Molecular Cloning and Nucleotide Sequence of the Cyclomaltodextrin Glucanotransferase Gene from the Alkalophilic *Bacillus* sp. Strain No. 38-2

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The cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) gene from the alkalophilic *Bacillus* sp. strain no. 38-2 was cloned in *Escherichia coli* using pBR322. A plasmid, pCS8, was isolated from a transformant producing CGTase and the cloned CGTase gene was found to be in a 5.3 kb DNA fragment. The nucleotide sequence of a 2.5 kb segment encoding the CGTase was determined. This segment showed an open reading frame which would encode a polypeptide of 712 amino acids. The pCS8 CGTase had the same enzymic properties as those of the extracellular CGTase produced by the alkalophilic *Bacillus* sp. strain no. 38-2. The nucleotide and amino acid sequences of this CGTase gene and gene product, respectively, have strong homology with those of the *Bacillus macerans* CGTase.

INTRODUCTION

Cyclodextrins (CDs) are closed ring structures in which six or more glucose units are joined by means of α -1,4 glucosidic bonds (Pulley & French, 1961). CDs are interesting molecules from the viewpoint of food and medical applications because of their unique properties. They are able to form inclusion complexes with a number of organic or inorganic molecules, thereby changing the physical and chemical properties of the included compounds (French, 1957). We have characterized a CD-forming enzyme (cyclomaltodextrin glucanotransferase, EC 2.4.1.19, CGTase) produced by a bacterium capable of growing in highly alkaline media (Nakamura & Horikoshi, 1976). This is a unique enzyme, especially in having a wide pH optimum, and enhanced pH and thermal stability, compared with other microbial CGTases (French, 1957; Okada *et al.*, 1973; Shiosaka *et al.*, 1973; Kitahata *et al.*, 1974; Bender, 1977). This CGTase is highly active on various substrates: about 80% of starch, 60% of glycogen, 50% of amylopectin β -limit dextrin, and 15% of maltose were converted to CDs (Nakamura & Horikoshi, 1976). To study this unique enzyme, we have started to clone the CGTase gene and to analyse the genetic information. This paper deals with the molecular cloning of the gene, the determination of its nucleotide sequence, and some enzymic properties of the gene product.

METHODS

Bacterial strains and plasmids. Alkalophilic *Bacillus* sp. strain no. 38-2 (ATCC 21783), a producer of an alkaline CGTase, was isolated in our laboratory (Yamamoto *et al.*, 1972). Other bacterial strains, *Escherichia coli* K12 strain HB101 (*pro leuB thiB1 lacY hsdR hsdM ara-14 galK2 xyl-5 mtl-1 supE44 F⁻ endoI recA Str^r*) and JM103 (Messing *et al.*, 1981) were used. The plasmids pBR322, pUC18 and pUC19 were used throughout.

Media. An alkaline medium (pH 10) containing 1% (w/v) soluble starch, 0.5% yeast extract, 0.5% polypeptone, 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O and 1% (w/v) Na₂CO₃ was used for the alkalophilic *Bacillus*. Sodium carbonate was separately sterilized in a tightly capped bottle. LB medium (Maniatis *et al.*, 1982) containing 0.1% soluble starch was used for *E. coli*.

Abbreviations: CGTase, cyclomaltodextrin glucanotransferase; CD, cyclodextrin; APase, alkaline phosphatase.

Preparation of DNA. Alkalophilic *Bacillus* sp. strain no. 38-2 was aerobically grown to the early stationary phase at 37 °C in the alkaline medium described above. Chromosomal DNA was purified by the method of Saito & Miura (1963). The vector pBR322 was purified by the method of Bolivar *et al.* (1977). Recombinant plasmids were detected by the boiling method (Davis *et al.*, 1980).

Construction of recombinant plasmids. pBR322 DNA was completely digested with *Bam*HI, and chromosomal DNA was partially digested with *Sau*3AI to obtain DNA fragments of 1–20 kb. After digestion, 1 µg plasmid and 3 µg bacterial chromosomal DNA were mixed, ligated with T4 DNA ligase overnight at 15 °C, and used for transformation (Lederberg & Cohen, 1974).

Analysis of CGTase activity. CGTase activity was detected as starch hydrolytic activity on LB/starch plates after exposure to a KI/I₂ indicator solution. β -CD-forming activity was measured by the reduction in the colour intensity of phenolphthalein after complex formation with β -CD (Kaneko *et al.*, 1987). The enzyme activity was determined as follows. A reaction mixture containing 40 mg soluble starch in 1.0 ml 50 mM-sodium phosphate buffer (pH 6.5) and 0.1 ml CGTase solution was incubated at 60 °C for 20 min. The reaction was stopped by adding 3.5 ml 30 mM-NaOH solution, and then 0.5 ml 0.02% (w/v) phenolphthalein in 5 mM-Na₂CO₃ solution was added. After standing for 15 min at room temperature, the reduction in *A*₅₅₀ was measured. One unit of enzyme activity was defined as the amount of enzyme that forms 1 mg β -CD min⁻¹.

Purification of CGTase. This was done as described by Nakamura & Horikoshi (1976). Alkalophilic *Bacillus* sp. strain no. 38-2 was grown at 37 °C. After 2 d cultivation, the extracellular CGTase was purified by starch adsorption and DEAE-Toyopearl 650M ion-exchange chromatography with a linear gradient of NaCl (0–0.15 M) followed by Sephacryl S-200 gel filtration.

Analysis of alkaline phosphatase (APase) and β -galactosidase activity. APase activity was determined as follows. The enzyme solution (pH 8.8) was mixed with 0.5 ml 0.1 M-*p*-nitrophenyl phosphate (PNPP), incubated at 30 °C for 15 min, and then 1 ml 2% (w/v) sodium carbonate was added. Then the *A*₄₂₀ was measured. β -Galactosidase activity was determined as follows. Enzyme solution (1 ml) was added to 0.2 ml *o*-nitrophenyl β -galactoside (ONPG) solution (4 mg ml⁻¹, pH 7.0) and incubated at 30 °C for 15 min. Then 1 ml 2% sodium carbonate was added and the *A*₄₂₀ was determined (Honda *et al.*, 1985).

Preparation of antiserum. Enzyme solution (1 ml, containing 1 mg protein) was mixed with 1 ml complete Freund's adjuvant and the mixture was injected into mature white rabbits. Booster injections of the samples were given in the same manner after four and six weeks, and the sera were collected a week after the last booster injection.

Amino acid sequence analysis. NH₂-terminal amino acid sequence determinations were done with an Applied Biosystems protein/peptide sequencer, model 477A (Edman & Henschen, 1975). Phenylthiohydantoin derivatives of amino acids were identified in an Applied Biosystems PTH analyser system, model 120A.

Amino acid composition. This 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. This was done using Sanger's dideoxy chain termination reaction (Sanger *et al.*, 1977) by the exonuclease deletion method with pUC18 and pUC19 (Henikoff, 1984).

Enzymes and chemicals. RNAase, lysozyme, ampicillin (Ap) and tetracycline (Tc) were purchased from Sigma. Restriction endonucleases, DNA polymerase I and T4 ligase were obtained from Toyobo, Osaka, Japan. The M13 sequence kit and the exonuclease deletion kit were from Takara Syuzo, Kyoto, Japan.

RESULTS

Cloning of the CGTase gene in E. coli

Ligation mixtures described above were used to transform *E. coli* HB101. The transformants were plated on the LB/starch plates and about 5×10^4 colonies of the transformants were screened. About 1×10^4 Ap^rTc^s transformants were obtained per µg DNA, and 15% of Ap^r transformants were Tc^s. After the exposure of LB/starch plates to a KI/I₂ indicator, one colony had a large halo. Analysis by paper chromatography (Kobayashi *et al.*, 1984) showed that this transformant had CGTase activity. The plasmid harboured by the transformant was designated pCS8. This plasmid could transform *E. coli* HB101 to Ap^rCGTase⁺ at a high frequency. The plasmid DNA was digested with several restriction endonucleases and the products analysed by agarose gel electrophoresis. The restriction map of the inserted DNA is shown in Fig. 1.

Homology between the cloned fragment and chromosomal DNA

To analyse the origin of the DNA inserted in pBR322, *Eco*RV-*Pvu*II-digested pCS8 was biotinylated (Langer *et al.*, 1981), and hybridized to *Eco*RV-*Pvu*II-digested chromosomal DNAs of the alkalophilic *Bacillus* sp. strain no. 38-2 and *E. coli* HB101, which had been

Fig. 1. Restriction map of pCS8. Restriction sites are indicated, and their coordinates given in kb. The medium and thick lines indicate the cloned fragment from alkalophilic *Bacillus* sp. strain no. 38-2. The thick line indicates the fragment containing the CGTase gene.

Fig. 2. Strategy for sequencing the CGTase gene of alkalophilic *Bacillus* sp. strain no. 38-2. A detailed restriction map of the *EcoRI*–*BclI* fragment 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). The sequence of the 3'–*HinfI*–*BclI* fragment (dashed line) has not been determined.

immobilized on a nitrocellulose sheet (Southern, 1975). The biotinylated plasmid hybridized to a 4.2 kb segment of DNA from alkalophilic *Bacillus* sp. strain no. 38-2, which was precisely identical in length to that of pCS8. However, no complementary sequences were detected in *E. coli* DNA fragments.

DNA sequence

We analysed the nucleotide sequence of a 2.5 kb *EcoRI*–*BclI* segment of pCS8. The DNA sequence was determined according to the strategy outlined in Fig. 2. The DNA 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 2136 bp which could encode a polypeptide of 712 amino acids. A putative ribosome-binding site, a GAGGAGG sequence that was complementary to the 3' end of *Bacillus subtilis* 16S ribosomal RNA, was observed upstream of the open reading frame (Fig. 3).

Expression and enzymic properties of CGTase produced by the *E. coli* transformant

(i) *Localization.* *E. coli* HB101(pCS8) was aerobically grown in LB broth for 24 h at 37 °C. The extracellular, periplasmic and cellular CGTase activities were assayed (Kato *et al.*, 1983). The CGTase activities localized in the extracellular, periplasmic and cellular fractions were 0.31, 3.42 and 2.09 U (ml culture)^{−1}, respectively, i.e. 5.3, 58.8 and 35.9%, respectively. As a reference, the distributions of a typical periplasmic protein, APase, and a typical cytoplasmic protein, β -galactosidase (β -gal), were determined. Neither enzyme activity was detected in the

EcoRI
GAATTCGCTTTTCATATAAAATGAACAAGAACACAT

CACTATACITACATAACAAGCTAAGGGCTATGCATTCTTACCTTACCCCGGTATGGAACAACCCCGGTATCTCTATTAGAGACGCGGGGT

TTTITATGAGCCGAGATGAAGGAGGTGATCCCCAAAGCGACGGACAGGCCTGTTATCCCCAAGCACTGTATACGATGAGGAGGTATAGT

10 20 30 40 50 60 70 80 90 (SD)
ATGAAAGATTTATGAAACTAACAGCCGATGGACACTCTGGTTATCCCTCAGCGTGGGCCTCTTGAGCCCGGTCCAGCAGCCCGGAT
MetLysArgPheMetLysLeuThrAlaValTrpThrLeuTrpLeuSerLeuThrLeuGlyLeuLeuSerProValHisAlaAlaProAsp
(-27) (1)

100 110 120 130 140 150 160 170 180
ACCTCGGATACCAACAAGCAGAATTTCAGCAGGATGTCATATATCAGATCTTCACCGACCGGTTCTCGGACGGCAATCCGGCCAACAAT
ThrSerValSerAsnLysGlnAsnPheSerThrAspValIleGlyrGlnIlePheThrAspArgPheSerAspGlyAsnProAlaAsnAsn

190 200 210 220 230 240 250 260 270
CCGACCGCGCGCATTTGACGGATCATGTACGAATCTTCGCTTATACCTGCGGCGGCGACTGGCAAGGCATCATCAACAAAATCAACGAC
ProThrGlyAlaAlaPheAspGlySerCysThrAsnLeuArgLeuTyrCysGlyGlyAspTrpGlnGlyIleIleAsnLysIleAsnAsp

280 290 300 310 320 330 340 *BclI* 350 360
GGTTATTTGACCGGCATGGGCATTACGGCCATCTGGATTTACAGCCCTGTCGAGAAATATCTACAGCGTGATCAACTACTCCGGCGTCCAT
GlyTyrLeuThrGlyMetGlyIleThrAlaIleIleTrpIleSerGlnProValGluAsnIleTyrSerValIleAsnTyrSerGlyValHis

370 380 390 400 410 420 430 440 450
AATACGGCTTATCAGGCTACITGGGCGGGGACTTCAAGAAGACCAATCCGGCCTACGGAACGATGCAGGACTTCAAAAACCTGATCGAC
AsnThrAlaTyrHisGlyTyrTrpAlaArgAspPheLysLysThrAsnProAlaTyrGlyThrMetGlnAspPheLysAsnLeuIleAsp

460 470 480 490 500 510 520 530 540
ACCGCGCATGCGCATAACATAAAGTCATCATCGACTTTGCACCGAACCATACATCTCCGGCTTCTTCGGATGATCCTTCTTTGCAGAG
ThrAlaHisAlaHisIleLysValIleIleAspPheAlaProAsnHisThrSerProAlaSerSerAspAspProSerPheAlaGlu

550 560 570 580 590 600 610 620 630
AACGGCCGCTTGACGATAACGGCAACCTGCTCGGCGGATACACCAACGATACCCAAATCTGTTCCACCATTATGGCGGCACGGATTTC
AsnGlyArgLeuTyrAspAsnGlyAsnLeuLeuGlyGlyTyrThrAsnAspThrGlnAsnLeuPheHisHisTyrGlyGlyThrAspPhe

640 650 660 670 680 690 700 710 720
TCCACCATGAGAACGGCAATTATAAAACCTGTACGATCTGGCTGACCTGAATCATTAACAACAGCAGCGTCGATGTGATCTGAAGGAT
SerThrIleGluAsnGlyIleTyrLysAsnLeuTyrAspLeuAlaAspLeuAsnHisAsnAsnSerSerValAspValTyrLeuLysAsp

730 740 750 *HincII* 760 770 780 790 800 810
GCCATCAAAATGIGGCICGACCTCGGGTTGACGGCATTGCGTGGACCGGTCAGCATATGCCATTGCGGCTGGCAGAAAGAGCTTTATG
AlaIleLysMetTrpLeuAspLeuGlyValAspGlyIleArgValAspAlaValLysHisMetProPheGlyTrpGlnLysSerPheMet

820 830 840 850 860 870 880 890 900
TCCACCATTAACAACTACAAGCGGCTTCAAACTTCGGCAATGGTTCCTTGGCGTCAATGAGATTAGTCCGGAATACCATCAATTGCGCT
SerThrIleAsnAsnTyrLysProValPheAsnPheGlyGluTrpPheLeuGlyValAsnGluIleSerProGluTyrHisGlnPheAla

910 920 930 940 950 960 970 980 990
AACGAGTCCGGGATGAGCCTGCCTCGATTTCCTGTTGCCGAGAAAGCCCGGCAAGTGTTCAGGGACAACACCGACAATATGTACGGCCTG
AsnGlySerGlyMetSerLeuLeuAspPheProPheAlaGlnLysAlaArgGlnValPheArgAspAsnThrAspAsnMetTyrGlyLeu

1000 1010 1020 1030 1040 1050 1060 1070 1080
AAAGCGATGCTGGAGGGCTCTGAAGTAGACTATGCCAGGTGAATGACCAGGTGACCTTCATCGACAATCATGACATGGAGCGCTTCCAC
LysAlaMetLeuGluGlySerGluValAspTyrAlaGlnValAsnAspGlnValThrPheIleAspAsnHisAspMetGluArgPheHis

1090 1100 1110 1120 1130 1140 1150 1160 1170
ACCAGCAATGGGACAGCAGGAAGCTGAGCAGGCGCTGGCCTTACCTGACCTCAGCGGTGTGCTGCCATCTATTACGGCAGCGAG
ThrSerAsnGlyAspArgArgLysLeuGluGlnAlaLeuAlaPheThrLeuThrSerArgGlyValProAlaIleTyrTyrGlySerGlu

1180 1190 1200 1210 1220 1230 1240 1250 1260
CAGTATATGTCIGGCGGGAATGATCCGGAACCGTGCTCGGATTCTTCTTCTCCACGACGACGACCGCATATCAAGTCATCCAAAAG
GlnTyrMetSerGlyGlyAsnAspProAspAsnArgAlaArgIleProSerPheSerThrThrThrAlaTyrGlnValIleGlnLys

1270 1280 1290 1300 1310 1320 1330 *BclI* 1340 1350
CTCGCTCCGCTCCGCAATCCAACCCGGCCATCGCTTACGGTTCCACACAGGAGCGCTGGATCAACAACGATGTGATCATCTATGAACGC
LeuAlaProLeuArgLysSerAsnProAlaIleAlaTyrGlySerThrGlnGluArgTrpIleAsnAsnAspValIleIleTyrGluArg

1360 1370 1380 1390 1400 1410 1420 1430 1440
AAATTCGGCAATAACGTGGCCGTTGTTGCCATTAAACGCAATATGAACACACCGGCTTCGATTACCGGCCTTGTCACTTCCCTCCCGCAG
LysPheGlyAsnAsnValAlaValAlaIleAsnArgAsnMetAsnThrProAlaSerIleThrGlyLeuValThrSerLeuProGln

1450 1460 1470 *EcoRI* 1480 1490 1500 1510 1520 1530
GGCAGCTATAACGATGTGCTCGGCGGAATCTGAACGGCAATACGCTAACCGTGGGTGCTGGCGGTGCAGCTTCCAACCTTACTTTGGCT
GlySerTyrAsnAspValLeuGlyGlyIleLeuAsnGlyAsnThrLeuThrValGlyAlaGlyGlyAlaAlaSerAsnPheThrLeuAla

1540 1550 1560 1570 1580 1590 1600 1610 1620
CCTGGCGGCACTGCTGTATGGCAGTACACAACCGATGCCACAGCTCCGATCAACGGCAATGTGCGGCCGATGATGGCCAAGGCAGGGGTC
ProGlyGlyThrAlaValTrpGlnTyrThrThrAspAlaThrAlaProIleAsnGlyAsnValGlyProMetMetAlaLysAlaGlyVal

1630 1640 1650 1660 1670 1680 1690 1700 1710
ACGATTACGATTGACGGCCGCGCTTCGGCTCGGCAAGGAACGGTTTACTTCGGTACAACGGCAGTCACTGGCGCGGACATCGTAGCTTGG
ThrIleThrIleAspGlyArgAlaSerAlaArgGlnGlyThrValTyrPheGlyThrThrAlaValThrGlyAlaAspIleValAlaTrp

1720 1730 1740 1750 1760 *EcoRV* 1770 1780 1790 1800
 GAAGATACACAAATCCAGGTGAAATCCTGCGGGTCCCTGGCGGCATCTATGATATCAGAGTTGCCAACGCAGCCGGAGCAGCCAGCAAC
 GluAspThrGlnIleGlnValLysIleLeuArgValProGlyGlyIleTyrAspIleArgValAlaAsnAlaAlaGlyAlaAlaSerAsn
 1810 1820 1830 1840 1850 1860 1870 1880 1890
 ATCTACGACAATTTCAGGTGCTGACCGGAGACCAAGTCCACCGTTCGGTTCGTAATCAACAATGCCACAACGGCGCTGGGACAGAATGTG
 IleTyrAspAsnPheGluValLeuThrGlyAspGlnValThrValArgPheValIleAsnAsnAlaThrThrAlaLeuGlyGlnAsnVal
 1900 1910 1920 1930 1940 1950 1960 1970 1980
 TTCCTCAGCGCAATGTCAGCGAGCTGGGCAACTGGGATCCGAACAACGGCGATCGGCCCGATGTATAATCAGGTCTGTACCAATACCCG
 PheLeuThrGlyAsnValSerGluLeuGlyAsnTrpAspProAsnAsnAlaIleGlyProMetTyrAsnGlnValValTyrGlnTyrPro
 1990 2000 2010 2020 2030 2040 2050 2060 2070
 ACTTGGTATTATGATGTCAGCGTTCGGGCAAGGCCAACGATTGAATTTAAATTCCTGAAAAAGCAAGGCTCCACCGTCACATGGGAAGGC
 ThrTrpTyrTyrAspValSerValProAlaGlyGlnThrIleGluPheLysPheLeuLysLysGlnGlySerThrValThrTrpGluGly
 2080 2090 2100 2110 2120 2130 2140
 GGCGCGAATCGCACCTTCACCAACCCCAACCGCGGCACGGCAACGGTGAATGTGAAGTGGCAGCCTTAATAGGCACCTTGAAGGTAAGCA
 GlyAlaAsnArgThrPheThrThrProThrSerGlyThrAlaThrValAsnValAsnTrpGlnPro*****
 (685)

*Hind*III

AGCGGCTCCGGGTAGAGGCTCGGGGCCGCTTGTTACGTTATGTGGGGAAAGTTGCTATAGGGTCTTGCTCCACACGCACGATTCTT

Fig. 3. The nucleotide sequence of the CGTase gene from alkalophilic *Bacillus* sp. strain no. 38-2. 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 arrowhead. The boxed amino acids have been determined by automated Edman sequencing of the purified CGTase. The sequence containing an inverted repeat structure downstream from the termination codon TAA is designated by $\leftarrow \rightarrow$.

Fig. 4. Ouchterlony double-diffusion analysis of CGTase. Well AB contained antibody to the purified CGTase from alkalophilic *Bacillus* sp. strain no. 38-2. Samples were added to the outer wells as indicated below. Well 1, periplasm fraction of *E. coli* HB101(pCS8); well 2, purified enzyme of alkalophilic *Bacillus* sp. strain no. 38-2; well 3, periplasm fraction of *E. coli* HB101(pBR322).

extracellular fraction, 78% (APase) and 3% (β -gal) of the total activities were detected in the periplasmic fraction, and 22% (APase) and 97% (β -gal) of the total activities were detected in the cellular fraction, respectively. Total CGTase activity of *E. coli*(pCS8) was 5.82 U (ml culture)⁻¹, which is about 70% of the enzyme activity produced by alkalophilic *Bacillus* sp. strain no. 38-2, cultivated for 3 d at 37 °C in alkaline medium. Although the production of the enzyme by alkalophilic *Bacillus* sp. strain no. 38-2 was glucose repressed, the synthesis of the CGTase in *E. coli* was not inhibited by addition of glucose.

(ii) *Immunological studies.* The CGTase of *E. coli* HB101(pCS8) was tested against antiserum prepared against the CGTase of *Bacillus* sp. strain no. 38-2. The CGTase of *E. coli* HB101(pCS8) gave a line of precipitation which fused with that for CGTase from *Bacillus* sp. strain no. 38-2. No reaction was observed with *E. coli* HB101(pBR322). The addition of 15 μ l antiserum in the activity assay system caused 100% inhibition of the CGTase activity (about 200 mU) from *E. coli* HB101(pCS8).

1	ATGAAAGATTTATGAAACTAACAGCCGTATGGACACTCTGGTTATCCCTCACGCTGGGCCTCTTGAGCCCGGTCCACGCAGCCCCGGAT	90
2	-----TCGCGGTAC-----GTTTGA--TCCTT-G-G--T-C-C-GAGTA-GG--T-----GA-T-CACTG--C-CATGG--T-A--C--	90
	S R Y R L T S L A S M A I S L A W S	
1	ACCTCGGTATCCAAACAGCAATTTACGACGGATGTCATATATCAGATCTTCACCGACCGGTTCTCGGACGGCAATCCGGCCAAACAAT	180
2	--GAGC--GGA-----GTC-----T-----C-----C-----TG-G-----C--G-----GG-CAG-A-G-----	180
	D V V V A D R T	
1	CCGACCGCGCGGCGCATTTGACGGATCATGTACGAATCTTCGCTTATCTGCGGCGGCGACTGGCAAGGCATCATCAACAAAATCAACGAC	270
2	--G-G--G-AT--G--CAG--CGACC-AT-G--T-GAAGC-C--T-T--G--A-----G--G--T--G--G--G--T-----	270
	A D S D R S K F D	
1	GGTATTTTGACCGGATGGGCATTACGGCCATCTGGATTTACAGGCCTGTCGAGAATATCTACAGCGTGATCAACTACTCGGGCGTCCAT	360
2	-----G-C--C--C-----L-----H-----A-----G--A-----AC-TC--C-----G--A-----TA-C	360
	V C L C A T K N	
1	AATACGGCTTACTACGGCTACTGGGCGCGGACTTCAAGAAGACCAATCCGGCCTACGGAACGATGACGACTTCAAAAACCTGATCGAC	450
2	-----T-----T-----A-----T-----C-A-----CGAC--T-T--GGATT-TGCC--T--TC--T-----T--T	450
	S Q D F A Q	
1	ACCGCGCATGCGCATAACATAAAGTCATCATCGACTTTGCACCGAACCATACATCTCCGGCTTCTTCGGATGATCCTTCTTTCAGAG	540
2	--GCTCAGCCTCATA-CATC--GGTCGGATCGACTTCGCC-C-AACCA--CGTC--CGGCGGAG-AGG-ACGA-C--CGG--C--C--	540
	L T L I T S R S D R L R P Q P V G R A G T N G	
1	AACGGCGCTTGTACGATAACGGCAACCTGCTCGGGGATACACCAACGATACCCAAAATCTGTTCCACCATTTAGGCGGCACGGATTTTC	630
2	-----TGCGC-----T-----TTCG-----CC--G--T--GGCCGGC--T-----T--A-C--G--G--C-----T	630
	A S A S A G N	
1	TCCACCATTTGAAACGGCATTTTATAAAACCTGTACGATCTGGCTGACCTGAATCATAACACAGCAGCGTCGATGTGTATCTGAAGGAT	720
2	-----G--G--AG--T-----C--G-----C-----C-----G--A--C--C-----A-GCTA-G--C--CT--T--AAGC	720
	D I N A M A G F S	
1	GCCATCAAAATGTGGCTCGACCTCGGGGTTGACGGCATTCGCGTGGACGCGGTCAAGCATATGCCATTCGGCTGGCAGAAAGGCTTTATG	810
2	--T--G--CC-T-----G-A-G--T--G-----G-----TT-T-----G-----GTAT--T-----A-A-----CT-T	810
	D L G M F Q Y T Y	
1	TCCACCATTAACAACTACAAG CCGGTCTTCAACTTCGGCGAATGGTTCTTGGCGTCAATGAGATTAGTCCGGAATACCATCAATTC	897
2	--T-G--T--GG-GG-G-TCAT-----A--T-CG-----G-----AT-----CGG--C-A--CCGACGGA--CA--AT-A-----	900
	S Y G G D H T Y A D Q T D G D N I K	
1	GCTAACGAGTCCGGGATGAGCCTGCTCGATTCCCGAAAGCCAGAAAGCCCGGCAAGTGTTCAGGGACAACACCGACAATATGTACGGC	987
2	--C-----AAG-----A-----G--C--T--AAT-C--G--G--A-TG--CG-----C-----A-G--C--A-G--AT	990
	N Q Y E V E K E T K D	
1	CTGAAAGCGATGCTGGAGGGCTCTGAAGTAGACTATGCCAGGAGAATGACCAGGTGACCTTCATCGACAATCATGACATGGAGCGTTTC	1077
2	--CT-T-A-G-----CCA--A-G--GTCG-A--C-A-T-CA-C--CA-TAT-----C-----T-----C--G--	1080
	Y E V A S T S Q D Y I N M D	
1	CACACCAAGCAATGGCGACAGACGGAAAGCTGGAGCAGGCGCTGGCCTTTACCTGACTTCACGCGGTGTGCCTGCCATCTATTACGGAAGC	1167
2	--GGTTGC--GG-TC--GT-CG-----GC-ACC-----A--T-----G-G--G-----C-----C-----A-----C-----CG	1170
	Q V A G S G T A T L T	
1	GAGCAGTATATGTCTGGCGGGAATGATCCGGACAACCGTGCTCGGATTCTTCTTCTCCACGACGACGACCGCATATCAAGTCATCCAA	1257
2	-----C--A-C-----ATGGC--C--CA-----G--GAT--GA-C--G--TAAT--CGG-----G--T--A--CG--T--G	1260
	T D G C N M M T N C G K	
1	AAGCTCGCTCCGCTCCGCAATCCAACCGGCCATCGCTTACGGTTCCACACAGGAGCGCTGGATCAACAACGATGTGATCATCTATGAA	1347
2	GCAT-G--G-----G--T-----T-----T-----T--GA-G--GACA-----G--T-----T--G--TAT---	1350
	A T T V L I	
1	CGCAAATTCGGCAATAACGTTGGTGTGCCATTAAACCGCAATATGAACACACCGGCTTCGATTACGGCCTTGTCACTTCCCTCCCG	1437
2	-----GC-G--CC--TT-G--G--G-----T--A--CTC-TC-G-CG-TTA-C-----T--G--T--GT-G--G--G--G--	1440
	S S A L S S A A Y P S L S	
1	CAGGGCAGCTATAACGATGTGCTCGGCGGAATCTGAACGGCAATACGCTAACCGTGGGTGCTGGCGGTGACGCTTCAACTTTACTTTG	1527
2	GC--CT--TCG-----AT-GAA--L--C--CT-----CT-CA-T-----AGC-----C--C--TCA--G--G--GC--	1530
	A T S N L S I S V T L	
1	GCTCTGGCGGCGACTGCTGTATGGCAGTACACAACCGATGCCACAGTCCGATCAACGGCAATGTGGCGCGGATGATGGCAAGGCAGGG	1617
2	--GG-C-----G--G-----G-GCCG-AA--GT-G--GCG-T-----G--T--C--CC--G--C--G--G--	1620
	A P E S A I T G Q P	
1	GTCACGATTACGATTGACGGCGCGGCTTCGGCTCGGCAA GGAACGGTTTACTTCGGTACAACGGCAGTCACTGGCGCGGACATCGTA	1704
2	AAT-TAC-G-----GC-TT-GCG-CACGGCG--C-----T-----GA-G-----G--G--C--T--C--G-----	1710
	N I V G F G G T A S G	

	A	Q	V	K	L	R	P	G	I	Y	D	I	R	A	N	A	A	A	
1	GCTTGGGAAGATACACAAATCCAGGTGAAAACTCTGCGGGTCCCTGGCGGCATCTATGATATCAGAGTTGCCAACGCGAGCCGGAGCAGCC	1794																	
2	AGC-----G--C--G--G--TA--C-GTC--A-C-AA--G-G-CG--AAACG-GCG-ATCG--CAAA-CGT-GT--CA-C---	1800																	
	S		K	A	V	P	K	A	A	K	T	G	V	S	K	T	S	S	T
	I	Y	D	N	E				V	I	N	T	A	L	Q				
1	AGCAACATCTACGACAATTCGAGGTGCTGACCGGAGACCAAGGTCACCGTTCGGTTCGTAATCAACAATGCCACAACGGCGCTGGGACAG	1884																	
2	-----T-CA-T-A-A-GC--A-T--A-----G-----T-----G-G-T--C-GG--TC-A--AT--CAATTAC--ACA	1890																	
	T	F	K	S	N				L	V	Q	N	N	Y	T				
	F	T	V	S	N			N				V	Y	Q					
1	AATGTGTTCTCAGCGGCAATGTCAGCGAGCTGGCCAACTGGGATCCGAACAACGCGATCGGCCCGATGTATAATCAGGTCTGTCTACCAA	1974																	
2	-----T-AT--TGTC-----C-C-GC-----C--C-----C-----A-----T--G-----C-----GA--GC-A-G	1980																	
	Y	V	A	A	T			K				I	A	K					
	T				Q	T	I	E	L		Q	S							
1	TACCCGACTTGGTATTATGATGTCAGCGTTCGGCAGGCCAAACGATTGAATTTAAATTCCTGAAAAAGCAAGGCTCCACCGTCACATGG	2064																	
2	-----T-C-----C-----G-----G--GAC--AGC-G-----TA-T-----GGC--GGT--G--G--T---	2070																	
	S					T	K	L	D	I	G	G							
	A	R	F		T	T	A	N	N	P	#								
1	GAAGCGCGCGGAATCGCACCTTCACCAACCCCAACCGCGGCACGGCAACGGTGAATGTGAAGTGGCAGCCTTAA	2139																	
2	-----G-GC--C-AT--G-A--G--G--GG-----GTA-GG-----CG--G-----AAA----	2145																	
	G	H	Y		A		V	G	T	D	N	#							

Fig. 5. Comparison of the nucleotide sequence and the deduced amino acid sequence of the CGTase genes of alkalophilic *Bacillus* sp. strain no. 38-2 and *B. macerans*. 1, *Bacillus* sp. strain no. 38-2; 2, *B. macerans* CGTase. Bars 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.

Table 1. Amino acid composition of the extracellular CGTase from the alkalophilic *Bacillus* sp. strain no. 38-2

Amino acid	From DNA sequence		Amino acid analysis (mol %)	Amino acid	From DNA sequence		Amino acid analysis (mol %)
	Position	Mol %			Position	Mol %	
Lys	23	3.3	3.5	Gly	64	9.4	10.3
His	12	1.7	1.7	Ala	57	8.3	8.3
Arg	23	3.4	3.2	Cys	2	0.2	—
Trp	13	1.9	2.0	Val	48	6.9	6.7
Asx*	110	16.0	16.0	Met	14	2.0	1.9
Thr	58	8.4	8.0	Ile	43	6.2	6.3
Ser	39	5.6	4.4	Leu	36	5.1	5.5
Glx*	48	6.9	7.5	Tyr	34	4.9	5.1
Pro	29	4.2	4.1	Phe	32	4.7	4.9

—, Not detected.

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

(iii) M_r . The M_r of pCS8-encoded CGTase (periplasmic) was estimated to be 68000 by the SDS-PAGE method of Weber *et al.* (1972).

Determination of amino acid composition and N-terminal amino acid sequence

The amino acid composition of the extracellular CGTase of alkalophilic *Bacillus* sp. strain no. 38-2 is presented in Table 1. Molar ratios obtained by amino acid analysis were closely consistent with those deduced from the DNA sequence. The NH₂-terminal sequence of the purified CGTase was determined by automated Edman degradation up to the 17th residue to be 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 position 82 in Fig. 3. Therefore, 27 amino acid residues (residues —27 to —1) may be a signal peptide which is removed during the secretion process.

DISCUSSION

We have now cloned and analysed the nucleotide sequence of the CGTase gene of alkalophilic *Bacillus* sp. no. 38-2. There is a single open reading frame of 2136 bp beginning with an ATG initiation codon at the nucleotide +1 and ending with a TAA termination codon at +2137. The sequence of the N-terminal 17 amino acids of the extracellular CGTase from alkalophilic *Bacillus* sp. no. 38-2 was determined with a peptide sequencer. This amino acid sequence was identical to that deduced from the DNA sequence (nucleotide positions 82 to 132, Fig. 3). This result suggested that the first 27 amino acid residues, from the initiator Met to the Ala at position 80 in the nucleotide sequence, represented a signal peptide which was removed during secretion of the CGTase. The amino acid sequence of this peptide, with several positively charged amino acids near the N-terminus followed by a hydrophobic amino acid core and alanine at the C-terminal end, is consistent with the signal peptides from other Gram-positive bacteria (Béguin *et al.*, 1985; Kato *et al.*, 1985; Murphy *et al.*, 1984; Palva *et al.*, 1981; Stephens *et al.*, 1984; Yang *et al.*, 1983). Localization of appreciable amounts of the enzyme in the periplasm suggested that this signal peptide is also functional in *E. coli*, but we have no information on the N-terminal sequence or the M_r of the periplasmic CGTase from the transformant. Thus, according to the nucleotide sequence, the mature CGTase comprises 685 amino acids and has an M_r of 75160. The Shine-Dalgarno (SD) sequence GAGGAGG (McLaughlin *et al.*, 1981), 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. The expression of the CGTase gene in *E. coli* was not affected by the orientation of the inserted fragment in pBR322. We assume that the promoter which works in *E. coli* exists upstream of the open reading frame; however, no clear promoter-like sequence was found in the *EcoRI*-*BclI* fragment.

The nucleotide sequence and the amino acid sequence of this CGTase have strong homology with the *Bacillus macerans* CGTase (Takano *et al.*, 1986). Fig. 5 shows the alignment between these sequences. In the amino acid sequences, 448 (63%) of the aligned amino acids are identical. The overall homology of the aligned nucleotide sequences is 64%.

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