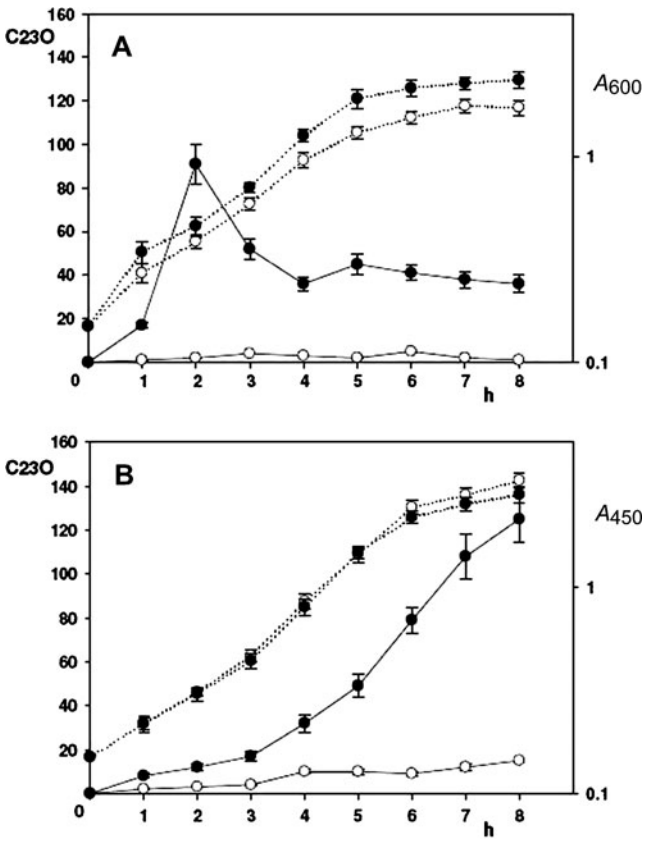

Fig. 3 Growth curves and induction time courses of C23O in *E. coli* (pBxylE) (**a**) and *S. maltophilia* (pBxylE) (**b**). *Errors bars* indicate standard deviations (n=3); *full lines*, C23O activity (for further details see "Materials and methods"); *dashed lines*, growth (A_{600} for *E. coli* or A_{450} for *S. maltophilia*); *open circles*, without arabinose addition; *closed circles*, with 0.02% arabinose

Depletion experiments of *araC–P_{BAD}* promoter in *S. maltophilia* system

To study essential genes of the Ara-inducible system, we considered of great importance whether the *araC–P_{BAD}* promoter can be repressed rapidly and efficiently in the *S. maltophilia*. Accordingly, depletion experiments were performed. *S. maltophilia* KJ (pBxylE) grown in the medium supplemented with Ara was shifted to Ara-containing and Ara-free LB medium. In Ara-containing culture, the C23O activity was continuously expressed as expected. However, the C23O activity in the Ara-free medium decreased rapidly and became undetectable after 30 min of culturing.

Discussion

The expression vector of pBBad22T has been shown to be compatible with the *S. maltophilia* system according to the following features: (a) It contains the pBBR1MCS-4 replicon (Kovach et al. 1995) of BHR characteristic, which can be replicated in a variety of Gram-negative bacteria including *S. maltophilia*. (b) Mobilization (mob) function of pBBad22T allows a conjugal delivery of the plasmid into *S. maltophilia*. Our previous studies showed that the heat-shock transformation and electroporation do not work well

Table 1 The induced C23O activity of *S. maltophilia* (pBxylE) upon the challenge of different Ara concentration

Ara,%	C23O activity[a]
0	10±1.0
0.001	10±1.2
0.002	19±2.0
0.005	24±2.1
0.01	32±2.9
0.02	32±3.2
0.05	38±2.8
0.1	62±7.0
0.2	70±5.9
0.5	72±6.4
1	65±5.6
2	69±8.0

[a] For details, see "Materials and methods"

for transferring the foreign plasmid into the *S. maltophilia*. On the other hand, the conjugation between *E. coli* S17-1 and *S. maltophilia* provides an efficient transformation (Hu et al. 2008). (c) A number of multiple restriction sites are available for cloning efficiency and adequate antibiotic resistance markers (Tet^R) for selection. (d) pBBad22T is not detrimental for cell growth and viability of *S. maltophilia*.

To survey the availability of the *araC–*P_{BAD} induction system in *S. maltophilia*, plasmid pBxylE had been generated. The C23O encoded by the *xylE* gene offers two advantages for the evaluation of the *araC–*P_{BAD} induction system. First, the C23O activity can be conveniently quantified by spectrometrically recording the amount of C23O-hydrolyzed product; second, unlike β-galactosidase or alkaline phosphatase, the C23O-hydrolyzed product is less accumulated owing to its short half-life. Therefore, the turn on or shut off of *araC–*P_{BAD} promoter can be sensitively and precisely monitored.

The application of the pBBad22T induction system in *E. coli* and *S. maltophilia* has been comparatively characterized in this study, and some differences were found. (a) The promoter of pBBad22T displays a less tight repression condition in the *S. maltophilia* system than in the *E. coli* in the absence of Ara (Fig. 3). The basal leakiness of pBBad22T in *S. maltophilia* is consistent with those in *X. campestris* pv. *phaseoli* and *P. aeruginosa* (Sukchawalit et al. 1999). (b) The induction patterns are quiet distinct between *E. coli* and *S. maltophilia*. In the *E. coli* system, the induction occurs once the inducer is added, which agrees well with the results of Guzman et al. (1995). The induction reaches a maximum 2 h after the addition of the inducer and then decreases. The decline after maximum expression is likely due to the decrease in the inducer concentration caused by Ara utilization in the *E. coli*. Nevertheless, the efficiency of Ara utilization is less prominent in the *S. maltophilia* system; hence, the Ara concentration can hardly change during the process of induction. This can account for the fact that the induced C23O activity increases after prolonged induction.

The *araC–*P_{BAD} system established in this study can be applied to a variety of *S. maltophilia* studies, including null mutant construction of essential genes, conditioned modulation of the assayed gene expression, and high-level expression and purification of the assayed protein.

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