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Molecular Cloning, DNA Nucleotide Sequencing, and Expression in *Bacillus subtilis* Cells of the *Bacillus macerans* Cyclodextrin Glucanotransferase Gene

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The gene for cyclodextrin glucanotransferase from *Bacillus macerans* was cloned in an *Escherichia coli* bacteriophage, λD69, and was recloned in a *Bacillus subtilis* plasmid, pUB110. Starting from an ATG initiation codon, a unique reading frame was shown to extend for 2,142 base pairs (714 amino acids). The nucleotide sequence revealed that the enzyme is composed of two identical subunits.

CD enzyme (cyclodextrin glucanotransferase, EC 2.4.1.19), a unique enzyme capable of degrading starch to cyclodextrins, is a very important enzyme in the food and pharmaceutical industries (10). Cyclodextrins are closed-ring structures in which six or more glucose units are joined by means of α-1,4 glucosidic bonds. The CD enzyme of *Bacillus macerans* has been purified, crystallized, and well characterized by many workers (4, 9).

To study the structure and mechanisms of action of the CD enzyme on starch and to obtain hyperproducers of CD enzyme for industrial use, we cloned the gene from *B. macerans* with an *Escherichia coli* and *Bacillus subtilis* vector system. The complete DNA nucleotide sequence of the gene was then determined, and the gene was expressed in *B. subtilis* cells.

B. macerans IAM 1243, which produces extracellular CD enzyme, was obtained from the Institute of Applied Microbiology, the University of Tokyo, Bunkyo-ku, Tokyo, Japan (4). *B. subtilis* 207-25 (m_{168}^- *hsrM recE4 amyE07 aroI906 leuA8 lys-21*) and *B. subtilis* NA64 (*amyR2 amyE*$^+$ *purB6 metB5*) are derivatives of *B. subtilis* Marburg 168 (14, 15). *B. macerans* chromosomal DNA (approximately 20 μg) was partially digested with the restriction enzyme *Sau*3AI. The DNA fragments (1 μg) were ligated with *Bam*HI-cleaved λD69 DNA (7). The resultant chimeric bacteriophage DNAs were packaged in vitro utilizing a commercial kit (Amersham International plc., Amersham, England), and the assembled phage were used to infect *E. coli* SM32 (SA500 *his pyrD Δlon-100 sulA gal rpsL*) (7). λ phages containing the CD enzyme gene were screened for the formation of halos around plaques resulting from the starch-hydrolyzing activity of the enzyme on L-broth agar plates containing 1% soluble starch. Four phage strains showed CD enzyme activity. One of them, λCD, had a 10.9-kilobase (kb) DNA insert and was chosen for further study (Fig. 1). After λCD DNA (10 μg) was partially digested with *Sau*3AI, 2- to 6-kb DNA fragments were isolated by agarose gel electrophoresis and inserted into the *Bam*HI site of pUB110. The constructed plasmids were then transferred into *B. subtilis* 207-25, an α-amylase deficient mutant, and kanamycin-resistant, halo-positive transformants were selected. Of 24 transformants, 6 were chosen at random, and the plasmids harbored in them were designated as pDS1, pDS3, pDS10, pDS11, pDS12, and pDS20, respectively. The physical maps of the inserted DNAs and the surrounding areas of pUB110 are summarized in Fig. 1. The DNA inserts in pDS12 and pDS20 were in the opposite orientation to those of the other plasmids, but all contained a common region of approximately 2,100 base pairs (bp), as shown by the arrows in Fig. 1.

Utilizing the strategy shown in Fig. 2, we sequenced 2,700

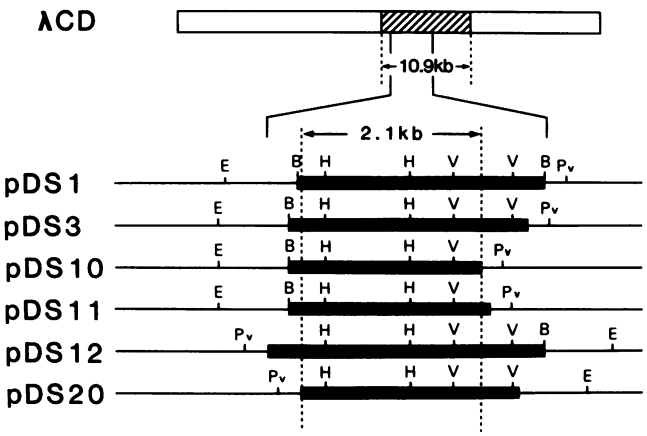

FIG. 1. Comparison of inserted DNA in pDS1, pDS3, pDS10, pDS11, pDS12, and pDS20 with λCD DNA in which the gene for *B. macerans* CD enzyme was first cloned. The common DNA region in the six plasmids is shown by a horizontal arrow which corresponds to a 2.1-kb DNA fragment. λCD phage particles were prepared by the method of Yamamoto and Alberts (13), and the DNA was then extracted by dialyzing against 50% formamide. Plasmids were prepared by the rapid alkaline method of Birnboim and Doly (1) and purified by ethidium bromide-CsCl equilibrium centrifugation, followed by agarose gel electrophoresis and subsequent recovery utilizing hydroxyapatite (11). ▭, λD69; ▨, 10.9-kb DNA region from *B. macerans* inserted into λCD DNA; ——, pUB110 DNA; and ▬, inserted DNA from λCD subcloned into pUB110. E, *Eco*RI site in pUB110; Pv, *Pvu*II site in pUB110; B, *Bam*HI site; V, *Eco*RV site; H, *Hin*dIII site.

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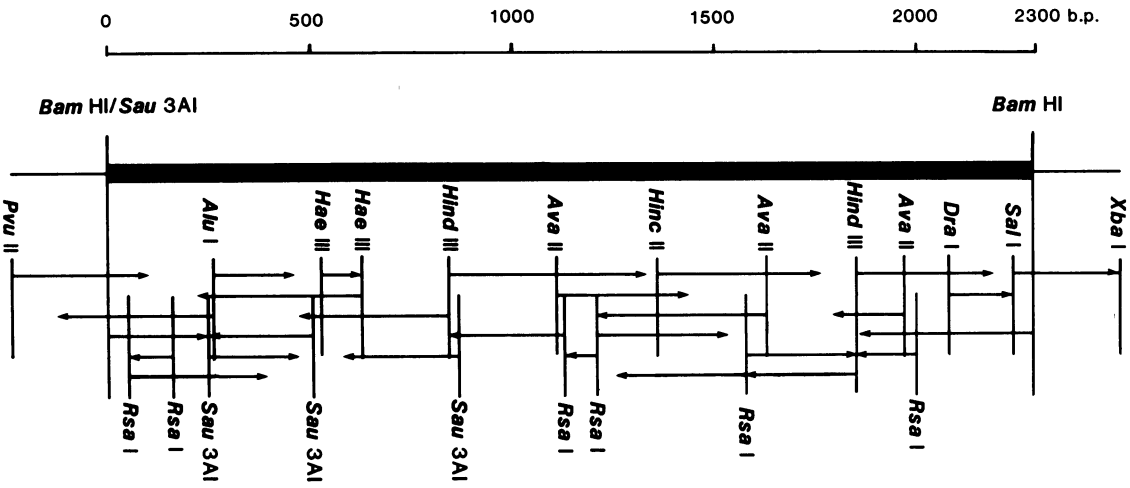

FIG. 2. Strategy for determining the DNA nucleotide sequence of the gene for *B. macerans* CD enzyme in pDS10. ▬, 2.3-kb inserted DNA containing the *B. macerans* CD enzyme structural gene; ——, pUB110 DNA. The direction and extent of sequence determination are shown by the horizontal arrows.

bp of the region containing the inserted DNA of pDS10 (the smallest of the recombinant plasmids) (Fig. 3). Included in the sequence were 2,289 bp of inserted DNA, the remainder being sequences around the *Bam*HI site of pUB110. There is a unique open reading frame of 2,142 bp beginning with an ATG initiation codon at nucleotides +1 to +3 and ending with a TAA termination signal at nucleotides 2143 to 2145 (714 amino acids with a molecular weight of 76,994).

The first two amino acids at the NH_2-terminal end of the extracellular CD enzyme from *B. macerans* were determined to be Ser-Pro. These amino acids correspond to nucleotide positions +82 to +87 in the analyzed DNA sequence. These results suggest that the first 27 amino acids, from the initiator Met to Ala, constitute a signal peptide involved in secretion of the protein. The deduced amino acid sequence of this peptide is typical of signal peptides of other exported proteins (several positively charged amino acids, followed by a hydrophobic amino acid core and a COOH-terminal Ala residue). Thus, the extracellular CD enzyme would be composed of 687 amino acids giving a molecular weight of 74,046.

TABLE 1. Predicted amino acid composition from DNA nucleotide sequence of *B. macerans* CD enzyme gene compared with that of a purified extracellular *B. macerans* CD enzyme[a]

Amino acids	No. of deduced amino acid residues		Mol%	
	Precursor protein	Assumed extracellular protein	Assumed extracellular protein	Purified extracellular CD enzyme
Ala	61	57	8.3	9.1
Arg	23	21	3.1	2.7
Asn	52	52	7.6 } 14.7	14.2
Asp	49	49	7.1	
Cys	0	0	0.0	0.0
Gln	21	21	3.1 } 5.9	6.4
Glu	19	19	2.8	
Gly	80	79	11.5	11.2
His	9	9	1.3	1.6
Ile	37	36	5.2	5.1
Leu	46	40	5.8	5.7
Lys	28	26	3.8	4.1
Met	14	12	1.7	1.8
Phe	32	32	4.7	4.8
Pro	25	24	3.5	3.6
Ser	52	47	6.8	6.9
Thr	68	67	9.8	9.5
Trp	14	13	1.9	1.7
Tyr	35	34	4.9	4.4
Val	49	49	7.1	7.2

[a] The amino acid composition of the purified enzyme was obtained from DePint and Campbell (3).

Upstream of the ATG initiation codon, the *Bam*HI site of pUB110 is located at nucleotides −35 to −31. A sequence for the ribosome-binding site, GAGGAGAGG, was found between this restriction site and the initiation codon at positions −16 to −8. However, a recognizable promoter was not found in this region. Therefore, the sequence TTGCTAGTAACATCTGACCGAGATTTTTT, at 38 to 10 bp upstream from the *Bam*HI site of pUB110 (−73 to −45 bp from the initiator ATG), seemed likely to serve as the promoter region.

B. subtilis 207-25 harboring pDS10 [*B. subtilis* 207-25(pDS10)] grown for 50 generations was plated onto an LG agar (14) plate containing 1% soluble starch (Fig. 4). The halos that appeared around colonies after exposure to a KI-I_2 indicator solution showed that starch had been degraded by the hydrolytic activity of CD enzyme. All *B. subtilis* colonies showed large halos. These results indicate that pDS10 is stably maintained and that the CD enzyme gene is efficiently expressed in *B. subtilis* grown on solid medium. No halo was detected around the colonies of *B. subtilis* 207-25 harboring pUB110.

Crude CD enzyme was prepared from the culture media of *B. macerans* IAM 1243 and *B. subtilis* 207-25(pDS10) and from an *E. coli* lysate after infection with λCD. The enzymes were incubated with soluble starch. The products were analyzed by paper chromatography (Fig. 5) and quantified by high-pressure liquid column chromatography (data not shown). The major product in all three cases was α-cyclodextrin, and a constant 6:3:1 ratio for α-, β-, and γ-cyclodextrins was also observed.

The amino acid composition for the projected extracellular form of the CD enzyme was calculated from the amino acid sequence deduced from the DNA nucleotide sequence and was compared with the composition of purified extracellular *B. macerans* CD enzyme (Table 1). As indicated, the moles

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                                                    ctggac taaaaggcat gcaatttcat aatcaaagag
                                                                                      -241

agcgaaaaag tagaacgaat gatgatattg accatgagcg aacacgtgaa aattatgatt tgaaaaatga taaaaatatt
                                                                                      -161

gattacaacg aacgtgtcaa agaaattatt gaatcacaaa aaacaggtac aagaaaaacg aggaaagatg ctgttcttgt
                                                                                       -81

aaatgagttg ctagtaacat ctgaccgaga ttttttgag caactgGATC GGCAGAACAT TTGAGAGGAG AGGTAGGACA
                                             BamHI/Sau3AI                              -1

Met Lys Ser Arg Tyr Lys Arg Leu Thr Ser Leu Ala Leu Ser Leu Ser Met Ala Leu Gly Ile Ser
ATG AAA TCG CGG TAC AAA CGT TTG ACC TCC CTG GCG CTT TCG CTG AGT ATG GCG TTG GGG ATT TCA
1                                                                                      66

Leu Pro Ala Trp Ala Ser Pro Asp Thr Ser Val Asp Asn Lys Val Asn Phe Ser Thr Asp Val Ile
CTG CCC GCA TGG GCA TCA CCC GAT ACG AGC GTG GAC AAC AAG GTC AAT TTC AGT ACG GAC GTC ATC
                                                                                     132

Tyr Gln Ile Val Thr Asp Arg Phe Ala Asp Gly Asp Arg Thr Asn Asn Pro Ala Gly Asp Ala Phe
TAT CAG ATT GTG ACC GAC CGC TTC GCG GAC GGG GAC AGG ACG AAC AAT CCG GCG GGG GAT GCG TTC
                                                                                     198

Ser Gly Asp Arg Ser Asn Leu Lys Leu Tyr Phe Gly Gly Asp Trp Gln Gly Ile Ile Asp Lys Ile
AGC GGC GAC CGA TCC AAT TTG AAG CTC TAT TTC GGG GGA GAC TGG CAG GGG ATT ATC GAC AAG ATT
                                                                                     264

Asn Asp Gly Tyr Leu Thr Gly Met Gly Val Thr Ala Leu Trp Ile Ser His Pro Val Glu Asn Ile
AAC GAC GGT TAT TTG ACC GGC ATG GGC GTC ACC GCC CTC TGG ATA TCC CAA CCT GTG GAA AAT ATC
                                                                                     330

Thr Ser Val Ile Lys Tyr Ser Gly Val Asn Asn Thr Ser Tyr His Gly Tyr Trp Ala Arg Asp Phe
ACC TCC GTC ATC AAG TAT TCC GGC GTT AAC AAT ACG TCT TAT CAC GGT TAC TGG GCG AGG GAT TTT
                                                                                     396

Lys Gln Thr Asn Asp Ala Phe Gly Asp Phe Ala Asp Phe Gln Asn Leu Ile Asp Thr Leu Thr Leu
AAG CAA ACC AAC GAC GCT TTC GGG GAT TTT GCC GAT TTT CAA AAT CTG ATT GAT ACG CTC ACG CTC
                                                                                     462

Ile Thr Ser Arg Ser Asp Arg Leu Arg Pro Gln Pro His Val Ser Gly Arg Ala Gly Thr Asn Pro
ATA ACA TCA AGG TCG GAT CGA CTT CGC CCC CAA CCA CAC GTC TCC GGC CGA GCA GGG ACG AAC CCC
                                                                                     528

Gly Phe Ala Glu Asn Gly Ala Leu Tyr Asp Asn Gly Ser Leu Leu Gly Ala Tyr Ser Asn Asp Thr
GGC TTC GCC GAG AAC GGT GCG CTG TAT GAT AAC GGT TCG CTG CTC GGC GCC TAC AGC AAT GAT ACG
                                                                                     594

Ala Gly Leu Phe His His Asn Gly Gly Thr Asp Phe Ser Thr Ile Glu Asp Gly Ile Tyr Lys Asn
GCC GGC CTT TTC CAT CAT AAC GGG GGG ACC GAT TTT TCC ACG ATT GAA GAC GGT ATT TAC AAG AAC
                                                                                     660

Leu Tyr Asp Leu Ala Asp Ile Asn His Asn Asn Asn Ala Met Asp Ala Tyr Phe Lys Ser Ala Ile
CTC TAC GAC CTG GCG GAC ATC AAC CAT AAC AAC AAC GCT ATG GAC GCT TAT TTT AAA AGC GCT ATC
                                                                                     726

Asp Leu Trp Leu Gly Met Gly Val Asp Gly Ile Arg Phe Asp Ala Val Lys Gln Tyr Pro Phe Gly
GAC CTT TGG CTC GGC ATG GGT GTG GAC GGG ATT CGT TTT GAC GCG GTG AAG CAG TAT CCT TTC GGC
                                                                                     792

Trp Gln Lys Ser Phe Val Ser Ser Ile Tyr Gly Gly Asp His Pro Val Phe Thr Phe Gly Glu Trp
TGG CAA AAA AGC TTC GTT TCC TCG ATT TAC GGC GGC GAT CAT CCG GTA TTT ACG TTC GGG GAA TGG
          Hind III                                                                   858

Tyr Leu Gly Ala Asp Gln Thr Asp Gly Asp Asn Ile Lys Phe Ala Asn Glu Ser Gly Met Asn Leu
TAT CTT GGC GCG GAT CAA ACC GAC GGA GAC AAC ATT AAA TTC GCC AAC GAA AGC GGG ATG AAC CTG
                                                                                     924

Leu Asp Phe Glu Tyr Ala Gln Glu Val Arg Glu Val Phe Arg Asp Lys Thr Glu Thr Met Lys Asp
CTG GAC TTT GAA TAC GCG CAG GAA GTG CGC GAA GTG TTC CGG GAC AAA ACG GAA ACG ATG AAG GAT
                                                                                     990

Leu Tyr Glu Val Leu Ala Ser Thr Glu Ser Gln Tyr Asp Tyr Ile Asn Asn Met Val Thr Phe Ile
CTC TAT GAG GTG CTG GCC AGC ACG GAG TCG CAA TAC GAC TAC ATC AAC AAT ATG GTG ACC TTC ATC
                                                                                    1056

Asp Asn His Asp Met Asp Arg Phe Gln Val Ala Gly Ser Gly Thr Arg Ala Thr Glu Gln Ala Leu
GAC AAC CAT GAT ATG GAC CGG TTC CAG GTT GCC GGT TCC GGT ACG CGG GCG ACC GAG CAA GCG TTG
                                                                                    1122
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Ala Leu Thr Leu Thr Ser Arg Gly Val Pro Ala Ile Tyr Tyr Gly Thr Glu Gln Tyr Met Thr Gly
GCG CTG ACG CTG ACT TCC CGC GGC GTG CCA GCC ATC TAC TAC GGC ACG GAG CAG TAC ATG ACC GGC
                                                                              1188

Asp Gly Asp Pro Asn Asn Arg Ala Met Met Thr Ser Phe Asn Thr Gly Thr Thr Ala Tyr Lys Val
GAT GGC GAC CCC AAC AAC CGG GCG ATG ATG ACC TCG TTT AAT ACC GGG ACG ACG GCT TAT AAA GTG
                                                                              1254

Ile Gln Ala Leu Ala Pro Leu Arg Lys Ser Asn Pro Ala Ile Ala Tyr Gly Thr Thr Thr Glu Arg
ATT CAG GCA TTG GCG CCG CTG CGT AAA TCC AAT CCG GCC ATC GCT TAT GGG ACG ACG ACA GAG CGC
                                                                              1320

Trp Val Asn Asn Asp Val Leu Ile Ile Glu Arg Lys Phe Gly Ser Ser Ala Ala Leu Val Ala Ile
TGG GTT AAC AAC GAT GTG TTG ATT ATT GAA CGC AAA TTC GGC AGC AGC GCC GCT TTG GTG GCG ATT
    Hinc II                                                                   1386

Asn Arg Asn Ser Ser Ala Ala Tyr Pro Ile Ser Gly Leu Leu Ser Ser Leu Pro Ala Gly Thr Tyr
AAT CGA AAC TCG TCC GCC GCT TAT CCG ATT TCG GGT CTG TTG AGT TCG CTG CCG GCG GGC ACT TAT
                                                                              1452

Ser Asp Val Leu Asn Gly Leu Leu Asn Gly Asn Ser Ile Thr Val Gly Ser Gly Gly Ala Val Thr
TCG GAT GTA TTG AAC GGA CTC TTA AAC GGC AAC TCC ATT ACC GTG GGC AGC GGC GGC GCC GTC ACC
                                                                              1518

Asn Phe Thr Leu Ala Ala Gly Gly Thr Ala Val Trp Gln Tyr Thr Ala Pro Glu Thr Ser Pro Ala
AAC TTT ACG CTG GCG GCC GGC GGC ACG GCG GTA TGG CAG TAC ACA GCG CCG GAA ACG TCG CCG GCG
                                                                              1584

Ile Gly Asn Val Gly Pro Thr Met Gly Gln Pro Gly Asn Ile Val Thr Ile Asp Gly Arg Gly Phe
ATC GGC AAT GTG GGT CCC ACC ATG GGC CAG CCG GGG AAT ATA GTG ACG ATT GAC GGC CGC GGC TTT
                                                                              1650

Gly Gly Thr Ala Gly Thr Val Tyr Phe Gly Thr Thr Ala Val Thr Gly Ser Gly Ile Val Ser Trp
GGC GGC ACG GCG GGC ACG GTT TAT TTC GGG ACG ACG GCG GTG ACC GGC TCC GGC ATC GTA AGC TGG
                                                                              1716

Glu Asp Thr Gln Ile Lys Ala Val Ile Pro Lys Val Ala Ala Gly Lys Thr Gly Val Ser Val Lys
GAG GAC ACG CAG ATT AAG GCG GTC ATA CCG AAG GTC GCG GCG GGC AAA ACG GGC GTA TCG GTC AAA
                                                                              1782

Thr Ser Ser Gly Thr Ala Ser Asn Thr Phe Lys Ser Phe Asn Val Leu Thr Gly Asp Gln Val Thr
ACG TCG TCC GGC ACC GCC AGC AAT ACA TTC AAA AGC TTC AAT GTA CTG ACG GGG GAT CAG GTC ACG
                                    Hind III                                  1848

Val Arg Phe Leu Val Asn Gln Ala Asn Thr Asn Tyr Gly Thr Asn Val Tyr Leu Val Gly Asn Ala
GTG CGT TTC CTG GTC AAT CAA GCC AAT ACC AAT TAC GGA ACA AAT GTT TAT CTT GTC GGC AAC GCC
                                                                              1941

Ala Glu Leu Gly Thr Trp Asp Pro Asn Lys Ala Ile Gly Pro Met Tyr Asn Gln Val Ile Ala Lys
GCC GAG CTC GGC ACC TGG GAC CCG AAC AAA GCG ATT GGG CCG ATG TAC AAT CAG GTG ATC GCC AAG
                                                                              1980

Tyr Pro Ser Trp Tyr Tyr Asp Val Ser Val Pro Ala Gly Thr Lys Leu Asp Phe Lys Phe Ile Lys
TAC CCG TCC TGG TAT TAC GAT GTC AGC GTG CCG GCG GGG ACA AAG CTG GAT TTT AAA TTT ATT AAA
                                                                              2046

Lys Gly Gly Gly Thr Val Thr Trp Glu Gly Gly Gly Asn His Thr Tyr Thr Thr Pro Ala Ser Gly
AAG GGC GGC GGT ACG GTG ACT TGG GAA GGC GGG GGC AAC CAT ACG TAC ACG ACG CCG GCC AGC GGC
                                                                              2112

Val Gly Thr Val Thr Val Asp Trp Gln Asn term
GTA GGG ACG GTG ACG GTG GAC TGG CAA AAT TAA GCGGCTAAGC GGCCGGCCTG AACGAGAGGC ATCCGGCAAA
                                                                              2185

AAACTGCGGC GGCCGGCAGT TAAAGTCGAC GTGCAAACGT GCCGGGGAGG ATTGTGAAAT ACAGGTGCGG gatccaggag
                          Sal I                                  Bam HI      2265

aacaaaaacg atttttgag gaaagttata aattattttc cgaacgatat ggcaagcaaa atattgctta tgcaacagtt
                                                                              2345

cataatgatg agcaaacccc tcacatgcat ttaggtgttg tgcctatgcg tgatggaaaa tgcaaggaaa aaatgtgttt
                                                                              2425

aatcgtcaag actgttatgg ctacagtaat cccgagcata tga
                                                                              2468
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FIG. 3. DNA nucleotide sequence of the gene for *B. macerans* CD enzyme. The nucleotide sequences were determined by the M13 cloning method (6) and the dideoxy chain termination method of Sanger et al. (8). The noncoding DNA strand from 5′ to 3′ is shown with its corresponding amino acid sequence. The numbering of nucleotides and amino acids begins at the potential initiator ATG (Met). The signal peptide (amino acids +1 to +27) is shown in italics, and the two amino acids at the NH_2-terminal region of extracellular *B. macerans* CD enzyme determined by dimethylaminoazobenzene isothiocyanate method (2) are boxed. The cleavage site between the signal peptide and extracellular mature CD enzyme is indicated by the vertical arrow. The probable promoter region and possible ribosome-binding site are underlined. The sequences containing inverted repeat structures downstream from the termination codon TAA are designated by ←→. The nucleotide sequence in pUB110 is shown by small letters.

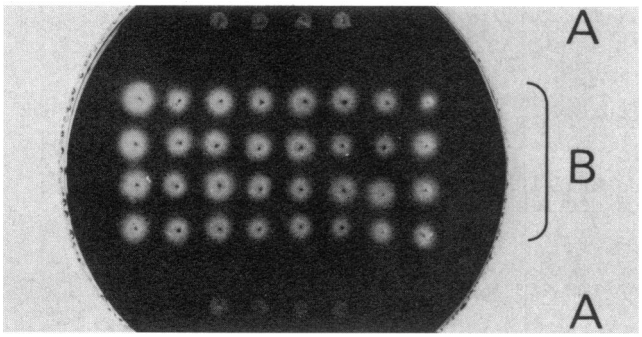

FIG. 4. Production of CD enzyme by *B. subtilis* 207-25(pDS10) on an LG agar plate (14) containing 1% soluble starch. *B. subtilis* 207-25(pDS10) was cultured overnight at 37°C. After incubation, a 0.1 M KI-I_2 indicator solution was sprayed on the plate. (A) *B. subtilis* 207-25(pUB110); (B) *B. subtilis* 207-25(pDS10).

percent of Asp and Asn and Glu and Gln in the former composition were calculated and compared favorably with those of Asp and Glu in the latter composition. The two amino acid compositions were similar, but they contained differences in some amino acids. These differences depended on the strain of *B. macerans*. Additionally, Cys could not be found in either amino acid composition.

The molecular weight of native *B. macerans* CD enzyme has been estimated to be 145,000 by gel filtration (4), but when the purified enzyme was subjected to denaturing conditions (1% sodium dodecyl sulfate or 1% sodium dodecyl sulfate with 20% 2-mercaptoethanol) before sodium dodecyl sulfate-disc gel electrophoresis, a 74,000-molecular-weight band was detected (4). The molecular weight of the probable extracellular CD enzyme encoded by the inserted DNA was 74,046. The two-amino acid sequence at the NH_2-terminal region of the enzyme was uniquely determined as Ser-Pro, and the amino acid composition predicted from the DNA nucleotide sequence corresponds with that of extracellular CD enzyme of *B. macerans*. Therefore, we suggest that *B. macerans* CD enzyme consists of two identical subunits with a molecular weight estimated to be 74,046.

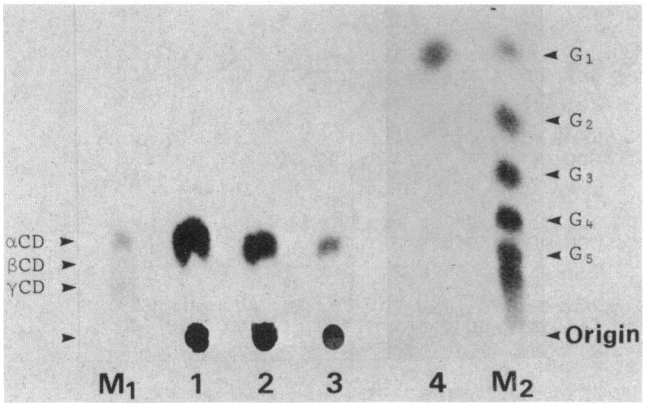

FIG. 5. Cyclodextrin production from soluble starch. Lanes: 1, extracellular CD enzyme of *B. macerans*; 2, extracellular CD enzyme of *B. subtilis* 207-25(pDS10); 3, CD enzyme from a λCD lysate of *E. coli*. The products were analyzed by paper chromatography, which was carried out at 60°C on Toyo Roshi no. 50 filter paper by an ascending technique with a 1-propanol–1-butanol–water (5:3:4, vol/vol) solvent system. Cyclodextrins were detected by the iodine method of Kobayashi et al. (5). α-, β-, and γ-CD represent α-, β-, and γ-cyclodextrin, respectively. As a control experiment, the hydrolysis products obtained from soluble starch with the extracellular α-amylase of *B. subtilis* NA64 are also shown (lane 4). This chromatography was carried out under the same conditions, but sugars were detected by the silver nitrate-sodium hydroxide reagent method (12). G_1, Glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose. Lanes M_1 and M_2 are standard markers.

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