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Nucleotide Sequence of the Penicillinase Repressor Gene *penI* of *Bacillus licheniformis* and Regulation of *penP* and *penI* by the Repressor

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Bacillus licheniformis **penicillinase genes, *penP* and *penI*, are coded on a 4.2-kilobase *Eco*RI fragment of pTTE21 (T. Imanaka, T. Tanaka, H. Tsunekawa, and S. Aiba, J. Bacteriol. 147:776–186, 1981). The *Eco*RI fragment was subcloned in a low-copy-number plasmid pTB522 in *Bacillus subtilis*. *B. subtilis* carrying the recombinant plasmid pPTB60 (Tc^r *penP*$^+$ *penI*$^+$) was chemically mutagenized. Of about 150,000 colonies, two *penI*(Ts) mutant plasmids, pPTB60D13 and pPTB60E24, were screened by the plate assay at 30 and 48°C for penicillinase. By constructing recombinant plasmids between wild-type and mutant plasmids, the mutation points were shown to be located in a 1.7-kilobase *Eco*RI-*Pst*I fragment. The *Eco*RI-*Pst*I fragments of the wild-type plasmid and two mutant plasmids were sequenced. A large open reading frame, composed of 384 bases and 128 amino acid residues (molecular weight, 14,983), was found. Since the mutation points were located at different positions in the protein coding region (Ala to Val for pPTB60D13 and Pro to Leu for pPTB60E24), the coding region was concluded to be the *penI* gene. A Shine-Dalgarno sequence was found 7 bases upstream from the translation start site (ATG). A probable promoter sequence which is very similar to the consensus sequence was also found upstream of the *penP* promoter, but in the opposite direction. A consensus twofold symmetric sequence (AAAGTATTA CATATGTAAGNTTT) which might have been used as a repressor binding region was found downstream and in the midst of the *penP* promoter and also downstream of the *penI* promoter. The regulation of *penP* and *penI* by the repressor is discussed.**

The penicillinase gene, *penP*, from *Bacillus licheniformis* 749/C has been cloned (1, 7) and sequenced (24). We have independently cloned *penP* and the repressor gene *penI* from both the wild-type and constitutive strains of *B. licheniformis* 9945A (14). By use of the *penP* signal sequence, many protein secretion vectors have been constructed in *Bacillus subtilis* (3, 10, 13).

Using penicillinase markers in *B. licheniformis* 749 that were transferrable by transformation into strain 9945A, Sherratt and Collins (25) genetically analyzed the penicillinase locus and proposed a model for the regulatory mechanism of penicillinase biosynthesis. According to their genetic analysis, one regulatory gene (repressor gene *penI*) is 90% linked to *penP*, and a second regulatory gene (antirepressor gene) is 50% linked to *penI*. Although biochemical and genetic analyses suggest that the *penI* gene might be contiguous with the carboxy terminus of the *penP* gene (15, 17, 18, 25), there is little direct experimental evidence to show that the *penP* and *penI* genes are transcribed as one functional unit.

The purpose of this paper is to describe the location of *penI* and the nucleotide sequences of *penI* and *penP* promoter regions. Based on the sequences, the expression mechanisms of *penP* and *penI* are discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. Genes *penP* and *penI*, which code for penicillinase and its repressor, respectively, are located on a 4.2-kilobase (kb) *Eco*RI fragment of pTTE11 and pTTE21 (Fig. 1).

Transformation. Transformation of *Escherichia coli* with plasmid DNA was done as described earlier (14). Transformants were selected on L agar (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract, 5 g of NaCl, and 15 g of agar in 1 liter of deionized water [pH 7.0]) plus 20 μg of tetracycline per ml. Transformation of competent *B. subtilis* cells was also performed as previously described (11). Transformants were selected on L agar plus 25 μg of tetracycline per ml.

DNA manipulations. Preparation of plasmid DNA, cleavage of DNA with restriction enzymes, ligation of DNA, agarose and polyacrylamide gel electrophoresis for DNA

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference
E. coli C600-1	*leu-6 thr-1 thi-1 supE44 lacY fhuA21 hsdM hsdR* Trp$^-$	14
pMB9	Tc^r	14
pTTE11	Tc^r *penP*$^+$ *penI10*	14
pTTE21	Tc^r *penP*$^+$ *penI*$^+$	14
pTTE60D13	Tc^r *penP*$^+$ *penID13*[a]	This work
pTTE60E24	Tc^r *penP*$^+$ *penIE24*[a]	This work
B. subtilis MI113	*arg-15 trpC2* r^-_M m^-_M	12
pTB522	Tc^r	13
pPTB50	Tc^r *penP*$^+$ *penI10*	13
pPTB60	Tc^r *penP*$^+$ *penI*$^+$	This work
pPTB60D13	Tc^r *penP*$^+$ *penID13*[a]	This work
pPTB60E24	Tc^r *penP*$^+$ *penIE24*[a]	This work

[a] Temperature-sensitive *penI*(Ts) mutant.

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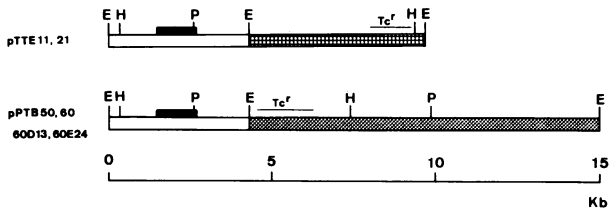

FIG. 1. Genetic structure of the plasmids. ▭, DNA fragment from *B. licheniformis*; ▦, pMB9; ▨, pTB52; ▬, penicillinase gene *penP*. *Eco*RI, *Hin*dIII, and *Pst*I cleavage sites are indicated by E, H, and P, respectively.

analysis, and isolation were all performed as described previously (13).

Detection of penicillinase-positive colonies on plates and the penicillinase assay. Penicillinase was assayed by the iodometric method as described previously (14). The method of detecting penicillinase-positive colonies on plates has also been described earlier (14).

DNA sequencing. DNA sequencing was performed by the Maxam-Gilbert (19) and dideoxy (22) methods, with an M13 sequencing kit (Takara Shuzo Co., Kyoto, Japan).

RESULTS

Isolation of *penI*(TS) mutant plasmids. The 4.2-kb *Eco*RI fragment of pTTE21 containing both *penP*$^+$ and *penI*$^+$ was subcloned in a low-copy-number plasmid pTB522. To isolate the temperature-sensitive *penI* mutant plasmid, *B. subtilis* MI113 carrying the recombinant plasmid pPTB60 (*penP*$^+$ *penI*$^+$) (Fig. 1) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) for 30 min and plated on L agar plus tetracycline. These colonies were replica plated and subjected to the plate test for penicillinase at 48°C. Of about 150,000 colonies, 200 colonies exhibited larger halos at 48°C than did the pTB60 (*penP*$^+$ *penI*$^+$) carrier of *B. subtilis*. These colonies were also used for the plate test at 30°C. Two colonies exhibited smaller halos than did the pPTB50 (*penP*$^+$ *penI*) carrier (Fig. 2). The two colonies were taken as candidates for *penI*(Ts) mutants and were designated strains D13 and E24.

Plasmid DNAs extracted from strains D13 and E24 were used to transform *B. subtilis* MI113. Since the Tc^r transformants showed the same phenotype as *penI*(Ts) mutants as mentioned above, these plasmids were considered to contain *penI*(Ts) and were named pPTB60D13 and pPTB60E24, respectively (Fig. 1).

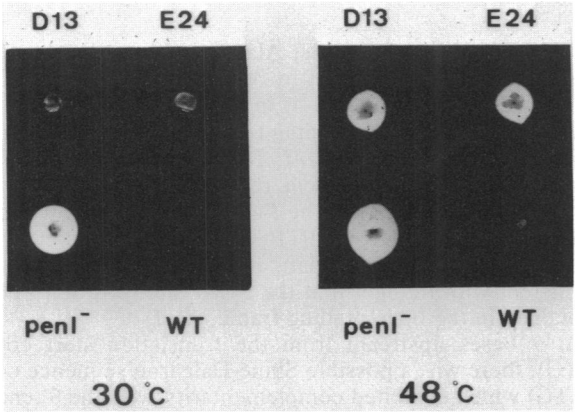

FIG. 2. Assay for penicillinase on polyvinyl alcohol plates. D13 and E24 are *B. subtilis* strains carrying pPTB60D13 and pPTB60E24, respectively (see text). penI$^-$, *B. subtilis*(pPTB50); WT, *B. subtilis*(pPTB60).

TABLE 2. Penicillinase production

Plasmid and temp (°C)	Time (h)	Activity (ODU)	Rate (ODU/h)	Ratio[a]	Derepression[b] (%)
pPTB60D13					
30	11.7	82	7.0	28.0	2
37	5.5	96	17.0	51.5	4
42	4.0	120	30.0	81.1	9
48	6.5	140	21.0	84.0	14
pPTB60E24					
30	11.7	66	5.6	22.4	2
37	4.6	92	7.0	21.2	1
42	4.0	130	33.0	89.2	10
48	6.2	310	49.0	196.0	33
pPTB50 (*penI*)					
30	11.7	4,100	350	1,400	100
37	5.0	2,900	480	1,450	100
42	4.6	1,800	320	865	100
48	6.8	1,000	150	600	100
pPTB60 (*penI*$^+$)					
30	12.0	3.0	0.25	1	<1
37	7.0	2.3	0.33	1	<1
42	6.0	2.2	0.37	1	<1
48	5.6	1.4	0.25	1	<1

[a] Ratio of penicillinase production rate (ODU per hour) to that of pPTB60 (*penI*$^+$) carrier at each cultivation temperature.

[b] Derepression of *penP* for pPTB50 (*penI*) carrier at each temperature was taken as 100%.

Penicillinase production by *B. subtilis* carrying a *penI*(Ts) plasmid. To characterize the temperature sensitivity of the mutant plasmids more precisely, penicillinase production in L broth by the plasmid carrier was examined as follows. *B. subtilis* MI113 carrying a plasmid (pPTB60D13, pPTB60E24, pPTB50, or pPTB60) was precultured overnight at 37°C, and a sample (1%) of each culture was inoculated into L broth plus tetracycline and cultivated at 30, 37, 42, and 48°C until the optical density at 660 nm was ≅1. The culture broth was assayed for total penicillinase activity (Table 2). Plasmids pPTB50 (*penI*) and pPTB60 (*penI*$^+$) were used as controls of complete derepression (full induction) and repression, respectively.

When the penicillinase production rate (in optical density units [ODU] per hour) for pPTB60 at each temperature was taken as 1, the relative values for pPTB60D13 and pPTB60E24 tended to increase with an increase in cultivation temperature, although the pPTB50 carrier exhibited the reverse tendency (Table 2). These results indicate that pPTB60D13 and pPTB60E24 have *penI*(Ts).

Derepression of the *penP* gene, i.e., temperature sensitivity of *penI*(Ts) at 30, 37, 42, and 48°C, is also shown in Table 2. pPTB60E24 was more typically temperature sensitive than was pPTB60D13, although the two *penI*(Ts) genes are leaky.

Location of *penI*(Ts) mutation. To determine the location of *penI*(Ts), some recombinant plasmids were constructed from pPTB60 and mutant plasmids (pPTB60D13 and pPTB60E24) (Fig. 1). When the 4.2-kb *Eco*RI fragment of pPTB60 was replaced by the corresponding fragment from mutant plasmids, the recombinant plasmids showed the *penI*(Ts) phenotype. Likewise, it was shown that two

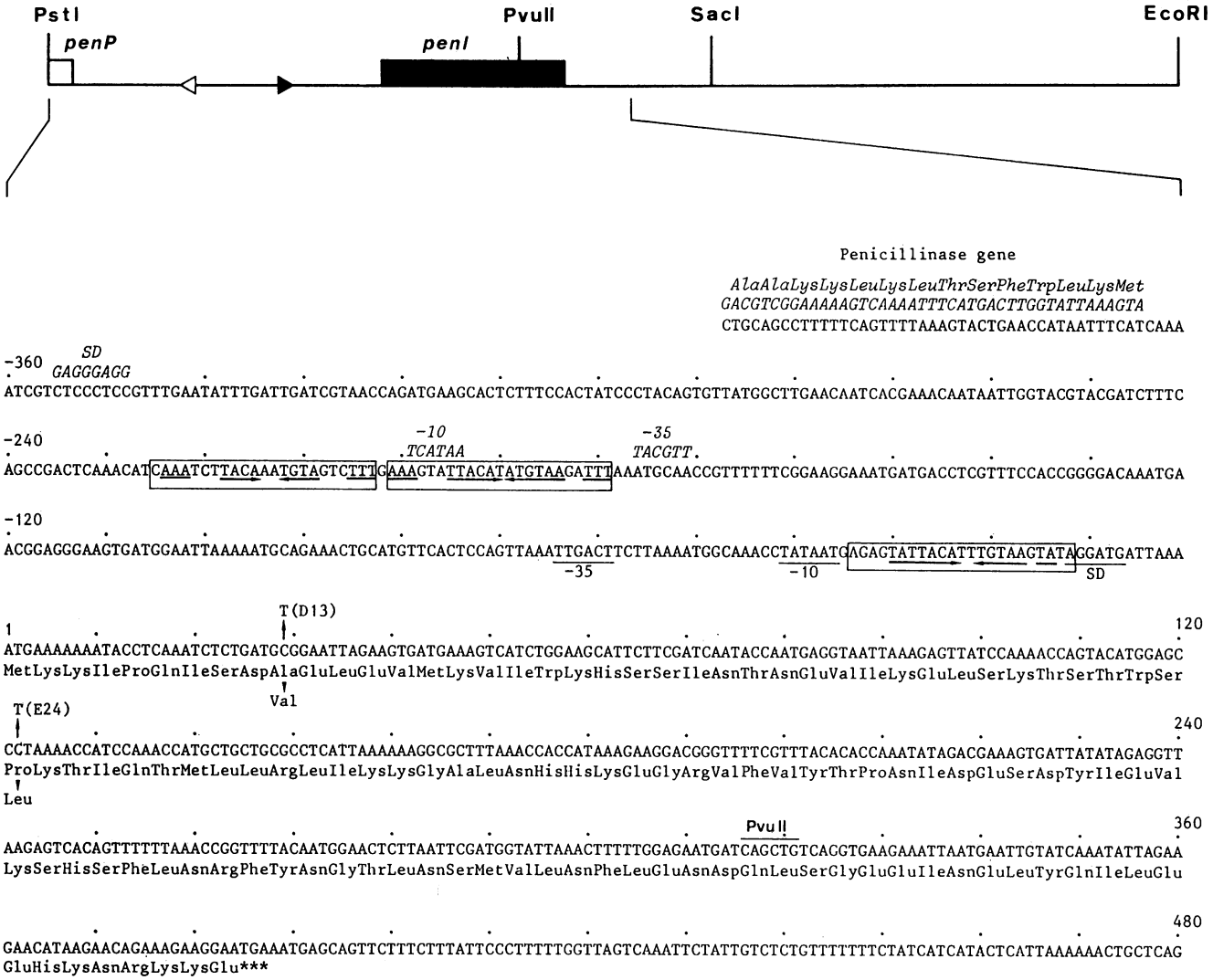

FIG. 3. Nucleotide sequences of pPTB60 and its *penI*(Ts) mutant plasmids. ◁ and ▶, Promoters of *penP* and *penI*, respectively. The position of the first nucleotide of the coding region is defined as +1. The amino acid sequence of the coding region is given beneath. The Shine-Dalgarno sequence and promoter (−35 and −10 regions) are shown. The nucleotide sequence, amino acid sequence, Shine-Dalgarno sequence, and promoter for the opposite direction are in italics. Twofold symmetric regions are surrounded by boxes. — ⟶ ⟵ —, Regions for the inverted repeat. Nucleotide substitutions for pPTB60D13 (strain D13) and pPTB60E24 (strain E24) are indicated by small vertical arrows. The corresponding substitutions of amino acid are indicated by arrowheads.

penI(Ts) mutations were located on a 1.7-kb *Eco*RI-*Pst*I fragment containing a part of the *penP* gene (Fig. 1).

Nucleotide sequences of *penI* and *penI*(Ts). To determine the nucleotide sequence of the *penI* region, 4.2-kb *Eco*RI fragments of pPTB60D13 and pPTB60E24 were transferred into pMB9 in *E. coli*. The recombinant plasmids were designated pTTE60D13 and pTTE60E24, respectively. All the nucleotide sequences of the 1.7-kb *Eco*RI-*Pst*I fragments from pTTE21 (*penI*$^+$) and the two *penI*(Ts) plasmids were determined. The sequence flanking the *penI* gene is shown in Fig. 3.

A large open reading frame extends 384 base pairs (bp) from nucleotide 1 to 384. It was confirmed that the whole nucleotide sequence was identical between pTTE21 (wild-type *penI*) and *penI*(Ts) mutant plasmids except for one base for each mutant; namely, cytosine at position 29 in the wild-type plasmid was replaced by thymine in pTTE60D13, and cytosine at position 122 was replaced by thymine in pTTE60E24. In other words, Ala at position 10 (Ala-10) of the wild type was replaced by Val-10 in pTTE60D13, and Pro-41 was replaced by Leu-41 in pTTE60E24. These results indicate that the protein coding region is the structural gene of penicillinase repressor *penI*. The protein coding region is composed of 128 amino acid residues (molecular weight, 14,983). In addition, when the *Pvu*II-*Eco*RI fragment shown in Fig. 3 was deleted from pPTB60, the plasmid exhibited the *penI* mutant phenotype (data not shown). The result is consistent with the fact that the *Pvu*II site (position 316) is contained in the open reading frame, *penI*.

At 7 bases upstream from the translation start codon (ATG), there was a possible Shine-Dalgarno sequence (AGGATG) which exhibited complementarity with the 3′ end of *B. subtilis* 16S rRNA, HO-UCUUUCCUCCACUAG—(21, 23). There was a typical promoter (TTGACT for the −35 region and TATAAT for the −10 region with 17-bp space) which closely resembled the consensus sequence (TTGACA

TABLE 3. Nucleotide sequences of protein binding regions

Regulatory protein	Regulated gene or operator	Sequence	Reference
TrpR	*trp* operon	ATCGAACTAGTTAACTAGTACGCA	8
	trpR	ATCGTACTCTTTAGCGAGTACAAC	
	aroH	AATGTACTAGAGAACTAGTGCATT	
	Consensus	ATCGTACTAGTTAACTAGTACANN[a]	
TetR (Tn*10*)	$tetAo_L$	ACTCTATCATTGATAGAGT	16
	$tetRo_R$	TCCCTATCAGTGATAGAGA	
	Consensus	NNNNTATCANTGATANNNN	
LexA	*recA*	TACTGTATGAGCATACAGTA	2, 26
	lexA	TGCTGTATATACTCACAGCA	
	lexA	AACTGTATATACACCCAGGG	
	Consensus	NNCTGTATATATATACAGNN	
LacI	*lac* operon	AATTGTGAGCGGATAACAATT	6
GalR	*gal* operon	AATTCTTGTGTAAACGATTCCACTAATT	5
λ *c*I Cro	Consensus	TATCACCGCCGGTGATA	9
CAP	Consensus	AANTGTGANNTNNNNCANATT	4
PenI	*penP*	AAAGTATTACATATGTAAGATTT	This work
	penP	CAAATCTTACAAATGTAGTCTTT	
	penI	AGAGTATTACATTTGTAAGTATA	
	Consensus	AAAGTATTACATATGTAAGNTTT[a]	

[a] Consensus sequence was determined in this table.

for the −35 region and TATAAT for the −10 region) for *B. subtilis* RNA polymerase $E\sigma^{55}$ (23). The probable *penI* promoter was located about 100 bp upstream of the *penP* promoter (20), but in the opposite direction.

DISCUSSION

To analyze the regulatory mechanism of the *B. licheniformis* penicillinase gene *penP*, we isolated two temperature-sensitive repressor genes, *penI*(Ts), and determined the nucleotide sequences of the repressor genes *penI* and *penI*(Ts). The sequences of wild-type *penI* and the two mutant temperature-sensitive genes were completely identical except for one base in each case.

The probable *penI* promoter was located about 100 bp upstream from the known *penP* promoter (20), and the directions of the two promoters were opposite (Fig. 3). Although it has been believed that the orientation of the penicillinase gene in *B. licheniformis* is *penO* (operator-gene)-*penP*-*penI* and that the *penP* and *penI* genes are transcribed as one unit (15, 17, 18, 25), the nucleotide sequence analysis of this region indicates that *penP* and *penI* genes are transcribed independently as different operons.

When we searched open reading frames in two strands for the 1.7-kb *Eco*RI-*Pst*I fragment (Fig. 1), there were two open reading frames capable of encoding a protein of 100 amino acids or more in this region. One was the sequence for *penI* (nucleotides 1 to 384), and the other followed the TGA termination codon of *penI* gene. The second open reading frame started from nucleotide 389 and went across the *Eco*RI fragment (Fig. 1 and 3). It appeared to be preceded by a Shine-Dalgarno sequence (AAGAA; nucleotides 379 to 384). There are more genes involved in the regulation of *penP* expression than in that of *penI* (15, 25). In this context, a polycistronic mRNA might contain another regulatory gene in addition to *penI*. Since the second open reading frame is not completed in the cloned *Eco*RI fragment (Fig. 1), the cloning of a fragment that completes the open reading frame is now in progress.

Since the penicillinase repressor, the peptide product of *penI*, must have bound to the operator site of the *penP* gene, a possible operator site was searched for in this region. The known nucleotide sequences that were recognized and bound by a specific regulatory protein in *E. coli* are listed in Table 3. These sequences have common features as follows: (i) the sequence is around 20 bp in size, (ii) the sequence contains a twofold symmetric region, and (iii) a group of operators which can be recognized by a specific regulatory protein (e.g., TrpR, TetR, LexA) exhibits the consensus sequence. Based on these features, we searched for such a nucleotide sequence in the promoter regions of *penP* and *penI* and found very similar symmetric sequences downstream and in the midst of the *penP* promoter and also downstream of the probable *penI* promoter (Fig. 3). The consensus sequence is AAAGTATTACATATGTAAG NTTT (Table 3). It is most likely that these sequences are recognized and bound by penicillinase repressor as operators for *penP* and *penI*. The idea leads to the argument that the expression of the *penI* gene is autoregulated as in the *trpR*, *tetR*, and *lexA* genes (Table 3) and that the repression of *penP* would be more strict than that of *penI*, because two repressor binding sequences are in tandem for *penP*, but only one for *penI*. Similarly, it was reported that LexA protein can bind to both the *recA* operator at one site and the

lexA operator at two tandem sequences as an autorepressor (26).

If a temperature-sensitive repressor was produced by a mutant plasmid, the amount of repressor protein would also increase at higher temperatures because of the autoregulation system and the repression level of *penP* would be relatively enhanced. This argument would account for the low level (33%) of derepression by *penI*(Ts) mutant plasmid pPTB60E24 at 48°C (Table 2).

To investigate this *penP-penI* system further, the isolation of repressor protein is now in progress.

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