Isolation and characterization of the gene encoding an aminopeptidase involved in the selective toxicity of ascamycin toward *Xanthomonas campestris* pv. *citri*

Tatsuhiko SUDO*, Kiwako SHINOHARA*‡, Naoshi DOHMAE†, Koji TAKIO†, Ron USAMI‡, Koki HORIKOSHI‡ and Hiroyuki OSADA*§

*Antibiotics Laboratory and †Division of Biomolecular Characterization, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-01, and ‡Department of Engineering, Toyo University, Nakanodai 2100, Kujirai, Kawagoe, Saitama 350, Japan

An aminopeptidase gene named *XAP* has been isolated from *Xanthomonas campestris* pv. *citri*, a plant pathogenic bacterium. The bacterium is one of the rare micro-organisms susceptible to ascomycin, an aminoacyl nucleoside antibiotic that inhibits protein synthesis. Sequence analysis reveals that the gene encodes a 311 amino acid protein with a calculated molecular mass of

35134 Da and approx. 50% identity for amino acids to the proline iminopeptidase from *Neisseria gonorrhoeae*. The *XAP* gene product, Xap, expressed in *Escherichia coli* has proline iminopeptidase activity as well as ascamycin dealanulating activity *in vitro*.

INTRODUCTION

An aminoacyl nucleoside antibiotic, ascamycin (Scheme 1), was originally isolated as a selective antibacterial antibiotic to *Xanthomonas* species, the plant pathogenic bacteria [1]. In contrast, a dealanylated derivative of ascamycin that is identical with AT265 has broad antibacterial activities [2,3]. In the process of elucidating the mechanisms of the selective action of ascamycin, we have previously identified and purified the 38 kDa protein named Xc-aminopeptidase from *Xanthomonas campestris* [3,4]. The selective toxicity of ascamycin to *X. campestris* was explained by the fact that Xc-aminopeptidase was localized in the membrane or periplasmic area of *X. campestris* and dealanylated the ascamycin to enter the bacterial cell [4,5]. Furthermore we have found that Xc-aminopeptidase has proline iminopeptidase activity [4,6]. To improve our understanding of the mechanism of action of ascamycin, cloning of the gene encoding Xc-aminopeptidase would be useful. Here we report the isolation and characterization of the gene *XAP* that encodes the aminopeptidase.

MATERIALS AND METHODS

Bacterial strains, vectors and media

Xanthomonas campestris pv. *citri* IFO 3781 (called *X. campestris* throughout this paper) was cultured as described previously [3]. *Escherichia coli* JM 109 was cultured at 37 °C in medium consisting of 1.6 % (w/v) Bacto tryptone, 1.0 % (w/v) Bacto yeast extract, 0.5 % NaCl, pH 7.0. pUC18 was used for the library construction and sequencing, and pGEX-3X (Pharmacia, Uppsala, Sweden) was used for the overproduction of the gene product.

Amino acid sequencing

Xc-aminopeptidase was purified by the procedure reported [4] and finally fractionated by SDS/PAGE [7], after which the protein was transferred electrophoretically to a poly(vinylidene difluoride) nitrocellulose filter. Direct N-terminal sequencing was attempted by using a protein sequencer (Perkin-Elmer 477A, Norwalk, CT, U.S.A.). In accordance with the method of

Scheme 1 Hydrolysis of ascamycin by Xc-aminopeptidase

Abbreviations used: GST, glutathione S-transferase; *XAP*, Xc-aminopeptidase gene; Xap, *XAP* gene product.

§ To whom correspondence should be addressed.

The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D82882.

Aebersold [8] the internal amino acid sequences were determined as follows. After digesting Xc-aminopeptidase on the nitrocellulose filter with *Achromobacter lyticus* protease I, the resulting peptides were separated by reverse-phase HPLC and sequenced by the protein sequencer.

Cloning and DNA sequencing

PCR-mediated amplification of the sequences flanked by the primers was performed [9]. A DNA probe was prepared with a digoxigenin-PCR labelling kit (Boehringer Mannheim GmbH). All DNA techniques were performed as described by Sambrook et al. [10]. DNA sequencing was done by the Sanger dideoxy method [11] with a Pharmacia ALF system in accordance with the conditions recommended by the manufacturer.

Expression of the gene product

To construct expression plasmids, 8 bp *Nco*I linker and 6 bp *Xba*I linker respectively were inserted into the *Sma*I site and the *Eco*RI site of the pGEX-3X vector. At the same time PCR-mediated mutagenesis was performed to make an *Nco*I site at the ATG start site of the cloned gene. Then the DNA fragment of the cloned *XAP* gene was ligated to the pGEX-3X vector. Xap, the *XAP* gene product, was extracted from *E. coli* JM109 harbouring pGEX-*XAP* (GST-*XAP* expression vector) as a fusion protein with glutathione S-transferase (GST).

Enzyme activities

Ascamycin dealanylating activity was detected by silica gel TLC as described previously [3]. Briefly, ascamycin was incubated for 2 h at 37 °C with whole-cell lysates and these samples were developed on a TLC plate [Merck F₂₅₄ silica TLC; solvent system 2-propanol/1 M ammonia (7:3, v/v)]. The spots of ascamycin were detected with UV irradiation (253 and 365 nm) and the ninhydrin test (ninhydrin reagent spray; Sigma Chemical Co.). Dealanyl ascamycin and L-alanine were detected by UV and ninhydrin respectively.

Proline iminopeptidase activity was measured as described [12]. Briefly, L-proline *p*-nitroanilide was incubated at 32 °C with each lysate prepared from *E. coli* containing GST or a fusion protein of GST and Xap (GST-Xap). The nitroaniline released was monitored at 410 nm with a spectrophotometer.

RESULTS AND DISCUSSION

The N-terminal amino acid sequence of Xc-aminopeptidase was determined with the purified enzyme. Direct N-terminal sequencing was attempted and 20 residues of the primary protein sequence, MRTLYPEITPYQQGSLKVDD, were identified. At the same time the protein transferred to a nitrocellulose filter was digested with *A. lyticus* protease I and the resulting peptide fragments were separated by reverse-phase HPLC. Two purified peptides were sequenced and found to be MRTLYPEITPYQ-QGSL and AQLQISPASGHSF. The former was revealed to be identical with that of the N-terminus and the latter was expected to be one of the internal sequences of Xc-aminopeptidase.

Oligonucleotides (21mer), ATGGG(C/T)AC(G/C)CTGTA-(C/T)CC(A/T/C/G)GAA and GGAGATCTGCAGCTGTG-CTTT respectively, corresponding to peptides MRTLYPE (N-terminal) and KAQLQIS (internal), were used as primers for PCR-mediated amplification. *X. campestris* genomic DNA was used as a template and a single fragment of approx. 850 bp was obtained (results not shown). The PCR-amplified fragment

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cggttaagcgcagaaggttgggtgcgctcttcagggttggcccatcacggcgaaactcttctcttcg -70
aatcgcatggcgttgcctgtgtgcgtgcgcagctgcgcatagcgggtttcccgacggacgcgc -1
ATG CGC AGC CTC TAT CCC GAG ATC ACG CCC TAC CAG CAG GGC AGC CTG AAG GTC 54
Met Arg Thr Leu Tyr Pro Glu Ile Thr Pro Tyr Gln Gln Gly Ser Leu Lys Val 18
GAC GAT CGC CAT ACG CTG TAC TTC GAG CAG TGC GGC AAT CGC CAC GGC AAG CCG 108
Asp Asp Arg His Thr Leu Tyr Phe Glu Gln Cys Gly Asn Pro His Gly Lys Pro 36
GTG GTG ATG TTG CAT GGC GGC CCC GGC GGC GGA TGC AAC GAC AAG ATG CGG CGC 162
Val Val Met Leu His Gly Gly Pro Gly Gly Gly Cys Asn Asp Lys Met Arg Arg 54
TTC CAC GAC CGC GCC AAG TAC CGC ATC GTG CTG TTC GAT CAG CGC GGT TCC GGC 216
Phe His Asp Pro Ala Lys Tyr Arg Ile Val Leu Phe Asp Gln Arg Gly Ser Gly 72
CGC TCT ACG CCG CAT GCC GAT CTG GTG GAC AAC ACC ACC TGG GAT CTG GTG GCC 270
Arg Ser Thr Pro His Ala Asp Leu Val Asp Asn Thr Thr Trp Asp Leu Val Ala 90
GAT ATC GAA CGC GTG CGC ACG CAT CTG GGC GTC GAT CGC TGG CAG GTG TTC GGC 324
Asp Ile Glu Arg Val Arg Thr His Leu Gly Val Asp Arg Trp Gln Val Phe Gly 108
GGC AGC TGG GGA TCC ACG CTG GCG CTG GCC TAC GCG CAG ACC CAT CCG CAG CAG 372
Gly Ser Trp Gly Ser Thr Leu Ala Leu Ala Tyr Ala Gln Thr His Pro Gln Gln 126
GTC ACC GAG CTG GTG CTG CGC GGT ATT TTC CTG CTG CGT CGC TTC GAA CTC GAA 426
Val Thr Glu Leu Val Leu Arg Gly Ile Phe Leu Leu Arg Arg Phe Glu Leu Glu 144
TGG TTC TAC CAG GAA GGT GCC AGC CGC CTG TTC CCG GAT GCG TGG GAG CAT TAC 486
Trp Phe Tyr Gln Glu Gly Ala Ser Arg Leu Phe Pro Asp Ala Trp Glu His Tyr 162
CTC AAC CGC ATT CCG CCG GTG GAA CGC GCC GAC TTG ATG TCT GCA TTC CAT CGC 540
Leu Asn Ala Ile Pro Pro Val Glu Arg Ala Asp Leu Met Ser Ala Phe His Arg 180
CGT CTC ACC AGC GAT GAC GAG GCC ACG CGT CTG GCT GCG GCC AAA GCC TGG AGC 594
Arg Leu Thr Ser Asp Asp Glu Ala Thr Arg Leu Ala Ala Lys Ala Trp Ser 198
GTG TGG GAA GGC GCC ACC AGC TTC CTG CAT GTC GAC GAG GAC TTC GTC ACC GGA 648
Val Trp Glu Gly Ala Thr Ser Phe Leu His Val Asp Glu Asp Phe Val Thr Gly 216
CAT GAA GAC CGC CAC TTT GCC CTG GCG TTC GCA CGC ATC GAA AAC CAT TAC TTC 702
His Glu Asp Ala His Phe Ala Leu Ala Phe Ala Arg Ile Glu Asn His Tyr Phe 234
GTC AAC GGC GGT TTC TTC GAG GTC GAA GAC AGT TGC TGC GCG ACG GAT CGC ATT 756
Val Asn Gly Gly Phe Phe Glu Val Glu Asp Ser Cys Cys Ala Thr Asp Arg Ile 252
GCC GAC ATT CCC GGC GTG ATC GTG CAC GGC CGC TAC GAT GTG GTG TGC CCG CTG 810
Ala Asp Ile Pro Gly Val Ile Val His Gly Arg Tyr Asp Val Val Cys Pro Leu 270
CAA AGC GCG TGG GAC CTG CAC AAG GCG TGG CCG AAG CAG TTG CAG ATC AGC CCG 864
Gln Ser Ala Trp Asp Leu His Lys Ala Trp Pro Lys Gln Leu Gln Ile Ser Pro 288
GCA TGC GGC CAC TCA CGC TTC GAA CCG GAA AAC GTC GAT GCC TTG GTG CGT GCC 918
Ala Ser Gly His Ser Ala Phe Glu Pro Glu Asn Val Asp Ala Leu Val Arg Ala 306
ACC GAC GGG TTT GCG TAA cgagtgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgc -981
Thr Asp Gly Phe Ala ***

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Figure 1 Nucleotide sequence and deduced amino acid sequence of Xc-aminopeptidase

The putative sequence for the ribosome-binding site is underlined and a palindromic sequence for transcriptional termination is indicated by arrows. The N-terminal amino acid sequence and an internal sequence of Xc-aminopeptidase, determined by the protein sequences, are boxed.

was cloned into the pUC18 vector for sequencing and it revealed that the fragment contained all the sequences corresponding to the two protein sequences derived from the fragments of purified Xc-aminopeptidase.

We have prepared *X. campestris* genomic DNA libraries including a *Pst*I library that was made from *X. campestris* genomic DNA digested with *Pst*I and ligated into pUC18. When the *Pst*I library was screened with the probe, one positive clone was obtained. The clone contained an insert of approx. 2.7 kb (pUC2.7 *Pst*I). The length of the insert was consistent with the result obtained from Southern hybridization with the probe against *X. campestris* DNA digested with *Pst*I. The open reading frame of the gene must be included in the insert, according to the physical map and the apparent molecular mass of Xc-aminopeptidase. The nucleotide sequences of both strands were determined by the dideoxy method; the deduced amino-acid sequence is represented in Figure 1. The boxed sequences are consistent with the sequences determined by peptide sequencing. A putative ribosome-binding site was located 11 nucleotides upstream from the ATG codon, and there was a palindromic sequence suggesting a termination structure downstream from the TAA stop codon of the gene (Figure 1). The structural gene encoded a 311 amino acid protein with a calculated molecular mass of 35134 Da: it was named *XAP*.

Although the sublocalization of Xap in *X. campestris* or *E. coli* was not determined in this work, Xc-aminopeptidase was expected to localize in the periplasmic area or the membrane

Figure 2 Expression of the gene product in *E. coli*

Western blotting was performed with rabbit polyclonal antibody raised against the purified Xc-aminopeptidase and alkaline phosphatase-conjugated anti-rabbit antibody as a second antibody. Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were also used as substrates for the detection process. Lane 1, extract from *E. coli* JM109 harbouring pUC2.7 *Pst*I; lane 2, *E. coli* JM109 harbouring pUC18; lane 3, *X. campestris*. Abbreviation: kD, kDa.

Xc	MRTLYPEITPYQQGSLKVDHRHTLYFEQCGNPHGKPVVMLHGGFGGCGNDKMRHFDPK	60
Ng	EKMYEIKQPFHSGYLQVSEIHQIYWEESGNPDGVPVIFLHGGFGAGASPECRGFNPDPV	223
Xc	YRIVLFDRQSGRSTPHADLVNTTDLVADIERVTHLGVDRWQVFGGSGWSTLALAYA	120
Ng	FRVIVIDQRCGRSHPYACAEEDNTTDLVADIEKVRMLGIGKWLFGGSGWSTLSLAYA	283
Xc	QTHPQQVTELVLRLRRFELEWYQEGA-SRLFPDAWEHYLNAIPPVERADLMSAFH	179
Ng	QTHPERVGLVLRGIFLCRPSETAWLNEAGGVSRIFYEQWQKFVAPIAENRRNRIEAYH	343
Xc	RRLTSDDEATRLAAAKAWSWEGATSFHLVDEDFVTGHEDAHFALAFARIENHYFVNGGF	239
Ng	GLLFHQDEEVCLSAKAWADWE---SYL-IRFEPEGVEDAYASLAIRLENHYFVNGGW	399
Xc	FEVEDSC-CATDRIADIPGVIVHGRYDVVCPQSAWDLHKAWPKQLQISPASGHSFAFEPE	298
Ng	LQGDKAILNIGKIRIHPITVIVQGRYDLCTPMQSAWELSKAFPEABLRVQAGHCAFDPP	459
Xc	NVDALVRATDGFA	311
Ng	LADALVQAVEDIL	472

Figure 3 Comparison of sequences of *X. campestris* pv. *citri* and *N. gonorrhoeae* proline iminopeptidases

The deduced sequence of Xap is aligned with the sequence for the *N. gonorrhoeae* proline iminopeptidase gene [12]. In the alignment, identical amino acids are indicated by lines and similar amino acids are indicated by dots.

according to our previous observations. However, there was no signal peptide sequence at the N-terminus nor a transmembrane domain in Xap. It has been reported that Gram-negative bacteria have a signal-peptide-independent secretion pathway [13,14]. *X. campestris* also has a membrane protein that assists in the secretion of proteins lacking an N-terminal signal peptide [15,16].

The Xap protein was expressed by inducing *E. coli* JM109 harbouring pUC2.7 *Pst*I with 1 mM isopropyl β -D-thiogalactopyranoside. The protein expressed in *E. coli* showed the same mobility with SDS/PAGE as that of purified Xc-aminopeptidase (Figure 2, lanes 1 and 3). The predicted amino acid sequence of the Xap protein was used in a similarity search of the GenBank computer database and revealed approx. 50 % identity with the proline iminopeptidase from *Neisseria gonorrhoeae* [12] (Figure 3). All computer programs were from

Xc	MRTLYPEITPYQQGSLKVDHRHTLYFEQCGNPHGKPVVMLHGGFGGCGNDKMRHFDPKARY-R	80
Ng	MESEKQPFHSGYLQVSEIHQIYWEESGNPDGVPVIFLHGGFGAGASPECRGFNPDPV-R	77
Bc	M-YTEG---FIDVTGGRVSQKF-DENGCGTPIVILHGGFGSSC-YSLGLKALAKD-RHVIYDQLGCGKSBRPM---	70
Lb	MQITEK---YLPF-GNQVTCRIVGEATDRAPLLLHGGFGSSSHNYFEVLDQVAEKSGRQVIMYDQLGCGNSIPDDQA	74
Xc	VDNTTDL---VADIERVTHLGVDRWQVFGGSGWSTLALAY-AOTHPQQVTELVLRLRRFELEWYQEGA-SRLFPD	157
Ng	EDNTTDL---VADIEKVRMLGIGKWLFGGSGWSTLSLAY-AOTHPERVGLVLRG-IFLCRPSETAWLNEAGGVSRIFYE	155
Bc	-DITLWRLDRFVEELAQKQALNLDVHILGHSGWSTLAAAY-CLTKPSGVKSVIFSPLCLSAPLWEQDKRNLKPLLDVQE	152
Lb	ETAYTAQT---WVKELENVREQLGLDQIHLGLQSGWGMALYLCQYQPKGVKSLILSSSTLASAKLWSQELHRLIKYLPKGEQA	156

Figure 4 Multiple alignment of the amino acid sequences of proline iminopeptidases

Xc, Xap isolated from *X. campestris* pv. *citri*; Ng, proline iminopeptidase from *N. gonorrhoeae* [12]; Bc, proline iminopeptidase from *B. coagulans* [18]; Lb, proline iminopeptidase from *L. delbrueckii* subsp. *bulgaricus* [17]. The three most conserved regions are boxed, and the serine residue marked with an asterisk corresponds to the catalytic site as suggested by Atlan et al. [17].

Figure 5 Enzymic activities of Xc-aminopeptidase *in vitro*

Cell-free lysates were prepared from *E. coli* containing *pGEX-XAP* (□), which is a Xap-coding plasmid, and from *E. coli* containing *pGEX-3X* (○), which is a mock plasmid, respectively. The proline iminopeptidase activities of the lysates were measured in 20 mM Tris/HCl, pH 7.5, containing 0.019 mM L-proline *p*-nitroanilide as a substrate. The increase in hydrolysed nitroaniline from proline was monitored at 410 nm with a spectrophotometer.

the Wisconsin Genetics Computer Group (Madison, WI, U.S.A.). The residue Ser-110 at the catalytic site and the surrounding region for some proline iminopeptidases are well conserved, as shown in Figure 4. [12,17]. However, in the other region the proline iminopeptidases from *Bacillus coagulans* [18] and from *Lactobacillus delbrueckii* subsp. *bulgaricus* did not show significant similarity.

The gene product was overexpressed in *E. coli* JM109 as a GST fusion protein (GST-Xap) to examine enzymic activity *in vitro*. The specific expression was confirmed by Western blotting with a rabbit polyclonal antibody [5] raised against Xc-aminopeptidase (results not shown). As shown in Figure 5, GST-Xap showed proline iminopeptidase activity with L-proline-*p*-nitroanilide as a substrate. Endogenous proline iminopeptidase activity of *E. coli* JM109 was negligible in this experiment.

Finally, the ascamycin dealanylating activity of Xap was detected on silica gel TLC by UV and the ninhydrin test. When ascamycin (R_f 0.72) was incubated with GST, no reaction occurred in the ascamycin spot on the TLC plate. In contrast, when ascamycin was incubated with GST-Xap, ascamycin was hydrolysed by Xap and gave two spots corresponding to dealanyl ascamycin (R_f 0.87) detected by UV and L-alanine (R_f 0.32) detected by ninhydrin (results not shown).

In this study, we have demonstrated direct evidence that a plant pathogenic bacterium *X. campestris* has a unique enzyme that might be involved in proline metabolism and sensitivity to ascamycin. As it was shown that *N. gonorrhoeae* has an enzyme

similar to Xc-aminopeptidase, ascamycin might become a good chemotherapeutic agent for gonorrhoea.

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