Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose P<sub>BAD</sub> Promoter

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Received 6 February 1995/Accepted 10 May 1995

We have constructed a series of plasmid vectors (pBAD vectors) containing the P<sub>BAD</sub> promoter of the araBAD (arabinose) operon and the gene encoding the positive and negative regulator of this promoter, araC. Using the phoA gene and phoA fusions to monitor expression in these vectors, we show that the ratio of induction/repression can be 1,200-fold, compared with 50-fold for P<sub>TAC</sub>-based vectors. PhoA expression can be modulated over a wide range of inducer (arabinose) concentrations and reduced to extremely low levels by the presence of glucose, which represses expression. Also, the kinetics of induction and repression are very rapid and significantly affected by the ara allele in the host strain. Thus, the use of this system which can be efficiently and rapidly turned on and off allows the study of important aspects of bacterial physiology in a very simple manner and without changes of temperature. We have exploited the tight regulation of the P<sub>BAD</sub> promoter to study the phenotypes of null mutations of essential genes and explored the use of P<sub>BAD</sub> vectors as an expression system.

In bacterial physiology studies, it is often useful to express a cloned gene from an inducible promoter and assess the effect of the expression or depletion of the gene product in mutants lacking the chromosomal gene. In these situations, it is highly desirable to use a system that can be efficiently shut off. This is particularly the case when the phenotype caused by the absence of a protein can be obscured by leakiness from a partially repressed promoter or when even low levels of a protein are detrimental to the cell. Also, it would be desirable to modulate the expression system to achieve synthesis levels similar to those of the wild-type gene. However, the available repertoire of Escherichia coli expression systems usually produce high levels of the corresponding cloned gene product (4, 13, 18, 45, 48) and in many cases still produce substantial levels of synthesis in uninduced or repressed conditions (4, 13, 15, 16, 48, 49). These systems include controllable expression vectors based on the strong inducible promoters P<sub>LAC</sub> (48), P<sub>TAC</sub> (13), P<sub>TRC</sub> (4), P<sub>l</sub> (4), and P<sub>R</sub> (18), and P<sub>T7</sub> (45). Some are better repressed than others, but induction of expression requires changes of temperature (18, 45) and produces very high levels of protein, resulting in conditions detrimental to cell growth and viability (17, 45).

We have been studying the function of several essential genes that encode membrane proteins involved in cell division of E. coli (7a, 22). To analyze their role, we have sought to deplete cells of the proteins and then examine the phenotype of cells so depleted under conditions that would minimize alterations in cell physiology. For these purposes, we wished to use a plasmid that would satisfy the following two conditions: (i) the synthesis of the proteins should be shut off rapidly and efficiently without changes of temperature, and (ii) expression before depletion should not produce very high levels of protein, which itself may give a phenotype or influence the phenotype of the depletion.

To achieve these conditions, we constructed a set of vectors (pBAD vectors) containing the P<sub>BAD</sub> promoter of the arabinose operon and its regulatory gene, araC (28, 41). The AraC protein is both a positive and a negative regulator (6, 30). In the absence of arabinose, transcription from the P<sub>BAD</sub> promoter is turned on; in its absence, transcription occurs at very low levels (28, 29). The uninduced levels can be reduced even further by growth in the presence of glucose. Glucose reduces levels of 3',5'-cyclic AMP, thus lowering expression of the catabolite-repressed P<sub>BAD</sub> promoter (32). The properties of the mechanism of expression and repression of P<sub>BAD</sub> by AraC have been studied extensively, and their interactions have been dissected at the molecular level (reviewed in reference 41). Additionally, plasmids described previously in which transcriptional or translational fusions to araB were used to set genes under P<sub>BAD</sub> and AraC control suggested that expression from this system was tightly regulated (12, 26, 38, 40).

Here, we describe the construction of pBAD vectors and the characteristics of repression, induction, and modulation of the expression of genes cloned into them. In addition, we show the application of the tight regulation of the vectors to the study of null mutations of essential genes and discuss the relevance of this and the vector's properties of expression for bacterial physiology.

MATERIALS AND METHODS

Bacterial strains and media. The E. coli strains used in this study were KS272 [F<sup>−</sup> ΔlasX74 galE galK thi rpsL ΔphoA (PvuII)] (44), LMG194 (KS272 Δara714 leu:Ta100), DH14 (3), LMG145 [KS272 fsl::phoAΔ681Δ50R (Kan<sup>+</sup>) pLMG180 (Amp<sup>+</sup>)], LMG89 (LMG145 pcm80 zul1:Ta100) (22), and DH5α (Bethesda Research Laboratories, Gaithersburg, Md.). The Δara714 deletion in LMG194 encompasses most of the araBAD operon (47) and was obtained from strain JPH3 (37).

The NYZ rich and M63 minimal media have been described (22). The M63 medium was always supplemented with a mixture of 18 amino acids (no methionine or cysteine) at a final concentration of 50 μg/ml (22). Unless otherwise indicated, glycerol, d-glucose, and l-arabinose (Plaschke Labs) were used at a concentration of 0.2%. The following antibiotics were used at the concentrations

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FIG. 1. Construction of pBAD vectors. (A) Maps of pBAD vectors with the relevant features of the plasmids. (B) MCSs of pBAD vectors. All sites are single, with the exception of shaded sites which are double. For MCS1 and MCS2, the double sites are BamH I, AccI, and PstI. In MCS3, BamH I is the only double site. The unique and double sites of MCS2 are applicable only to pBAD18. Vectors with MCS2* contain the same double sites as those of pBAD18 (MCS2) and the following additional double sites: pBAD18-Cm, EcoRI; pBAD18-Kan, SmaI and HindIII (PstI becomes unique); pBAD28, NheI and EcoRI; pBAD30, NheI; and pBAD33, NheI and EcoRI (PstI becomes unique). (C) Steps in construction of pBAD vectors (see Materials and Methods). Abbreviations: 5'SS and rrnB T1T2, parts of the 5' SS rRNA and the strong ribosomal rrnB terminators, respectively; ori, origin of replication; +1, site of initiation of transcription; ATG, initiation of translation codon; bla9 and cat9, truncated bla and cat genes; ER1, EcoRI; H3, HindIII; MCSa, MCS of M13mp18; MCSb, MCS of pUC9; T4 pol, T4 polymerase; MCS, multicloning site; SD, Shine-Dalgarno box.
FIG. 1—Continued.

Plasmid pLMG161 was constructed by cloning a 900-bp EcoRI (KpnI-KpnI) fragment from plasmid pMC103 (7a) into the same sites of pBAD18 (unpublished results). This fragment carries the complete ftSQ gene, including the poorly efficient fosQ SD box (33), and an additional 12 bp that precede the EcoRI site. pLMG163 was obtained by inserting a 5,250-bp KpnI-KpnI fragment from plasmid pZQ::TnphoA91 (7a) into the internal KpnI site of ftSQ from plasmid pLMG161. This cloning resulted in replacement of the 3' end of the fosQ gene by the fosQ-phoA91 fusion with the rest of the IS50-deleted TnphoA (7a) and expression of the fusion from the PBAD promoter. pLMG160 was constructed in the same manner as pLMG161 but cloned into pBAD18 instead of pBAD18 (unpublished results). Also, the fosQ-phoA91 fusion was cloned into pLMG160 to yield pLMG162, as was done with the pLMG163 plasmid. The resulting phoA fusion in pLMG162 contains the SD box and the first 5 residues of the araB gene located before the EcoRI site of the pBAD18 vector (Fig. 1B, MCS 3), followed by the SD box of fosQ and the fosQ-phoA91 coding sequence. The expression of the fosQ-phoA91 fusion from pBAD18 is higher than that obtained from pBAD18; however, no AraB fusion protein is produced since the 5' end of araB is fused out of frame with respect to fosQ. Similarly, higher levels of alkaline phosphatase (AP) activity were observed when the...
m of growth, 0.5-ml aliquots were taken and pulse-labeled for 1 min with 200 μCi of [35S]-methionine per ml (wild-type AP cloned into pBAD22) were grown at 37°C in glycerol-minimal medium supplemented with 0.02% arabinose. When cultures reached the mid-log phase of growth, 0.5-ml aliquots were taken and pulse-labeled for 1 min with 200 μCi of [35S]-methionine per ml (Ara lanes). Subsequently, 4 ml of the cultures was washed once with glycerol minimal medium containing 0.2% glucose by a brief centrifugation of 2 min. After resuspension of the cultures in the same glucose-containing medium, they were returned to the initial culturing conditions, and 0.5-ml fractions were pulse-labeled immediately (0′) and at the indicated times (in minutes). Labeled cultures were arrested by the addition of 0.05% unlabeled methionine, subsequently immunoprecipitated with anti-AP and anti-OmpA antibodies, and analyzed by 10% polyacrylamide gel electrophoresis and fluorography as described previously (22, 44). The positions of precursor (p) and mature (m) AP and OmpA are indicated.

Induction of plasmid pDB3, which expresses wild-type AP very efficiently, seems to cause some difficulty for the export of AP as judged by the elevated amount of AP polyacrylamide gel electrophoresis and fluorography as described previously (22, 44). The positions of precursor (p) and mature (m) AP and OmpA are indicated.

The band at the bottom corresponds to the dye front.

FIG. 2. Synthetic rate of wild-type AP production from P_BAD: kinetics of repression by glucose. Cultures of ara+ and ara mutant strains with plasmid pDB3 (wild-type AP cloned into pBAD22) were grown at 37°C in glycerol-minimal medium supplemented with 0.02% arabinose. When cultures reached the mid-log phase of growth, 0.5-ml aliquots were taken and pulse-labeled for 1 min with 20 μCi of [35S]-methionine per ml (Ara lanes). Subsequently, 4 ml of the cultures was washed once with glycerol minimal medium containing 0.2% glucose by a brief centrifugation of 2 min. After resuspension of the cultures in the same glucose-containing medium, they were returned to the initial culturing conditions, and 0.5-ml fractions were pulse-labeled immediately (0′) and at the indicated times (in minutes). Labeled cultures were arrested by the addition of 0.05% unlabeled methionine, subsequently immunoprecipitated with anti-AP and anti-OmpA antibodies, and analyzed by 10% polyacrylamide gel electrophoresis and fluorography as described previously (22, 44). The positions of precursor (p) and mature (m) AP and OmpA are indicated.

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The band at the bottom corresponds to the dye front.

RESULTS

Construction and characteristics of the vectors. Details on the construction of pBAD vectors are shown in Fig. 1C and Materials and Methods. In steps 1 to 6 (Fig. 1C), four plasmids were constructed by several steps of cloning and site-directed mutagenesis to eliminate a 5′ fragment from araB and avoid production of any araB fusions, to eliminate downstream sequences from araC, and to exchange polylinker among pUC vectors. The resulting 4.6-kb pBAD18 plasmid (Fig. 1) contains the araC gene and the P_BAD promoter followed by an MCS (MCS 2) which can be used to clone any gene containing translation initiation sequences. Immediately downstream of the MCS are the strong mRN transcription terminators. The plasmid also carries a ColE1 origin of replication, an M13 intragenic region for phage packaging and production of single-stranded DNA, and an ampicillin resistance gene. pBAD18-Cm (Fig. 1) is a Cm′ Ap′ derivative of pBAD18 that contains the chloramphenicol-resistant gene from pACYC184 (9) inserted into the ampicillin gene, whereas pBAD18-Kan (Fig. 1) has a kanamycin resistance gene from pUC4K (Pharmacia Biotech) inserted in the same fashion. As with pBAD18s, these last two plasmids should be used to clone genes that contain their own SD box. Also, in both of these two plasmids (pBAD18-Cm and -Kan), the chloramphenicol- and kanamycin-resistant markers are surrounded by bla sequences which could promote recombination when these plasmids are used in combination with other compatible ampicillin-resistant plasmids. Recombination can be avoided by the use of recA strains; however, this recombination can also be useful to intentionally exchange markers among the plasmids. This is also applicable to pBAD30 and pBAD33 (Fig. 1).

The pBAD24 vector (Fig. 1) is used to clone genes which lack sequences for initiation of translation. This vector contains an optimized SD sequence (42, 43) between the Nhel and EcoRI sites of the MCS as well as a translation start codon (ATG) at the NcoI site. A Kozak sequence, relevant for expression in eukaryotic cells (25), is also present before the ATG. If this start codon is to be used, the desired frame can be obtained by digesting with the appropriate enzyme and filling in with Klenow fragment as depicted in Fig. 1B. Besides the sequences for translation initiation, the only other difference between pBAD24 and pBAD18 is that in the former, the AccI and PstI sites of the MCS are unique in the plasmid. The complete sequences of these two plasmids have been deposited in the EMBL/GenBank/DDBJ databases (see Materials and Methods).

pBAD28 (Ap′ Cm′), pBAD30 (Ap′), and pBAD33 (Cm′) (Fig. 1) are derivatives of pBAD18 that contain the pBR322-compatible p15A origin of replication from the pACYC184 vector (9). These plasmids can be used to reduce gene expression (due to lower copy number) or, in conjunction with pBAD18 or pBAD24, to stably coexpress different genes.

Repression from P_BAD is rapid and efficient. In Fig. 2, we show that the P_BAD promoter is turned off rapidly and efficiently. Cultures of strains KS272 (ara+Cm′) and LMG194 (ara) carrying the wild-type AP enzyme cloned into pBAD22 were grown in minimal medium supplemented with arabinose and glycerol and subsequently shifted to glucose-containing medium. At the indicated times, samples were pulse-labeled with 35S-methionine, immunoprecipitated with anti-AP and anti-OmpA (internal control) antibodies, and analyzed by fluorography. In the cultures of the repressed ara+ strain, the amount of pulse-labeled AP protein decreased rapidly in the first minutes, became negligible by 5 min, and was below detection at subsequent times. In the ara strain cultures, repression of AP synthesis was slower and did not decrease significantly until 20
to 40 min. Nondetectable levels of AP synthesis were then maintained for at least 2 h (see Fig. 3, Glu lanes) for both strains. Thus, P_BAD is rapidly and efficiently repressed, particularly in ara^+ strains. A likely explanation for the difference in repression kinetics between the two strains is that ara^+ strains lower the concentration of inducer as they grow since they can metabolize arabinose and use it as a carbon source. In contrast, in ara mutant strains, the intracellular concentration of inducer remains elevated since arabinose cannot be degraded. This is particularly relevant in this experiment where submaximal concentrations of arabinose were used for induction (see also Fig. 4B). We also point out that ara mutant strains that are araD alone must be avoided since arabinose is toxic for these strains (19).

The P_BAD promoter has a very fast induction rate. The converse time course labeling experiment to the one shown in Fig. 2 was performed to determine how fast the P_BAD promoter is induced (Fig. 3). Here, cultures of strains were grown under repressing conditions for several hours and then shifted to inducing conditions. Upon induction, samples were pulse-labeled and analyzed by immunoprecipitation and fluorography. First, it is evident that after several hours of growth in repressing conditions, just prior to induction (Fig. 3, Glu lanes), the expression of AP is totally repressed. Subsequently, as early as 1 min after the addition of arabinose, significant amounts of labeled AP, which continued to increase over the following 2 to 4 min, were observed. Induction was maintained at a high level as judged by the total amount of AP and the AP/OmpA protein ratio (ara^+ strain). After 10 min of induction, the ara mutant strain displayed a tendency to decrease the rate of AP synthesis, probably as a result of the deleterious effects of high-level AP expression. The timing of the induction is only approximated since it can be observed that samples in lanes 0' show significant amounts of AP due to induction of the promoter during the 2-min wash and resuspension, in which case induction still occurs very soon after the addition of arabinose. Thus, these results indicate that the P_BAD promoter has a very fast rate of induction.

Ratio of induction/repression from pBAD vectors. To estimate the levels of expression from the araC-P_BAD promoter system in induced and repressed states, we determined the enzymatic activity of an FtsQ fusion to AP (FtsQ–AP-91) as well as that of wild-type AP cloned into pBAD vectors. FtsQ is an E. coli cell division membrane protein that is normally expressed at very low levels (7a, 33). AP fusions to the periplasmic domain of the protein exhibit normal specific activities of AP, which make them suitable to estimate the levels of expression (7a). Plasmids pLMG162 and pLMG163 express the ftsQ–phaA-91 fusion, which retains the ftsQ SD box, from the P_BAD promoter of vectors pBAD18s and pBAD18 (see Materials and Methods). The only difference between the two constructs is that pLMG162, the pBAD18s vector provides an additional SD box and 5 residues from the araB gene, and this plasmid produces higher levels of the FtsQ–AP-91 fusion than those produced by pLMG163. The mechanism of translation of the fusion in pLMG162 is not known. pDB3 contains the wild-type phoA gene devoid of its SD box, expressed from the P_BAD promoter, and an optimized SD box provided by pBAD22. This plasmid produces high levels of AP and in conjunction with pLMG162 and pLMG163, which produce medium and low AP activity levels, respectively, allows a careful assessment of the ratio of induction/repression of the P_BAD promoter.

Table 1 shows that the ratio of induction/repression for wild-type AP under the control of araC-P_BAD was about 250-fold in rich medium and about 1,300-fold in minimal medium. Similarly, the induction/repression ratios for the FtsQ–PHA–91 fusion on plasmid pLMG162 ranged from about 280-fold in rich medium to 1,000-fold in minimal medium. Therefore, the efficiency of repression from the P_BAD promoter can be from 2 to even 3 orders of magnitude. In strains carrying the plasmid pLMG163, the induction/repression ratios varied from the overall lowest (118-fold) to the overall highest (1,855-fold). These estimates are probably inaccurate because of the low expression of the ftsQ–phaA–91 fusion from the weak SD box-containing pLMG163 plasmid, which resulted in low levels of AP activity at maximum induction and repression levels very close to those of the negative control strain that carries the pBAD22 vector. The ftsQ–phaA–91 fusion was also expressed from the P_TAC promoter in the vector pDH80, which differs from pBAD vectors only in the promoter region (Fig. 1C). This P_TAC-derived plasmid, pLMG281, is directly comparable to pLMG163. The P_TAC promoter was maintained in a repressed state by the lac^+ gene provided by the DHB4 strain (3) and was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at concentrations that provide maximum induction. pLMG281 (P_TAC) produced higher levels of AP activity than those of pLMG163 (P_BAD) after induction and in the repressed state. The highest induction/repression ratio for this P_TAC-derived plasmid was 50-fold (Table 1). Thus, the efficiency of repression from P_BAD, which is about 12- to 40-fold greater than that...
TABLE 1. Induction/repression ratios of expression of AP fusions and wild-type enzyme from the P_BAD promoter

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Strain</th>
<th>Rich medium*</th>
<th>Minimal medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inducing^b</td>
<td>Repressing^c</td>
</tr>
<tr>
<td>pBAD22</td>
<td>KS272 (ara^+ )</td>
<td>0.33</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>LMG194 (ara)</td>
<td>0.36</td>
<td>0.41</td>
</tr>
<tr>
<td>pLMG163 = pBAD18/ FtsQ-AP-91</td>
<td>KS272 (ara^- )</td>
<td>47.3</td>
<td>0.40</td>
</tr>
<tr>
<td>pLMG162 = pBAD18/ FtsQ-AP-91</td>
<td>KS272 (ara^- )</td>
<td>71.2</td>
<td>0.52</td>
</tr>
<tr>
<td>pDB3 = pBAD22/wt AP</td>
<td>KS272 (ara^- )</td>
<td>427</td>
<td>1.80</td>
</tr>
<tr>
<td>pLMG281 = pDH60 (P_{araC}/FtsQ-AP-91)</td>
<td>LMG194 (ara^- )</td>
<td>580</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>DHB4 (lacF)</td>
<td>2,382</td>
<td>10.32</td>
</tr>
</tbody>
</table>

* Diluted cultures were grown for four to six generations to the mid-logarithmic phase in NZY (rich) medium or glycerol-minimal medium supplemented with either 0.2% glucose or 0.2% arabinose to ensure maximum induction. AP enzymatic activity was determined in these cultures as indicated in Materials and Methods. The rich NZY medium is very similar to LB medium (39). The only difference is that in NZY, NZ amine is used instead of tryptone. Enzyme levels from pBAD vectors are comparable in both media. However, LB medium may give higher expression levels than NZY medium does from the uninduced P_{araC} promoter, depending on the tryptophan batch.

^ Induction/repression ratios were calculated by dividing the number of units of AP activity obtained from inducing conditions (medium plus arabinose for strains containing plasmids pBAD, pLMG163, pLMG162, and pDB3 and medium plus IPTG for the strain with plasmid pLMG281) by that obtained from repressing conditions (medium plus glucose for strains containing plasmids pBAD22, pLMG163, pLMG162, and pDB3 and medium without IPTG for the strain with plasmid pLMG281). The average ratios for pBAD plasmid-containing strains grown in rich and minimal media were 220 ± 74 and 1,182 ± 441, respectively.

^ The difference in induction and repression levels between the P_BAD and P_{araC} promoters was calculated by comparing the average levels from ara^- and ara strains with pLMG163 with the levels of strain DHB4 with pLMG281. Accordingly, P_{araC} has 4.5-fold (rich medium) and 2.5-fold (minimal medium) higher levels of induction than P_BAD and 12.4-fold (rich medium) and 46.7-fold (minimal medium) less repressed than P_BAD.

^ wt, wild type.

^ Cultures of strains containing pDB3 were first grown in rich or minimal medium with only glycerol as the carbon source to the beginning of the log phase, and then arabinose or glucose was added and growth was continued for about four generations before determination of AP activity.

^ For this strain, rich NZY medium or 0.2% glucose minimal medium was used in inducing or repression conditions. For induction, 5 mM IPTG was used, and for repression, cultures were grown in the absence of inducer.

of P_{araC} (Table 1), is due primarily to lower uninduced levels from the P_BAD promoter.

These results indicate that the repression of the P_BAD promoter is very efficient and that, under some conditions, it can yield striking levels of repression. However, this tight control does not mean that the protein levels in the repressed state are always zero, since the levels of expression in repressed states are relative to the levels of expression at maximum induction. Thus, as shown in Table 1, the P_BAD promoter is turned off to levels close to zero only when the expression of the gene at maximum induction is not too high (pLMG163). When levels of expression are high, the repressed promoter can produce as much as 11 U of AP activity (pDB3). Induction and repression levels do not appear to be consistently different between ara^- and ara strains for all plasmids. pLMG163-containing strains gave different levels of induction, while in pDB3-containing strains, similar levels were observed. However, these results were obtained at maximum induction. It is possible that at lower concentrations of arabinose, all ara^- strains would yield lower induction levels than ara mutant strains would (see Fig. 4B). Also, results shown in Table 1 indicate that the P_BAD promoter is expressed more efficiently in minimal medium than in rich medium.

These observations are of particular importance for studies of null mutations in essential genes in which the complementing P_BAD-driven gene expression is required to be shut off as efficiently as possible. If such complementing, arabinose-induced expression gives high levels of the protein, enough protein could be produced in the repressed state to complement the null mutation and its phenotypic effect would not be observed. This problem could be circumvented by lowering the overall expression of the gene from the pBAD-derived plasmid, for instance, by making initiation of translation less efficient (use of a nonoptimal SD box) (42, 43), by using pACYC derivatives or pCNB mutants to decrease the plasmid copy number (31), or, when possible, by recombining the P_BAD-driven gene into the chromosome.

The araC-P_BAD promoter system can be modulated. A useful property of a controllable expression system is the possibility to obtain different levels of expression by partial induction of the promoter. Thus, we assessed the susceptibility to modulation of pBAD vectors by measuring the extent of induction from P_BAD at different concentrations of inducer. Figure 4A shows a plot of the amount of AP enzymatic activity produced by cultures of strain DHB4 (ara/pBAD22-phoA) as a function of induction at different arabinose concentrations. A linear relationship between induction and expression is clearly observed for arabinose concentrations that extend over 2 logarithms. There is virtually no induction at concentrations equal to 1.33 μM (8 U of AP activity) or lower, and the curve plateaus between 133 (2,000 U of AP activity) and 1,330 μM. A comparison of this latter value with the levels of AP produced by the cultures repressed with glucose (6 U of AP activity) shows that the ratio of induction/repression is about 300-fold. Based on estimates that correlate the enzymatic activity of phoA fusions with the number of molecules per cell (7a), the observed ratio of induction/repression, in this particular case 62,000, would correspond to (60 ± 15)/(20,000 ± 5,000) molecules per cell.

These results indicate that the expression from the araC-P_BAD promoter system can be modulated over a wide range of inducer concentrations, starting with conditions that yield effective repression of expression. Similar results were obtained with strains carrying the pLMG162 and pLMG163 plasmids with the FtsQ-AP-91 fusion (Fig. 4), although the curves are displaced down as a result of the lower overall efficiency of expression of these fusions with respect to that of wild-type AP. Figure 4B shows a comparison of the induction curves of ara^-
Values obtained under repressing conditions with glucose. Mutant strain is LMG194/pLMG163. Growth in rich or minimal medium is indicated. Unconnected points at the beginning of the curves, before 0, indicate cultures of Strains DHB4 (ara-1)/pLMG162 (FtsQ-AP)/pDB3 (wt AP) and LMG194 (ara-1)/pLMG163 (FtsQ-AP) were assayed for AP enzymatic activity as indicated in Materials and Methods. (A) Strains DHB4 (ara-1)/pDB3 (wt AP) and LMG194 (ara-1)/pLMG162 (FtsQ-AP) were grown in rich medium. (B) The ara-1 strain is KS272/pLMG163, and the ara mutant strain is LMG194/pLMG163. Growth in rich or minimal medium is indicated. Unconnected points at the beginning of the curves, before 0, indicate values obtained under repressing conditions with glucose.

FIG. 4. Modulation of expression from the araC-PBAD system. Overnight cultures of ara+ and ara strains carrying pDB3 (pBAD22/wt AP), pLMG162 (pBAD18s/FtsQ–AP-91), or pLMG163 (pBAD18s/FtsQ–AP-91) were diluted to 10-2 and grown at 37°C in glycerol-minimal medium or rich medium supplemented with 0.2% glucose or with arabinose at the indicated concentrations. When cultures reached the mid-log phase, they were transferred to 4°C and assayed for AP enzymatic activity as indicated in Materials and Methods. (A) Strains DHB4 (ara-1)/pDB3 (wt AP) and LMG194 (ara-1)/pLMG162 (FtsQ-AP) were grown in rich medium. (B) The ara-1 strain is KS272/pLMG163, and the ara mutant strain is LMG194/pLMG163. Growth in rich or minimal medium is indicated. Unconnected points at the beginning of the curves, before 0, indicate values obtained under repressing conditions with glucose.

FIG. 5. Essentiality of the ftsL gene in pcnB+ and pcnB mutant strains. Strains LMG145 (pcnB+) and LMG89 (pcnB), both containing the chromosomal insertion ftsL::Tphoa/L81ΔIS50 complemented by the plasmid pLMG180 (wild-type ftsL cloned into pBAD18 [22]) were grown on plates containing rich or minimal medium supplemented with 0.2% arabinose, ampicillin (30 μg/ml), kanamycin (20 μg/ml), and 5-bromo-5-chloro-3-indolyl phosphate (XP). Single colonies from both strains were streaked on plates with four divisions, each containing a different medium as indicated. Colonies were first streaked on minimal medium sections and incubated at 37°C for several hours. Later, colonies were streaked on sections containing rich medium and incubated for an additional 12 to 16 h at the same temperature. Medium abbreviations: rich+ARA, NZY medium supplemented with 0.2% l-arabinose, ampicillin (30 μg/ml), and XP (40 μg/ml); rich+GLU, same as rich+ARA but supplemented with 0.2% α-glucose instead of arabinose; min+ARA, M63 minimal medium supplemented with 0.2% l-arabinose, 0.2% glycerol, ampicillin (30 μg/ml), and XP (40 μg/ml); rich+GLU, same as min+ARA but supplemented with 0.2% α-glucose instead of arabinose.

Application of pBAD vectors to the study of null mutations of essential genes. We have exploited the tight regulation of the araC-PBAD promoter system to determine the essentiality of the ftsQ and ftsL cell division genes and to study the phenotype of their null mutations (7a, 22). In these experiments, chromosomal ftsQ or ftsL null mutations were complemented by pBAD18 plasmids containing the ftsL or ftsQ genes. The viability of the mutants depends on the presence of arabinose to induce the expression of the corresponding genes. ftsQ and ftsL encode bitopic membrane proteins present in 25 to 50 copies per cell (7a, 22). Thus, the araC-PBAD promoter system provided efficient repression of ftsQ or ftsL in glucose such that it failed to produce enough copies of the proteins to complement the chromosomal null mutation. Moreover, depletion experiments in which the expression of the genes was shut off by the addition of glucose allowed us to examine the null mutant phenotype, which was a fast and efficient block in cell division and septum formation that produced multinucleate asoapellite filaments (7a, 22).

It was also previously observed that depletion of FtsL in minimal medium was not very effective and varied with different culture conditions (22). This was attributed to a less-efficient shutoff of the PBAD promoter in this medium as compared with that in rich medium. However, similar to the case shown in Table 1 for ftsQ fusions, the expression of ftsL-phaA fusions cloned into pBAD18 is repressed very efficiently and at about the same levels in rich and minimal media but induced to much higher levels in the latter (data not shown). Thus, it is likely that reducing the total amount of the complementing wild-type FtsL would permit observation of the null phenotype.
in minimal medium. In Fig. 5, the essentiality of ftsL was tested in ftsL null mutant strains complemented by a pBAD-ftsL plasmid on rich and minimal medium plates containing arabinose or glucose. One of the null mutants harbored a mutation in the pcnB gene in addition to the ftsL mutation. The pcnB mutants (31) have a significantly lower copy number of pBR origin-containing plasmids and make them unstable, resulting in a mixture of low-copy-plasmid and plasmidless cells in the culture. Essentially was then inferred from the ability of the strains to form colonies after streaking on the plates. It is shown that both strains grew in arabinose-containing media and failed to grow in rich medium with glucose. However, in glucose minimal medium, the pcnB mutant strain did not grow, while the pcnB strain grew well. These observations indicate that the ftsL gene is indeed essential in both media and that lowering the gene dosage of the complementing ftsL gene in a pcnB mutant strain allowed the observation of the null phenotype in glucose minimal medium. It seems possible that the effect of lowering the gene dosage could also be achieved with careful modulation of the P Bad promoter. These results also indicate that in addition to the very good repression provided by the P Bad promoter, it is important to avoid protein overproduction prior to depletion since, after repression, the observation of the depletion phenotype will depend on how much of the already existing protein is diluted and turned over through growth and subsequent cell divisions. Consistently, one of the best conditions for depletion in broth occurs when stationary, saturated, induced cultures of Ara− strains (where arabinose has been partially or totally depleted) are diluted into medium containing 0.2% glucose.

Additional evidence for the usefulness of the pBAD vectors in the characterization of null phenotypes of essential genes has been provided by studies with the fhl gene (36), ftsI (7), and the protein secretion genes secE (34), secD (37), and secF (37).

High levels of expression from pBAD vectors. The results shown in Table 1 and Fig. 3 and 4 indicate that high levels of expression can be obtained when genes are cloned under araC-P Bad control, particularly when the gene possesses a good natural SD sequence or when an optimized one is provided by pBAD22 or pBAD24. Thus, the FtsQ–AP-91 fusion produces 47 to 593 U of AP activity when the system contains the wild-type ftsQ SD sequence (Table 1), which deviates from the SD consensus sequence (33, 43). When the system is provided with an additional SD box from the araB gene, which better fits the SD consensus, 427 to 1,363 U of AP activity is produced. More than 5,000 U of AP activity can be obtained after maximal induction when wild-type phoA is expressed from the P Bad promoter and uses the optimized SD sequence present in pBAD22 (Table 1 and Fig. 4). In previous studies, Johnston et al. (24) used the araC-P Bad promoter system from Salmonella typhimurium for high-level expression of proteins in E. coli cells. They obtained overproduction levels corresponding to almost 15% of the total protein, which provided an abundant source for purification and direct visualization. Also, the P Bad promoter system present in pBAD18 has been used as an expression system for peptide libraries (11).

Comparison of the expression of the FtsQ–AP-91 fusion from the P Bad or P Tac promoters (Table 1, pLMG163 versus pLMG281) indicates that P Tac is 2.5- to 4.5-fold stronger than the P Bad promoter. Therefore, promoters based on P Lac (i.e., P Tac, P LacCm, and P TxyC) (4, 13, 48) may be more suitable for hyperexpression purposes than P Bad. However, problems that arise from massive overproduction of some proteins from these promoters (1, 16) can be relieved by the use of the P Bad promoter, while reasonably high yields of protein can still be obtained. For instance, expression of the malF gene and a truncated malG gene from the P Bad promoter at maximum induction is slightly deleterious to the cells, although growth is close to normal. However, when the same genes are expressed from the P Tac promoter on the comparable vector pHIB60 (Fig. 1), fully induced by IPTG, expression is lethal (data not shown).

Other problems that result from hyperproduction of certain proteins, such as the formation of inclusion bodies, have also been relieved by expression from the P Bad promoter. This was the case for the IS911 transposase, which formed inclusion bodies when produced from P Tac. When the protein was produced from the pBAD18 vector, inclusion bodies were no longer observed and high quantities of the soluble transposase from IS911 could be purified (37a).

DISCUSSION
We have constructed a series of vectors based on the P Bad promoter and its regulator araC that provide versatile features for genetic engineering techniques and for expression purposes. The most important characteristics of this system are that it can be used to (i) achieve very low levels of uninduced expression, (ii) obtain moderately high levels of expression in the presence of inducer, and (iii) modulate expression over a wide range of inducer concentrations.

The tight control of expression provided by the araC-P Bad promoter system is an important characteristic that is mostly absent in other available expression systems (4, 13, 15, 38). This feature has been indispensable in the isolation and study of null mutations in essential genes and in the evaluation of the depletion phenotype of these genes. In fact, the efficient repression available with this system is very useful in complementation studies of any null mutations, whether in essential or in nonessential genes, since leakiness can obscure results in both cases. The tight control also helps to avoid deleterious effects due to the uninduced expression of toxic genes. Additionally, we showed that the repression of expression of genes cloned into pBAD vectors is rapid and efficiently maintained for at least several hours (Fig. 2 and 3). A practical consequence of this finding, besides providing an efficient shutoff of gene expression, is that rapidity helps in the evaluation of primary effects of the depletion of a protein. If secondary effects result from depletion, they will be more likely to be observed at later times after shutdown of the gene.

In general, genes cloned under the control of the araC-P Bad promoter system are efficiently repressed. This has been the case for the essential genes ftsQ (7a), ftsL (22) (Fig. 5), ftsI (7), ffh (36), secD and secF (37), secE (34), and lepA (12) and for the nonessential genes dshA (31a), phoA (Fig. 2 to 4), and malF (data not shown). However, the levels of expression of P Bad-controlled genes may not be zero in the repressed state (Table 1), and that a particular gene is not turned off efficiently is a possibility. The P Bad promoter on pBAD vectors is repressed from 200- to 1,200-fold, which provides an excellent range to repress most genes.

There are several factors that contribute to an efficient repression of gene expression. These include the concentration of arabinose used in inducing conditions (the lower the better), the ability of the strain to degrade arabinose (ara− strains are better), and the physiological state of the culture due to the type of medium and amount and type of carbon sources present in the medium. Regarding the latter, since the araC-P Bad promoter system is subject to catabolite repression (32), levels of cyclic AMP will have a marked influence on expression. This could be important for strains growing in minimal medium with mixtures of carbon sources. Additionally, alter-
native compounds can be used to improve and expand the levels of repression from P_{BAD}; for instance, glucose-6-phosphate, which catabolite represses more efficiently than glucose, could be used to lower further the levels of repression. Also, the nonmetabolizable analog of L-arabinose, D-fucose, antagonizes arabinose induction by AraC (2, 28) and can be added to cultures containing arabinose to inhibit induction and lower the level of expression from P_{BAD}. Thus, different mixtures of arabinose and D-fucose can be used to obtain yet another level of modulation of expression with pBAD vectors, while mixtures of glucose and D-fucose could also achieve lower levels of repression as compared with those of glucose alone (2, 29).

Finally, once the P_{BAD} promoter has been repressed, the efficiency of repression depends mainly on the specific properties of posttranscriptional control of expression of each gene. For genes that yield very high levels of protein from the P_{BAD} promoter, repression may require lowering the overall expression of the gene (by lowering plasmid copy number and/or efficiency of initiation of translation).

Most of the available expression systems are not well repressed (4, 13, 15, 48). However, there are several systems that provide good repression (16, 20, 21, 35). Among them are vectors based on the lambda phage P_{1} promoter and its temperature-sensitive cI repressor allele, cI857 (15, 18, 35), and runaway replication plasmids (21, 23, 27). Still, the use of pBAD vectors will be advantageous in situations where (i) the high temperature required for P_{1} and some runaway vectors is not desired (23, 27), (ii) rapid shutoff is needed (e.g., runaway plasmids take several generations to be lost), (iii) analysis of recovery from a depleted state by turning on (very rapidly) a previously repressed promoter is desired, and (iv) modulation of expression is required.

Cagnon and coworkers (5) have also reported vectors allowing expression from the P_{BAD} promoter. However, the development of these vectors was oriented more towards biotechnological goals of high-level expression, which is in contrast to ours which were developed for physiological studies.

Using wild-type phoA and phoA fusions, we have estimated that the induction/repression ratio from the P_{BAD} promoter is from 200- to 1,200-fold, compared with a ratio of 50-fold for the P_{TAC} promoter (Table 1). In studies of the regulation of the arabinose operon (29), it was determined that the wild-type chromosomal araC-P_{BAD} promoter system can be activated 1,200-fold in vivo. These measurements, which were obtained with L-arabinose isomerase assays and log-phase cultures grown in minimal medium, are very similar to the induction/repression ratios that we obtained in minimal medium under similar culture conditions (Table 1). On the other hand, we found three- to fourfold less induction in rich NZY medium than in minimal medium (Table 1). This was also observed in rich LB medium and with AP fusions to two additional cell division proteins (data not shown). Similarly, Cagnon et al. obtained induction values lower than 1,200-fold when they measured β-galactosidase activity from the P_{BAD} promoter in LB broth (5). While we do not know the basis of this effect, it could be explained as the result of properties of the rich media, such as producing lower plasmid copy number and the presence of medium-related substances that could induce partial catabolite repression or inhibition, etc. The effect of lower activation in rich media was also observed with the P_{TAC} promoter (Table 1). However, in this case, the induction/repression ratio remained constant (i.e., 50-fold) as a result of post-translational effects that increase in expression of the protein. However, repression in minimal medium. Our results with phoA fusions also indicated that P_{TAC} is at least 2.5-fold stronger and 12-fold less repressed than P_{BAD}. The leakiness and high strength of the P_{TAC} promoter has been widely observed (4, 13, 15). In fact, the higher expression from P_{TAC} is strongly supported by the observations that expression of mult-multG (data not shown) and the IS911 transposase gene (37a) from induced P_{TAC} promoter is lethal but perfectly viable when the same genes are expressed in pBAD vectors.

Finally, a great and often-overlooked disadvantage of many expression systems, including those repressed more efficiently, is that their promoters are too strong when they are induced and expression is not modulated (15, 18, 21, 23, 27, 35). In many cases, the resulting protein overproduction from these systems negates the physiological relevance of the experiments, and it becomes only useful to purify and visualize proteins. Among the two most common and drastic overproduction problems are lethality (as in the case of overproduction of membrane proteins, cytoplasmic proteins like β-galactosidase [17], some cell division proteins [14], or toxic products [16]) and formation of inclusion bodies (37a, 46). In E. coli, protein hyperproduction to 30% of the total protein can lead to RNA and ribosome destruction, accumulation of heat shock proteins, and lethality (17). However, overproduction can create more subtle but nonetheless important problems. For instance, in complementation studies, a mutated gene expressed from a multicopy plasmid may complement the corresponding chromosomal mutation only when overexpressed from the plasmid. In this case, the mutated gene may encode a protein that is significantly deficient in the required function, but not totally defective, which fails to complement when expressed at levels closer to those of the wild type. However, overproduction of the mutated protein may produce enough of it, such that even though it is defective, the process can be driven to complementation. Another example is in depletion experiments, when protein overproduction before depletion (besides increasing the time needed for depletion) may be detrimental for cell growth or interfere with some processes which in turn can affect the analysis of the depletion phenotype. We have shown that moderately high expression and modulation can be achieved with pBAD vectors (Table 1 and Fig. 4). While maximum levels are high enough to permit most studies that require overproduction, they are not as high as those obtained from strong inducible promoters, and some of the detrimental effects of overproduction like lethality and formation of inclusion bodies have been alleviated by replacing the vector in use with pBAD plasmids (37a) (see Results). This is particularly the case when the excellent modulation of the P_{BAD} promoter expression is taken advantage of by inducing with arabinose concentrations that vary by as much as 2 orders of magnitude (Fig. 4).

Thus, there are many advantages in using a system that is easily modulated and contains a moderately strong promoter in conjunction with tight regulation. pBAD vectors represent a simple and useful expression system for efficient repression, modulation, and moderately high expression, which should permit more careful experimentation on biological processes in bacteria.

**ACKNOWLEDGMENTS**
We thank Patricia Conley, David Blair, Rick Stewart, and Dana Boyd for providing pPCI and related plasmids and pDHB60. We gratefully acknowledge Mike Chandler, Peter Shatz, and members of the Beckwith laboratory particularly, Joe Pogliano and Dana Boyd, for sharing their results on pBAD vectors and helpful comments. We also thank Terri Luna for medium preparation and Ann McIntosh for administrative assistance.

This work was supported by grants from the American Cancer Society and from the National Institute of General Medical Sciences to J.B., a grant from the Swiss National Science Foundation to D.B., and
by an American Cancer Society postdoctoral fellowship to L.M.G. J.B. is an American Cancer Society Research Professor.

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