Molecular cloning, characterization and nucleotide sequence of the gene for secreted α -amylase from *Xanthomonas campestris* pv. *campestris*

NIEN-TAI HU,^{1*} MING-NI HUNG,^{1,2} A-MIN HUANG,^{1,2†} HUEI-FUNG TSAI,^{1,2‡}
BIH-YING YANG,^{1§} TEH-YUAN CHOW³ and YI-HSIUNG TSENG²

^{1,2}Agricultural Biotechnology Laboratories¹ and Graduate Institute of Botany², National Chung Hsing University, 250 Kuo Kuang Road, Taichung, Taiwan 40227, Republic of China

³Institute of Botany, Academia Sinica, Nankang, Taiwan, Republic of China

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α -Amylase (1,4- α -D-glucan glucanohydrolase, EC 3.2.1.1) of apparent molecular mass 45 kDa was secreted by *Xanthomonas campestris* pv. *campestris* grown in medium containing starch or maltose. We isolated its structural gene from a recombinant λ library and located it on a 2.7 kb DNA fragment. Nucleotide sequencing of the fragment revealed a potential ORF encoding a protein of 475 amino acid residues, including a potential signal sequence of 35 amino acids. The signal processing site was confirmed by N-terminal amino acid sequence analysis of the exported α -amylase. The deduced amino acid sequence of the mature protein is very similar to that of the α -amylase of *Aeromonas hydrophila*. It also contains all four amino acid sequences highly conserved in the α -amylases from a wide range of organisms. Expression of the *amy* gene in *Escherichia coli* was poor from its own promoter, but was enhanced by the upstream promoter on the vector. The α -amylase synthesized in *E. coli* was located in the periplasm.

Introduction

Extracellular proteins produced by Gram-negative bacteria have to traverse a cell envelope composed of two membranes. One of the unsolved problems is how these proteins are translocated across this complex barrier. Depending upon whether an N-terminal signal sequence is present in the unprocessed exported proteins, they can be divided into two categories (Hirst & Welch, 1988). Either genetic or biochemical evidence has been reported in different cases to suggest that a majority of the extracellular proteins with an N-terminal signal sequence were exported via the periplasm (Andro *et al.*, 1984; Hirst & Holmgren, 1987; Howard & Buckley, 1983). On the other hand, in the case of the haemolysin of *Escherichia coli*, no periplasmic intermediate was detect-

ed in excretion mutants (Gray *et al.*, 1986). Comparison of the N-terminal amino acid sequence of the extracellular haemolysin with that of the amino acid sequence predicted from the nucleotide sequence of the gene indicated that the protein was exported without any processing at its N-terminus (Felmlee *et al.*, 1985). Instead, the signal required for export resides at the C-terminus (Gray *et al.*, 1986). It was proposed that the haemolysin was exported through a haemolysin translocator at junctions between the inner and outer membranes (Holland *et al.*, 1990).

Among other proteins (Daniels *et al.*, 1984), an α -amylase that hydrolyses starch endolytically at random (Tseng & Peng, 1985) is exported into the milieu by the Gram-negative bacterium *Xanthomonas campestris* pv. *campestris* (Dow *et al.*, 1987; Tseng & Peng, 1985). Protein export mutants of *X. campestris* pv. *campestris* have been isolated by Dow *et al.* (1987) and from our laboratory (Hu *et al.*, 1992). Accumulation of α -amylase in the periplasm was observed in these mutants (Dow *et al.*, 1987; Hu *et al.*, 1992), suggesting that α -amylase may be exported via the periplasm. In this study, the gene encoding the α -amylase (*amy*) of *X. campestris* pv. *campestris* was cloned and sequenced. A probable N-terminal signal sequence of 35 amino acid residues was predicted from the deduced amino acid sequence.

* Author for correspondence. Tel. 4 287 4754; fax 4 286 1905.

† Present address: Department of Pathology, School of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China.

‡ Present address: Department of Plant Pathology, University of Kentucky, Lexington, Kentucky, USA.

§ Present address: Department of Biology, University of Maryland, Baltimore, Maryland, USA.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M32874.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant characteristics	Source or reference
<i>X. campestris</i> pv. <i>campestris</i> XC1701	Rif ^r	Spontaneous mutant of XC17*
<i>E. coli</i> JM101	$\Delta(lac-pro) supE thi F'(traD36 proAB lacI^q-Z\Delta M15)$	Messing (1979)
BHB2688	<i>recA</i> (<i>Eam4 b2 red3 imm434 cIts Sam7</i>)	Hohn & Murray (1977)
BHB2690	<i>recA</i> (<i>Dam15 b2 red3 imm434 cIts Sam7</i>)	Hohn & Murray (1977)
BNN45	<i>hsdR supE44 supF thi met</i>	C.-H. Lee, Indiana University, USA.
NM538	<i>supF hsdR trpR lacY</i>	Frischauf <i>et al.</i> (1983)
NM539	<i>supF hsdR lacY</i> (P2 <i>cox3</i>)	Frischauf <i>et al.</i> (1983)
pUC13	Ap ^r	Vieira & Messing (1982)

* Bacterial strain obtained from S.-T. Hsu of National Chung Hsing University, Taiwan, Republic of China.

Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media. Luria broth (Miller, 1972) was used for culturing all the bacteria. Solid M9 medium (Miller, 1972), supplemented with thiamin ($10 \mu\text{g ml}^{-1}$), was used for growing *E. coli* JM101. Minimal medium XOL [1^{-1} : 0.7 g K_2HPO_4 , 0.2 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g MnCl_2 , 0.1 g MgCl_2 , pH 7.15] (Chu & Tseng, 1981) supplemented with 0.125% tryptone, 0.125% yeast extract and 0.2% soluble starch was used for induction of α -amylase production by *E. coli* or *X. campestris* pv. *campestris*. When soluble starch was included at 2% (w/v) and kept at 4 °C till the medium turned opaque, starch hydrolysis could be detected directly as the clearing of the starch. Nozu supplement (Arber *et al.*, 1983), comprising 0.3% glucose, 7.5 μM - CaCl_2 , 4 μM - FeSO_4 and 2 mM- MgSO_4 , was included in Luria broth to raise the phage titre of the liquid lysate.

Construction of EMBL3 library of XC1701. Total DNA of XC1701 was partially digested with *Sau3AI*, treated with calf intestinal phosphatase and ligated with λ EMBL3 DNA cleaved with *Bam*HI and packaged *in vitro* with extracts prepared from the lysogens *E. coli* BHB2688 and *E. coli* BHB2690 (Silhavy *et al.*, 1984). Infection of *E. coli* NM539 (Frischauf *et al.*, 1983) with the packaged DNA yielded 1.2×10^4 p.f.u..

Transformation. The procedures of Cohen *et al.* (1972) for preparing *E. coli* competent cells and transformation were followed with slight modifications. Cells were treated with 100 mM- CaCl_2 at 4 °C for 20 min. Ampicillin ($50 \mu\text{g ml}^{-1}$) and tetracycline ($15 \mu\text{g ml}^{-1}$) were used to select for plasmid transformants. Insertional inactivation of the *lacZ* gene on pUC13 was screened by including 50 μM -IPTG and 0.005% X-Gal.

Cellular fractionation. The periplasmic fraction of *E. coli* was prepared as described by Neu & Heppel (1965). For XC1701, the supernatant from centrifugation at 16000 g for 10 min was collected as the extracellular fraction. The pelleted cells were washed with distilled water twice, treated with lysozyme ($200 \mu\text{g ml}^{-1}$) in 20% (w/v) sucrose–30 mM-Tris/HCl, pH 8.0–1 mM-EDTA at 4 °C for 30 min and pelleted by centrifugation at 25000 g for 10 min. The supernatant containing periplasmic materials was collected. The pelleted spheroplasts were

broken by resuspending in 10 mM-Tris/HCl, pH 8.0, and centrifuged at 25000 g for 15 min to collect the supernatant as the cytoplasmic fraction.

Enzyme assay. The extracellular, periplasmic or cytoplasmic fraction was incubated with 1% starch in 27.5 mM-sodium/potassium phosphate buffer, pH 7.15 at 37 °C, and the reducing sugar produced was determined according to the method of Nelson (1944). One unit of α -amylase activity was defined as 1 μg of glucose produced per min under these conditions.

SDS-PAGE and in situ activity stain. Extracellular proteins prepared from 30–40% ammonium sulphate precipitation were separated by SDS-PAGE on a 12% (w/v) acrylamide gel. After electrophoresis, the gel was washed in 5 mM-potassium phosphate buffer, pH 7.15, twice at room temperature for 1 h each, and twice at 37 °C for 80 min each (Tai *et al.*, 1985). Detection of α -amylase was performed by incubating the washed gel in 27.5 mM-sodium/potassium phosphate buffer, pH 7.15, containing 1% starch at 37 °C for 2–4 h, rinsing with water, blot drying and fumigating with iodine vapour. α -Amylase activity appeared as a clear band against a blue background.

Nucleotide sequence determination and analysis. DNA sequences were determined using the dideoxy chain termination method of Sanger *et al.* (1977). Sequences of both strands were determined, either from M13 subclones or from deleted plasmids. Deletion of pB16 was accomplished with exonuclease III (ExoIII) and S1 nuclease treatment (Henikoff, 1984). Sequences were analysed with Release 6.01 of PC/GENE (IntelliGenetics). Tfasta (Pearson & Lipman, 1988) was used to search in the Genetics Computer Group (GCG) for homologous sequences.

N-Terminal amino acid sequence analysis of extracellular α -amylase. The extracellular α -amylase was prepared from an early stationary phase culture of the parental strain of *X. campestris* pv. *campestris*. Proteins collected from 30–40% ammonium sulphate precipitation were fractionated by DEAE-cellulose chromatography. The α -amylase, whose activity appeared in the flow-through, was then concentrated followed by separation on an SDS-polyacrylamide gel. A 45 kDa protein stained with Coomassie Blue (Fig. 1a, upper band) coincided with the α -amylase activity stain. After being electroblotted onto a polyvinylidene difluoride (PVDF) membrane, this 45 kDa protein was analysed for its N-terminal amino acid sequence using the Edman degradation method.

Results

Characterization of the α -amylase of *X. campestris* pv. *campestris*

X. campestris pv. *campestris* XC1701 was grown in XOL medium supplemented with 0.2% starch to late exponential phase and the culture supernatant was fractionated using ammonium sulphate precipitation. The maximal starch-hydrolytic activity was obtained in the fraction precipitated with 30–40% ammonium sulphate, followed by that precipitated with 40–60% ammonium sulphate. HPLC analysis showed that the reaction products were glucose and maltose (not shown). Glucose constituted approximately 10% of the total product, suggesting that the starch was hydrolysed by an α -amylase activity. Separation of the ammonium-sulphate-precipitated proteins on an SDS-polyacrylamide gel, followed by activity staining showed a major starch-hydrolysing activity with a molecular mass of 45 kDa (Fig. 1).

Isolation of the amy gene from an EMBL3 library

A λ EMBL3 library of XC1701 DNA was constructed and was plated out on a lawn of *E. coli* NM539 on starch medium. When the plaques were exposed to I_2 vapour, three out of approximately 300 plaques showed clear zones. These three recombinant phage, after plaque purification, were found to contain the same cloned DNA fragment. One of them was named Amy1 and was studied further.

The restriction map of Amy1, which contains a cloned fragment of 15 kb, is shown in Fig. 2(a). Different fragments (Fig. 2b) were subcloned into plasmid vectors and transformants assayed for enzymic activity. These experiments indicated that the *amy* gene was located on a 2.7 kb *Bam*HI fragment adjacent to the left arm of the λ EMBL3 vector (Fig. 2b).

Expression of the cloned amy gene in *E. coli*

The Amy⁺ subclone of Amy1, pB16 (Fig. 2b), contained a 2.7 kb *Bam*HI fragment in pUC13. When the cloned fragment was reversed in its orientation with respect to the *lac* promoter on the vector, the starch-hydrolytic activity conferred to the *E. coli* host was significantly reduced, but not abolished. This suggested that the *amy* gene promoter might be recognized by the *E. coli* RNA polymerase. Nevertheless, expression of the *amy* gene was probably enhanced by the promoter located on the vector. Inclusion of IPTG caused a fivefold increase in the α -amylase activity produced by JM101(pB16),

Fig. 1. Analysis of the α -amylase prepared from XC1701 on a 12% SDS-polyacrylamide gel. Gel (a) was stained with Coomassie Brilliant Blue; (b) was stained *in situ* for starch hydrolytic activity following renaturation. The clear band in (b) represents the area where starch-hydrolytic enzyme activity migrated. Protein standards were included in the lane marked M.

Fig. 2. (a) Restriction map of the recombinant phage Amy1. L and R represent the left and the right arms, respectively, of λ EMBL3. (b) Subcloning experiment for locating the *amy* gene on Amy1. The fragments included in each subclone, shown as horizontal lines, are aligned with the restriction map of Amy1 in (a). (c) Restriction map of pB16. The ORF revealed by the sequencing data is represented by the arrow. Restriction sites are represented as follows: B, *Bam*HI; E, *Eco*RI; S, *Sal*I; Bn, *Ban*II; Hc, *Hinc*II; P, *Pst*I; RV, *Eco*RV; St, *Stu*I.

indicating that the upstream *lac* promoter on pB16 might be responsible for the enhancement (data not shown). α -Amylase synthesized in *E. coli* was found primarily in the periplasm, in contrast to that synthesized in *X. campestris* pv. *campestris*, which was secreted extracellularly (Table 2).

CTGCTG GACGTCACGG CATCCATACC
GGCAACCACC GCCGCCATTC ATCAGCACCG CGCGCAGTGC ATCGGTGCGA GTGCGACCGT GCACGCCCTC GGCATCGAAC CGGCCGCACG
TTTCGCGGCT GTTGTGCAGT GAATACGTAT GCACAGGGTC AATGCTGCGC CGCGATGCGC GCTTAGCATC ACCCGCGGCC GCACGCCGTG
-----> <-----
GGGCGCGCTT TCCGGAACAG GGGCCGGAGG GCATTGCTGC ATGCATCGCT GTGCGGATGC GTGCGTCCAC GTGATCAGGA GACACACCCG

ATG CAC GCC ACC TCT CGC CCC TGT CCA CGC ACG TTC TGG CAA CGC GCT CAC CAG TTG CTG TTG ATC GCG CTG ACC 25
M H A T S R P C P R T F W Q R A H Q L L L I A L T

TTG TTG CTG ACC ACC GCC AGC GCA CAG GCC GAC GTC ATC CTG CAT GCG TTC AAC TGG CCC TAC GCC ACG GTA GAA 50
L L L T T A S A Q A D V I L H A F N W P Y A T V E

GCG CGC GCC AAG CAG ATC GCC GAT GCC GGG TAT CGC AAG GTA CTG GTC GCA CCG GCC TAT CGC TCC GAA GGC AGT 75
A R A K Q I A D A G Y R K V L V A P A Y R S E G S

GCC TGG TGG GCG CGC TAC CAG CCG CAG GAT ATC CGC CTG ATC GAC AAT CCA CTC GGC GAC ACC ACC GCG TTC GCG 100
A W W A R Y Q P Q D I R L I D N P L G D T T A F A

CGC ATG GTG CAG GCA CTG GCA AAC AAC GGT GTG GAA ACC TAT GCC GAC GTG GTG TTC AAC CAG ATG GCC AAC GAA 125
R M V Q A L A N N G V E T Y A D V V F N H M A N E

GCA GCA ACG CGC TCG GAC CTC AAT TAC CCC GGC AGC GCG GTG CTG GCG CAG TAC GCA GCC AAT CCT GGG CGC TAC 150
A A T R S D L N Y P G S A V L A Q Y A A N P G R Y

GAT GCA CTG CGC CTG TTC GGC ACG GTG CAG TCC AAC TTT TTG AGC GCC AGT GAT TTC GGG CCG GCG CAA TGC ATC 175
D A L R L F G T V Q S N F L S A S D F G P A Q C I

AGC AAC TAC AAC GAT GCC TTC CAG GTG CGC AAT TAC CGC ATC TGC GGC GGC GGC AGC GAT CCG GGC CTG CCG GAT 200
S N Y N D A F Q V R N Y R I C G G G S D P G L P D

CTG CTC GGC AAC GAT TGG GTG GTG CAG CAG CAG CGC GCC TAC CTG CAG GCG CTC AAG GGC CTG GGC GTC ACC GGC 225
L L G N D W V V Q Q R A Y L Q A L K G L G V T G

Region 2
TTC CGC GTG GAC GCG GCC AAG CAG ATG ACC TTC GAC CAT CTC AAC CGC GTT TTC GAT GCC GGC ATC CGC TCC GGT 250
F R V D A A K H M T F D H L N R V F D A G I R S G

Region 3
GTG TAC GTG TTT GGT GAA GTG ATC ACC GGC GGT GGC AGC GGC AAT GGC GAC TAC GAC CAG TTC CTG GCG CCG TAT 275
V Y V F G E V I T G G G S G N G D Y D Q F L A P Y

CTG CAA TCC ACC CCG CAT GCC GCC TAC GAT TTC CCG CTG TTC AAC GCG GTA CGC AAT GCC TTC GGT GTG GGT GCG 300
L Q S T P H A A Y D F P L F N A V R N A F G V G A

Region 4
AGC ATG CAA CAG TTG GTG GAC CCG GCG TCC ACC GGG CAG GCC TTG CCG GGC AAT CGC GCA GTC ACC TTT GCC GTA 325
S M Q Q L V D P A S T G Q A L P G N R A V T F A V

ACG CAT GAC ATC CCC AAT AAC GCG GGC TTC CGC TAC GCG ATT CTC GAC CCG GTC GAT GAA ACG CTG GCC TAT GCG 350
T H D I P N N A G F R Y A I L D P V D E T L A Y A

TAC TTG CTG GGC CGC AAC GGC GGC GTG CCG ATG GTC TAC ACC GAC AAC AAC GAA AGC GGC GAC AAC CGC TGG GTC 375
Y L L G R N G G V P M V Y T D N N E S G D N R W V

AAT GCC TAT CTG CGC GAT GAT CTG CGC CGC ATG ATT GGC TTC CAC AAC GGC GTG CAG GGC AGC GAC ATG CAG GTG 400
N A Y L R D D L R R M I G F H N G V Q G S D M Q V

CTG TCA TCC AGT GCC TGC CAC ATC CTG TTC CGC CGC GGC AGC CTC GGC ATC GTG GGC ATC AAC AAG TGC GGC AAC 425
L S S S A C H I L F R R G S L G I V G I N K C G N

CCG GTC AAC ACC ACC GTC GCC ATG AAC GGC AGC GTG TTG TTC TGG AAT GCG GAT TAC GTC GAT GCA CTG GGT TCC 450
P V N T T V A M N G S V L F W N A D Y V D A L G S

↓
GGC ACC GTG GTG CGC ATC TCC AGT GGC TCG TAC ACC TTC ACC CTG CCG GCG CGG CAG GCG CGT ATG TGG CGA CGT 475
G T V V R I S S G S Y T F T L P A R Q A R M W R R

TGA TTTTGCAGAC GCGCTGCGTT TGCAGGAGCG CTGCAGCACG GTCTGTTTTT CGTAGGTGCC ATCCAGGCGA CTAGGTAACA TCAGCGGATC
*

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the *amy* gene of *X. campestris* pv. *campestris*. In the 5' untranslated region, the potential -10 and -35 regions are in bold type and underlined. The potential ribosome-binding site is in bold type only. The dyad symmetrical structure of the putative operator is shown by the dashed arrows. In the translated region, the N-terminal amino acid sequence determined from extracellular α -amylase is underlined. Sequences highly conserved in other α -amylases are boxed. A downward arrow near the C-terminus marks the left-hand position of the deletion mentioned in the text. The asterisk denotes a stop codon.

	1				50
Aeoamy		MKNTAG	ILAIAGMLIA	PLAHADVILH	AFNWKYSEVT
Amy	MHATSRPCPR	TFWQRAHQLL	LIALTLLLT	ASAQADVILH	AFNWPYATVE
	51				100
Aeoamy	AKADLIK GAG	YKQVLIS PPL	KSSGNEW WAR	YQPQDLRLVD	SPLGNKQDLE
Amy	ARAKQIADAG	YRKVLVAPAY	RSEGS AWWAR	YQPQDIRLID	NPLGDTT AFA
	101				150
Aeoamy	QLIA A MQARG	IAV YADVV LN	HMANESW KRN	DLNYPG TELL	GQYA ANPDY Y
Amy	RMVQALANNG	VET YADV VFN	HMANEA ATRS	DLNYPG SAVL	AQYA ANPG RY
	151				200
Aeoamy	SRQ RLFG DLG	QN LLSAS DFH	PEGCITD WSD	PGHVQY WRLC	GGAGDK GLPD
Amy	DAL RLFG TVQ	SN FLSAS DFG	PAQCIS NYND	AFQVR NYRIC	GGGSD PGLPD
	201				250
Aeoamy	LDPNN WVVSQ	QQAYLK KAL KG	MGIKG FRVDA	VKHMSDY QIN	AVFTPEIK QOG
Amy	LLGND WV VQ	QRAYLQ AL KG	LGVTG FRVDA	AKHMT FDHLN	RVFDAGIR SG
	251				300
Aeoamy	MHV FGEVI TT	GGAGST DYER	FLKPYLD NSG	QGAYD FPLFA	SLRGAL GYGG
Amy	VYV FGEVI TG	GGSGNGD YDQ	FLAPYL QSTP	HAAYD FPLFN	AVRNA FGVGA
	301				350
Aeoamy	SM NQLAD PGA	YGQAL PGNRA	VTFAITH DIP	TNDG FRYQIL	NQ TEK LAYA
Amy	SMQQL VDPAS	TGQAL PGNRA	VTFAV THDIP	NNAG FRYAIL	DPVDE T LAYA
	351				400
Aeoamy	YLLGRD G GSP	LVYSDH GETQ	DKDGLRW QDY	YLRSDLK GMI	RFHNAV Q GP
Amy	YLLGRNG G VP	MVYTDN NESG	DN...RW NA	YLRDDLR MI	GFHNGV Q GSD
	401				450
Aeoamy	MQLIGSG DCF	VLFRKG KQGL	VG VNKC DYEQ	EYWLD TAKFE	LNWYR NYKDV
Amy	MQVLSS SACH	ILFRRG SLGI	VG INKC GNPV	NTTVAM NGSV	LFWNAD YVDA
	451		479		
Aeoamy	LDQSAVIN VQ	SQWVRV AMPA	RRPPLAA E*		
Amy	LGS GT VVRIS	SGSYT FTLPA	RQARM WRR*		

Fig. 4. Comparison between the amino acid sequence of α -amylase of *X. campestris* pv. *campestris* (Amy) and that of *A. hydrophila* (Aeoamy). Identical amino acid residues are in bold type.

Table 2. Distribution of α -amylase produced by *X. campestris* pv. *campestris* XC1701 and by *E. coli* JM101(pB16)

Bacterial strains	α -Amylase activity [units (ml culture) ⁻¹]		
	Extracellular	Periplasmic	Cytoplasmic
<i>X. campestris</i> pv. <i>campestris</i> XC1701	520.8	2.2	12.3
<i>E. coli</i> JM101(pB16)	0.5	2.8	0.8

Nucleotide sequence analysis of the amy gene

α -Amylase activity was abolished by the deletion of 150 bp from the right-hand *Bam*HI site as shown in Fig. 2(c), indicating that the *amy* gene is located near the right end (as shown in Fig. 2c) of the 2.7 kb fragment. When a

fragment extending from the *Ban*II site to the right-hand *Bam*HI site was subcloned downstream of a T7 promoter, T7 RNA polymerase-driven transcription produced an α -amylase of 45 kDa *in vivo* (data not shown). The DNA sequence of this fragment revealed an ORF of 475 amino acids (Fig. 3). Location of the deletion mentioned above was determined and is marked in Fig. 3.

Nakajima *et al.* (1986) identified four conserved regions, while comparing the amino acid sequences of 11 different α -amylases from a wide range of organisms. All these four regions exist in the putative α -amylase amino acid sequence of *X. campestris* pv. *campestris* (Fig. 3). When the EMBL-GenBank Data Library was searched for homologous sequences, the α -amylase of *Aeromonas hydrophila* (Gobius & Pemberton, 1988) was found. Comparison of the two amino acid sequences showed 70% homology (Fig. 4). A signal sequence of 35 amino

(a)	Region 1	Region 2	Region 3	Region 4
	121	233	259	328
<i>X. campestris</i>	DVVfNH	GFevDAAKH	EVIt	FavtHD
	122	210	233	297
<i>A. oryzae</i>	DVVaNH	GlRiDtvKH	EVlD	FevNHD
	106	238	267	331
<i>B. stearothermophilus</i>	DVVfDH	GFRLDAvKH	Eyws	FVDNHD
	117	208	244	306
<i>Drosophila</i>	DVVfNH	GFRvDAAKH	EVID	FVDNHD
	116	213	248	312
Mouse (pancreatic)	DaViNH	GFRLDAAKH	EVID	FVDNHD
Consensus	DVVFNH	GFRLDAAKH	EVID	FVDNHD
(b)				
	83	189		395
<i>X. campestris</i>	WWaRYQP	IsNYNDafQVRNyRi	lRrMigFhNgVqG	
	80	169		401
<i>Drosophila</i>	WWERYQP	IsNYNDaneVRNCeL	IyNMVaFRNtVgs	
	79	174		415
Mouse (pancreatic)	WWERYQP	IdNYNDaYQVRNCRL	IRNMVaFRNVVNG	
	79	177	418	
Mouse (salivary)	WWERYQP	IENYqDAaQVRDCRL	IRNMVaFRNVVNG	
	74	169		410
Rat	WWERYQP	InNYNDanQVRNCRL	IRNMVaFRNVVNG	
	64	161		403
Hog	WWERYQP	IEsYNDpyQVRDCqL	IRNMVwFRNVVDG	
	79	177		418
Human (pancreatic)	WWERYQP	IENYNDatQVRDCRL	IRNMViFRNVVDG	
	79	177		418
Human (salivary)	WWERYQP	IENYNDatQVRDCRL	IRNMVnFRNVVDG	
Consensus	WWERYQP	IENYNDA QVRDCRL	IRNMV FRNVVDG	
(c)				
		363		
<i>X. campestris</i>	LAYAy1LgRngGvPmVy			
		393		
<i>B. amyloliquefaciens</i>	LAYAFILTResGYPqVF			
		398		
<i>B. stearothermophilus</i>	LAYAFILTRqeGYPCVF			
		391		
<i>B. licheniformis</i>	LAYAFILTResGYPqVF			
Consensus	LAYAFILTR GYP VF			

Fig. 5. Comparison of the α -amylase of *X. campestris* pv. *campestris* (a) with those of *Aspergillus oryzae* (Toda *et al.*, 1982), *B. stearothermophilus* (Nakajima *et al.*, 1985), *Drosophila* (Boer & Hickey, 1986) and mouse (Hagenbuechle *et al.*, 1980) in the four conserved regions (Nakajima *et al.*, 1986); (b) with seven animal enzymes; and (c) with three *Bacillus* enzymes. The numbers above the last residues designate the locations of each region in each α -amylase. Those amino acids appearing in over half of the compared sequences were taken as the consensus. A blank indicates absence of the consensus using the above criterion. Lower case letters represent unique sequences that are different from the consensus.

acids was predicted at the N-terminus of the α -amylase of *X. campestris* pv. *campestris* by the algorithm of von Heijne (1986).

The overall G+C content of the *Ban*II–*Bam*HI fragment is 63.4%, which is in good agreement with the high G+C content of the DNA of *X. campestris*

(Bradbury, 1984). Within the *amy* coding sequence, the third base of each codon exhibits a strong bias (80%) toward G or C, as is typical for coding sequences from organisms with high G+C contents (Liu *et al.*, 1990).

The predicted initiation codon is preceded with a likely ribosome-binding site, AGGAGA (Fig. 3). Located at 117 and 152 nucleotides upstream from the initiation codon, there is a putative -10 sequence, TAGCAT, and a likely -35 sequence, TGCACA, respectively (Fig. 3). This promoter may not be efficiently recognized in *E. coli*, as the expression of *amy* in *E. coli* was enhanced by the upstream *lac* promoter or T7 promoter. An inverted repeat of 25 nucleotides was located four nucleotides downstream from the putative -10 region (Fig. 3). The location and the dyad symmetrical structure of this 25 nucleotide region resemble those of the *lac* operator (Watson *et al.*, 1987). It was observed that the synthesis of α -amylase in *X. campestris* pv. *campestris* was induced by starch or maltose (H.-F. Tsai, & Y.-H. Tseng, unpublished results). This 25 bp inverted repeat may be involved in the negative regulation of *amy* gene expression in *X. campestris* pv. *campestris*.

N-Terminal amino acid sequence of purified α -amylase

The extracellular α -amylase of *X. campestris* pv. *campestris* was purified as described in Methods. The N-terminal amino acid sequence was determined and is shown in Fig. 3 (underlined amino acid sequence). This result strongly suggested that the α -amylase of *X. campestris* pv. *campestris* was processed at the predicted signal sequence processing site.

Discussion

We have cloned and expressed an *amy* gene of *X. campestris* pv. *campestris* in *E. coli*. The expressed α -amylase activity was not likely to be that of *E. coli*, since (i) the *E. coli* host exhibited no detectable starch-hydrolytic activity under our culture conditions (data not shown); (ii) the α -amylase of *E. coli* has an apparent molecular mass of 66 kDa (Freudlieb & Boos, 1986), unlike the 45 kDa α -amylase identified here; and (iii) the N-terminal amino acid sequence of the processed protein predicted from the derived amino acid sequence of the cloned gene matched that of the extracellular α -amylase purified from *X. campestris* pv. *campestris*.

The amino acid sequence derived from the nucleotide sequence of the *amy* gene of *X. campestris* pv. *campestris* not only showed the four regions highly conserved in eleven different prokaryotic and eukaryotic α -amylases

(Nakajima *et al.*, 1986), but also depicted 70% homology with the amino acid sequence of the α -amylase of *Aeromonas hydrophila* (Gobius & Pemberton, 1988). Furthermore, the postulated catalytic residues, Glu-230 and Asp-297, of Taka-amylase A of *Aspergillus oryzae* (Matsuura *et al.*, 1984) are both conserved in the α -amylase of *X. campestris* pv. *campestris* (Glu-256 and Asp-328 in Fig. 5a). In addition to the four conserved regions (Nakajima *et al.*, 1986), comparisons of the α -amylase of *X. campestris* pv. *campestris* with those of other organisms (as summarized in Figs 5b and c) revealed three more regions, which were similar to those of animal enzymes (Hagenbuechle *et al.*, 1980; MacDonald *et al.*, 1980; Nakamura *et al.*, 1984; Boer & Hickey, 1986; Pasero *et al.*, 1986), and one other region, which was similar to that of *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus* and *Bacillus licheniformis* α -amylases (Takkinen *et al.*, 1983; Nakajima *et al.*, 1985; Yuuki *et al.*, 1985). Even though the α -amylase of *X. campestris* pv. *campestris* is neither closely related to the animal nor to the *Bacillus* counterparts, it contained discrete conserved amino acid sequences from both categories.

Translocation of proteins across membranes occurs in both prokaryotes and eukaryotes. In the case of prokaryotic-transported proteins, the significance of the N-terminal signal sequence in the translocation of periplasmic or outer membrane proteins is well recognized (Oliver, 1985; Randall *et al.*, 1987). As to extracellular proteins of Gram-negative bacteria, it has been shown that the N-terminal signal sequence is not sufficient to direct a protein across the outer membrane (Hirst & Welch, 1988). Replacement with the signal sequence of an extracellular protein, the B subunit of *E. coli* enterotoxin, did not make β -lactamase leave the periplasm (Hirst & Welch, 1988). The N-terminal signal sequence of the *amy* gene predicted from the deduced amino acid sequence revealed all the characteristics of a typical signal sequence: a central hydrophobic region preceded by a positively charged N-terminus and followed by a polar C-terminus with a consensus processing site (Ala-X-Ala) (von Heijne, 1985). N-Terminal amino acid sequence analysis of the exported α -amylase showed that the precursor was indeed cleaved at the predicted processing site. These results suggest that processing of the N-terminal signal sequence of this extracellular protein does not differ from those of envelope proteins. The appearance of α -amylase activity in the periplasm of *E. coli* further supported the functional homology of the N-terminal signal sequence of extracellular protein with those of periplasmic proteins of *E. coli*.

Like *X. campestris* pv. *campestris*, *A. hydrophila* is also Gram-negative and secretes α -amylase extracellularly. Alignment of the two α -amylase sequences exhibits a

high degree of relatedness throughout. It is tempting to speculate that the high relatedness between the two α -amylase sequences may reflect the conservation of sequences required for the secretion process, in addition to those required for enzyme activity. Such sequences indeed exist; for instance, the nine N-terminal amino acids of the predicted mature protein are identical in both α -amylases. However, in the absence of other molecular data to show the relatedness of the two micro-organisms, one has to be cautious in making such a speculation.

The genes encoding two other extracellular enzymes, protease and endoglucanase, produced by *X. campestris* pv. *campestris* have been sequenced (Gough *et al.*, 1990; Liu *et al.*, 1990). Each deduced amino acid sequence predicted a potential N-terminal signal sequence. However, comparison of the two amino acid sequences of the mature protein with that of the α -amylase of *X. campestris* pv. *campestris* determined in this study did not reveal any consensus sequence. In studying the secretion of extracellular pullulanase by *Klebsiella oxytoca*, Pugsley *et al.* (1991) have recently demonstrated that the secretion intermediate, before it was translocated across the outer membrane, existed in a certain higher-ordered structure. This suggests that the signal(s) required for secretion across the outer membrane may not be one linear sequence of amino acid residues on the secreted protein, but could also involve some particular protein conformation.

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