Construction of a Chimeric Series of *Bacillus* Cyclomaltodextrin Glucanotransferases and Analysis of the Thermal Stabilities and pH Optima of the Enzymes

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The cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) gene from the alkalophilic *Bacillus* sp. strain no. 17-1 was cloned in *Escherichia coli*. The cloned CGTase gene consisted of a single open reading frame which would encode a polypeptide of 713 amino acids, and the first 27 amino acid residues comprised a signal peptide. The nucleotide sequence and the amino acid sequence of this CGTase (CGTase 17-1) gene had strong homology with those of the CGTase (CGTase 38-2) gene previously cloned in our laboratory from the alkalophilic *Bacillus* sp. strain no. 38-2, although the enzymic properties of the CGTase 17-1 were distinct from those of the CGTase 38-2. To analyse those enzymic properties further, we constructed 12 chimeric CGTases using three restriction nuclease sites and compared the enzymic properties of the chimeric CGTases. The N-terminal part of the enzyme was important for heat stability, and the pH-activity profile was influenced by both the N- and the C-terminal parts. A third segment was less important for enzymic properties.

INTRODUCTION

Cyclomaltodextrin glucanotransferase (EC 2.4.1.19, CGTase) produces a mixture of alpha-, beta- and gamma-cyclodextrins from starch or related carbohydrates. We have reported the enzymic properties of a CGTase produced by the alkalophilic *Bacillus* sp. strain no. 38-2 (Nakamura & Horikoshi, 1976), and analysed the nucleotide sequence of the CGTase (CGTase 38-2) gene from this strain (Kaneko *et al.*, 1988). We have also isolated a CGTase (CGTase 17-1) from alkalophilic *Bacillus* sp. strain no. 17-1 (Yamamoto *et al.*, 1972). Here we report the cloning of the CGTase gene from strain 17-1. We compare the gene and amino acid sequences of CGTase 38-2 and CGTase 17-1, and their properties, and describe the construction of chimeric enzymes using the CGTase genes from the two strains. Through analysis of the properties of the chimeric enzymes, the role of various enzyme segments in the structure and function of CGTase 38-2 and CGTase 17-1 proteins were examined.

METHODS

Bacterial strains and media. Alkalophilic *Bacillus* sp. strain no. 17-1 (ATCC 31007) was isolated in our laboratory from soil (Yamamoto *et al.*, 1972). *Escherichia coli* K12 strain HB101 (F⁻ *hsd-20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*), was used as a host strain (Messing *et al.*, 1981). The alkaline medium (pH 10) contained 1% (w/v) soluble starch, 0.5% yeast extract, 0.5% polypeptone, 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O, and (sterilized separately) 1% Na₂CO₃. LB-starch medium (Maniatis *et al.*, 1982) containing 0.2% soluble starch was used for growth of *E. coli*.

DNA isolation and cloning. *Bacillus* sp. 17-1 was grown aerobically at 37 °C in the alkaline medium described above. Chromosomal DNA was isolated by the method of Saito & Miura (1963). Plasmid DNA was purified by the method of Bolivar *et al.* (1977). A boiling method was used for rapid isolation of plasmids (Davis *et al.*, 1980).

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Abbreviation: CGTase, cyclomaltodextrin glucanotransferase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M28053.

The cloning strategy was as follows. After complete digestion with *Pst*I, plasmid pUC19 DNA (1 µg) and chromosomal DNA (3 µg) were mixed and ligated with T4 DNA ligase. The ligated DNA was used to transform *E. coli* HB101 by the method of Lederberg & Cohen (1974). The transformants were plated on the LB-starch plates, containing 50 µg ampicillin ml⁻¹. CGTase activity was detected as starch hydrolytic activity on LB-starch agar plates using I₂/KI indicator solution.

Amino acid sequence and amino acid composition. The CGTase enzyme was purified as described by Nakamura & Horikoshi (1976). The N-terminal amino acid sequence was determined by the method of Edman & Henschen (1975) using a Protein/Peptide Sequencer model 477A, and the PTH analyser model 120A system (Applied Biosystems). Amino acid composition was determined as described by Spackman *et al.* (1958) with a Hitachi amino acid analyser, model 835, after hydrolysis with 6 M-HCl (Moore & Stein, 1963).

DNA sequencing. Sequencing was done using the dideoxy chain-termination reaction (Sanger *et al.*, 1977) by the exonuclease deletion method (Henikoff, 1984) with pUC18 and pUC19.

Construction of the chimeric enzymes. Plasmid pUCP1, containing the CGTase gene from alkalophilic *Bacillus* sp. 17-1, was obtained in the present study (see Results and Fig. 1). The 3.6 kb *Hind*III fragment containing the CGTase gene from alkalophilic *Bacillus* sp. 38-2 (Kaneko *et al.*, 1988) was inserted into pUC19, the orientation of the CGTase gene being the same as that of pUCP1. The resulting plasmid (CGTase⁺, Ap^r, 6.1 kb) was designated pCS100.

Using three aligned restriction nuclease sites, the *Nde*I, *Eco*47III and *Bal*I sites in both the CGTase 17-1 gene (pUCP1) and the CGTase 38-2 gene (pCS100), 12 chimeric CGTase genes were constructed (see Figs 5 and 6).

Plasmid pCGT1 contained a chimeric gene (SUUU). 'S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively. For example, 'SUUU' indicates the chimeric enzyme in which the N-terminal segment was derived from CGTase 38-2 gene and the second, third, and fourth segments were derived from CGTase 17-1. Plasmid pCGT1 was constructed by ligation of a 3.6 kb *Nde*I(I)–*Nde*I(II) fragment [N-terminal segment (1S) of the CGTase 38-2 gene and most of plasmid pUC19] of pCS100 and a 2.3 kb *Nde*I(2)–*Nde*I(3) fragment [the second, third and fourth segments (2U, 3U, 4U) of the CGTase 17-1 gene] of pUCP1.

Plasmid pCGT2 (SSUU) was constructed by ligation of a 4.7 kb *Eco*47III(II)–*Eco*47III(I) fragment (1S, 2S) of pCS100 and a 3.0 kb *Eco*47III(2)–*Sma*I fragment (3U, 4U) of pUCP1. Plasmid pCGT3 (SSSU) was constructed by ligation of a 4.6 kb *Sma*I–*Bal*I fragment (1S, 2S, 3S) of pCS100 and a 2.0 kb *Bal*I(2)–*Bal*I(3) fragment (4U) of pUCP1. Plasmid pCGT4 (SSUS) was constructed by ligation of a 5.7 kb *Bal*I(3)–*Bal*I(2) fragment (1S, 2S, 3U) of pCGT2 and a 1.5 kb *Bal*I–*Sma*I fragment (4S) of pCS100. Plasmid pCGT5 (USSS) was constructed by ligation of a 4.5 kb *Nde*I(1)–*Nde*I(2) fragment (1U) of pUCP1 and 2.5 kb *Nde*I(II)–*Nde*I(I) fragment (2S, 3S, 4S) of pCS100. Plasmid pCGT6 (USSU) was constructed by ligation of a 5.5 kb *Sma*I–*Bal*I fragment (1U, 2S, 3S) of pCGT5 and a 2.0 kb *Bal*I(2)–*Bal*I(3) fragment (4U) of pUCP1. Plasmid pCGT7 (USUU) was constructed by ligation of a 4.5 kb *Nde*I(1)–*Nde*I(2) fragment (1U) of pUCP1 and a 2.3 kb *Nde*I(II)–*Nde*I(3) fragment (2S, 3U, 4U) of pCGT2. Plasmid pCGT8 (UUUS) was constructed by ligation of a 5.5 kb *Sma*I–*Bal*I(2) fragment (1U, 2U, 3U, partially digested with *Bal*I) of pUCP1 and a 1.5 kb *Bal*I–*Sma*I fragment (4S) of pCS100. Plasmid pCGT9 (UUSS) was constructed by ligation of a 5.2 kb *Sma*I–*Eco*47III(2) fragment (1U, 2U, partially digested with *Eco*47III) of pUCP1 and a 1.4 kb *Eco*47III(I)–*Eco*47III(II) fragment (3S, 4S) of pCS100. Plasmid pCGT10 (USUS) was constructed by ligation of a 6.8 kb *Bal*I(3)–*Bal*I(2) fragment (1U, 2S, 3U) of pCGT7 and a 1.5 kb *Bal*I–*Sma*I fragment (4S) of pCS100. Plasmid pCGT11 (SUUS) was constructed by ligation of a 3.6 kb *Nde*I(I)–*Nde*I(II) fragment (1S) of pCS100 and a 2.5 kb *Nde*I(2)–*Nde*I(I) fragment (2U, 3U, 4S) of pCGT8. Plasmid pCGT12 (SUSS) was constructed by ligation of a 3.6 kb *Nde*I(I)–*Nde*I(II) fragment (1S) of pCS100 and a 2.1 kb *Nde*I(2)–*Nde*I(I) fragment (2S, 3S, 4S) of pCGT9.

CGTase assay. CGTase activity was measured as dextrinizing power using amylose as a substrate, according to the method of Fuwa (1954), with a slight modification. In the standard assay, the reaction mixture, containing 50 µl enzyme solution (suitably diluted with distilled water) and 0.3 ml 0.2% amylose in 10 mM-phosphate buffer (pH 6.0), was incubated at 60 °C for 10 min. The reaction was stopped with 0.5 ml of 1 M-acetic acid and 0.5 ml 0.02% I₂/0.2% KI solution. Distilled water was added to make up a final volume of 10 ml, and absorbance at 700 nm was measured. One unit of enzyme was defined as the amount of enzyme which produced a 10% reduction in the intensity of blue colour of the amylose-iodine complex per minute under the conditions described.

Enzymes and chemicals. RNAase, lysozyme, and ampicillin (Ap) were purchased from Sigma. Restriction endonucleases, DNA polymerase I and T4 ligase were obtained from Toyobo, Osaka, Japan. The M13 sequence kit and exonuclease deletion kit were from Takara Syuzo, Kyoto, Japan. All other chemicals used were of reagent grade.

RESULTS AND DISCUSSION

Cloning and sequencing of the CGTase gene from alkalophilic Bacillus sp. strain no. 17-1

Cloning in *E. coli*. About 4 × 10⁴ colonies of the transformants were screened; one transformant showed amylolytic activity on LB-starch plates using the I₂/KI indicator, and

Fig. 1. Restriction map of plasmid pUCP1. Restriction sites are indicated, and their coordinates given in kb. The medium and thick lines indicate the cloned fragment from alkalophilic *Bacillus* sp. 17-1. The thick line indicates the fragment containing the CGTase gene.

Fig. 2. Strategy for sequencing the CGTase gene of alkalophilic *Bacillus* sp. 17-1. A detailed restriction map of the *EcoRI*–*MboI* region of pUCP1 encoding the CGTase gene and the sequencing strategy are shown. The arrows below the line represent the direction and extent of sequence determinations done by the dideoxy chain-termination method of Sanger *et al.* (1977). Extra *MboI* sites were located at positions 249, 306, 667, 676, 739, 1036, 1192, 1338, 1505, 1725, 1834, 2022 and 2203 in Fig. 3. The *MboI* site at position 2422 (Fig. 3) is shown here.

showed CGTase activity using a paper chromatography method (Kobayashi *et al.*, 1984). The plasmid thus obtained was designated as pUCP1; it contained a 5.5 kb insert of *Bacillus* sp. 17-1 DNA. A restriction map of the 5.5 kb insert of pUCP1 is shown in Fig. 1.

To determine the origin of the DNA inserted in pUC19, the 5.5 kb insert DNA was biotinylated (Langer *et al.*, 1981), and hybridized to *PstI*-digested chromosomal DNA of *Bacillus* sp. 17-1 and *E. coli* HB101, immobilized on a nitrocellulose sheet (Southern, 1975). The biotinylated segment hybridized to a 5.5 kb chromosomal DNA segment of *Bacillus* sp. 17-1 identical in length to that contained in pUCP1. No complementary sequences were detected in *E. coli* chromosomal or plasmid DNA fragments.

DNA sequence. We determined the DNA sequence of a 2.9 kb *EcoRI*–*MboI* region of pUCP1 (Fig. 2). The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. Analysis of the sequence showed that there was a single open reading frame of 2139 bp which could encode a polypeptide of 713 amino acids. The SD sequence AGGAGG, which is highly complementary to the 3' end of *B. subtilis* 16S rRNA (Murray & Rabinowitz, 1982), was observed 6 bp upstream of the initiation codon.

Determination of amino acid composition and N-terminal amino acid sequence. The N-terminal amino acid sequence of CGTase 17-1 was determined up to the 17th residue to be NH₂-Ala-Pro-Asp-Thr-Ser-Val-Ser-Asn-Lys-Gln-Asn-Phe-Ser-Thr-Asp-Val-Ile. This amino acid sequence was identical to that deduced from the DNA sequence starting at nucleotide position 82 in Fig. 3. The initial 27 amino acid residues (residues –27 to –1) seemed to represent a signal

EcoRI

GAATTCGCTTTCATTTACAATATTGTTGAAATCAAACACTCGC

ATAATTTAAGGGCCATGCATTCGGTCACCGCACACCCGGTATGGAACAACCCCGGTTTCTCTTCAGGAGATGCCGGGGTTTTTGTGTGCC

CTATATTTTCGTACAGGAGGTGATTCCCGCAGCTTCGGTTTGATCATTGAACCAACCCCATCTATAACTGCTACATAGGAGGAATACG

10 20 30 40 50 60 HindIII 70 80 ↓ (SD) 90
 ATGAAAAAATCTCCAAACTTACAACCGCTCTTGCCCTTAGCTTGCCCTGGCGCTAAGCTTGCTCGGCCAGCCCATGCAGCCCGGAT
 MetLysLysIleSerLysLeuThrThrAlaLeuAlaLeuSerLeuSerLeuAlaLeuSerLeuLeuGlyProAlaHisAlaAlaProAsp
 (-27) (1)

100 110 120 130 140 150 160 170 180
 ACCTCGGTATCCAACAAGCAAAATTTACGACGGATGTCATTATCAAATCTTTACGGACCGTTTCTCCGACGGCAATCTGCCAACAC
 ThrSerValSerAsnLysGlnAsnPheSerThrAspValIleTyrGlnIlePheThrAspArgPheSerAspGlyAsnProAlaAsnAsn

190 200 KpnI 210 220 230 240 250 260 270
 CCGACCGCGCCGGCTTTGACGGTACCTGCACCAACCTGAGGCTTACTGCGCGGCGACTGGCAAGGATCATCAACAAAATCAACGAC
 ProThrGlyProAlaPheAspGlyThrCysThrAsnLeuArgLeuTyrCysGlyGlyAspTrpGlnGlyIleIleAsnLysIleAsnAsp

280 290 300 310 320 330 340 350 360
 GGCTACCTGACCGGCATGGGCGTTACCGCCATCTGGATCTCCAGCCGGTCGAGAACATCTACAGCGTCATCAATTATCCGGCGTGAAT
 GlyTyrLeuThrGlyMetGlyValThrAlaIleTrpIleSerGlnProValGluAsnIleTyrSerValIleAsnTyrSerGlyValAsn

370 380 390 400 410 420 430 440 450
 AACACCGCATACCAACGGCTATTGGGCTAGAGACTTCAAGAAAACGAACCCGGCTTACGGCAGCATTGCGGATTTCCGAATCTCATCGCT
 AsnThrAlaTyrHisGlyTyrTrpAlaArgAspPheLysLysThrAsnProAlaTyrGlyThrIleAlaAspPheGlnAsnLeuIleAla

460 470 480 490 500 510 520 530 540
 GCCGCCCATGCCAAGACATCAAAGTCATCATCGACTTCGCACCGAACACACGCTCTCCCGCTTCTTGGACCAGCCGCTGTTTGGCGGAG
 AlaAlaHisAlaLysAsnIleLysValIleIleAspPheAlaProAsnHisThrSerProAlaSerLeuAspGlnProSerPheAlaGlu

550 560 570 580 590 600 610 620 630
 AACGGCAAGCTGTACAACAACGGCAGGACGAAGCGGATACCGAACGATACGCACAATTTATCCATCATAACGGGGGCACCGATTTC
 AsnGlyLysLeuTyrAsnAsnGlyArgAspGluGlyGlyTyrThrAsnAspThrHisAsnLeuPheHisHisAsnGlyGlyThrAspPhe

640 650 660 670 680 690 700 710 720
 TCGACGACGAGAACGGGATTTCAAAAACCTGTACGATCTTGGCGATCTGAACCAACAACAGCAGCATGGATACCTACCTGAAGGAT
 SerThrThrGluAsnGlyIleTyrLysAsnLeuTyrAspLeuAlaAspLeuAsnHisAsnAsnSerThrValAspThrTyrLeuLysAsp

730 740 750 760 770 780 790 800 810
 GCCATTAATATGTGGCTCGATCTGGGCATTGACGGCATTGCAATGGACGGGTAAACATATGCCGTTTGGGCTGGCAGAGAGCTTCATG
 AlaIleLysMetTrpLeuAspLeuGlyIleAspGlyIleArgMetAspAlaValLysHisMetProPheGlyTrpGlnLysSerPheMet

820 830 840 850 860 870 880 890 900
 GCCACCGTCAACAATTACAACGGGCTTTCACCTTCGGCGAATGGTTCTCGGGTAAACGAAGTGAGTGGCGAGAATCATAAGTTCCGCC
 AlaThrValAsnAsnTyrLysProValPheThrPheGlyGluTrpPheLeuGlyValAsnGluValSerAlaGluAsnHisLysPheAla

910 920 930 940 950 960 970 980 990
 AACGTTTCCGGCATGAGCCTGCTGGACTTCCGCTTCGCGCAGAAGGTTCCGCCAGGTATTCAAGGACAACACCGACAATATGTACGGACTG
 AsnValSerGlyMetSerLeuLeuAspPheArgPheAlaGlnLysValArgGlnValPheLysAspAsnThrAspAsnMetTyrGlyLeu

1000 1010 1020 1030 1040 1050 1060 1070 1080
 AAATCCATGCTAGAGGGCTCTGCGACCGATTACGCGCAGATGGAGGATCAGGTACGTTTCATCGATAACCACGACATGGAACGTTTCCAC
 LysSerMetLeuGluGlySerAlaThrAspTyrAlaGlnMetGluAspGlnValThrPheIleAspAsnHisAspMetGluArgPheHis

1090 1100 1110 1120 1130 1140 1150 1160 1170
 AATAACAGCGCAACCGCGGAAGCTGGAGCAAGCGCTCGCCTTACGTTAACTTCCGCGGCGTGCCAGCCATCTATTGGAACCGAG
 AsnAsnSerAlaAsnArgArgLysLeuGluGlnAlaLeuAlaPheThrLeuThrSerArgGlyValProAlaIleTyrTyrGlyThrGlu

1180 1190 1200 1210 EcoRI 1220 1230 1240 1250 1260
 CAATACATGTCGGCGGGAACGATCCCGACAACCGGGCTCGAATTCCTTCTTCTCTACCACGACTACGGCTTATCAGGTACGTAATAAAG
 GlnTyrMetSerGlyGlyAsnAspProAspAsnArgAlaArgIleProSerPheSerThrThrThrAlaTyrGlnValSerLysLys

1270 1280 1290 1300 1310 1320 1330 1340 1350
 CTGGCGCCTCTGCGTAAATCCAATCCGGCCATCGCTTATGGGACGACGAGGAGCGCTGGATAAACAACGATGTGTGATCTATGAGCGC
 LeuAlaProLeuArgLysSerAsnProAlaIleAlaTyrGlyThrThrGlnGluArgTrpIleAsnAsnAspValLeuIleTyrGluArg

1360 1370 1380 1390 1400 1410 1420 1430 1440
 AAGTTCGGCAACAACGTAGCCGTCATCGCGTCAACCGCAACGTGAACACGTCGGCCTCCATTACGGGACTGGTAACCTCACTGCCGGCC
 LysPheGlyAsnAsnValAlaValIleAlaValAsnArgAsnValAsnThrSerAlaSerIleThrGlyLeuValThrSerLeuProAla

1450 1460 1470 1480 1490 1500 BamHI 1510 1520 1530
 GGCAGCTACACCGACGTGCTGGCGGCTCTTGAACGGTAACAATTTGACCGTCGGCTCCGGCGGATCCGCTTCCATCTTCACGCTGGCA
 GlySerTyrThrAspValLeuGlyGlyLeuLeuAsnGlyAsnAsnLeuThrValGlySerGlyGlySerAlaSerIlePheThrLeuAla

1540 1550 1560 1570 1580 1590 1600 1610 1620
 GCGGGCGGTACGGCGGTGGCAATACACACCGGCCGCTGACTGCCCAACCATCGGGCAGCTAGGACCGATGATGGCCAAACACGCGCA
 AlaGlyGlyThrAlaValTrpGlnTyrThrThrAlaValThrAlaProThrIleGlyHisValGlyProMetMetAlaLysProGlyAla

1630 1640 1650 1660 1670 1680 1690 1700 1710
 GCCGTGACATTACGGCGCGGTTTTCGGTGCAACCAAGGCACGGTATCTTCCGGCAGCAGCGGAGTTACCGCGCTAATATTACGGCT
 AlaValThrIleAspGlyArgGlyPheGlyAlaThrLysGlyThrValTyrPheGlyThrThrAlaValThrGlyAlaAsnIleThrAla

1720 1730 1740 1750 1760 1770 1780 1790 1800
 TGGGAAGACACGCAGATCAAAGTGAAATCCCTGCCGTTGCCGAGGCGTATACAATATCAAAATCGCCAACAGTGCCGGAACCTCAAGC
 TrpGluAspThrGlnIleLysValIleProAlaValAlaGlyGlyValTyrAsnIleLysIleAlaAsnSerAlaGlyThrSerSer
 1810 1820 1830 1840 1850 1860 1870 1880 1890
 AATGTGCATGACAACCTCGAAGTGCTGAGCGGGGATCAGGTCAGCGTGCGCTTTGTGGTGAACAACGCCACCGCGCTCGGCCAGAAC
 AsnValHisAspAsnPheGluValLeuSerGlyAspGlnValSerValArgPheValValAsnAsnAlaThrThrAlaLeuGlyGlnAsn
 1900 1910 SacI 1920 1930 1940 1950 1960 1970 1980
 GTATATCTGGCAGGCAGCGTAAGCGAGCTCGGCAACTGGGACCGGCCAAAGCCATCGGTCCGTTGTACAACAGGTCATCTACCAATAC
 ValTyrLeuAlaGlySerValSerGluLeuGlyAsnTrpAspProAlaLysAlaIleGlyProLeuTyrAsnGlnValIleTyrGlnTyr
 1990 2000 2010 2020 2030 2040 2050 2060 2070
 CCAACCTGGTACTATGACGTACCGTTCGCCCGGCCAAAACGATCGAATTAAATTCCTGAAAAACAGGGCTCGACGGTAACGTGGGAA
 ProThrTrpTyrTyrAspValThrValProAlaGlyLysThrIleGluPheLysPheLeuLysLysGlnGlySerThrValThrTrpGlu
 2080 2090 2100 2110 2120 2130 2140
 GCGGGCTCGAACCATACCTTACCGCACCAACGAGCGGCACGCCACCATAAACGTGAACGGCAACCGTAAGTTCAAGAACGACTAGCT
 GlyGlySerAsnHisThrPheThrAlaProThrSerGlyThrAlaThrIleAsnValAsnTrpGlnPro***
 (686)
 TCTGCCGAAGAACGGCCCTAAACAAAACAGCTCCGGATATGATCCCGAGCTGGTTTTGTTTCATGCAGGTGATAATTCCAGTAGCAAT
 AGCCCCGTTACGCGAAGCATCTCACAGTTCCTCTGAAGTGCATGAGCGATTCCCAAAGAGAGAGCGTGCTCAGCAGGCAGTAACCTCAGCAT
 MboI
 TCCTCTGCATTGCATGAGCGATTCCCAAAGGAGAGCGTCTCAACAAGTAGTGACTCAGCAAGCAGACGGGACAACCTCAACGATC

Fig. 3. Nucleotide sequence of the CGTase gene from alkalophilic *Bacillus* sp. 17-1. The DNA sequence is given in the direction 5' to 3', numbered from nucleotide 1 at the putative initiation site. The proposed ribosome-binding site (Shine-Dalgarno, SD) is underlined with a dotted line. The predicted amino acid sequence is given below the DNA sequence. The deduced position of processing of the signal peptide is indicated by an arrow. The boxed amino acids have been determined by automated Edman sequencing of the purified CGTase.

Table 1. Amino acid composition predicted from the DNA nucleotide sequence of the CGTase from alkalophilic *Bacillus* sp. 17-1 compared with that determined from the purified enzyme

Amino acid	From DNA sequence		Amino acid analysis (mol%)	Amino acid	From DNA sequence		Amino acid analysis (mol%)
	No.	Mol%			No.	Mol%	
Lys	31	4.5	5.2	Gly	65	9.6	10.1
His	13	1.9	2.1	Ala	62	9.1	10.1
Arg	19	2.7	3.1	Cys	2	0.3	—
Trp	13	1.9	0.2	Val	48	6.9	7.6
Asx*	101	14.7	14.7	Met	13	1.9	1.0
Thr	66	9.5	8.9	Ile	38	5.6	5.6
Ser	42	6.1	5.9	Leu	39	5.6	6.3
Glx*	43	6.3	6.0	Tyr	32	4.7	4.8
Pro	27	3.9	4.8	Phe	32	4.6	4.9

—, Not detected.

* Asx represents Asp and Asn; Glx represents Glu and Gln.

peptide which was removed during the secretion process. Thus, the mature CGTase comprised 686 amino acids and had an M_r of 74140. The amino acid composition of CGTase 17-1 was calculated from the deduced amino acid sequence and compared with the composition of purified CGTase. Molar ratios obtained by these analyses were closely consistent (Table 1).

Comparison of the nucleotide sequences and deduced amino acid sequences of the CGTase genes of alkalophilic Bacillus sp. strain no. 17-1 and strain no. 38-2. We previously cloned and analysed the nucleotide sequence of the CGTase gene of *Bacillus* sp. 38-2 (Kaneko *et al.*, 1988). Fig. 4 shows the nucleotide sequence and amino acid sequence alignment between the CGTase 17-1 and

1.	ATGAAAAAAATCTCCAAACTTACAACCGCTCTTGCCTTGTCCCTGCGCTAAGCTTGTCTCGGCCAGCCCATGCAGCCCGGAT	90
2.	-----G-T-TATG-----A---G---TATGGA-A--CT-G--A-----CA---GG--C-CT-GA---G-T--C-----	90
	R F M A V W T W T G S V	
1.	ACCTCGGTATCCAACAAGCAAATTTACGACCGGTATCATTTATCAAATCTTTACGGACCGTTTCTCCGACGGCAATCCTGCCAACAC	180
2.	-----G-----A-----G-----C--C-----G-----G-----G-----T	180
1.	CCGACCGGCCCGGCTTTGACGGTACCTGCACCAACCTGAGGCTTTACTGCGGCGGCGACTGGCAAGGGATCATCAACAAAATCAACGAC	270
2.	-----G--A-----AT-A--T--G--T--TC-CT-A-----C-----	270
	A S	
1.	GGCTACCTGACCGGCATGGGCGTTACCGCCATCTGGATCTCCGACCGGTCGAGAATCTACAGCGTCATCAATTATCCGGCGTGAAT	360
2.	--T--TT-----A---G-----T--A-----T-----T-----G---C--C-----CC--	360
	I H	
1.	AACACCGCATACCACGGCTATTGGGCTAGAGACTTCAAGAAAACGAACCGGCTTACGGCACGATTGCCGATTCCAGAATCTCATCGCT	450
2.	--T--G--T--T-----C-----GC-G-----G--C--T-----C-----A-----GCAG--C---A-A--C--G---AC	450
	M Q K D	
1.	GCCGCCCATGCCAAGAATCAAAAGTCATCATCGACTTCGCACCGAACACACGTCCTCCGCTTCCTTGGACCAGCGCTGTTTGCGGAG	540
2.	A---G-----GC-T-----A-----T-----T-----G---S---D---T--T--C---A---	540
	T H	
1.	AACGGCAAGCTGTACAACAACGGCAGGACGAAGGCGGATACGAACGATACGCACAATTTATTCATCATAACGGGGGACCGGATTTT	630
2.	-----CGCT-----G-T-----ACCTGCTC-----C-----C-A--C-G-----C--T--C---G---C	630
	R D R L L Q Y	
1.	TCGACGACCGAGAACGGGATTTACAAAACTGTACGATCTTGCCGATCTGAACCACAACAACAGCAGATGGATACCTACCTGAAGGAT	720
2.	--C--C--TT-----C---T-----G--T--C-----G--C--C---GTG--T-----	720
	I S V	
1.	GCCATTAATATGGGCTCGATCTGGGCTTACGGCATTCGAATGGACGCGTAAACATATGCGGCTTGGCGAGAGAGCTTTCATG	810
2.	-----C-----C--C--GG-----CG-----C--G---A-----A-----T---	810
	V V	
1.	GCCACCGTCAACAATTACAAGCCGGTCTTCCACTTCGGCGAATGGTTCTCGGGTAAACGAAGTGAAGTGCAGAGATCATAAGTTCCGC	900
2.	T---A-T-----C-----A-----T-----T--C--C--T--GA-T---C-G--AT--C--C-A---T	900
	S I N I P Y Q	
1.	AACCTTTCGGCATGAGCCTGCTGGACTTCGCGTTCGCGCAGAAGTTTCGCCAGGTATTCGAAGGACAACACCGACAATATGTACGGACTG	990
2.	---AG-----G-----C--T-----CG--T--C-----CC--G--A--G---G-----C---	990
	E P A R	
1.	AAATCCATGCTAGAGGCTCTGCGACCGATTACGCGCAGATGGAGGATCAGGTACAGTTCATCGATAACACGACATGGAACGTTTCCAC	1080
2.	--G-G-----G-----AAGTA--C--T--C--G--A--T--C-----G--C-----C--T--T-----G-----	1080
	A E V V N	
1.	AATAACAGCGCCAACCGCGGAAGCTGGAGCAAGCGCTGCGCTTACGTTAACTTCCGCGGCGTGCCAGCCATCTATTATGGAACCGGAG	1170
2.	--CC-G--AT-G-G--A-A-----G-----G-----T--CC-G---A-----T-----T-----C---G---	1170
	T S N G D S	
1.	CAATACATGTCGGCGGGAACGATCCCGACAACCGGGCTCGAATTCCTTCTTCTCTACACGACTACGGCTTATCAGGTCAGTAAAAAG	1260
2.	--G--T-----T-----G-----T-----G-----C--G--A-----G--C--A-----TCC	1260
	I Q	
1.	CTGGCGCTCTGCGTAAATCCAATCCGCGCATCGCTTATGGGACGACGCGAGGCGCTGGATAAAACAACGATGTGTTGATCTATGAGCGC	1350
2.	--C--T--G--C--C-----C-----C--TT-C--A-----C-----A--C-----A---	1350
	S I	
1.	AAGTTCGGCAACAACGTAGCCGTATCGCCGTCAACCGCAACGTGAACACGTGCGGCTCCATTACGGGACTGGTAACCTCACTGCCGGCC	1440
2.	--A-----T-----G-----TG-T--A--T-----TA-----AC---T--G-----C--C--T--C--T--C--C---CAG	1440
	V I M P Q	
1.	GGCAGCTACACCGAGTCTGGGCGGCTTTGAACGGTAACAATTTGACCGTCGGCTCCGGGGGATCCGCTTCCATCTTACGCTGGCA	1530
2.	-----T-A--T-----C-----AA-TC-----C--T--CGC-A-----G--TG-T--TG-A-----A--N-----T	1530
	N I T A A N	
1.	GCGGGCGGTACGGCGTGTGGCAATACACCACGGCGTGACTGCGCAACCATCGGGCACGTAGGACCGATGATGGCCAAACAGGCGCA	1620
2.	C-T-----C--T--T--A-----G-----A--C--AT--CC--A--T--G--T--A-----CA-T--C--C-----GG---G--TC	1620
	P D A I N N A V	

1.	A V	G F G A T K	N T	
1.	GCCGTCACCATTCAGCGCCGGTTCGGTGCAACCAAGGCACGGTATACTTCGGCACGACGGCAGTTACCGGCGCTAATATTACGGCT			1710
2.	A-GA-T-G-----C-CG-C-CGGCAA	--A-----T-----T-A-----C--T-----GG-C-CGTA--		1707
	T I	A S A R Q	D V	
1.	TGGGAAGACACGCAGATCAAAGTGAAATCCCTGCCGTGCGCGAGGGGTATACAATATCAAAATCGCCAACAGTGCCGGAACCTCAAGC			1800
2.	-----T--A--A-----C-G-----TGCGG--CC-T-CC--A-C--TG-----G-G-T-----GCA--G-AG-C--			1797
		Q	L R P I D R V A A A	
1.	V H	S	S	V
1.	AATGTGCATGACAACCTTCGAAGTGTGAGCGGGGATCAGGTACGGTCGCGCTTTGTGGTGAACAACGCCACCACCGCGCTCGGCCAGAAC			1890
2.	--CA-CT-C-----T-----G-----C--A--C-----T-----G--C--AA-C-----T-----A--G-----G--A-----T			1887
	I Y	T	T	I
1.	Y A S		A K	L I
1.	GTATATCTGGCAGGCAGCGTAAGCGAGGTCGGCAACTGGGACCGGCCAAAGCCATCGGTCCGTGTACAACCAGGTCATCTACCAATAC			1980
2.	--G-T-C--CA-G-----AT-C-----G-----T-----AA--C--G-----C--A-----T-----T-----G-----V-----			1977
	F T N	N N	M	V
1.	CCAACCTGGTACTATGACGTACCGTTCCCGCGGCAAAACGATCGAATTTAAATTCCTGAAAAAACAGGGCTCGACGGTAACGTGGGAA			2070
2.	--G-T-----T-----T-----G--A-----T-----G--A-----C--C--C--A-----			2067
		S	Q	
1.	S H	A	I	#
1.	GGCGGCTCGAACCATACCTTCACCGCACCAACGAGCGGCACGGCCACCATAAACGTGAACCTGGCAACCGTAA			2142
2.	-----G--T--GC-----A-C-----C-----A--GG-G--T-----G--T-----			2139
	A R	T	V	#

Fig. 4. Comparison of the nucleotide sequence and the deduced amino acid sequence of the CGTase 17-1 gene (1) and the CGTase 38-2 gene (2). Dashes represent identical nucleotides. The deduced amino acids that are different are indicated below and above the nucleotide sequence. The nucleotides are numbered taking initiation A as 1. The position of processing of the signal peptide is indicated by an arrow.

Table 2. Comparison of CGTase 17-1 and CGTase 38-2

	CGTase 17-1	CGTase 38-2
Amino acid residues	713	712
(Signal peptide)	(27)	(27)
M_r of mature enzyme	74 140	75 160
Homology		
Nucleotide		77%
Amino acid		83%
Heat stability*	53 °C	65 °C
pH-activity profile	narrow	broad
Optimum pH	6	6, 9

* Temperature at which 50% loss of activity occurred (see Fig. 7 and text).

CGTase 38-2 genes. In the amino acid sequences, 593 (83%) of the aligned amino acids were identical. The overall homology of the aligned nucleotide sequence was 77%. However, the enzymic features, especially the heat stability and optimum pH, of the two CGTases were distinct (Table 2). To analyse these differences further, we constructed chimeric CGTases using the two genes and compared their enzymic properties.

Construction and comparison of chimeric CGTases

Construction of chimeric CGTases. As described in Methods, 12 chimeric CGTase genes were constructed (Fig. 5). Schematic representations of the chimeric CGTases are shown in Fig. 6. *E. coli* HB101 cells harbouring the various chimeric genes were grown aerobically in 10 ml LB broth for 24 h at 37 °C, then harvested and sonicated. The lysates were dialysed against running tap water and these crude extracts were used as the enzyme preparations. Their activities were 270–440 units per ml broth; *E. coli* harbouring pUC19 had no dextrinizing activity. SDS-polyacrylamide gel electrophoresis and Western blot analysis indicated that the 14 enzyme proteins (CGTase 17-1, CGTase 38-2 and 12 chimeric CGTases) had almost the same M_r as expected.

Fig. 5. Construction of chimeric CGTases. Restriction endonuclease sites which were used for construction of chimeric CGTases are indicated. The thick lines indicate the fragments containing the CGTase genes from alkalophilic *Bacillus*, and thin lines indicate the vector plasmid pUC19.

Fig. 6. Schematic representation of chimeric CGTase genes. The restriction endonuclease sites used were *NdeI*, *Eco47III* and *BstI*, and their amino acid residues were numbered taking initiation Met as 1. 'S' and open boxes represent regions from CGTase 38-2; 'U' and filled boxes represent regions from CGTase 17-1.

Heat stability of chimeric CGTases. With respect to the heat stability of the chimeric CGTases, their N-terminal segment was most important. As shown in Fig. 7, among the 14 CGTases, those enzymes (S---) which contained the N-terminal segment derived from CGTase 38-2 were more stable than those (U---) which contained the N-terminal segment derived from CGTase 17-1 ('S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively). The second segment also had some role in increasing heat stability. Among the enzymes (S---)

Fig. 7. Comparison of heat stability of chimeric CGTases. Each enzyme in 10 mM-phosphate buffer (pH 6.0) was treated at 40, 50, 55, 60, 65 and 70 °C for 30 min. The residual activities were measured by the standard method (see Methods). The temperatures in each figure represent the point of 50% loss of activity. 'S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively (see Fig. 6).

whose first (N-terminal) segment was from CGTase 38-2, those chimeric CGTases (SS—) which contained a second segment from CGTase 38-2 were most stable. The third and the fourth segment apparently did not contribute to heat stability.

The homologies of the first, second, third and fourth segments were 86.1%, 86.0%, 81.4% and 77.7%, respectively. It is interesting that the heat stability was changed distinctly by substituting the two most homologous segments, the first and second segments. Thus, heat stability may be influenced by only a few amino acid substitutions. Several properties are known to influence the heat stability of proteins. Insertions of helix-breaking amino acids (proline, threonine, etc.) into an alpha-helix decreases heat stability, and insertion of helix-forming amino acids (glutamic acid, alanine, etc.) into an alpha-helix increases heat stability. The secondary structures of the two CGTases were predicted by Chou-Fasman's method and the numbers of helix-forming and helix-breaking amino acid residues in the alpha-helix regions were compared. There were fewer helix-breaking and more helix-forming amino acid residues in the alpha-helix region of CGTase 17-1 than those of CGTase 38-2, suggesting that the number of helix-breaking and helix-forming amino acids may not be the primary influence on heat stability of the CGTases. Hydrophobic interaction inside the protein molecule is also important in stabilizing protein structure. Comparing the first and second segments of CGTase 38-2 and CGTase 17-1, amino acid substitution from CGTase 17-1 to CGTase 38-2 increased its hydrophobic properties a little. These substitutions might be important for heat stability.

Fig. 8. Comparison of pH-activity profiles of chimeric CGTases. The pH was adjusted with the following buffer systems: sodium acetate (pH 4.0, 5.0 and 6.0); potassium phosphate (pH 5.0, 6.0, 7.0, 8.0 and 9.0); glycine/NaOH (pH 8.0, 9.0, 10.0 and 11.0). 'S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively (see Fig. 6).

pH-activity profiles of chimeric CGTases. The pH-activity profile depended on both the N-terminal and C-terminal segments. As shown in Fig. 8, the chimeric CGTases (S--S) which contained the N- and C-terminal segments derived from CGTase 38-2 showed two pH optima, at pH 6 and pH 9, and those enzymes (U--U) which contained the N- and C-terminal segments from CGTase 17-1 showed only one pH optimum, at pH 6. Those chimeric enzymes which contained the N-terminal segment from CGTase 38-2 and the C-terminal segment from CGTase 17-1 (S--U), or which contained the N-terminal segment from CGTase 17-1 and the C-terminal segment from CGTase 38-2 (U--S), showed intermediate pH profiles. There was no change of pH profile upon substitution of the third segment except with the chimeric enzymes USSS and USUS.

Yamane *et al.* (1984) reported that the α -amylase produced by *B. natto* (*B. subtilis*) IAM1212 had a deletion of about 100 amino acid residues near the C-terminal region, but this deletion did not affect enzyme activity. Fukumori *et al.* (1987) reported that the cellulase of alkalophilic *Bacillus* sp. no. 1139 had a size of 92 kDa and that the large C-terminal part (about 46 kDa) of the cellulase was not necessary for enzyme activity. In contrast, the CGTases studied here consisted of about 720 amino acid residues, and in our unpublished findings CGTases lacking 30 amino acid residues from the C-terminal end showed no enzyme activity. These results suggest that the C-terminal part of the enzyme is important for CGTase activity. The change of pH-activity profile on substitution of the C-terminal segment might be related to this phenomenon.

Our analysis of the chimeric CGTases suggests that the N- and/or C-terminal segment(s) might be important for the CGTase specificity. The third segment might not be essential for the enzyme specificity.

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