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Functional Expression of Two *Bacillus subtilis* Chromosomal Genes in *Escherichia coli*

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*Eco*RI-cleaved deoxyribonucleic acid segments carrying two genes from *Bacillus subtilis*, *pyr* and *leu*, have been cloned in *Escherichia coli* by insertion into a derivative of the *E. coli* bacteriophage λ. Lysogenization of pyrimidine- and leucine-requiring auxotrophs of *E. coli* by the hybrid phages exhibited prototrophic phenotypes, suggesting the expression of *B. subtilis* genes in *E. coli*. Upon induction, these lysogens produced lysates capable of transducing *E. coli pyr* and *leu* auxotrophs to prototrophy with high frequency. Isolated DNAs of these bacteriophages have the ability to transform *B. subtilis* auxotrophs to *pyr* and *leu* independence and contain *Eco*RI-cleaved segments which hybridize to corresponding segments of *B. subtilis*.

Methods have been developed recently which allow the insertion of deoxyribonucleic acid (DNA) segments from different sources into *Escherichia coli* replicons and the amplification of the inserted segments in the bacterium. When ligation to the replicon is performed with a heterogeneous population of exogenous DNA segments, it is important to be able to select *E. coli* clones containing the desired specific DNA segments. Genes that code for enzymatic functions can be selected by isolation of hybrid *E. coli* clones that have acquired the corresponding function, provided that the exogenous DNA can be expressed in *E. coli*. Such a procedure has been already used in several laboratories for the selection of *E. coli* bacteria with cloned segments of the *E. coli* chromosome itself; one example is the isolation of *trp* genes (6). Interspecific hybrid DNAs have also been generated in vitro. For example, a plasmid gene coding for ampicillin resistance in *Staphylococcus aureus* (3) and a phage gene, thymidylate synthetase of the *Bacillus subtilis* bacteriophage φ3T (5), have been ligated to pSC101 and expressed in *E. coli*. A segment of yeast (*Saccharomyces cerevisiae*) DNA has been cloned on phage λ and complements histidine-requiring *E. coli* auxotrophs. In this case, the functional nature of the cloned yeast DNA has not been identified (17). Using the above approach, we describe here the expression of two *B. subtilis* chromosomal segments carrying *pyr* and *leu* markers in *E. coli* and verify that the hybrid phages grown in *E. coli* contain sequences homologous to those in *B. subtilis*.

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MATERIALS AND METHODS

Bacterial and bacteriophage strains. *E. coli* and *B. subtilis* strains used are identified in Table 1. λ bacteriophage used was derived from λ*plac* and λ*gti* and supplied by R. Davis (Fig. 1). This phage carries the *c*I857 allele which codes for temperature sensitive *c*I repressor; thus lysogenic bacteria will grow at 30°C but not at 42°C.

DNA and enzymes. λDNA was prepared as described in reference 4. *B. subtilis* DNA was prepared from SB 1070 as described in reference 10. *Eco*RI restriction enzyme and T4 ligase were prepared as described in reference 5. Ribonucleic acid (RNA)-polymerase was a gift from D. Brutlag.

Preparation of hybrid bacteriophage λ and *B. subtilis* pools. *Eco*RI endonuclease reactions were performed in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), 5 mM $MgCl_2$, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 37°C with two additions of enzyme. The completeness of *B. subtilis* DNA digestion was defined by reduction of *lys*-transforming activity to less than 0.1%. This resulted in a gel electrophoresis pattern similar to that observed by Harris-Warrick et al. (9). The completeness of phage DNA digestion was defined by reduction of transfection efficiency to 0.1%. The DNA was heated to 60°C for 5 min immediately before the T4 ligase reactions to dissociate the λ cohesive ends and to inactivate the *Eco*RI endonuclease. T4 ligase reactions were in 50 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, and 0.1 mM NAD at 25°C for 3 h with one addition of enzyme. The reactions were terminated by making the buffer 20 mM in EDTA. The completeness of T4 ligase sealing was checked under an electron microscope for the appearance of covalently closed circular molecules and the restoration of transfection activity on *E. coli*.

TABLE 1. *E. coli and B. subtilis strains*

Strains	Genotypes	Source
E. coli		
W5445	C600, *pro rpsL thrB supE44 lac tonA rK⁻ mK⁺*	Stanford
AT2538	*thi-1 pyrE60 argE3 his-4 proA2 thr-1 leu-6 met-1 xyl-5 ara-14 galK2 lacY1 rpsL31 supE44*	A. L. Taylor
Hfr300YA149	*pyrF40 thi-1 relA1*	Jacob
MA1008(3050-U6)	*thi-1 pyrC46 relA1 lacZ43*	W. Maas
CA158	Hfr Hayes *lac pyrD thi*	J. Beckwith
B. subtilis		
SB1070	*thyA thyB*	Stanford
SB863	*aroB trpC tyrA hisA cys-1 leu str*	Stanford
SB270	*pyrE*	Stanford
SB5	*trpC pyrE his*	Stanford
SB319	*met pyrD*	Stanford
GSY289	*trp pyrD*	Anagnostopoulos
M8(168MIUM8)	*trp pyrB*	Gooder
M16(168MIUM16)	*trp pyrB*	Gooder
M13(168MIUM13)	*trp pyrC*	Gooder
S5	*trp pyrA his*	Nester

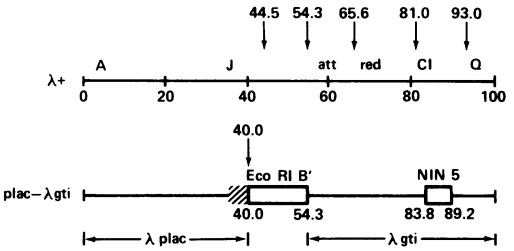

FIG. 1. *EcoRI map of λ⁺ and λplac-λgti hybrid phage. EcoRI sites (1) are indicated by arrows. Substitution of the lac operon in λplac is indicated by shaded area. λplac-λgti is a phage derivative made and given as a generous gift by R. Davis. λplac-λgti has two deletions, NIN5 and EcoRIB' segments (indicated by blanks), and one EcoRI site at 40% position and produces two segments upon cleavage. All the original RI sites of the λ have been either mutated or substituted.*

Calcium-treated cells were transfected by the method of Mandel and Higa (12) (except for dilution of DNA to 1 μg/ml in 10 mM Tris–1 mM EDTA before mixing with competent cells of strain W5445) and plated on tryptone agar plates. Plaques representing independent viable hybrids were pooled by scraping the plates. The phage pool was treated with $CHCl_3$ (2%, vol/vol) at 37°C for 30 min and filtered through a membrane filter (0.45-μm-pore diameter; Millipore Corp.). To minimize the selective pressure on proportions of phage genotypes, no further growth was allowed.

Transduction. Recipient auxotrophic *E. coli* grown overnight in L-broth were resuspended in 10 mM $MgCl_2$ (0.5 volume) and shaken at 30°C for 1 h to deplete amino acids. The starved recipients were then mixed with phages from the hybrid pool at a multiplicity of infection of 0.01 and incubated for 1 to 2 h at 30°C. The cells were then plated onto appropriate selective media and incubated at 30°C. Prototrophic lysogens appeared on day 2 of incubation.

Preparation of high-frequency transductant lysates and curing of λ prophage from prototrophic lysogens. The lysogenic culture was grown with gyratory shaking (300 rpm) at 30°C until the cell density reached a Klett turbidity reading (filter no. 66 red [640 to 700 nm]) of 70 (about 10^8 cells per ml). The culture was induced at 42°C for 20 min, and incubation was continued at 37°C with shaking (or for 5 min at 42° and then returned to 30°C for 4 h to cure the lysogens) (18) until the cells lysed. $CHCl_3$ (2%, vol/vol) was added, and the mixture was incubated at 37°C for 30 min with shaking to insure complete lysis. After centrifugation at 25,000 rpm for 15 min to remove cell debris, the lysate was sterilized by membrane filtration (Millipore).

High-frequency transductant lysate spot tests. Sterile lysates prepared from lysogens were diluted 10-fold in 10 mM $MgCl_2$ and spotted onto selective media previously spread with a lawn of the appropriate starved *E. coli* auxotrophs. High-frequency transductant lysates gave confluent growth on the spot after 48 h at 30°C.

Transformation. *B. subtilis* cells were brought to competence and transformed as described in reference 16.

Agarose gel electrophoresis and transformation with DNA recovered after separation. The procedure was followed as described in reference 9.

In situ hybridization on plaques. The method of Kramer et al. (11) was followed.

Hybridization on the electrophoretic pattern of *Eco*RI-cleaved DNA. The method of Southern (15) was followed.

These experiments were conducted prior to the formal establishment of NIH guidelines. However, construction of hybrid DNA and all operations involving DNA isolation were carried out in a P2-level containment laboratory.

RESULTS

Cloning of *pyr* and *leu* genes and complementation of *E. coli* auxotrophs. Fully

*Eco*RI-cleaved λ*plac*-λ*gti* DNA was added to *B. subtilis Eco*RI segments to a ratio of 1:1 (wt/wt) at 30 μg/ml and ligated with T4 ligase. The ligated mixture was used to transform competent *E. coli* W5445. Plaques obtained from the transformation contained 30 to 50% hybrid phages (bacteriophage λ with *B. subtilis* DNA insertion), judging by in situ hybridization on plaques (11), and a hybrid pool representing 3×10^3 independent plaques was obtained. The advantage of using λ*plac*-λ*gti* is that the hybrid phages do not need helper for integration, thus increasing the chance of lysogenic expression. These hybrids were used to infect *E. coli* auxotrophs AT2538 and W5445 for all available amino acid markers. Prototrophic lysogens appeared only for *pyr* in AT2538 and *leu* in W5445. Prototrophic lysogens were found at frequencies (defined as number of prototrophic lysogens per total number of input phages) of 10^{-5} for *pyr* and 10^{-4} for *leu* (Table 2). The prototrophic lysogens isolated in this manner appeared at approximately the same frequencies as spontaneous revertants (number of prototrophs per total number of input cells) of the *pyr* (10^{-7}) and *leu* (10^{-6}) markers on selective plates. Lysogens were distinguished from revertants by testing for temperature sensitivity at 42°C. Results are summarized in Table 2.

A control experiment on the origin of the active segments was done by the following procedures. Fully *Eco*RI-cleaved λ*plac*-λ*gti* DNA, without addition of *B. subtilis* segments, was religated with T4 ligase. A similar "hybrid" pool of 10^3 independent phages was generated and used to infect *E. coli* auxotrophs. Twenty prototrophic colonies from each marker were tested. As expected, no prototrophic lysogens were found. The nonlysogenic prototrophs comprise and cannot be distinguished from spontaneous reverse mutations similar to those which complicate the experiment with *B. subtilis* DNA, e.g., the 3/10 *leu*$^+$ nonlysogens indicated in Table 2.

TABLE 2. *Prototrophic lysogenization of pyr and leu markers*

Gene marker	Prototrophic colonies appearing[a]	Temperature-sensitive colonies[b]	Lysogenization frequency[c]
pyr	4	3/4	10^{-5}
leu	50	7/10	10^{-4}

[a] Strains AT2538 *pyr* and W5445 *leu* were used as recipients for infection with phage from the hybrid pool at a multiplicity of infection of 0.01.

[b] In both cases, all three of three tested temperature-sensitive clones were lysogenic.

[c] Prototrophic lysogens per input phage after subtraction of temperature-resistant prototrophic revertants.

The *pyr* and *leu* markers were located on hybrid λ DNA. This has been shown by three lines of evidence. Three colonies from each prototrophic temperature-sensitive lysogens were used for the following experiment.

(i) Cured prototrophic lysogens became auxotrophic. A short heat-pulse treatment can cure *E. coli* lysogens of the λ prophage (18) which results in loss of *pyr* and *leu* markers from the prototrophic lysogens. Loss of prophage was independently tested by loss of immunity to λ*b2cI* and loss of temperature sensitivity (i.e., growth at 42°C). The frequency of curing under the described condition is 30%.

(ii) Purified hybrid bacteriophages transduced *E. coli pyr* or *leu* auxotrophs at high frequencies. The phages induced from prototrophic lysogens were purified through three steps of single-plaque isolation. At each step, a stable transducing frequency (number of prototrophic lysogens per total number of input phages) of 3% was obtained (Table 3). This represents an enrichment of 10^3 for *pyr* and 10^2 for *leu* markers from the original hybrid pools. These results show that *B. subtilis pyr* and *leu* genes were carried on the λ DNA and could complement *E. coli* auxotrophs. Thus, some genes could function when transferred between these two species.

(iii) Hybrid bacteriophage DNA transformed *B. subtilis* auxotrophs. The efficiency of transformation was measured with intact hybrid phage DNA at limited DNA concentrations (Table 4). DNAs from SB1070 and λ*plac*-λ*gti* were used as standards and controls. Hybrid bacteriophage DNA could transform *B. subtilis* auxotrophs to *pyr* and *leu* independence at approximately the same frequency as the *B. subtilis* DNA: 10^{-5} transformants per *B. subtilis* genome equivalent. This result not only confirms that the *pyr* and *leu* genes were incorporated in the λ chromosome, but also suggests the *B. subtilis* origin of these genes.

Characterization of the cloned *B. subtilis* segments. DNA from the hybrid bacteriophages was cleaved with *Eco*RI, and the segments were characterized by gel electrophoresis, using *Eco*RI-cleaved phage SPP1 DNA (7) and λ*plac*-λ*gti* DNA as standards. Results are shown in Fig. 2. λ*pyr* DNA contained one *Eco*RI segment that was not present in the λ parent, λ*plac*-λ*gti*, whereas λ*leu* DNA contained two additional segments. This presumably resulted from incomplete *Eco*RI cleavage of the donor *B. subtilis* DNA prior to ligation with λ*plac*-λ*gti*. Limited *Eco*RI cleavage of λ*leu* DNA produced only one additional segment equal in length to the

TABLE 3. *Transduction of λpyr phage in E. coli pyrimidine-requiring mutants*

Recipient	Marker	Map position (min)	Transducing frequency[a] (%)
MA1008	*pyrC*	23	<0.01[b]
CA158	*pyrD*	21	3
Hfr300YA149	*pyrF*	28	5
AT2538	*pyrE*	81	3

[a] All the transducing frequencies were the average of three transduction experiments, with the total number of input phage being 10^3, 10^4, and 10^5 per 10^7 bacteria (0.2 ml).

[b] Experimental condition did allow lysogen formation by the eosin-methylene blue agar test for lysogeny (8).

TABLE 4. *Transformation of the cloned pyr segment into B. subtilis pyrimidine-requiring mutants*[a]

Recipient	Marker	Transformation per ng of λ*pyr* DNA/transformation per ng of SB1070 DNA
S5	*pyrA*	0
M13	*pyrC*	0
M16	*pyrB*	0
M8	*pyrB*	60
GSY289	*pyrD*	63
SB319	*pyrD*	70
SB5	*pyrE*	66
SB270	*pyrE*	0.4

[a] All the transformations were done at limiting DNA concentrations (at these concentrations, number of transformants increased linearly as DNA concentrations increased) with intact λ*pyr* DNA. SB1070 DNA was used as a standard to normalize transformation efficiency.

sum of two segments (data not shown). A further cleavage resulted in the pattern of Fig. 2. It is likely that the *Eco*RI targets of these inserted segments are not cleaved with equal efficiency (9).

The *pyr* and *leu* markers were not located on the same DNA segment. λ*pyr* DNA could not transform *B. subtilis leu* auxotrophs to *leu* independence, and λ*leu* DNA could not transform *pyr* auxotrophs (data not shown).

Evidence of *B. subtilis* sequences in the hybrid phage DNA. *B. subtilis* DNA was cleaved by *Eco*RI endonuclease, separated electrophoretically using 0.6% agarose gels, and transferred to nitrocellulose paper according to the methods of Southern (15). ^{32}P-labeled RNA complementary to either of the hybrid phage DNAs was hybridized to this electrophoretic pattern of *B. subtilis* segments, and successful hybridization was detected by autoradiography. Autoradiographic bands corresponding to the size of the inserted *pyr* and *leu* segments in the λ hybrids were clearly seen on the *B. subtilis* *Eco*RI electrophoretic pattern (Fig. 2). Gels representing *Eco*RI segments of the two hybrid phage DNAs running parallel to the above two were sliced into 5-mm pieces (15 pieces per gel) and assayed for transforming activity. Figure 2 (h) shows the pattern of transforming activity for *pyr* and *leu* markers. It is evident that the size of the segment matched exactly with the biological activity and the hybridization for both markers. The 0.7-kb segment in λ*leu* lacked transforming activity for the one particular mutant (SB863) used, but it did hybridize with *Eco*RI-cleaved *B. subtilis* DNA at exactly the same size.

These results confirm that the segments of *B. subtilis* DNA inserted in λ phage and cloned subsequently in *E. coli* retain the same size as the original *Eco*RI segments of *B. subtilis*.

Linkage and complementation studies of the cloned *B. subtilis pyr* segment. The ge-

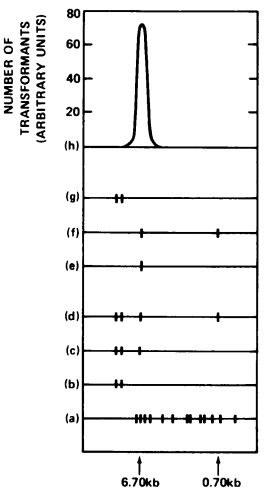

FIG. 2. *Agarose gel electrophoresis of DNA from the hybrid phage and nucleic acid hybridization. EcoRI-cleaved DNA of hybrid phages were run on 0.6% agarose gel, and the bands were compared with those of SPP1 DNA and parental λplac-λgti DNA. Nucleic acid hybridization was performed as described in the text. a, EcoRI SPP1, size standard; b, EcoRI λplac-λgti, parent (note two bands); c, EcoRI λpyr, note extra band at 6.7 kb; d, EcoRI λleu, note two extra bands (see text); e, autoradiograph pattern of complementary RNA of λpyr on EcoRI B. subtilis DNA; f, autoradiograph pattern of complementary RNA of λleu on EcoRI B. subtilis DNA; g, autoradiograph pattern of complementary RNA of λpyr (or λleu) on EcoRI λplac-λgti DNA; h, transforming activity of EcoRI λpyr (or λleu) DNA into B. subtilis pyrimidine-requiring mutants.*

netic map of *B. subtilis* pyrimidine-requiring mutants (13) and the "genetic length" of the cloned *pyr* segment (corresponding to a physical length about 6.7 kb) are described in Fig. 3. Transformation studies with *B. subtilis* pyrimidine-requiring mutants (Table 4) showed that the cloned *pyr* segment contained at least three structural genes: *pyrD*, *pyrF*, *pyrE*, and part of *pyrB* from the pyrimidine operon (Fig. 3). Positions of the ends to the right of SB270 and to the left of M8 were uncertain due to the lack of mutants for mapping in these regions; however, the cloned segment did not exceed M16 (zero transformation with M16, M13, and S5) to the left. A decreased transformation efficiency of SB270 (Table 4) suggests that the right end is near SB270 if we invoke edge effects (9). Transduction studies with *E. coli* pyrimidine-requiring mutants (Table 3) showed that the cloned segment can complement *pyrD*, dihydro-orotate dehydrogenase (EC 1.3.3.1), *pyrE*, orotidylate pyrophosphorylase (EC 2.4.2.10), and *pyrF*, orotidylate decarboxylase (EC 4.1.1.23) mutants in the lysogenic states, but not *pyrC*. *pyrA* and *pyrB* were not tested. None of the three different *E. coli* markers that can be complemented are linked to one another: *pyrD* is located at 21 min, *pyrF* at 28 min, and *pyrE* at 81 min (2). Since the λ integration site is normally unique on the *E. coli* chromosome (if λatt is not deleted), this indicates that the complementation of the three genetic mutations was not site specific. Also, it is less likely that the complementation was due to suppression of mutations, because a bona fide suppressor would have to suppress three mutations at three different map positions. Although we still lack direct chemical evidence that the cloned *B. subtilis* DNA has been faithfully transcribed and translated in *E. coli*, the functional evidence strongly suggests that protein synthesis directed by *B. subtilis* genes can be utilized by *E. coli* for biosynthesis.

DISCUSSION

We have shown that two hybrid λ phages containing *Eco*RI-cleaved segments from *B. subtilis* DNA carrying *pyr* and *leu* markers can be expressed in *E. coli*. The ability to propagate foreign DNA segments in *E. coli* cells, especially sequences coding for known proteins, promises to illuminate our understanding of gene regulation in vivo.

The pyrimidine pathway has been studied thoroughly in both *B. subtilis* and *E. coli*; we use this as an example to demonstrate the potential of a uniquely defined DNA segment in studying gene regulation. The *pyr* DNA-containing phage, when integrated into *E. coli* mutants

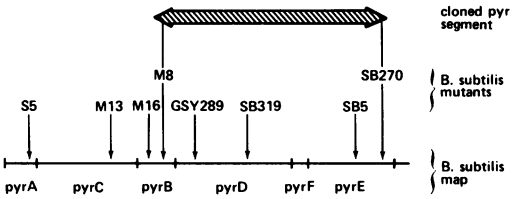

FIG. 3. *Genetic composition of the cloned pyr segment on the B. subtilis map. Map position of the markers and B. subtilis mutants indicated are those of Potvin et al. (13; personal communication). The shaded lines indicate the cloned pyr segment.*

lacking dihydro-orotate dehydrogenase, orotidylate pyrophosphorylase, or orotidylate decarboxylase as lysogens, complements these auxotrophic characters. As discussed in Results, this indicates that the inserted *B. subtilis pyr* segment has probably been transcribed and translated in *E. coli*. The transcription of such a segment is almost certainly started from the *B. subtilis* DNA, since λ promoters are known to be repressed in the lysogenic state. This brings us to the question: does the transcription begin at a correct *B. subtilis* promoter? The inserted *pyr* segment, judging by transformation experiments with *B. subtilis* mutants, does not contain the first three genes in the operon. If transcription begins at a correct promoter, this suggests that a promoter exists inside the *pyr* operon and near the beginning of the structural gene for DHO-DHase (*pyrD*). These three mutations in *E. coli* complement to the same degree (transducing frequencies all between 3 to 5%); thus, they probably belong to a single transcription unit. Once the DNA segment is recognized by *E. coli* RNA polymerase, the genes downstream should then be transcribed and translated until a normal stop signal appears. This possible explanation could be tested by constructing λ*pyr* derivatives with polar mutations or deletions within the operon and then assessing the level of complementation.

Our indirect evidence that proteins coded by *B. subtilis* DNA have been synthesized and utilized in *E. coli* cells deserves further inquiry into whether these proteins are accurate products, e.g., by direct isolation of the proteins coded by the *B. subtilis* DNA from the prototrophic lysogens and protein sequence analysis.

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