Molecular Cloning and Expression of Cellulase Genes of Alkalophilic 
Bacillus sp. Strain N-4 in Escherichia coli

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The genes for cellulases of alkalophilic Bacillus sp. strain N-4 were cloned in Escherichia coli with pBR322. Plasmids pNK1 and pNK2 were isolated from the transformants producing carboxymethyl cellulase, and the carboxymethyl cellulase genes cloned were in 2.0- and 2.8-kilobase-pair HindIII fragments, respectively. On the DNA level, the pNK1 fragment had a different restriction map from that of the pNK2 fragment, but the genomic hybridization experiments showed partial homology among these fragments. A total of 74 and 34% of the enzyme activities were observed in the periplasmic space of E. coli carrying the plasmids pNK1 and pNK2, respectively. The carboxymethyl cellulase thus produced had broad pH activity curves (pH of 5 to 10.9) and was stable up to 75°C.

Cellulase has been detected in many strains of microorganisms and is important not only from an industrial point of view but also from an academic standpoint so that a multienzyme system can be studied. Recently, the structural genes for the cellulase of Clostridium thermoascus and Cellulomonas fimii were cloned with a phase (3) and a plasmid vector (12) in Escherichia coli.

In our laboratory, many alkalophilic bacteria which grow well at the pH range (10.0 to 11.0) have been isolated. These bacteria produce many kinds of extracellular enzymes which have optimum pH in the alkaline region (5). One of these, alkalophilic Bacillus sp. strain N-4, produced several cellulases (5; K. Horikoshi, M. Nakao, Y. Kurono, and N. Sashihara, Can. J. Microbiol., in press). The enzymes had strong activity toward carboxymethyl cellulose (CMC) and very weak activity toward avicel. We have started to clone the CMCase genes to analyze their genetic information for the multicomponents of the cellulase. This paper deals with the molecular cloning and expression of the CMCase genes of alkalophilic Bacillus sp. strain N-4 in E. coli. Some enzymatic properties will also be discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Alkalophilic Bacillus sp. strain N-4, which is a producer of alkaline CMCase, was isolated from soil (Horikoshi et al., in press). The best growth and maximum enzyme production were observed at pH 10.0. The optimal pH of CMCase was between 5.0 and 11.0. E. coli K-12 HB101 (pro leuB1 lacY hsdR hsdM ara 14 galKZ Xyl-5 mtl-l supE44 F- endol recA Strr) and the plasmid pBR322 were used throughout.

Media. Brain heart infusion medium was used for E. coli. The alkaline medium contained 10 g of starch, 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of polypeptone, 1 g of K2HPO4, 0.2 g of MgSO4·7H2O, and 10 g of Na2CO3 in 1 liter of deionized water (pH 10.0). Sodium carbonate was sterilized separately.

Preparation of DNA. Alkalophilic Bacillus sp. strain N-4 was aerobically grown to the early stationary phase at 37°C in the alkaline medium described above. Bacterial chromosomal DNA was purified by the method of Saito and Miura (9). The vector pBR322 was purified by the method of Bolivar et al. (1). Recombinant plasmids were detected by the miniscreening method (4).

Construction of recombinant plasmids. DNAs were digested with HindIII at 37°C for 1 h (plasmid DNA) or for 14 h (chromosomal DNA). After the digestion, 1 µg of plasmid and 3 µg of bacterial chromosomal DNA were mixed and ligated with T4 DNA ligase overnight at room temperature. This ligation mixture was used for transformation (7).

Assay of CMCase. The enzyme solution (0.1 ml) was mixed with 0.5 ml of 1% CMC solution (made up with 0.1 M glycine-NaOH-NaCl buffer [pH 10.0] and with 0.05 M acetate buffer [pH 5.5]). After 10 min of incubation at 40°C, 1 ml of dinitrosalicylic acid solution (11) was added, and the mixture was heated in a boiling water bath for 5 min. Then, 4 ml of water was added, and the absorbance of the sample was measured at 510 nm. One unit of enzyme activity is defined as the amount of enzyme which liberates 1 mg of reducing sugar expressed as glucose per min under the above conditions.

Preparation of antiserum. Alkalophilic Bacillus sp. strain N-4 was grown in CMC medium for 2 days at 37°C. The cells were removed by centrifugation, and the supernatant was precipitated by the addition of 4 volumes of ethanol and suspended in a small volume of buffer. One milliliter of the sample (ca. 4.8 U of CMCase activity) was mixed with 1 ml of complete Freund adjuvant, and the mixture was injected into mature white rabbits. Booster injections of the samples were given in the same manner after 4 and 6 weeks, and the sera were collected a week after the last booster injection.

Gel electrophoresis. Electrophoresis of the plasmids was performed on a 1% agarose slab gel. DNA was detected with a short-wavelength UV transilluminator (Ultra-Violet Products, Inc.), and the molecular weights were estimated. As molecular size references, DNA fragments of λ-phage digested with HindIII were used.

Nick translation and hybridization of DNA digests. DNA was labeled by nick translation, using E. coli DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, Md.) in the presence of [32P]dATP (8). The hybridization technique used was essentially the same as that described previously (10).

Analysis of CMCase distribution. Fractionation of extracellular, periplasmic, and intracellular CMCase was carried out by a modified method (6) of Cornells et al. (2).
Enzymes and chemicals. RNase, lysozyme, DNase, and ampicillin (Ap) were purchased from Sigma Chemicals Co., St. Louis, Mo. Restriction endonucleases, DNA polymerase I, T4 ligase, and agarose were obtained from Bethesda Research Laboratories and used according to the methods suggested by the manufacturer. The CMC, with a degree of substitution of 0.65 and an average molecular weight of 110,000, was obtained from Nakarai Chemical Co., Kyoto, Japan.

RESULTS

Cloning of alkalophilic Bacillus sp. strain N-4 CMCase gene in E. coli. The chromosomal DNA of alkalophilic Bacillus sp. strain N-4 and plasmid pBR322 DNA were digested with HindIII restriction endonuclease and ligated with T4 DNA ligase. The ligation mixtures were used to transform E. coli HB101, and ca. 2 × 10⁸ Amp⁴ Te⁴ transformants per µg of DNA were obtained (10% of Amp⁴ transformants showed Te⁴). The CMCase activity could be detected directly on the plates because a shallow crater was formed around a colony producing CMCase on an LB agar plate containing CMC. Eight transformants produced a shallow crater around a colony. Plasmid pNK1 was obtained from an Ap⁴ CMCase⁺ transformant which contained a 2.0-kilobase-pair (kbp) HindIII fragment. The other recombinant plasmid, pNK2, was also isolated from Ap⁴ CMCase⁺ transformants containing 2.8-kbp HindIII fragments. These plasmids could transform E. coli HB101 to Ap⁴ CMCase⁺ at a high frequency.

Restriction maps of pNK1 and pNK2. The DNA of the hybrid plasmids were digested with several restriction endonucleases. The digests were analyzed by agarose gel electrophoresis. The restriction maps of these plasmids are shown in Fig. 1. The HindIII fragments were digested with PstI, BamHI, and EcoRI.

Homology among the fragments and chromosomal DNA. To analyze the origin of DNA inserted in the pBR322 plasmid, ³²P-labeled fragments of pNK1 and pNK2 were hybridized to restriction enzyme-digested chromosomal DNAs of alka-

FIG. 1. Restriction maps of plasmids pNK1 and pNK2. Restriction sites are indicated, and their coordinates are given in kbp.

FIG. 2. Homology between the cloned fragments and chromosomal DNAs. (A) Agarose (1%) gel electrophoresis of digests of various DNAs. Lane 1, λ-DNA digested with HindIII (0.1 µg); lane 2, 2.8-kbp HindIII fragment (0.1 µg); lane 3, 2.0-kbp HindIII fragment (0.1 µg); lane 4, alkalophilic Bacillus sp. strain N-4 DNA digested with HindIII (3 µg); lane 5, E. coli DNA digested with HindIII (3 µg). (B) Hybridization analysis of the Southern transfer of the DNAs from the gel A. The ³²P-labeled 2.0-kbp HindIII fragment was used as a probe. Lanes 1 through 5 are the same as described in A.

FIG. 3. Homology between the cloned fragments and chromosomal DNAs. (A) Agarose (1%) gel electrophoresis of digests of various DNAs. Lane 1, λ-DNA digested with HindIII (0.1 µg); lane 2, 2.0-kbp HindIII fragment (0.1 µg); lane 3, 2.8-kbp HindIII fragment (0.1 µg); lane 4, alkalophilic Bacillus sp. strain N-4 DNA digested with HindIII (3 µg); lane 5, E. coli DNA digested with HindIII (3 µg). (B) Hybridization analysis of the Southern transfer of the DNAs from the gel A. The ³²P-labeled 2.8-kbp HindIII fragment was used as a probe. Lanes 1 through 5 are the same as described in A.
TABLE 1. Distribution of CMCases in E. coli HB101* carrying plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>CMCase activity* in the following fraction:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extracellular (%)</td>
</tr>
<tr>
<td>pNK1</td>
<td>50 (4.4)</td>
</tr>
<tr>
<td>pNK2</td>
<td>90 (13.9)</td>
</tr>
<tr>
<td>pBR322</td>
<td>0</td>
</tr>
</tbody>
</table>

* E. coli strains were aerobically grown in brain heart infusion broth (Difco) for 24 h at 37°C.

** CMCase activity (at pH 10.0) was expressed as milliunits per milliliter of the broth.

lophobic Bacillus sp. strain N-4 and E. coli HB101 which had been immobilized on nitrocellulose sheets. Radioactively labeled fragments of pNK1 and pNK2 hybridized to the fragments of unlabeled pNK1 and pNK2, respectively (lanes 2 and 3 in Fig. 2 and 3), and also hybridized to the 2.0- and 2.8-kbp fragments from alkalophilic Bacillus sp. strain N-4 (lane 4 in Fig. 2 and 3). There was partial homology among pNK1 and pNK2 fragments. However, no sequences complementary to fragments of pNK1 and pNK2 were detected in E. coli DNA fragments (lane 5 in Fig. 2 and 3).

Expression and localization of the CMCases in E. coli. E. coli HB101 carrying pNK1 and pNK2 plasmids were aerobi- cylcally grown in brain heart infusion broth for 24 h at 37°C. The extracellular, periplasmic, and cellular CMCase activities were assayed. A significant amount of the enzymatic activity was found in the periplasmic space in E. coli carrying the plasmids (Table 1). The synthesis of CMCase in E. coli was constitutive, and no effect of CMC supplement was observed.

Immunological studies. The periplasmic CMCases of E. coli HB101 carrying pNK1 and pNK2 plasmids were studied with antiserum prepared against an alkalophilic Bacillus sp. strain N-4 crude CMCase fraction. Periplasmic fractions of E. coli HB101 carrying the plasmids gave lines of precipitation that fused with that for CMCase from the alkalophilic Bacillus sp. strain N-4 (Fig. 4). No reaction was observed with the periplasmic fraction of E. coli HB101 carrying pBR322. In the activity assay system, the addition of 20 μl of antiserum caused 100% inhibition of the CMCase activity (ca. 10 mU) of E. coli HB101 carrying pNK1 or pNK2.

Some enzymatic properties of the periplasmic CMCases produced by E. coli HB101 carrying pNK1 or pNK2. (i) Effects of pH. Stability of the pNK1- and pNK2-encoded enzymes were investigated in buffer solutions of various pH values. The mixtures were incubated at 60°C for 10 min. The enzymes were stable from pH 5 to 11. The optimum pH values for enzyme actions were between 5.0 and 10.9 (Table 2).

(ii) Thermal stability. The enzymes were heated at various temperatures for 10 min, and the residual activity was measured at pH 5.5 and 10.0. The enzymes were stable up to 75°C.

(iii) Molecular weight of the enzymes. The molecular weights of pNK1- and pNK2-encoded CMCase were ca. 58,000 and 50,000, respectively (Sephacryl S-200 chromatography).

DISCUSSION

It is well known that some cellulases produced by microorganisms have multienzyme systems. Several attempts have been made to analyze the multicomponents of these enzymes. Two possibilities to be considered are: (i) processing or modification during production occurs with proteases or other enzyme systems; (ii) the DNA contains genes for multienzymes. We have started the cloning of the genes for cellulases to analyze the genetic information for the production of cellulases of the alkalophilic Bacillus sp. strain N-4. Two plasmids, pNK1 and pNK2, were constructed. On the DNA level, the pNK2 fragment was different from the pNK1 fragment in its restriction map, but there was partial homology between these fragments on the basis of results of genomic hybridization experiments. It is quite possible, given the extensive homology of the pNK2 insert with that of pNK1, that the cellulase gene is duplication present on another HindIII fragment. Another interesting result is that the plasmid-encoded cellulases have very broad pH activity curves, as was observed for the enzymes of alkalophilic Bacillus sp. strain N-4. It seems that the plasmid-encoded enzymes are not a mixture of several enzymes, because the 2.0- or 2.8-kbp DNA fragments are too small to encode multienzymes. The distribution of activity for pNK2 is

TABLE 2. Effect of pH on the activity of CMCase

<table>
<thead>
<tr>
<th>pH*</th>
<th>pNK1 (%)</th>
<th>pNK2 (%)</th>
<th>Crude enzyme from N-4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>18.4</td>
<td>30.3</td>
<td>27.8</td>
</tr>
<tr>
<td>5.0</td>
<td>81.0</td>
<td>82.4</td>
<td>98.1</td>
</tr>
<tr>
<td>7.5</td>
<td>96.7</td>
<td>79.7</td>
<td>86.4</td>
</tr>
<tr>
<td>8.0</td>
<td>106.8</td>
<td>89.1</td>
<td>103.3</td>
</tr>
<tr>
<td>9.2</td>
<td>102.3</td>
<td>87.9</td>
<td>92.3</td>
</tr>
<tr>
<td>10.1</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>10.9</td>
<td>107.7</td>
<td>100.0</td>
<td>119.9</td>
</tr>
<tr>
<td>11.7</td>
<td>36.8</td>
<td>62.1</td>
<td>54.7</td>
</tr>
<tr>
<td>12.8</td>
<td>7.4</td>
<td>10.6</td>
<td>14.7</td>
</tr>
</tbody>
</table>

* Acetate buffers were used for the assays at pH 4.4 and 5.0. Tris-hydrochloride buffers were used for the assays at pH 7.5 and 8.0. Glycine buffers were used for the assays at pH 9.2, 10.1, 10.9, 11.7, and 12.8.

* The enzymes were used at 10 mU, and the enzyme activity at pH 10.1 was expressed as 100%.

FIG. 4. Ouchterlony double-diffusion analysis. The well (A) contained antibody to alkalophilic Bacillus sp. strain N-4 crude enzyme. Samples were added to the outer wells as indicated below. Well 1, periplasm fraction of E. coli HB101 carrying pBR322 (40 μg); well 2, crude enzymes of alkalophilic Bacillus sp. strain N-4 (20 μg); well 3, periplasm fraction of E. coli HB101 carrying pNK1 (12 μg); well 4, periplasm fraction of E. coli HB101 carrying pNK2 (40 μg).
clearly different from that of pNK1. Maybe this is a cell-bound form of the enzyme. As a result, two of the cellulase genes of alkalophilic Bacillus sp. strain N-4 were cloned in E. coli with pBR322. It will be interesting to compare these genes, because the cloned fragments had partial homology.

ACKNOWLEDGMENT

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LITERATURE CITED


