Establishment of an Arabinose-Inducible System in *Stenotrophomonas maltophilia*

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ABSTRACT

Stenotrophomonas maltophilia is an opportunistic pathogen and has gained increasing attention as a cause of healthcare-associated infection. In this study, a pBBad22T-derived conditioned arabinose-inducible expression system was evaluated in *S. maltophilia*. *S. maltophilia* cannot grow well when arabinose 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 arabinose. Accordingly, the established arabinose inducible system can provide a convenient tool for the study of *S. maltophilia*.

Keywords: *Stenotrophomonas maltophilia*, *xylE*, pBBad22T, arabinose

Abbreviations: A, absorbance; Ap, ampicillin; BHR, broad host range; C23O, catechol 2,3-dioxygenase; *E., Escherichia;* kb, kilobase(s); Km, kanamycin; LB, Luria-Bertani; MCS, multiple cloning site(s); R, resistance/resistant; *S.*, *Stenotrophomonas*; Tet, tetracycline; *xylE,* gene encoding C230.

INTRODUCTION

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 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 arabinose-containing medium to a no arabinose-containing medium, allows us to examine the null mutant phenotype.

97 In *E. coli,* the P_{BAD} promoter is induced in the presence of $_L$-arabinose and in the
98 absence of glucose. AraC is a cAMP-catabolite repressor protein that can bind to the
99 operator sequence upstream P_{BAD} and repress the expression of *araBAD* genes (Hahn
100 *et al*. 1984). Guzman et al. have developed an *araC*-P_{BAD} controlled expression vector
101 that is tightly regulated in enteric bacteria (Guzman *et al*. 1995). Some broad host
102 range *araC*-P_{BAD}-derived systems have been reported, including the plasmids of
103 pCF430, pJN105, pBBad18-series, and pBBad22-series (Newman and Fuqua 1999;
104 Sukchawalit *et al*. 1999). Among them, the pCF430 and pJN105 have been utilized in
105 the plant pathogen *Agrobacterium tumefaciens* (Newman *et al*. 1999). The application
106 of pBBad18- and pBBad22-serie plasmids in *Pseudomonas aeruginosa* and
107 *Xanthomonas campestris* pv. *phaseoli* has also been characterized (Sukchawalit *et al*.
108 1999).

109 *Stenotrophomonas maltophilia* is an aerobic, ubiquitous Gram-negative bacillus
110 and has gained increasing attention as a cause of nosocomial infection (Nyc and
111 Matejkova, 2010). A compatible inducible expression system for *S. maltophilia* is of
112 great urgency. In this study, we described the application of the broad host range
113 pBBad22T vector and established the induction parameters for the expression of
114 cloned gene in the *S. maltophilia* system.

115

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 LB medium. Tetracycline was added, if needed, at a concentration of 30 mg/L. The XOL medium (Yang *et al*. 2002) contained the basal salts (per litre): K_2HPO_4, 0.7 g; KH_2PO_4, 0.2 g; $(NH_4)_2SO_4$, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnCl_2$, 0.001g; pH 7.15. Carbon sources were autoclaved separately and added before use.

Growth conditions

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

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 Co. 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-arabinose-inducible BHR expression vector with the BAD promoter and *araC* gene of *Escherichia 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 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 arabinose was added to the culture at a concentration of 0.02%. A control group without additive was simultaneously assayed. Samples were taken at an interval of 1 h and the C23O activity was determined. Meanwhile, the growth of bacteria was also monitored by recording the A_{600nm} for *E. coli* and the A_{450nm} for *S. maltophilia*.

159

160 *Determination of the optimal inducer concentration*

161 An overnight culture was used as a starting inoculum to give 0.15 optical density at
162 450 nm. The culture was further grown for 0.5 h. Then, the distinct amounts of
163 $_{L}$-arabinose were added and incubation was continued for 4 h. For investigation of
164 $_{L}$-arabinose induction potency, the C23O activity was determined.

165

166 *C23O activity determination*

167 C23O activity was measured in intact cell as described previously (Lin *et al*. 2009).
168 Activity assays were performed in a buffer with 50 mmol/L sodium phosphate buffer,
169 10% acetone and 0.1 mol/L pyrocatechol as the substrate. Hydrolysis of pyrocatechol
170 was examined by spectrophotometric analysis, with readings recorded at 10-s
171 intervals for 3 min at the wavelength of 375 nm. The rate of hydrolysis was calculated
172 by using 44,000 $M^{-1}cm^{-1}$ as extinction coefficients. One unit of enzyme activity
173 was defined as the amount of enzyme that converts 1 nmole substrate per minute.
174 The specific activity of the enzyme was defined in terms of units per 3.6×10^8 cells
175 (assuming that an A_{450nm} of 1 corresponds to 3.6×10^8 cells/ml).

176
177

178 *Depletion experiment*

S. maltophilia(pBxylE) was culture in the LB medium supplemented with 0.2% arabinose at 37°C. Overnight cultures were washed by a fresh LB medium once and subsequently diluted to an A_{450nm} of 0.15 in the fresh LB medium containing 0.2% arabinose and in the medium without additive, respectively. The C23O activity of *S. maltophilia*(pBxylE) was monitored before cell-washing, immediately after cell-washing, and during the period of further 1.5-h culture at an interval of 0.5 h.

RESULTS

The arabinose utilization in E. coli and S. maltophilia

The arabinose utilization by *S. maltophilia* was evaluated so as to establish the arabinose-induction system in *S. maltophilia*. In the meantime, the arabinose 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% arabinose 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% arabinose continued with prolonged culture, indicating that *E. coli* can metabolize arabinose and use it as a sole carbon source. In contrast, the growth of *S. maltophilia* in 0.4% arabinose was suppressed after 9 h, demonstrating that arabinose 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 L-arabinose inducing conditions proposed by Sukchawalit's group as the reference, the induction kinetics were firstly investigated by the induction of 0.02% L-arabinose; meanwhile, an additive-free control was also carried out. Without the addition of arabinoses, 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 arabinose and, thereafter, the activity decreased monotonously (Fig. 3). However, the uninduced 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 the optimal induction parameter of pBBad22T in S. maltophilia

Based on the results of growth curve and induction time course, the forth hour after inducer addition is an appropriate point time for the following assay of optimum inducing concentrations. The C23O activity displayed an increase as the L-arabinose concentration exceeded 0.002%. Moreover, the C23O activity changed insignificantly

when more than 0.2% arabinose was added (Table 1). In conclusion, the applied induction condition of pBBad22T for *S. maltophilia* is the inducer concentration ranging from 0.01% to 0.5% for an induction time of 4 h.

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

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

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

DISCUSSION

In this present study, the expression vector of pBBad22T has been shown to be compatible with the *S. maltophilia* system according to the following features: (i) It owns the pBBR1MCS-4 replicon (Kovach *et al.* 1995) of broad host range characteristic, which can replicate in a variety of Gram-negative bacteria including *S. maltophilia*. (ii) Mobilization (*mob*) function of pBBad22T allows a conjugal delivery of the plasmid into *S. maltophilia*. Our previous studies have shown that heat-shock transformation and electroportation do not work well for transferring the foreign plasmid into the *S. maltophilia* system. Instead, the conjugation between *E. coli* S17-1 and *S. maltophilia* provides an efficient transformation (Hu *et al*. 2008). (iii) A number of multiple restriction sites are available for cloning efficiency and adequate antibiotic resistance markers (Tet^R) for selection. (iv) It 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. Firstly, the C23O activity can be conveniently quantified by spectrometrically recording the amount of C23O-hydrolyzed product. Secondly, unlike β-galactosidease or alkaline phosphatase,

274 the C23O-hydrolyzed product is less accumulated owing to its short half life.
275 Therefore, the turn on or shut off of *araC-*P_{BAD} promoter can be sensitively and
276 precisely monitored.

277 The application of the pBBad22T induction system in *E. coli* and *S. maltophilia*
278 has been comparatively characterized in this study. Some distinct differences can be
279 concluded. (i) The promoter of pBBad22T displays a less tight repression condition in
280 the *S. maltophilia* system than in the *E. coli* system in the absence of arabinose (Fig.3).
281 The basal leakiness of pBBad22T in *S. maltophilia* is consistent with those in *X.*
282 *campestris* pv. *phaseoli* and *P. aeruginosa* (Sukchawalit *et al*. 1999). (ii) The
283 induction patterns are quiet distinct between *E. coli* and *S. maltophilia*. In the *E. coli*
284 system, the induction occurs once the inducer is added, which agrees well with the
285 results made by Guzman et al. 1995. The induction reaches a maximum at 2 h after
286 the addition of the inducer and then decreases monotonously with time. The decline
287 after maximum expression is likely due to the decrease in the inducer concentration
288 owing to the arabinose utilization in the *E. coli* system. Nevertheless, the efficiency of
289 arabinose utilization is less prominent in the *S. maltophilia* system; hence, the
290 arabinose concentration hardly changes during the process of induction. This can
291 account for the fact that the induced C23O activity increases after prolonged
292 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.

ACKNOWLEDGEMENTS

This research was supported by grant CMU-99-S-42 from the China Medical University and grant NSC 98-2320-B-039-011-MY3 from the National Science Council.

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388 **Legends to figures**

389 **FIG. 1. Construction of pBxylE.** The 1.3-kb *xylE* gene from pX1918GT was PCR

390 amplified and ligated to yT&A vector, yielding pTxylE. For construction of pBxylE,

391 a1.3-kb HindIII-EcoRI fragment from pTxylE was gel-purified and ligated to

392 HindIII/EcoRI-treated pBBad22T.

393

394 **FIG. 2. Growth curves of *E. coli* and *S. maltophilia*.** (A) Strains *E. coli* and *S.*

395 *maltophilia* (B) Strains *E. coli*(pBxylE) and *S. maltophilia*(pBxylE). Cells from

396 overnight cultures were separately harvested and then inoculated into XOL containing

397 glucose or arabinose at a final concentration of 0.4%. Cell growth was measured by

398 monitoring A_{600nm} for *E. coli* and A_{450nm} for *S. maltophilia* at intervals of 1 h. The

399 errors bars indicate standard deviations (n=3).

400

401 **FIG. 3.** Growth curves and induction time courses of *E. coli*(pBxylE) and *S.*

402 *maltophilia*(pBxylE). (A) strain *E. coli*(pBxylE) (B) strain *S. maltophilia*(pBxylE).

403 The errors bars indicate standard deviations (n=3). One unit (U) of enzyme activity

404 was defined as the amount of enzyme that converts 1 nmole substrate per minute.

405 A_{450nm}, optical density at 450 nm. Symbols: ○, without arabinose addition; ●, 0.02%

406 arabinose.

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

Arabinose conc. (%)	C23O activity (U/A_{450nm})
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

Fig. 1

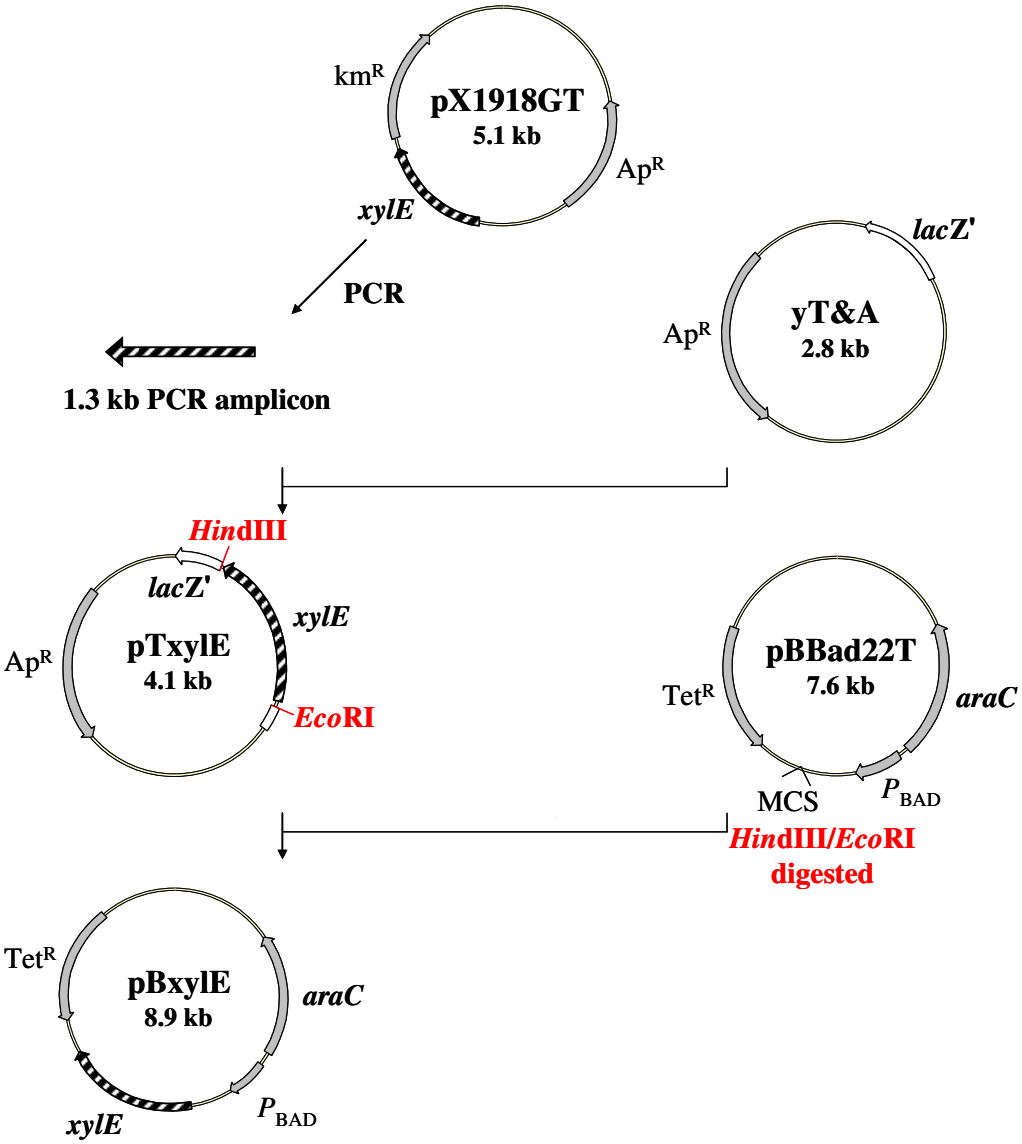
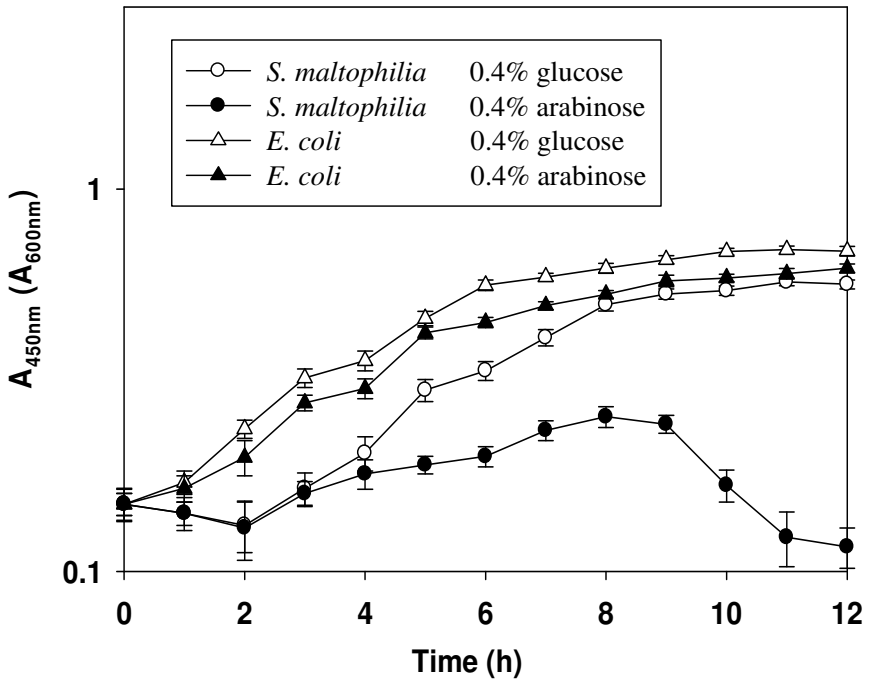

km^R
pX1918GT
5.1 kb
Ap^R
xylE
PCR
1.3 kb PCR amplicon
lacZ'
Ap^R
yT&A
2.8 kb
HindIII
lacZ'
xylE
Ap^R
pTxylE
4.1 kb
EcoRI
pBBad22T
7.6 kb
Tet^R
araC
MCS
P BAD
HindIII/EcoRI
digested
Tet^R
pBxylE
8.9 kb
araC
xylE
P BAD

Fig. 2

(A)


S. maltophilia 0.4% glucose
S. maltophilia 0.4% arabinose
E. coli 0.4% glucose
E. coli 0.4% arabinose
A450nm (A600nm)
1
0.1
0
2
4
6
8
10
12
Time (h)

(B)

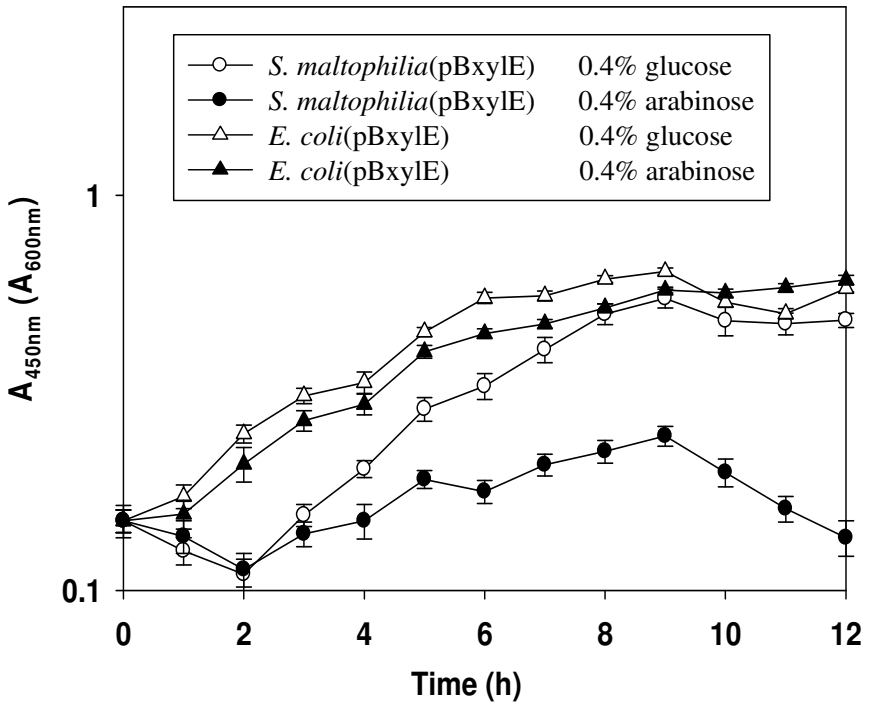
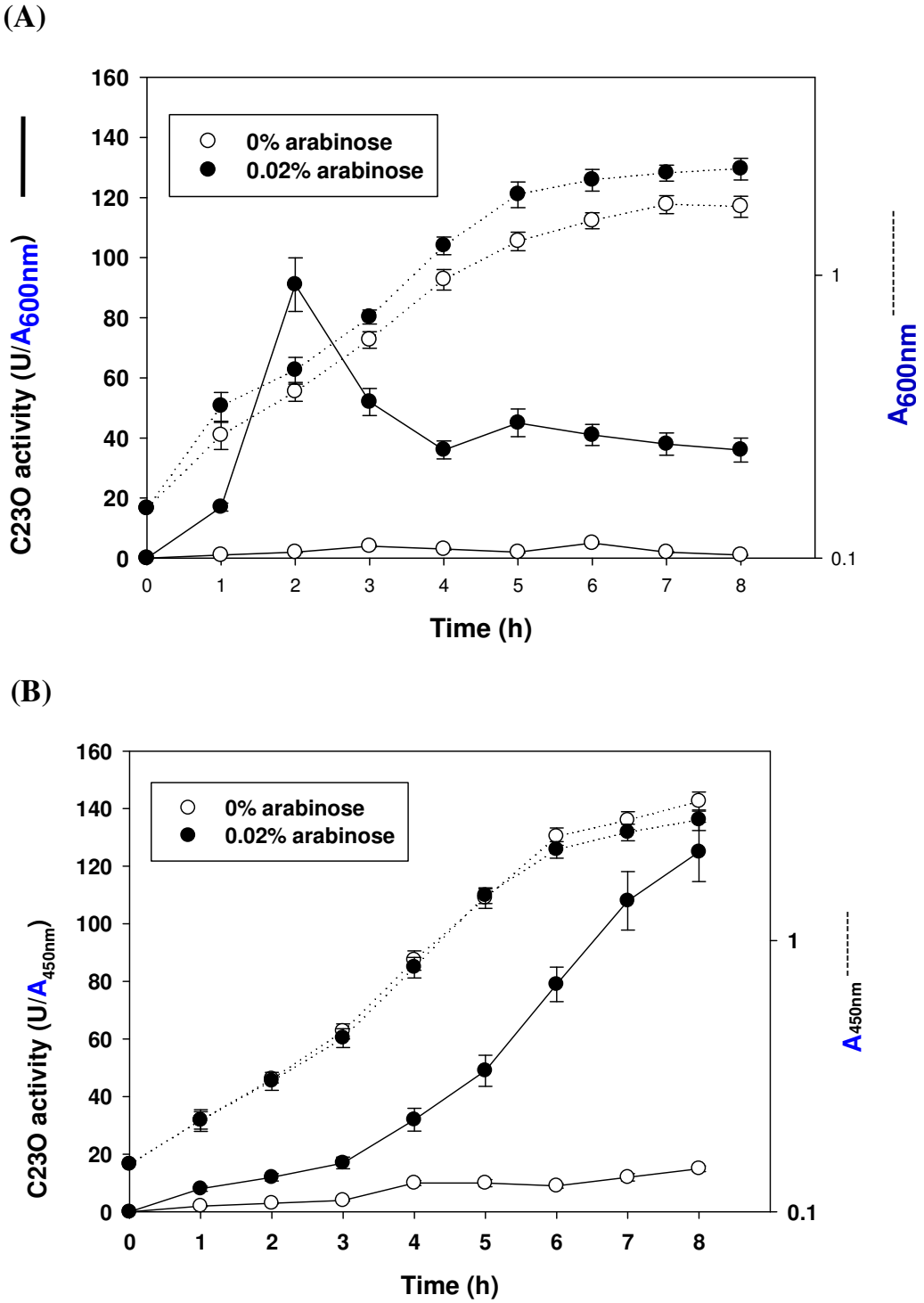

S. maltophilia(pBxylE) 0.4% glucose
S. maltophilia(pBxylE) 0.4% arabinose
E. coli(pBxylE) 0.4% glucose
E. coli(pBxylE) 0.4% arabinose
A450nm (A600nm)
1
0.1
0
2
4
6
8
10
12
Time (h)

Fig. 3


(A)
0% arabinose
0.02% arabinose
C23O activity (U/A600nm)
A600nm
Time (h)
(B)
0% arabinose
0.02% arabinose
C23O activity (U/A450nm)
A450nm
Time (h)