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Genetic Organization of the Recombinant *Bacillus pasteurii* Urease Genes Expressed in *Escherichia coli*

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The genetic organization of the urease gene cluster from an alkalophilic *Bacillus pasteurii* was determined by subcloning and Tn5 transposon mutagenesis of a 10.7 kilobasepair cloned fragment. A region of DNA between 5.0 and 6.0 kb in length is necessary for urease activity. *In vitro* transcription-translation analysis of transposon insertion mutants of the cloned urease genes demonstrated that the major (M_r 67,000) and minor (M_r 20,000) structural peptides of urease are encoded at one end of the urease gene cluster and at least 3 additional polypeptides are encoded by adjacent DNA sequences.

Urease (EC 3.5.1.5), which hydrolyses urea to ammonia and carbon dioxide, has important roles in environmental transformations of nitrogenous compounds including aspects of nitrogen metabolism of microorganisms, plants, and herbivores (4, 10, 16). Additionally, it plays important roles in the development of certain human and animal diseases and in clinical diagnosis of blood urea (3, 17, 21). The best studied urease is that from jack bean, a hexameric protein composed of six identical 90,000 Da subunits containing two nickel atoms per subunit (2). Recently, the genetic organization of several bacterial urease gene clusters have been studied (8, 9, 18, 20, 23) and the bacterial enzymes have been shown to differ from the plant ureases in subunit composition and molecular weight (16). The protein from *Bacillus pasteurii* was the first bacterial urease purified (13). Similarly, isolation of a 10.7 kb DNA fragment from the alkalophilic *Bacillus pasteurii* ATCC 11859 was the first reported cloning and expression of a urease gene cluster (11). Christians and Kaltwasser reported the *Bacillus pasteurii* urease was a homotetramer of a 65,000 subunit (6); however, *Bacillus pasteurii* urease expressed in *Escherichia coli* was shown to consist of four identical subunits (M_r 67,000) and an additional, distinct subunit (M_r 20,000) (14).

In this report we present the physical restriction map and genetic organization of the *Bacillus pasteurii* urease gene cluster.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *Bacillus* urease gene cluster characterized in this study was located on the previously described plasmid pGU66 (11), constructed by inserting a *Hin*dIII fragment of *Bacillus pasteurii* ATCC 11859 chromosomal DNA into the *E. coli-B. subtilis* shuttle vector, pGR71. The 10.7 kb *Hin*dIII fragment from pGU66 containing the urease genes was inserted into the same site of pBR322 and denoted pBU11. *E. coli* HB101 (*sup*E44, *rec*A, *ara*14, *pro*AZ, *lac*Y1, *gal*KZ) was used for routine transformation (22) and *E. coli* JM109, *E. coli* LE392, and *E. coli* 271 (*rec*A, *rm*, *rk*) for transposon mutagenesis. Plasmid vectors pBR322 (Amp^R, Tet^R), pBR328 (Amp^R, Tet^R, Cm^R), and their derivatives were extracted by a miniprep procedure (22).

Culture and Selection of Urease Positive Strains

For transformation and general culture, *E. coli* strains were grown in Luria broth (LB) containing ampicillin (60 μg/ml) or appropriate antibiotics. For the selection of urease positive colonies, a modified Christensen's urea plate was used. The medium consisted of 0.1% peptone, 0.08% yeast extract, 0.2% KH_2PO_4, 0.5% NaCl, 0.0012% phenol red, 0.1% glucose, 2% filtered urea, and 60 μg/ml ampicillin (pH 6.8). The urea R broth is composed of 0.1% yeast extract, 0.045% KH_2PO_4, 0.048% Na_2HPO_4, 0.0012% phenol red, and ampicillin. Urease positive colonies were selected by their color reaction, turning the plates or broth from yellow to red. All cultures were grown at 37°C and aerated by shaking.

Subcloning

Restriction endonuclease digestion and ligation of isolated DNA fragments were performed according to the enzyme supplier's recommendations. The sizes of DNA fragments were analyzed by agarose gel electrophoresis. Isolation of desired DNA fragments was perfomed by elution onto a DEAE-cellulose membrane (Schleicher and Schuell NA4S) according to the method of Dretzen (7).

Transposon Mutagenesis

The phage stock used in transposon mutagenesis was obtained by infection of *E. coli* LE392 with lambda 467 (λ::Tn5) carrying kanamycin resistance. *E. coli* 271 (*sup*^{O}, λ^{S}) carrying pBU11 was infected with λ::Tn5 to M.O.I=0. 1 by the method of de Bruijn and Lupski (5). The pBU11 plasmids containing inserted transposons were isolated from kanamycin and ampicillin resistant transductants by the method of Birnboim and Doly (1), and transformed into *E. coli* DH1 or *E. coli* JM109 using LB plates containing kanamycin and ampicillin. *E. coli* DH1 (pBU 11::Tn5) and *E. coli* JM109 (pBU11::Tn5) transformants possessing plasmids with the transposon were screened for urease activity by the urea R broth assay. The site of transposon insertion was estimated by electrophoresis on 0.7% agarose gel following digestion of mutant plasmids with *Bgl*II, *Bam*HI, and *Kpn*I.

Gel Electrophoresis

For restriction mapping, DNA fragments were analyzed by electrophoresis on 0.7% or 0.8% agarose gels and the sizes were estimated in comparison to *Hin*dIII digestion fragments of phage λ. The polypeptides synthesized by *in vitro* transcription-translation were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (12). The running gel was comprised of a 10~18% acrylamide gradient and the stacking gel used 4.5% polyacrylamide gel. After separation, the gel was fixed in 7% acetic acid and 5% glycerol, dried, and exposed to X-ray film. Low range prestained SDS-PAGE standards (Bio-Rad Co.) consisting of phosphorylase b (M_r 106, 000), bovine serum albumin (M_r 80,000), carbonic anhydrase (M_r 32,500), soybean trypsin inhibitor (M_r 27,500), and lysozyme (M_r 18,500) were used as references to estimate the molecular weight.

***In Vitro* Transcription-Translation**

Mutant pBU11::Tn5 plasmids for *in vitro* transcription-translation analysis were isolated by large-scale preparation (22), and purified by Superose 6 HR (1.0×30 cm) chromatography. The DNA peak was precipitated with ethanol in the presence of sodium acetate and then dissolved with TE buffer (pH 8.0). *In vitro* transcription-translation was performed by using a procaryotic DNA-directed translation kit and cell-free coupled transcription-translation system of Amersham (Arlington Heights, IL) according to the supplier's recommendation. To each reaction, 30 μCi of L-[^{35}S]methionine was added.

Autoradiography

To calculate the amount of ^{35}S incorporated into each polypeptide produced by *in vitro* transcription-translation, the polypeptides were precipitated with trichloroacetic acid (TCA), collected on a nitrocellulose membrane, and added to a xylene-based scintillant for scintillation counting. To each lane of the gel, 4×10^5 cpm of labelled polypeptide was loaded. The gel was dried and exposed to X-ray film (Eastman Kodak) for 20 hrs.

RESULTS

Subcloning of *B. pasteurii* Urease

pGU66 (11), containing the *B. pasteurii* urease gene cluster in a *B. subtilis-E. coli* shuttle vector pGR71, was found to be unsuitable for further characterization. A partial *Hin*dIII digestion fragment of pGU66 carrying the urease gene cluster was inserted into the *Hin*dIII site of pBR322 and designated pBU11. When transformed into the host strain *E. coli* HB101, pBU11 was found to be present in high copy number and the two resistance markers facilitated further analysis. Restriction mapping of pBU11 was carried out by double digestion with many kinds of restriction enzymes as shown in Fig. 1. Seven *Eco*RI sites and three *Pst*I sites were located, but no restriction sites for *Sal*I, *Spe*I, and *Nde*II were found. All transformants subcloned with complete or partially digested *Eco*RI fragments were urease negative. To minimize the inserted fragment size and to

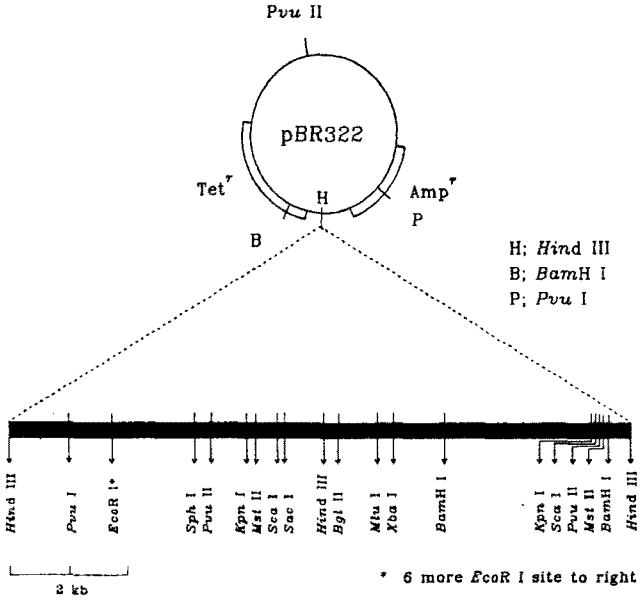

Fig. 1. Restriction physical map of pBU 11.
B. pasteurii DNA fragment containing the urease gene cluster inserted in pBR322.

estimate the region of pBU11 containing the urease genes, selected *Hin*dIII, *Bam*HI, and *Pvu*II fragments were subcloned into pBR328. As shown in Fig. 2, the size of DNA required for expression of the urease gene cluster was estimated to be at least 4.0 kb, extending from the left most *Hin*dIII site to the middle *Bam*HI site. Even the subcloned *Bam*HI fragment only very slowly yielded a positive color on Christensen's urea plate.

Transposon Mutagenesis

To determine the size of the region comprising the cloned urease genes in *E. coli* and to examine the relationships between the urease genes and plasmid encoded polypeptides, pBU11 was mutagenized by Tn5 transposon mutagenesis. The sites of insertion were determined by restriction analysis with *Bam*HI, *Bgl*II, and *Kpn*I. As shown in Fig. 3, the minimum size of DNA that must be free of transposon insertion to observe urease activity is about 5.0 kb. This region is located from the first *Eco*RI site to the *Bam*HI site. Importantly, the pUB11/Tn5-J34 mutant, where the transposon inserted just before the first *Bam*HI site, expressed a very weak activity.

***In Vitro* Transcription-Translation Analysis**

The peptides encoded by pBU11 and Tn5 transposon-mutagenized pBU11 mutant plasmids were analy-

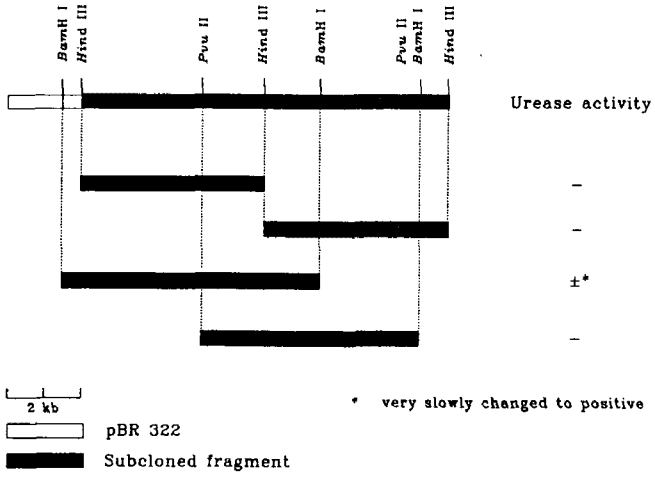

Fig. 2. Localization of *B. pasteurii* urease gene from a gene cluster cloned by subcloning.

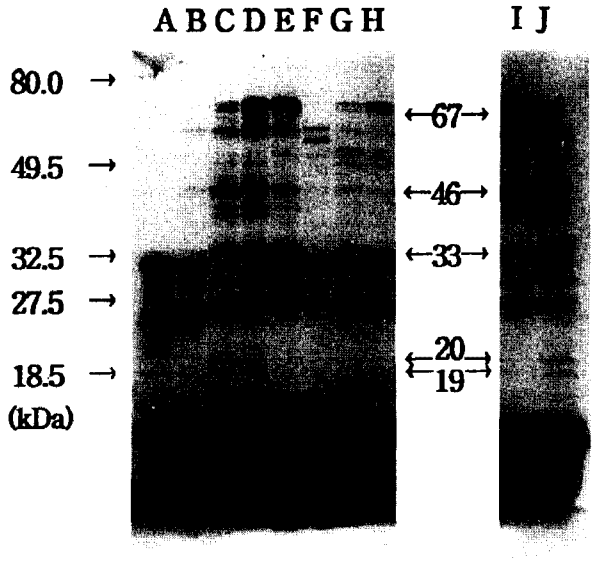

Fig. 4. Autoradiograph of electrophoresed ^{35}S-labelled polypeptides from *in vitro* transcription-translation of transposon insertion mutants.

Lane A; pAT153, Lane B; pBR322, Lane C; pBU11, Lane D; pBU 11-J6, urease activity +, Lane E; pBU11-J21, urease activity −, Lane F; pBU11-J1, urease activity −, Lane G; pBU11-J32, urease activity −, Lane H; pBU11-J3, urease activity −, Lane I; pBU11-J34, urease activity −, Lane J; pBU11-J12, urease activity +.

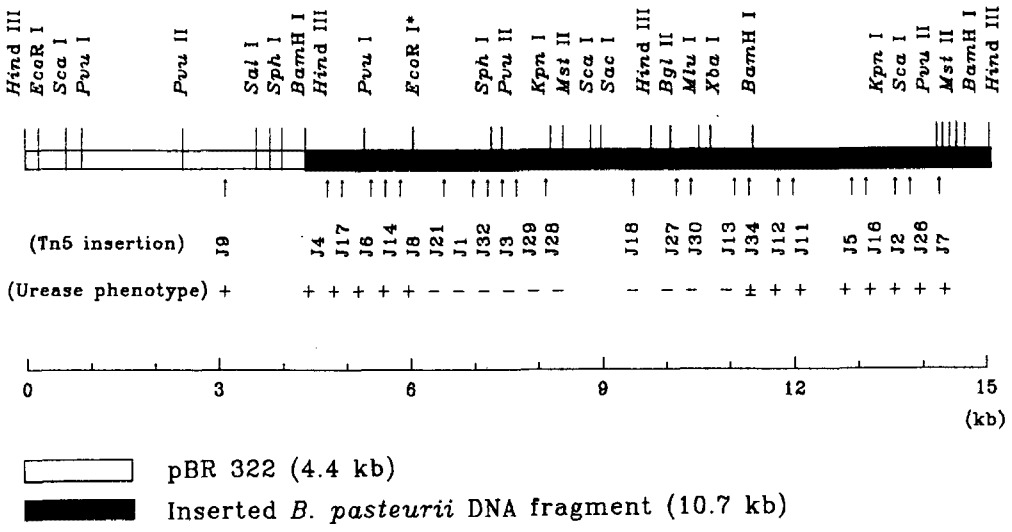

Fig. 3. Transposon (Tn5) mutagenesis of *B. pasteurii* urease gene.

Kanamycin-resistant colonies of *E. coli* (pBU11::Tn5) were isolated and screened for urease activity (+; positive, −; negative, ±; very slowly change to +). Sites of insertion of Tn5 into pBU11(↑) were determined by digests with *Bgl*II, *Bam*HI, and *Kpn*I.

zed by *in vitro* transcription-translation methods. The patterns of polypeptides produced were compared by SDS-PAGE and autoradiography (Fig. 4). pBU11 and the urease-positive pBU11/Tn5-J6, pBU11/Tn5-J12 mutant DNA encoded polypeptides of M_r 67,000, 61,000, 46,000, 33,000, 30,000, 27,000, 20,000, and 19,000. Excluding the polypeptides encoded by pBR322, at least 5 polypeptides of M_r 67,000, 46,000, 33,000, 20,000, and 19,000 were found to be associated with the DNA responsible for urease activity. Among the polypeptide patterns produced from the urease-negative mutants, the leftmost insertion (pBU11/Tn5-J21) eliminated the band of M_r 20,000, whereas the adjacent insertion (Tn5-J1) eliminated both this peptide and the band of M_r 67,000. Insertions located more to the right (Tn5-J32 and -J3) led to partial restoration of the M_r 67,000 band. Additional effects on the M_r 46,000, 33,000, and 19,000 bands are also observed. Notably, the urease-negative mutant pBU11/Tn5-J34 did not produce the 20,000 and 19,000 polypeptides despite the presence of the other polypeptide bands such as major 67,000 band.

DISCUSSION

A 10.7 kb *Hin*dIII fragment of DNA containing the *Bacillus pasteurii* urease gene cluster, previously cloned in *Escherichia coli* using the pGR71 vector (11), was transferred to pBR322 and called pBU11. The size of the urease gene cluster in this construct was estimated by subcloning and a physical map of the fragment was constructed (Fig. 1 and 2). To more accurately estimate the locations, organization, and length of the genes in the urease cluster, transposon mutagenesis of the plasmid was accomplished. It was estimated that the expression of urease activity requires about 5.0~6.0 kb of DNA from the first *Eco*RI site to the *Bam*HI site (Fig. 3). Insertion just before the *Bam*HI site reduced the urease activity, consistent with subcloning data that the subcloned *Bam*HI fragment has very weak activity. Analogous transposon analysis previously had estimated the length of microbial urease gene clusters as 4.4~6.0 kb in *Providencia stuartti* (20), 4.0~6.5 kb in *Proteus mirabilis* (9). In order to identify the products associated with the *B. pasteurii* urease gene cluster, the polypeptides encoded by pBU11 and transposon mutants of this plasmid were examined by SDS-PAGE after *in vitro* transcription-translation in the presence of ^{35}S-methionine and autoradiograpy (Fig. 4). The urease-related polypeptides encoded by pBU11 possess M_r 67,000, 46,000, 33,000, 20,000, and 19,000. Previously, the urease of *B. pasteurii* was shown to be composed of two subunits of M_r 67,000 and 20,000 (14), identical in size to two of the peptides observed here. The genes encoding these structural peptides appear to be located toward the leftmost region of the cloned DNA region. The additional urease-related polypeptides of M_r 46,000, 33,000, and 19,000, suspected to play a role in nickel incorporation, nickel transport, or other function, are presumed to be encoded by the DNA toward the right side of the cloned region. These results suggest that the urease gene cluster of *B pasteurii* has two structural genes and several accessory genes similar to that found in the urease gene clusters of *Providencia stuartii* (20) or *Klebsiella aerogenes* (19) which are each composed of 3 structural genes and several accessory genes. Our results are further bolstered by very recent sequence results and deletion analysis of the urease gene complex in a thermophilic *Bacillus* sp. strain TB-90 (15). That work indicates a requirement for three accessory genes in addition to the structural genes for urease in that strain (15).

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