Cloning and Nucleotide Sequence of the Penicillinase Antirepressor Gene penJ of Bacillus licheniformis

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The penicillinase antirepressor gene, penJ, of Bacillus licheniformis ATCC 9945a was cloned in Escherichia coli by using pMB9 as a vector plasmid. The penicillinase gene, penP, its repressor gene, penI, and penJ were encoded on the cloned 5.2-kilobase HindIII fragment of the recombinant plasmid pTTE71. The penJ open reading frame was composed of 1,803 bases and 601 amino acid residues (molecular weight, 68,388). A Shine-Dalgarno sequence was found 7 bases upstream from the translation start site. Since this sequence was located in the 3'-terminal region of the penJ gene, penJ might be transcribed together with penI as a polycistronic mRNA from the penJ promoter. Frameshift mutations of penJ were constructed in vitro from pTTE71, and the penJ mutant gene was introduced into B. licheniformis by chromosomal recombination. The transformant B. licheniformis UI73 (penP+ penI+ penJ) turned out to be uninducible for penicillinase production, whereas the wild-type strain (penP+ penI+ penJ) was inducible. Only when these three genes (penP, penI, and penJ) were simultaneously subcloned in Bacillus subtilis did the plasmid carrier exhibit inducible penicillinase production, as did wild-type B. licheniformis. It was concluded that penJ is involved in the penicillinase induction. The regulation of penP expression by penI and penJ is discussed.

The penicillinase gene, penP, of Bacillus licheniformis 749/C has been cloned (3, 7) and sequenced (20). We have independently cloned penP and the repressor gene, penI, from both the wild-type and constitutive strains of B. licheniformis ATCC 9945a (13) and determined the nucleotide sequence of penI and the flanking regions (8, 12). In the process, a new open reading frame, designated penJ, was found immediately downstream of the penI gene.

Genetic analysis of the B. licheniformis 9945a system by transformation (25) has given evidence of the complexity of the penicillinase system. The structural gene, penP, is very closely linked to penI, slightly linked to another regulator gene (R1), and not linked at all to a further regulator gene (R2) (5, 25). However, the role of the R1 and R2 gene products has not been made clear yet.

The purpose of this paper is to describe the cloning and nucleotide sequence of the antirepressor gene penJ (see below) that might correspond to the positive regulator gene R1. The regulation of penicillinase induction by the products of penI and penJ is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. The penicillinase gene, penP, and its repressor gene, penI, from B. licheniformis ATCC 9945a are located on a 4.2-kilobase (kb) EcoRI fragment of pTTE21 (Fig. 1). Since a large open reading frame was found at 5 base pairs downstream from the penI gene (8), the newly found protein-coding region was named penJ.

Transformation. Transformation of Escherichia coli with plasmid DNA was done as described previously (13). 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]) containing 20 μg of tetracycline or ampicillin per ml. Transformation of competent Bacillus subtilis cells was also performed as previously described (9). B. subtilis transformants were selected on L agar containing 25 μg of tetracycline per ml. Transformation of B. licheniformis competent cells with chromosomal DNA was done as described previously (13).

DNA manipulations and analyses. Preparation of plasmid DNA, cleavage of DNA with restriction enzymes, repair and ligation of DNA, gel electrophoresis for DNA analysis, and isolation were all performed as described previously (11). Chromosomal DNA of B. licheniformis was prepared as mentioned previously (13). DNA was sequenced by the Maxam-Gilbert method (16) and the dideoxy method (18), with an M13 sequencing kit (Takara Shuzo Co., Kyoto, Japan). The sequencing was done on both strands, and all restriction sites were sequenced across.

Penicillinase assay. Penicillinase was assayed by the iodometric method as described previously (13). The method of detecting penicillinase-positive colonies on LP plates (L agar containing 0.75% [wt/vol] polyvinyl alcohol) has been described previously (13). The inducibility of penicillinase production was examined by cultivating microbial cells either on LPC plates (LP plus 5 μg of cephalosporin C [an inducer] per ml) or in liquid medium.

Computer analysis of hydrophatic characteristic of the protein. The AC program to assess hydropathy, as described by Kyte and Doolittle (14), was translated to BASIC and implemented on an NEC PC-8001 computer (Nippon Electric Co., Tokyo, Japan). The mean hydrophathy of a protein was evaluated by a moving segment of 9 amino acid residues along the sequence as mentioned previously (15).

RESULTS

Cloning of the entire penJ gene. Since only the 5'-terminal region of penJ existed on the 4.2-kb EcoRI fragment of pTTE21, we attempted to clone the entire penJ gene. The chromosomal DNA of B. licheniformis FD0120 (penP+ penJ+)}
penI+ penJ+) was cleaved by HindIII and ligated with the HindIII digest of pMB9. The ligation mixture was used to transform E. coli C600-1. Plasmids extracted from ampicillin-resistant (penP+) colonies were analyzed by treatment with HindIII and agarose gel electrophoresis. The smallest recombinant plasmid was screened and named pTTE71. The plasmid was found to be composed of pMB9 and the cloned 5.2-kb HindIII fragment which included the region 1.4 kb downstream from the EcoRI site in the penJ gene (Fig. 1).

**Nucleotide sequence of penJ.** All the nucleotide sequences of penI (8) and the following region down to the HindIII site (right-hand side in Fig. 1) were determined. The complete nucleotide sequence of penJ and its deduced amino acid sequence are shown in Fig. 2.

The penJ open reading frame extends 1,803 base pairs, from nucleotides 1 to 1803, and codes for 601 amino acid residues (molecular weight, 68,388). The sequence AGAAAGAAGG was found at -16 to -7 base pairs upstream from the initiation codon of penJ. It might be a possible Shine-Dalgarno sequence which exhibits complementarity with the 3' end of B. subtilis 16S rRNA, designated AGCUUCUCUGUCCAG- (17, 19). This sequence was located in the 3'-terminal portion of the penJ gene (Fig. 2). These results indicate that penJ could be transcribed together with penI as a polycistronic mRNA from the penI promoter, located at positions -452 to -424 in Fig. 2 (8).

**Role of penJ in B. licheniformis.** To analyze the role of penJ in penicillinase regulation, we attempted to construct a penJ mutant gene as follows. Plasmid pTTE71 (penJ+) was partially digested by EcoRI, and the cohesive ends were paired with the large fragment of E. coli DNA polymerase I. The 10.5-kb linear DNA was electrophoretically isolated and self-ligated. The ligation mixture was used to transform E. coli C600-1. A new plasmid, pTTE73 (penJΔE), whose EcoRI site in the penJ region (Fig. 1) was eliminated, could be isolated from the ampicillin-resistant transformants. Likewise, another mutant plasmid, pTTE74 (penIΔJ), was constructed by ScaI cleavage of pTTE71 and treatment with T4 DNA polymerase. Both penI mutations were frameshift mutations.

By using these plasmids, transformation of B. licheniformis competent cells by recombination of chromosomal DNA was carried out as follows. Strain C01P1013 (penP+ penP+ penJ+ met-1) was transformed with a mixture of chromosomal DNA of strain M015-1 (penP+ penP+ Met+) and a BamHI digest of one of the various plasmids (pTTE71, pTTE73, and pTTE74) (Table 2). Penicillinase-positive transformants (penP+) were screened from the Met+ transformants. Among them, constitutive strains (penI), irrespective of the presence or absence of penJ, could be easily distinguished from inducible strains (wild type) by their larger and clear halos on LP plates. Inducible strains could form larger halos on LPC plates than on LP plates, and uninducible strains could form the same-sized halos on LP and LPC plates. Therefore, inducible strains could be distinguished from uninducible strains by their larger halos on LPC plates.

When pTTE71 (penP+ penI+ penJ+) was used as donor DNA, most of the penP+ transformants were inducible, and no uninducible strains could be found. In contrast, when pTTE73 (penP+ penI+ penJ+) and pTTE74 (penP+ penI+ penJ+) were used, the majority of penP+ transformants were uninducible (Table 2). These results indicate that penJ is one of the regulatory genes for penP and that the gene product PenJ must be involved in the induction of penicillinase.

Patterns of recombination in the pen locus are shown in Fig. 3. Although it has been shown by hetero- and meopolyploid analyses (13) that penI0 is a repressor-deficient mutant gene, localization of the mutation locus has not been determined yet. penJΔE gave rise to some inducible recombinants with penI0, but penJΔS did not (Table 2). These facts suggest that the mutation point in penI0 is fairly close to that in penJΔS (Fig. 3).

**Expression of penJ in B. subtilis.** To examine the role of penJ in more detail, 5.2-kb HindIII fragments of pTTE71 and pTTE73 were cloned into the HindIII site of pTBS522 in B. subtilis. The recombinant plasmids thus obtained were designated as pPTB71 (penP+ penI+ penJ+) and pPTB73 (penP+ penI+ penJ+), respectively.

The result of the penicillinase plate assay for the plasmid carriers of B. subtilis is shown in Fig. 4. The growth of the pPTB73 (penJ) carrier (spot A) was slightly inhibited on the LPC plate, despite the normal growth pattern for the pTTE71 (penJ+) carrier (spot B) on the same plate. The

![FIG. 1. Physical map of the pen locus of B. licheniformis ATCC 9945a and the cloned regions in plasmids. Genes (penP, penI, and penJ) are indicated by boxes. Plasmids pTTE21, pTTE71, and pPTB77 carry 4.2-kb EcoRI, 5.2-kb HindIII, and 3.9-kb FspI fragments of the pen locus, respectively. Δ, Mutation points of penJ mutant plasmids (pTTE73 and pTTE74).](http://jb.asm.org/)

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**TABLE 1. Bacterial strains and plasmids**

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tr>
<td>E. coli C600-1</td>
<td>leu-6 thr-1 thi-1 supE44 lacY fhuA21 hisD42 Trp-</td>
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<td>Ml113</td>
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<td></td>
</tr>
<tr>
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<td>pepAI met-1</td>
<td>13</td>
</tr>
<tr>
<td>C01P1013</td>
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<td>13</td>
</tr>
<tr>
<td>M015-1</td>
<td>pepAI penI10 penP81 penP83</td>
<td>13</td>
</tr>
<tr>
<td>UI73</td>
<td>pepI penIΔE</td>
<td>This work</td>
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<td></td>
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<tr>
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<td>Tc'</td>
<td>13</td>
</tr>
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<td>pTTE21</td>
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<td>This work</td>
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<tr>
<td>pPTB77</td>
<td>Tc' penP+ penI+ penJΔS</td>
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</tr>
</tbody>
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**FIG. 1. Physical map of the pen locus of B. licheniformis ATCC 9945a and the cloned regions in plasmids. Genes (penP, penI, and penJ) are indicated by boxes. Plasmids pTTE21, pTTE71, and pPTB77 carry 4.2-kb EcoRI, 5.2-kb HindIII, and 3.9-kb FspI fragments of the pen locus, respectively. Δ, Mutation points of penJ mutant plasmids (pTTE73 and pTTE74).**
The complete sequence of the *penI* coding region is given below the nucleotide sequence. The Shine-Dalgarno (SD) sequence and promoter (−35 and −10 regions) are shown. *SacI*, *EcoRI*, *FspI*, and *HindIII* sites are also shown.

![Diagram](http://jb.asm.org/Downloadedfrom)

**FIG. 2.** Nucleotide sequence of the *penI* gene. The sequence is presented from the *penI* promoter region to the right-hand *HindIII* site in Fig. 1. The complete sequence of the *penI* coding region was reported previously (8). The position of the first nucleotide of the *penI* coding region is defined as +1. The amino acid sequence of the coding region is given below the nucleotide sequence. The *Shine-Dalgarno* (SD) sequence and promoter (−35 and −10 regions) are shown. *SacI*, *EcoRI*, *FspI*, and *HindIII* sites are also shown.

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pPTB71 carrier exhibited a larger halo than did the pPTB73 carrier on the LPC plate, although their halos on the LP plate were of the same size.

To exclude the involvement of regions other than penP, penI, and penJ, a smaller recombinant plasmid carrying the 3.9-kb FspI fragment in the HindIII site of pTBS22 was also constructed and was named pPTB77 (Fig. 1). Since B. subtilis carrying pPTB77 showed a large halo on the LPC plate (Fig. 4, spot C), it was concluded that only three genes (penP, penI, and penJ) are necessary and sufficient for the basic regulation of penicillinase expression in terms of repression and induction.

Comparison of the penicillinase induction in B. subtilis and B. licheniformis. It is well known that induction of the penicillinase in B. licheniformis occurs slowly and requires two or three generations for the maximum induction level to be reached (24). This prolonged induction is a typical characteristic in the regulation of penicillinase. To compare the induction patterns in B. subtilis and B. licheniformis, we examined penicillinase production by both strains. B. subtilis M1113 carrying a plasmid (pPTB71 or pPTB73), B. licheniformis FD0120 (penJ+), and B. licheniformis UI73 (penJ/ΔE) were precultured overnight. A sample of each preculture was inoculated into fresh L broth and cultivated at 37°C until the optical density at 660 nm was ca. 0.1; 5 μg of cephalexin C per ml was then added to each culture, and the cultivation was continued for several hours. The culture broth was assayed for penicillinase activity (Fig. 5).

Penicillinase production by the penJ mutants B. subtilis M1113 (pPTB73) and B. licheniformis UI73 could not be increased by the inducer, whereas enzyme production by the strains carrying the wild-type penJ gene, B. subtilis M1113 (pPTB71) and B. licheniformis FD0120, was induced to the same level. In both the penJ+ strains, about 2.1 generations were required for the maximum induction level to be reached after the addition of cephalexin C. The difference in time for full induction of enzyme (Fig. 5) could be explained by the difference in growth rates of these two strains.

![FIG. 3. Patterns of recombination in the pen locus. Crossovers in regions 1 and 2 were of recombinant genotype penP+ pen10 penJ+ and were of constitutive phenotype; crossovers in regions 1 and 3 were of genotype penP+ penI+ penJ+ and were inducible; and crossovers in regions 1 and 4 were penP+ penI+ penJ+ΔE or penJ+ΔS and were uninducible. ×, Mutation.](image)

![FIG. 5. Kinetics of penicillinase induction in B. licheniformis and B. subtilis. Cephalexin C as an inducer was added at time zero. Symbols: C, wild-type penJ+ strains [B. licheniformis FD0120 and B. subtilis M1113 (pPTB73)]; O, penJ mutants [B. licheniformis UI73 and B. subtilis M1113 (pPTB73)].](image)

**TABLE 2. Transformation of B. licheniformis C01P1013**

<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>No. of penicillinase-positive transformants</th>
<th>No. of Met-positive transformants</th>
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</thead>
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<tr>
<td>M015-1 + pTTE71</td>
<td>52/176</td>
<td>1/176</td>
</tr>
<tr>
<td>M015-1 + pTT73</td>
<td>73/175</td>
<td>4/175</td>
</tr>
<tr>
<td>M015-1 + pTT74</td>
<td>20/94</td>
<td>3/94</td>
</tr>
</tbody>
</table>

*p penP+ penI+ penJ+.

*p penP+ penI+ penJ+.

*p penP+ penJ+.

*p penP+ penJ+.

**DISCUSSION**

We have identified a new regulatory gene, penJ, which is involved in the induction of penP (encoding penicillinase) in B. licheniformis. The penJ gene is located at 5 base pairs downstream of penI (encoding the repressor), and these two genes might be transcribed as a polycistronic mRNA from the penI promoter. Expression of penP is negatively controlled by the penJ product and positively controlled by the penJ product as the antirepressor not only in B. licheniformis but also in B. subtilis.

PenJ is a large protein (molecular weight, 68,388) in comparison with other regulatory proteins in bacteria. The molecular weights of the negative regulatory proteins such as the repressor range from 6,800 for P22 Cro (22) to 38,350 for LacI (6), whereas those of the positive factors and the positive and negative regulatory proteins range from 23,619 for catabolite gene activator protein (1) to 94,000 for MalT (23). The higher the molecular weight, the more complex are the functions of the regulatory protein that tend to appear. In fact, the largest regulatory protein, MalT, can interact with catabolite gene activator protein and activate transcription of the malPQ operon (4). From this point of view, it might be understandable that the PenJ protein is involved in the
induction of penP expression by interacting with the PenI protein (repressor; molecular weight, 14,983 [8]).

We searched for a specific amino acid sequence in the PenJ protein that was similar to the consensus sequence (Ala-N-N-N[hydrophobic]-Gly-N[hydrophobic]-N-N-N-N-Val[Ile]) found in many DNA-binding proteins (21). In fact, such amino acid sequences were found (Ala-Ile-Leu-Ala-Gly-Thr-Pro-Ser-Val-Ser-Ile [333 to 343]) and (Ala-Leu-Ile-Ala-Gly-Leu-Val-Leu-Thr-Phe-Val [336 to 326]), the direction of the latter sequence being from the carboxy to the amino terminus as shown for a repressor (2). Therefore, the PenJ protein might interact with DNA.

According to Sherratt and Collins (25) and Collins (5), three genes are required for penicillinase regulation in B. licheniformis. The penicillinase gene, penP, is 90% linked to a negative regulatory gene (penI), 50% linked to another regulatory gene (R1), and not linked at all to a further regulatory gene (R2). The penI gene has been identified and sequenced previously (8, 13). The penI gene is located upstream of the penP gene, and the transcription from the penI promoter is in the opposite direction. In addition, the penJ gene is transcribed together with the penI gene. In this context, the R1 gene might correspond to the penJ gene. It is well known that it takes a long time (about 2 to 3 h) for the full induction of penP after the addition of inducer (24). The third regulatory gene, R2, might play a role in this gradual induction. However, its function is not clear yet.

In summary, the following speculation on the penicillinase regulatory mechanism is presented (Fig. 6). In the absence of inducer, the synthesis of penicillinase, PenP, is repressed by the binding of PenI repressor to the operator region in the middle of and downstream of the penP promoter (8). In addition, PenI binds to the operator region of penI and regulates the synthesis of PenI itself and PenJ. When the inducer (cephalosporin C) is added to the culture medium, the synthesis of PenP can be induced by the cooperative functions of endogenous inducer, PenI (antirepressor), and PenI (repressor). However, whether or not the primary inducer in the external environment can penetrate into the cytoplasm is obscure. If the primary inducer cannot penetrate, it must regulate some secondary effector (endogenous inducer) in the cell, which can act at the genetic level. This kind of indirect regulation might explain the long time lag in penicillinase induction.

If PenJ protein is sensing the primary inducer, it might be expected to be a membrane protein. Therefore the hydrophobic character of PenJ was evaluated (Fig. 7). The portions above the solid line in the figure denote hydrophobic segments, whereas those projecting below the line are hydrophilic segments. The average degree of hydrophobicity of many soluble proteins is around −0.4 (14). In comparison...
with this value, three highly hydrophobic segments were found in the amino-terminal region of PenJ. This fact suggests that PenJ is a membrane-bound protein. However, the real localization and function of PenJ remain to be investigated.

In the work described in this paper, we found another open reading frame, starting from position 1,900 (downstream of penJ) (Fig. 2). A possible Shine-Dalgarno sequence GGAGG (17, 19) at positions 1,888 to 1,892 is located in front of the open reading frame. Therefore the open reading frame might be a member of the penI operon, because no apparent terminator sequence is found between penJ and the following reading frame. The open reading frame, however, is not terminated in the cloned 5.2-kb HindIII fragment. To investigate whether the open reading frame is a penP regulatory gene such as R2 mentioned above, cloning of the entire open reading frame is now in progress.

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