Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 439–443, January 1984
Biochemistry

Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*

(hybrid promoter/isopropyl β-D-thiogalactoside induction)

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Communicated by Mark Ptashne, October 11, 1983

ABSTRACT **The *Escherichia coli lac* operator has been placed on the 3′ side of the promoter for the penicillinase gene of *Bacillus licheniformis*, creating a hybrid promoter controllable by the *E. coli lac* repressor. The *E. coli lac* repressor gene has been placed under the control of a promoter and ribosome-binding site that allows expression in *Bacillus subtilis*. When the penicillinase gene that contains the *lac* operator is expressed in *B. subtilis* on a plasmid that also produces the *lac* repressor, the expression of the penicillinase gene can be modulated by isopropyl β-D-thiogalactoside (IPTG), an inducer of the *lac* operon in *E. coli*. A similar system was constructed from a promoter of the *B. subtilis* phage SPO-1 and the leukocyte interferon *A* gene, which allowed the controlled expression of interferon in *B. subtilis*. These two examples show that a functional control system can be introduced into *B. subtilis* from *E. coli*.**

The ability to regulate transcription of a gene in *Escherichia coli* by using a number of easily controllable promoter systems has been important for the development of foreign protein expression systems as well as for the study of expression of native *E. coli* proteins. The *lac* repressor operator system is one such system. Repression of *lac* operon transcription is a result of binding of the *lac* repressor to the DNA sequence comprising the *lac* operator, thus preventing RNA polymerase from binding to the promoter (1). Induction occurs through the binding of an inducer to the *lac* repressor, which causes a conformational shift in the repressor, decreasing the affinity of the repressor for the operator (1). In *Bacillus subtilis* (or other Gram-positive organisms), no analogous system has been described. The only transcriptionally controlled promoters that have been characterized use a different mechanism. Gene regulation in these systems is controlled by σ factors, which are proteins that bind to the RNA polymerase and determine the recognition site for RNA initiation (2). Such systems cannot be used for the controlled expression of another gene easily.

To take advantage of the desirable characteristics of an operon whose transcription is easily induced, we have transferred the regulatory elements of the *lac* operon into *B. subtilis*. We report here that the *lac* operator–repressor control system can be transferred into *B. subtilis* and that the *lac* repressor and operator function as transcriptional regulatory elements in this microorganism. The penicillinase gene of *Bacillus licheniformis* system was used as a model system. In this system, the expression of penicillinase is modulated by isopropyl β-D-thiogalactoside (IPTG), an inducer of the *lac* operon in *E. coli*. To further illustrate the use of this transferred control system, we demonstrated IPTG-modulated expression of leukocyte interferon in *B. subtilis*.

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MATERIALS AND METHODS

Strains and Plasmids. *E. coli* strain MM294 (F⁻ *supE44 endA1 thi-1 hsdR4*) was used for all constructions unless otherwise indicated (3). *E. coli* strain 3300 (Hfr *thi-1 lacI22 relA1 spoT1* λ⁻) was obtained from the *E. coli* Genetic Stock Center (CGSC 808). *B. subtilis* strain I168 (*trpC2*) was provided by James Hoch. Plasmids pBR322 (4), pBS42 (5), pUB110 (6), and pBSA105 (7) were used for construction of the derivative plasmids described here.

Plasmid Constructions. The procedures used for isolation of plasmid DNA, cleavage of restriction fragments, isolation of DNA fragments from gels, kinase treatment of DNA fragments, ligation with T4 DNA ligase, and transformation of *E. coli* and *B. subtilis* were as described (8). "Primer repair" reactions were done as described (9). Antibiotic concentrations for selection of transformants were as follows: chloramphenicol, 12.5 μg/ml; neomycin, 20 and 10 μg/ml (*E. coli* and *B. subtilis*, respectively); ampicillin, 20 μg/ml.

Hybrid Promoters. The construction of the hybrid promoter of the penicillinase promoter and the *lac* operator, designated pac-I, and of the hybrid promoter of the phage SPO-1 and the *lac* operator, designated spac-I, have been described in detail elsewhere (7). Fig. 1 presents the nucleotide sequences of these two hybrid promoters and shows relevant restriction sites.

Assays. Cultures to be assayed for penicillinase were grown overnight in L broth containing 0.5% glucose and chloramphenicol at 10 μg/ml in the presence or absence of 1 mM IPTG. Appropriate dilutions of the total cell broth were made in 0.1 M Na phosphate buffer (pH 7.0) and the amount of penicillinase enzymatic activity was determined by following the conversion of penicillin to penicilloic acid by a published procedure (10). Detection of penicillinase activity on polyvinyl alcohol plates was accomplished as described (10).

Cultures to be assayed for interferon expression were grown in L broth containing 0.5% glucose and neomycin at 10 μg/ml in the presence or absence of 1 mM IPTG. The cells were harvested by centrifugation, lysed by treatment with lysozyme and detergent, and assayed for interferon activity using a bioassay as described (5).

RESULTS

Expression of the *lac* Repressor in *B. subtilis*. The promoter and ribosome-binding site of the penicillinase gene of *B. licheniformis* were linked in the plasmid pIQ45 to the *E. coli lac* repressor gene so that it might be expressed in *B. subtilis* (Fig. 2). The first two amino acids of the two genes are identical. When the *lac* repressor gene was digested with *Hph* I, then repaired to flush ends with the Klenow fragment of

Abbreviations: IPTG, isopropyl β-D-thiogalactoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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EcoRI
GAATTCGGTG GAAACGAGGT CATCATTTCC TTCCGAAAAA ACGGTTGCAT TTAAATCTTA CATATGTAAT

      <<<< <<  lac Op  >>>>>>    Sau3A                *****              met
ACTTTCAATT GTGAGCGGAT AACAATTCCG GATCAATCAA ATATTCAAAC GGAGGGAGAC GATTTTGATG
      ---- ---------- ----------
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EcoRI
GAATTCTACA CAGCCCAGTC CAGACTATTC GGCACTGAAA TTATGGGTGA AGTGGTCAAG ACCTCACTAG

GCACCTTAAA AATAGCGCAC CCTGAAGAAG ATTTATTTGA GGTAGCCCTT GCCTACCTAG CTTCCAAGAA

AGATATCCTA ACAGCACAAG AGCGGAAAGA TGTTTTGTTC TACATCCAGA ACAACCTCTG CTAAAATTCC

                                                              <<< <<< lac Op
TGAAAAATTT TGCAAAAAGT TGTTGACTTT ATCTACAAGG TGTGGCATAA TGTGTGGAAT TGTGAGCGGA
                                                  --------- ----------

  >>>>>>HindIII  **** *    XbaI EcoRI met
TAACAATTAA GCTTAAGGAG GTGTATCTAG AATTCATG
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FIG. 1. Nucleotide sequences of the pac-I (*Upper*) and spac-I (*Lower*) promoters. The −35 and −10 regions of the promoters are overlined, probable ribosome-binding sites are indicated by *s, the palindromic sequences of the *lac* operator are indicated by << >>, and the initiation codons are indicated by "met." Sequences not derived from the parent promoter are underlined.

DNA polymerase I, the nucleotides for the first two codons of the gene were removed. Subsequent digestion with *Bst*EII produces a DNA fragment containing the amino-terminal portion of the *lac* repressor gene but lacking the first two codons. A DNA fragment containing the promoter, ribosome-binding site, and the first two codons of the penicillinase gene was created using a "primer repair" reaction, followed by digestion with *Eco*RI. These two fragments were ligated into the previously constructed plasmid pIQ2, which already contained the carboxyl-terminal portion of the *lac*

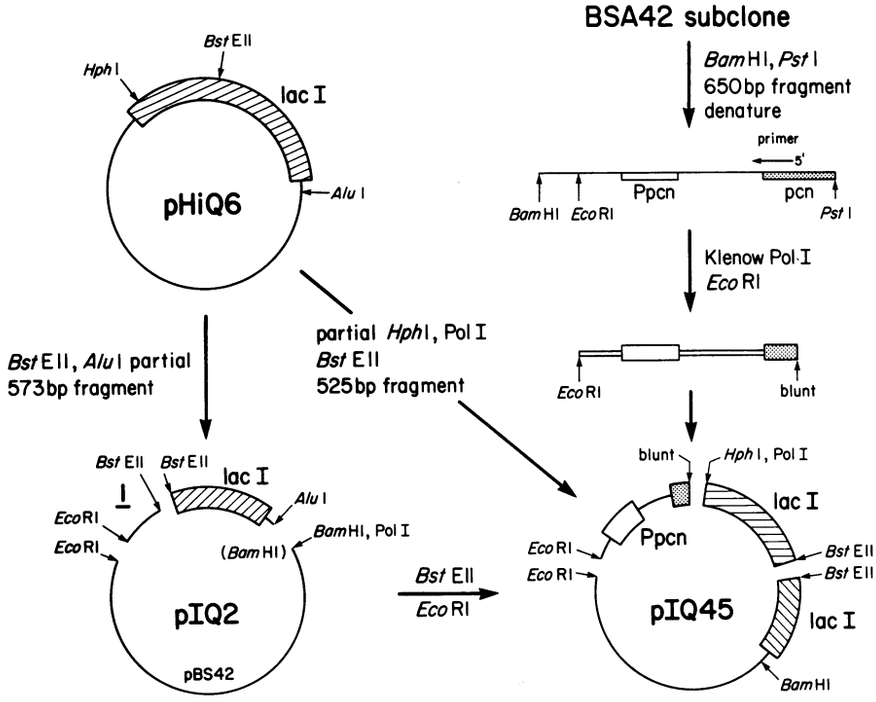

FIG. 2. Construction of pIQ45. Construction of the *lac* repressor gene under the control of the penicillinase promoter and the ribosome-binding site is diagrammed. Plasmid pHiQ6, containing the *lac* repressor, and plasmid pBS42, containing the pBR322 and pUB110 origins of replication and the pC194 chloramphenicol acetyltransferase gene, have been described (5, 11). The carboxyl portion of the *lac* repressor gene was subcloned into pBS42 in a three-part ligation, creating pIQ2. Fragment 1 used in the construction was simply a convenient *Eco*RI/*Bst*EII restriction fragment whose *Bst*EII site had the same sequence as the *Bst*EII site in the *lac* repressor. This allowed the *Bst*EII site to be conserved for the subsequent construction. Plasmid pIQ2 was constructed in strain MM294, with selection for chloramphenicol resistance. Plasmid pIQ45 was constructed by ligating three fragments, one containing the penicillinase promoter, ribosome-binding site, and first two amino acids of the coding sequence, a second containing the amino terminus of the *lac* repressor gene (minus the first two amino acids), and a third containing the pBS42 vector and the carboxyl portion of the *lac* repressor gene. The derivation of the three fragments is shown in the figure. The pBSA42 subclone contains the promoter and amino terminus of the penicillinase gene as described (7). The sequence of the primer used for the primer repair reaction was 5′ T-T-T-C-A-T-C-A-A-A-A. Plasmid pIQ45 was constructed in strain 3300 (lac I^-), with selection for chloramphenicol resistance on X-Gal indicator plates. Ppcn, penicillinase promoter; pcn, penicillinase-encoding region; lac I, *lac* repressor coding region.

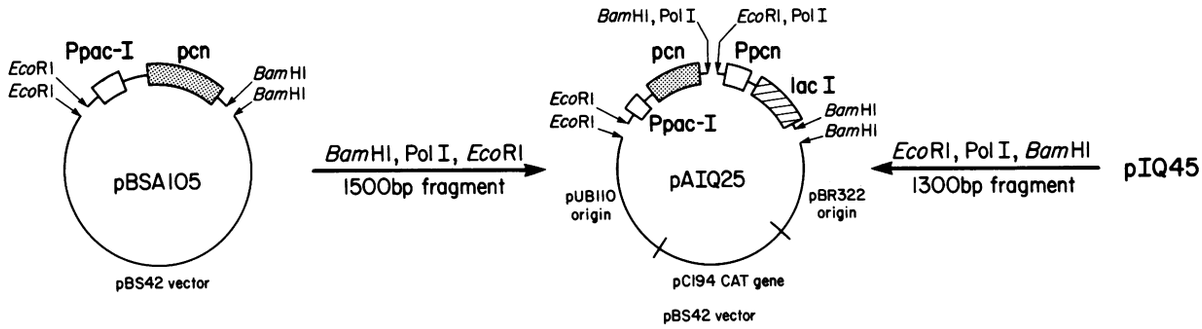

FIG. 3. Construction of pAIQ25. Placement of the penicillinase gene under the control of the pac-I promoter and the *lac* repressor gene under the control of the penicillinase promoter together on a plasmid is diagrammed. Plasmid pAIQ25 was constructed by ligating three fragments, one was the pBS42 vector, the second contained the *lac* repressor derived from pIQ45, and the third contained the penicillinase gene derived from pBSA105. Plasmid pBSA105 contained the penicillinase structural gene under the control of the hybrid pac-I promoter (7). Plasmid pAIQ25 was constructed in strain MM294 with selection for ampicillin resistance. Ppac-I, pac-I promoter; Ppcn, penicillinase promoter; pcn, penicillinase-encoding region; lac I, *lac* repressor coding region.

repressor gene distal to the *Bst*EII site. Transformants were selected in *E. coli* strain 3300 (lac I^-), and white colonies on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) indicator plates indicated the construction of a functional *lac* repressor on the plasmid.

Inducible Expression of the Penicillinase Gene in *B. subtilis*. To determine whether the *lac* repressor and the hybrid promoters could function together to control gene expression in *B. subtilis*, the *lac* repressor from pIQ45 was placed on a plasmid together with the penicillinase gene under the control of the hybrid promoter pac-I. The construction of such a plasmid, designated pAIQ25, is shown in Fig. 3. After transformation of pAIQ25 into *B. subtilis* strain I168 with selection for chloramphenicol resistance, colonies were grown on polyvinyl alcohol indicator plates with or without 1 mM IPTG. A photograph of such plates after staining is shown in Fig. 4. These plates show that the expression of penicillinase is induced in the presence of IPTG. The amount of penicillinase enzymatic activity produced was quantitated in liquid cultures, and the results indicate that a 100-fold induction of penicillinase can be obtained in the presence of IPTG, with the production of 60 units/ml in cultures grown in the absence of IPTG and 6,000 units/ml in cultures grown in the presence of IPTG. Polyacrylamide gel analysis of the proteins recovered from the supernatants of these cultures showed a prominent protein of M_r 33,000 in the media of IPTG-induced cultures (data not shown). The size of this protein correlates with the known M_r of the *B. licheniformis* penicillinase (12).

Inducible Expression of Leukocyte Interferon *A* in *B. subtilis*. Another construction was made in order to determine whether the intracellular accumulation of a heterologous gene product could also be regulated in *B. subtilis* by the *lac* repressor. The human leukocyte interferon *A* gene under the control of the spac-I promoter was isolated from pSPIF-III.

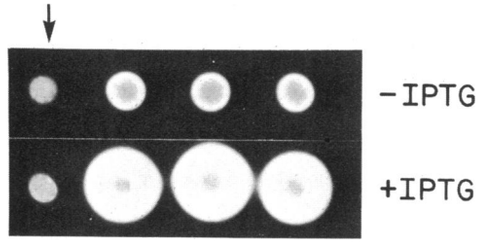

FIG. 4. Assay for penicillinase activity on polyvinyl alcohol plates. *B. subtilis* colonies carrying plasmid pAIQ25 were plated on LB plates containing 0.8% polyvinyl alcohol and chloramphenicol at 10 μg/ml. The plates were stained and developed as described (10). The arrow indicates colonies carrying the parent plasmid, pBS42.

Plasmid pSPIF-III has been described; basically, it has the spac-I promoter and Shine–Dalgarno region shown in Fig. 1 linked to the interferon gene (5). This interferon gene was placed on a plasmid together with the *lac* repressor gene as outlined in Fig. 5. This plasmid, designated pLIQ-1, was transformed into *B. subtilis* strain I168. Transformants were assayed for interferon production after growth in the presence or absence of IPTG. As shown in Fig. 6, addition of IPTG to the medium causes a rapid increase in the level of interferon in the cells.

DISCUSSION

This paper describes the expression of the *E. coli lac* repressor in *B. subtilis* and its use in controlling transcription from hybrid promoters that contain the *E. coli lac* operator sequence. Production of both penicillinase, under the control of the pac-I promoter, and leukocyte interferon, under control of the spac-I promoter, was induced in the presence of IPTG. Both the *lac* repressor and operator are necessary for this induction; when the *lac* repressor is not present in the cells, expression is constitutive, and IPTG has no inducing effect on promoters that do not contain the *lac* operator (data not shown). The most likely interpretation of these results is that the *lac* repressor is being expressed in a functional state in *B. subtilis* to control transcription from these hybrid promoters. Also important is the observation that IPTG can enter *B. subtilis* in sufficient amounts to derepress the hybrid promoters.

The penicillinase and SPO-1 promoters were used to construct the hybrid promoters primarily because the nucleotide sequences and sites of transcription initiation of both had been determined (12–14). The two hybrid promoters differ slightly in the relationship of the RNA polymerase recognition site and the *lac* operator sequence. The spac-I promoter has the *lac* operator positioned at the start of transcription initiation, just as found in the *lac* promoter. Also, the sequence between the −10 portion of the RNA polymerase site and the *lac* operator is identical to that of the *lac* promoter. The pac-I promoter has the *lac* operator positioned four nucleotides upstream of the transcription initiation site of the original penicillinase promoter (13). Whether these differences have effects on the induction of these promoters cannot be determined from these studies; more careful measurements of the induction ratios would need to be made to determine whether they are similar to those observed for such hybrid promoters used in *E. coli* (15). However, it does appear that the exact spacing of the *lac* operator with respect to the RNA initiation site is not critical for the functioning of a repressible hybrid promoter.

The ability to control the transcription of a gene in *B. sub-*

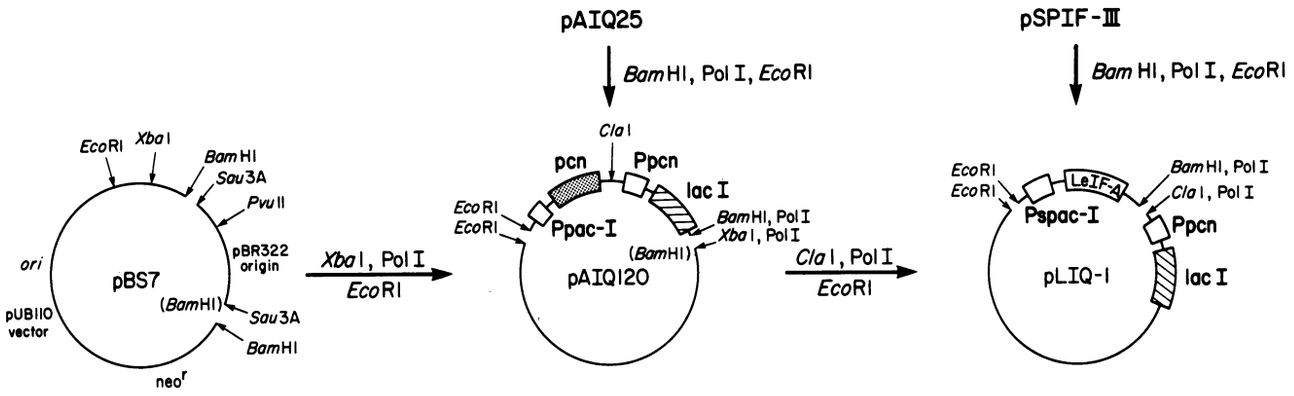

FIG. 5. Construction of pLIQ-1. The vector plasmid, pBS7, is the neomycin-resistance-conferring plasmid pUB110, with the pBR322 origin inserted into the unique *Bam*HI site of pUB110 as shown. Plasmid pAIQ120 was constructed by the ligation of a restriction fragment of pAIQ25 that contained the penicillinase gene and the *lac* repressor gene to the pBS7 vector plasmid by using the restriction sites indicated. Plasmid pLIQ-1 was derived from pAIQ120 by replacing the penicillinase gene and its pac-I promoter with the human leukocyte interferon *A* gene under the control of the spac-I promoter. The interferon gene was derived from pSPIF-III (5). Both pAIQ120 and pLIQ-1 were constructed in strain MM294 with selection for neomycin resistance. Restriction sites in parentheses were re-created by the ligation. Pspac-I, spac-I promoter; Ppac-I, pac-I promoter; pcn, penicillinase-encoding region; lac I, *lac* repressor coding region; LeIF-A, leukocyte interferon *A* coding region.

tilis by using hybrid promoters and the *lac* repressor should be useful for a wide variety of purposes. It is often desirable to regulate the expression of a protein and be able to induce its synthesis rather than rely on constitutive expression because the expression of high levels of proteins can have deleterious effects on growth of the cells (16). This may be true for the expression of the *B. licheniformis* penicillinase gene. This gene cannot be transformed into *B. subtilis* on a high-copy plasmid under the control of either its natural promoter or the pac-I promoter (unpublished results; ref. 17). However, when the transcription of the gene was placed under the control of the *lac* repressor, as on plasmid pAIQ25, the gene could then be transformed into *B. subtilis*. This controllable system now gives us a potential tool to study the effect of high levels of expression of the penicillinase gene on *B. subtilis*. Also, the use of this transcriptionally controlled system might have advantages over previously described *B. subtilis* expression systems that appear to be regulated at the translational level (18, 19), as the presence of very strong unregulated promoters can be incompatible with plasmid replication in *E. coli* (20) and perhaps in *B. subtilis* (21).

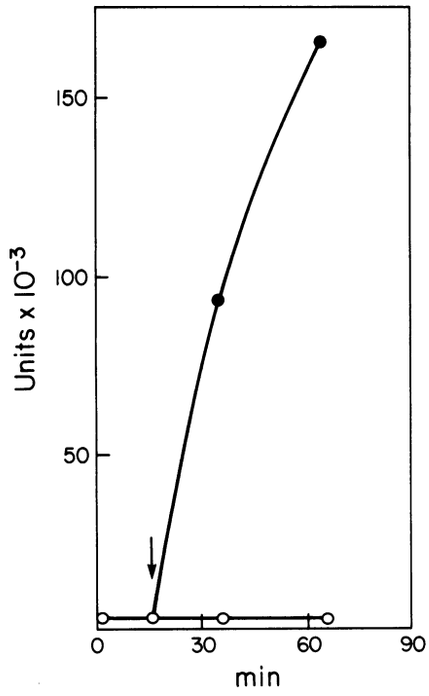

FIG. 6. Induction curve of pLIQ-1. *B. subtilis* strain I168 transformed with plasmid pLIQ-1 was grown in liquid culture to an OD_{600} of ≈0.2. The culture was then divided in half and 1 mM IPTG was added to one portion (indicated by the arrow). Triplicate samples were removed to assay for interferon activity at the indicated times. Values shown are means of triplicate determinations and have been corrected for differences in optical densities of the cultures. Thus, the values reported are units/ml per optical density unit. ●, IPTG added; ○, no IPTG.

One exciting feature of this system is that it offers the potential of regulated expression systems in other bacteria for which DNA transfer techniques exist. This sytem should be transferable to any organisms in which the pBR322 or pUB110 origins of replication function; alternatively, the genes could easily be moved to plasmids of different host ranges. The promoters and ribosome-binding sites used to express the *lac* repressor gene and to construct the hybrid promoters should be functional in a wide range of both Gram-positive and Gram-negative organisms. Even if new promoters needed to be created for expression in some alternative organism, this process should reduce the efforts that would otherwise be needed to isolate and characterize appropriate gene control systems that are native to that organism.

We would like to acknowledge Dennis Kleid for encouragement and useful discussions and Herman de Boer for generously providing some of the plasmids and DNA fragments used in the constructions.

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