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Characterization of the first angiotensin-converting like enzyme in bacteria: Ancestor ACE is already active

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Abstract

Angiotensin-converting enzyme (ACE) is a metallopeptidase that converts angiotensin I into angiotensin II. ACE is crucial in the control of cardiovascular and renal homeostasis and fertility in mammals. In vertebrates, both transmembrane and soluble ACE, containing one or two active sites, have been characterized. So far, only soluble, single domain ACEs from invertebrates have been cloned, and these have been implicated in reproduction in insects. Furthermore, an ACE-related carboxypeptidase was recently characterized in *Leishmania*, a unicellular eukaryote, suggesting the existence of ACE in more distant organisms.

Interestingly, *in silico* databank analysis revealed that bacterial DNA sequences could encode putative ACE-like proteins, strikingly similar to vertebrates' enzymes. To gain more insight into the bacterial enzymes, we cloned the putative ACE from the phytopathogenic bacterium, *Xanthomonas axonopodis* pv. *citri*, named *Xc*ACE. The 2 kb open reading frame encodes a 672-amino-acid soluble protein containing a single active site. *In vitro* expression and biochemical characterization revealed that *Xc*ACE is a functional 72 kDa dipeptidyl-carboxypeptidase. As in mammals, this metalloprotease hydrolyses angiotensin I into angiotensin II. *Xc*ACE is sensitive to ACE inhibitors and chloride ions concentration. Variations in the active site residues, highlighted by structural modelling, can account for the different substrate selectivity and inhibition profile compared to human ACE. *Xc*ACE characterization demonstrates that ACE is an ancestral enzyme, provoking questions about its appearance and structure/activity specialisation during the course of evolution.
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1. Introduction

Angiotensin-converting enzyme (ACE, dipeptidyl-peptidase A, kininase II, E.C. 3.4.15.1, DCP1) is a dipeptidyl-carboxy-peptidase that belongs to the M2-metalloprotease family. In mammals, this zinc-dependent metalloprotease participates in blood pressure elevation (review in (Corvol and Eyries, 2004)), generating the vasopressor angiotensin II (Ang II) from the inactive angiotensin I (Ang I), and by inactivating the vaso-dilator bradykinin. In vertebrates, somatic (sACE) and testicular (germinal) ACE (tACE) have been characterized. These two separate isoforms are transcribed from two alternate promoters within a single gene (Howard et al., 1990), which arose from duplication of an ancestral gene (Hubert et al., 1991). Con-sequently, sACE possesses two domains (termed N-domain and C-domain, respectively) bearing the highly conserved gluzincin motif HExxH, whereas tACE possesses only one, corresponding to the C-terminal domain of sACE. Crystal structures of both the human N-domain (Corradi et al., 2006) and tACE (Natesh et al., 2003) have shown them to be homologous helical ellipsoids harbouring two large cavities connected by a narrow catalytic channel containing the catalytic zinc and the HexxH motif. Somatic ACE N- and C-domain active sites have distinct enzymatic specificities with respect to *in vivo* and/or synthetic substrates. Indeed, the C-domain is highly selective for Hippuryl-Histidyl-Leucine (HHL) (Wei et al., 1991a) whereas the N-domain efficiently cleaves the hemoregulatory peptide Ac-Ser-Asp-Lys-Pro (AcSDKP) (Rousseau et al., 1995) and Ang 1-7 (Deddish et al., 1998). In addition, ACE inhibitors (Dive et al., 1999) and chloride ion dependence (Jaspard et al., 1993) can discriminate between the two domains. Both sACE and tACE possess a C-terminal transmembrane domain and are membrane-anchored isoforms, though a post-translational shed-ding releases active solubilized ACE into the extracellular milieu (Eyries et al., 2001; Oppong and Hooper, 1993). A single domain ACE homologue, ACE2, has also been characterized in the human (Donoghue et al., 2000; Tipnis et al., 2000) and its structure determined (Towler et al., 2004). ACE2 is an essential regulator of heart function (Crackower et al., 2002) and has also been identified as a functional receptor for the SARS corona-virus (Li et al., 2003).

In invertebrates, however, functional ACE-related enzymes exist in insects and in annelids (which belong to the Ecdysozoa and Lophotrochozoa groups, respectively). Interestingly, and though *in silico* evidence suggest the presence of a two-domain ACE-related protein in mosquitoes (Burnham et al., 2005), all the cloned genes encode soluble, single active site enzymes (Tatei et al., 1995; Taylor et al., 1996; Wijffels et al., 1996). In the fruit fly *Drosophila melanogaster*, two homologues have been cloned and named *AnCE* (Cornell et al., 1995) and *ACER* (Tatei et al. 1995) (or *Race*), of which AnCE has had its structure determined (Kim et al., 2003). AnCE and ACER show about 40% amino-acid sequence identities with vertebrate ACEs, share similar enzymatic properties (Coates et al., 2000), acting on a broad range of substrates and play a key role in development (Crackower et al., 2002) and reproduction (Ekbote et al., 1999; Hurst et al., 2003). Molecular and biochemical data

from the leech ACE homologue have related it to the N-domain of mammalian ACE (Riviere et al., 2004). However, no functional ACE is present in the genome of the nematode *Caenorhabditis elegans* (*C. elegans*), despite ACN-1, a homologue that lacks ACE-like proteolytic activity, is crucial in development (Brooks et al., 2003). Recently, a dicarbox-ypeptidase, LdDCP, was cloned in a pathogenic unicellular eukaryote, *Leishmania donovani* (Goyal et al., 2005). LdDCP, like ACE, is able to cleave a carboxyterminal dipeptide from HHL and is inhibited by captopril, suggesting that ACE may have already been present before the appearance of multicellular organisms. However, LdDCP was not reported to hydrolyse AngI and does not display sequence homology with ACE enzymes. These observations raise questions about the presence of a true ACE in more distant species.

Very surprisingly, *in silico* databank analysis revealed that DNA sequences in bacterial genomes have been annotated as encoding potentially functional ACE-like enzymes. All these sequences display a great similarity despite phylogenetic distance (for references see http://tolweb.org and (DeLong and Pace, 2001)). The striking sequence conservation in the active region of these putative ACE-like peptidases suggests that the protease activity would be conserved in these ancestral, prokaryotic species. Among them, *Xanthomonas axonopodis* pv. *citri* (*X. citri*) is a phytopathogen gamma-proteobacteria in whose genome an ACE-like encoding sequence has been annotated. In order to gain more insight into stages in ACE evolution and to investigate its putative activity in unicellular organisms, we report the cloning and biochemical characterization of the expressed recombinant enzyme. We have also generated a homology-based model of *Xc*ACE using the structure of tACE to understand the interactions of residues in the active site. This ACE-like enzyme in *X. citri* is, to our knowledge, the first bacterial ACE-like enzyme ever characterized.

2. Materials and methods

2.1. In silico analysis

The protein sequence of the leech ACE (*Tt*ACE, Genbank accession no. **45272589**), the most distant known ACE characterized at the time of the present study, was submitted to similarity searches using the BLAST program suite (tblastn; default parameters) (http://www.nih.nlm.ncbi.gov/blast) against all bacterial genomic sequences available at the NCBI server at the time of the study. Alignments were performed using the ClustalW (default parameters) software (http://www.ebi.ac.uk/clustalw/). The distance matrix generated into the tree file of ClustalW was used in the drawtree program of the PHYLIP suite and an unrooted tree diagram was generated (http://bioweb.pasteur.fr/seqanal/interfaces/drawtree-simple).

2.2. Bacterial strain

X. citri strain N1 was from the culture collection of the Laboratory of Plant Pathology, Faculty of Agriculture, Shi-zuoka University, Shizuoka, Japan (Talaga et al., 1996).

2.3. DNA extraction

Bacterial DNA was obtained as previously described (Davis et al., 1980).

2.4. RNA extraction

Total RNA was extracted using the SV total RNA isolation system (Promega).

2.5. Protein extraction

X. citri was cultured in YP medium under agitation at 30 °C until an optical density of 0.3 at 620 nm and centrifuged (5000 ×g, 30 min). Cells were washed in water, centrifuged, and the pellet resuspended in water in one hundredth of the culture volume. The resulting suspension was passed twice through a French pressure cell at 15,000 lb/in.^{2} (10^8 Pa) and unbroken cells and debris were removed by 5 min centrifugation at 5000 ×g.

2.6. RT-PCR

To assess the presence of a messenger RNA correponding to the *XcACE* gene, total RNAs (30–40 ng) were treated with RNAse-free DNAse I and reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen). The same amount of nucleic acids (170 ng) was further used in PCR reactions containing 2% DMSO using XcInt-S1 (5′-GCTGCAGCAGATCCCACAGA-3′) and XcInt-AS1 (5′-CTATTGCGTGGGCGTAGGC-3′) as primers. Water was used instead of cDNA as a negative control. PCR products were resolved on an agarose gel stained with ethidium bromide.

2.7. Cloning of XcACE, construction of the pXcACE vector and expression in mammalian cell cultures

2.7.1. Cloning

Full-length *XcACE* cDNA sequence (NP 641553; GenBank accession no. gi: **21241971**) was obtained by PCR on *X. citri* DNA (see Section 2.4). Sense and antisense primers (*XcACE*FL-S1 5′-CTGACCTATTCGGATGCGCTCAAGGAT-3′ and *XcA-CE*FL-AS2 5′-CTCTAGCCTTCGGCGGACTTCACTG-CAAAGGAC-3′) were used (2 pmol each) in a PCR reaction containing 400 ng DNA, 2% DMSO, 20 μM dNTPs, 5 U *Pfu* DNA polymerase (Promega) in 50 μL total volume. Reaction cycling parameters were 94 °C, 3 min; 94 °C, 40 s, 61 °C, 1 min, 72 °C, 4 min for 35 cycles. The expected 2.2 kb product was blunt-end subcloned into the EcoRV site of pcDNA3.1 (Invitrogen) and sequenced with *XcACE* and pcDNA3 specific primers.

2.7.2. Construct

Heterologous expression of *XcACE* was carried out using mammalian CHO-K1 cells (Invitrogen). Because the start codon is GTG for *XcACE*, it was necessary to change it into the standard ATG, a sequence recognized as an initiation codon by CHO-K1 cells. This substitution mutation (T11A) was realised by PCR using the *Xc*FLAS2.2 (5′-GCT CTA GCC TTC GGC GGA CTT CAA TGC AAG-3′) antisense oligonu-cleotide and resulted in an in-frame ATG initiation codon. The PCR product was subcloned in pcDNA3.1 and the obtained p*XcACE* plasmid sequenced as described above (see Section 2.7.1).

2.7.3. Expression

CHO-K1 cells were stably transfected with p*XcACE* using lipofectamine 2000 reagent (Invitrogen), and were selected for resistance to Geneticin. Individual resistant colonies producing large amounts of *Xc*ACE were cloned by limiting dilution techniques as described (Wei et al., 1991b).

2.8. Biochemical characterization of XcACE

2.8.1. Semi-purification

Both native and recombinant *Xc*ACE were partially purified by ion exchange chromatography. Briefly, native *Xc*ACE was obtained from bacterial protein extract, and secreted recombi-nant *Xc*ACE from concentrated culture medium of transfected CHO cells (fraction A). The fraction A samples were first desalted on a HiPrep Fast Desalting Column (Amersham Pharmacia Biotech), then fractionated onto a FPLC MonoQ anion-exchange column system (Pharmacia). The resulting fractions showing specific ACE activity were pooled and concentrated. The resulting fractions were named native *Xc*ACE (n*Xc*ACE) and recombinant *Xc*ACE (r*Xc*ACE), respec-tively. For n*Xc*ACE purification steps, each pooled fractions containing specific ACE activity were analysed by SDS-PAGE.

2.8.2. Western blot

Both n*Xc*ACE and r*Xc*ACE (see Section 2.8.1) were submitted to western blot experiment using a polyclonal antibody (HKCE) raised against the human full-length sACE.

2.8.3. Enzymatic assays

ACE activity was assayed using various substrates: HHL (5 mM), as a human ACE C-domain specific substrate, AcSDAcKP (Acetyl-Serine-Aspartyl-Acetyl-Lysine-Proline) (2.5 mM), as an N-domain specific substrate, Ang I (100 μM), as both an N- and a C-domain ACE substrate. Assays were performed at 37 °C for 30 or 60 min. Hydrolysis of [Leu5]-enkephalinamide ([Leu5]-enk-NH2) (0.8 mM, incubated for 330 min), as an amidated substrate and [Leu5]-enkephalin ([Leu5]-enk) (0.8 mM, incubated for 30 min), were compared to determine whether *Xc*ACE also exhibits endopeptidase activity. The rate of hydrolysis of all substrates used was determined and quantified using reverse phase HPLC (Waters Co, Milford, MA, USA) as previously described (Azizi et al., 2000; Houard et al., 1998; Wei et al., 1991b). The specificity of the reaction was assessed in the presence of 10 μM captopril. The effect of chloride ions (NaCl concentrations from 0 to 1.0 M) on HHL hydrolysis was determined under standard conditions.

2.8.4. Inhibition of XcACE activity by various ACE inhibitors

The inhibition potentials of various tight binding ACE inhibitors (captopril, delaprilat, fosinoprilat, lisinopril, perindoprilat, RXP
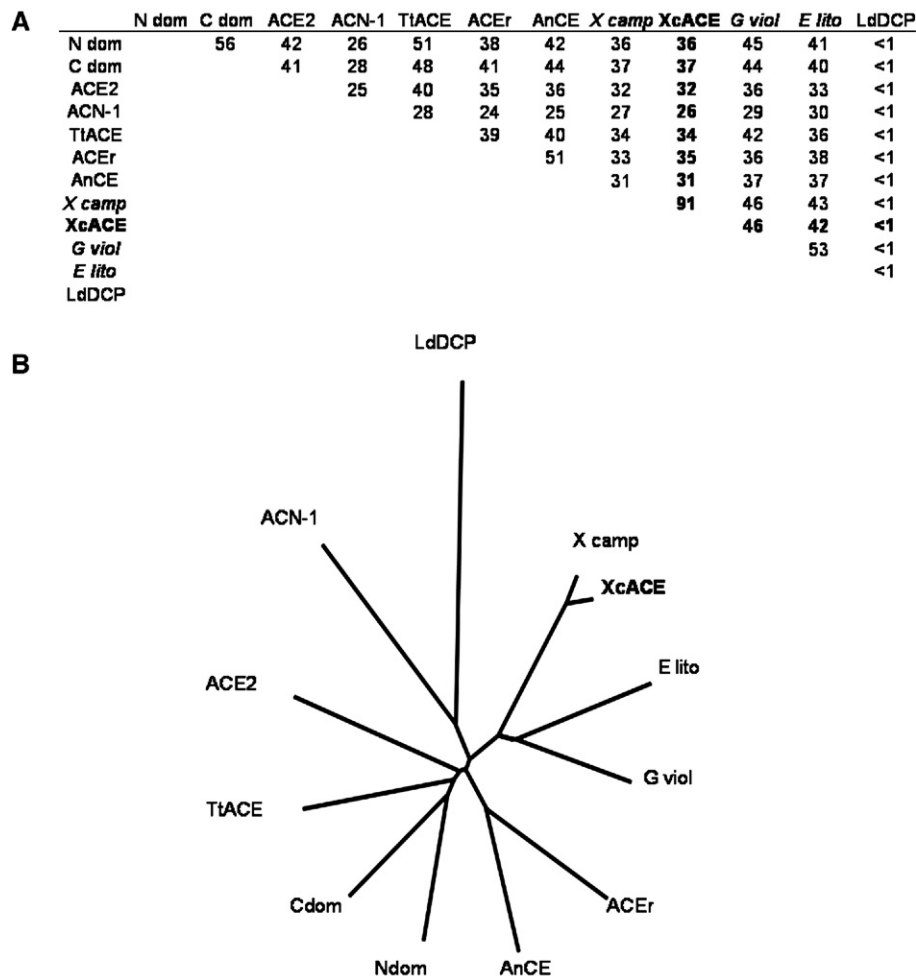
A

	N dom	C dom	ACE2	ACN-1	TtACE	ACEr	AnCE	X camp	XcACE	G viol	E lito	LdDCP
N dom		56	42	26	51	38	42	36	36	45	41	<1
C dom			41	28	48	41	44	37	37	44	40	<1
ACE2				25	40	35	36	32	32	36	33	<1
ACN-1					28	24	25	27	26	29	30	<1
TtACE						39	40	34	34	42	36	<1
ACEr							51	33	35	36	38	<1
AnCE								31	31	37	37	<1
X camp									91	46	43	<1
XcACE										46	42	<1
G viol											53	<1
E lito												<1
LdDCP												

B

Fig. 1. Homology of the bacterial ACE related to various ACEs in the phylogenetic tree. (A): Percentage of identity in amino acids between *Xc*ACE and various ACE-like enzymes primary sequences described or deduced from genomic sequences: Ndom, human N-domain ACE (gi: **4732026**, residues 1–583); Cdom, human C-domain ACE (gi: **4732026**, residues 641–1306); ACE2, human ACE2 (gi: **71658783**); ACEr, *Drosophila melanogaster* ACEr (gi: **73921650**); X camp, *Xanthomonas campestris* pv. *campestris* predicted dipeptidyl-carboxypeptidase (gi: **66769433**); E lito, *Erythrobacter litoralis* predicted dipeptidyl-carboxypeptidase (gi: **61100845**); G viol, *Gloeobacter violaceus* predicted dipeptidyl-carboxypeptidase (gi: **37522712**). (B): unrooted cladogram indicating evolutionary relationships between the different ACE homologues among the living kingdom.

407 and enalaprilat) were assessed by determining the IC$_{50}$ values for the fluorogenic substrate Mca-Ala-Ser-Asp-Lys-DpaOH hydrolysis in standard conditions as described previously (Dive et al., 1999). Compounds were pre-incubated with n*Xc*ACE (see Section 2.8.1) for 1 h and reactions initiated by the addition of 12 μM substrate.

2.9. Modelling of XcACE

A model of *Xc*ACE was generated by the ESyPred3D server (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) (Lambert et al., 2002) using testis ACE (ProteinDataBank pdb code **1O8A**) as a model. This model was compared with the testis ACE structures (Natesh et al., 2003; Natesh et al., 2004) and the N-domain structure (Corradi et al., 2006) and AnCE (Houard et al., 1998) using the program Coot (Emsley and Cowtan, 2004). Figures were generated with Pymol (DeLano, 2002).

2.10. XcACE activity during bacterial growth

To examine *Xc*ACE localization, ACE activity in medium and in bacterial extracts was monitored along during bacterial growth using HHL as substrate as described above.

3. Results

3.1. Presence of ACE orthologues in bacterial genomes

Among 465 bacterial strains genomes screened, 26 sequences produced significant similarity (*E* value <0.5) with leech *Tt*ACE. Further analysis revealed that at least 22 sequences within 19 different bacterial strains within 8 generas potentially encode putative ACE-like enzymes. These species are ecologically as well as phylogenetically distant, since they are found amongst Cyanobacteria (*Gloeobacter violaceus*, gi: **37522712**, gi: **35213714**), Acidobacteria (*Solibacter usitatus*, gi: **67858770**,
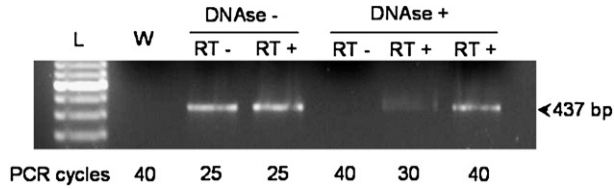
Fig. 2. RT-PCR assay of the XcACE transcript. cDNA was generated and submitted to PCR (cycling parameters: 94 °C, 2 min; 94 °C, 45 s, 61 °C, 30 s, 72 °C, 1 min, for 25, 30 or 40 cycles, 72 °C, 4 min).Treatments of the different samples are indicated above the corresponding lane. The size of the expected band is indicated. L: DNA molecular weight marker, W: water control. The number of PCR cycles is indicated under each sample.

gi: **66769433**) and Proteobacteria of the subdivision alpha (*Erythrobacter litoralis*, gi: **61100845**, gi: **60736774**), delta (*Anaeromyxobacter dehalogenans*, gi:**66855040**) and gamma (*X. citri*, gi: **21241971**, gi: **21107345**; *Xanthomonas oryzae*, gi: **37931607**, gi: **58583162**; *Xanthomonas campestris pv campestris*, gi:**21230574**, gi: **21112152**; *Shewanella oneidensis*, gi: **24374038**, gi: **24348510**) proteobacteria. All these predicted proteins share between 38 and 98% primary sequence identity between them, indicating a wide variety among the potential bacterial ACEs (Fig. 1 and Supplementary data). Nevertheless, all these sequences exhibit key ACE features such as the presence of two highly conserved consensus VCHASAWDFY and GANPGFHEA motifs surrounding a putative M2-catalytic site HexxH (data not shown).

3.2. Molecular characterization of XcACE

We cloned an ACE homologue in the phytopathogenic bacterium *X. citri* corresponding to the sequence XAC1217 (gi: **21107356**, gene ID **1155288**). The transcription of the *XcACE* gene was suggested by the sequence conservation upstream the start codon and is assessed by the presence of a specific 437-bp RT-PCR product in DNAse-treated, reverse-transcribed total RNA (Fig. 2). The protein encoded by this gene, referred to as

*Xc*ACE (*Xanthomonas citri* angiotensin-converting enzyme), is a 672 amino-acid protein. Hydrophobicity profile (SignalP V1.1 at www.cbs.dtu.dk/services/SignalP) indicates cleavage of a putative signal peptide between residues 26 and 27 of the native protein (…RDA↕AP…). There does not appear to be a motif encoding a transmembrane domain at the C-terminus or elsewhere in the mature molecule, suggesting that *Xc*ACE should exist only in a soluble form. Because of the mutation inserted, the recombinant proenzyme is slightly different from the native one in the N-terminal end (r*Xc*ACE: MQGPPVNPRLL *vs* n*Xc*ACE: MNPRLL). However, this difference does not change predictions about the signal peptide (hydrophobicity profile and cleavage site) (data not shown), indicating that the mature recombinant and native proteins have identical primary sequences. The mature enzyme has a theoretical molecular weight of 72 kDa and an isoelectric point of 5.72. Its primary structure includes all the residues implied in zinc (H^{429}, H^{433} and E^{457}), lisinopril (H^{391}, A^{392}, E^{430}, K^{556}, H^{558}, Y^{565} and Y^{568}) (Natesh et al., 2003) and captopril (Q^{290}, K^{556}, Y^{565}, H^{391}, H^{558} and Y^{568}) (Natesh et al., 2004) binding. Thus the soluble, secreted enzyme should possess a single active site like the other ACEs found in invertebrates. Whereas two chloride atoms were observed bound to the human tACE, *Xc*ACE appears to be more similar to the N-domain in this respect, as it only has the chloride II binding site conserved (Y^{230} and R^{567}), and only one amino acid among the three implicated in the binding of the first chloride atom (W^{530}). The *Xc*ACE primary sequence has between 31 and 37% identity with the other known ACEs (human, *Drosophila* and leech) and 26% with *C. elegans* ACN-1 (Fig. 1 and Supplementary data).

3.3. Biochemical characterization of XcACE

3.3.1. Semi-purification
The semi-purification of *Xc*ACE procedure using FPLC (Fig. 3A) resulted in a fraction containing a protein band at the expected size for *Xc*ACE (∼75 kDa), as revealed by SDS-PAGE analysis of the different fractions obtained after each purification step (data not shown).
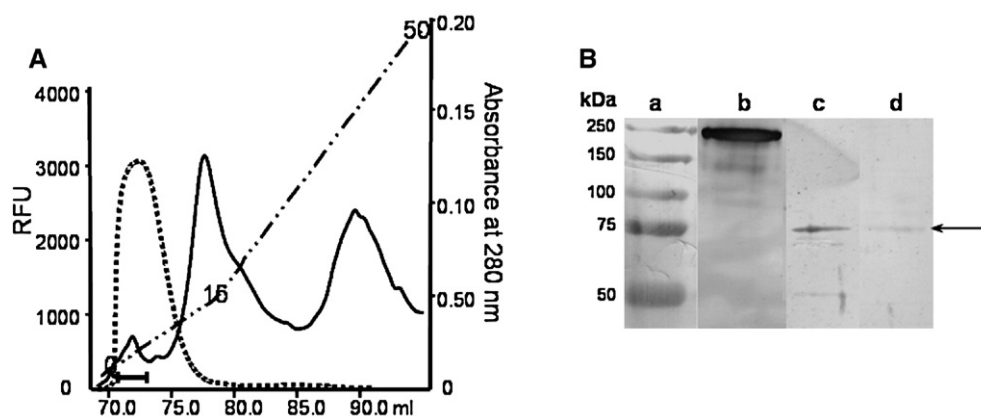

Fig. 3. Semi-purification and Western blot of *Xc*ACE. (A): FPLC profile of the last purification step (see Section 2.8.1), absorbance at 280 nm (full line), the gradient (dashed line) was from 0 to 50 mS/cm, arbitrary units of ACE activity (dotted line), the horizontal bar indicates the fractions pooled for further analysis. (B): Native and recombinant *Xc*ACE were analysed by SDS-PAGE followed by Western blot with the antiserum *HKCE*. Lane a, molecular-mass standards; lane b, human somatic ACE; lane c, native *Xc*ACE; lane d, recombinant *Xc*ACE. The band corresponding to XcACE is indicated (arrow).
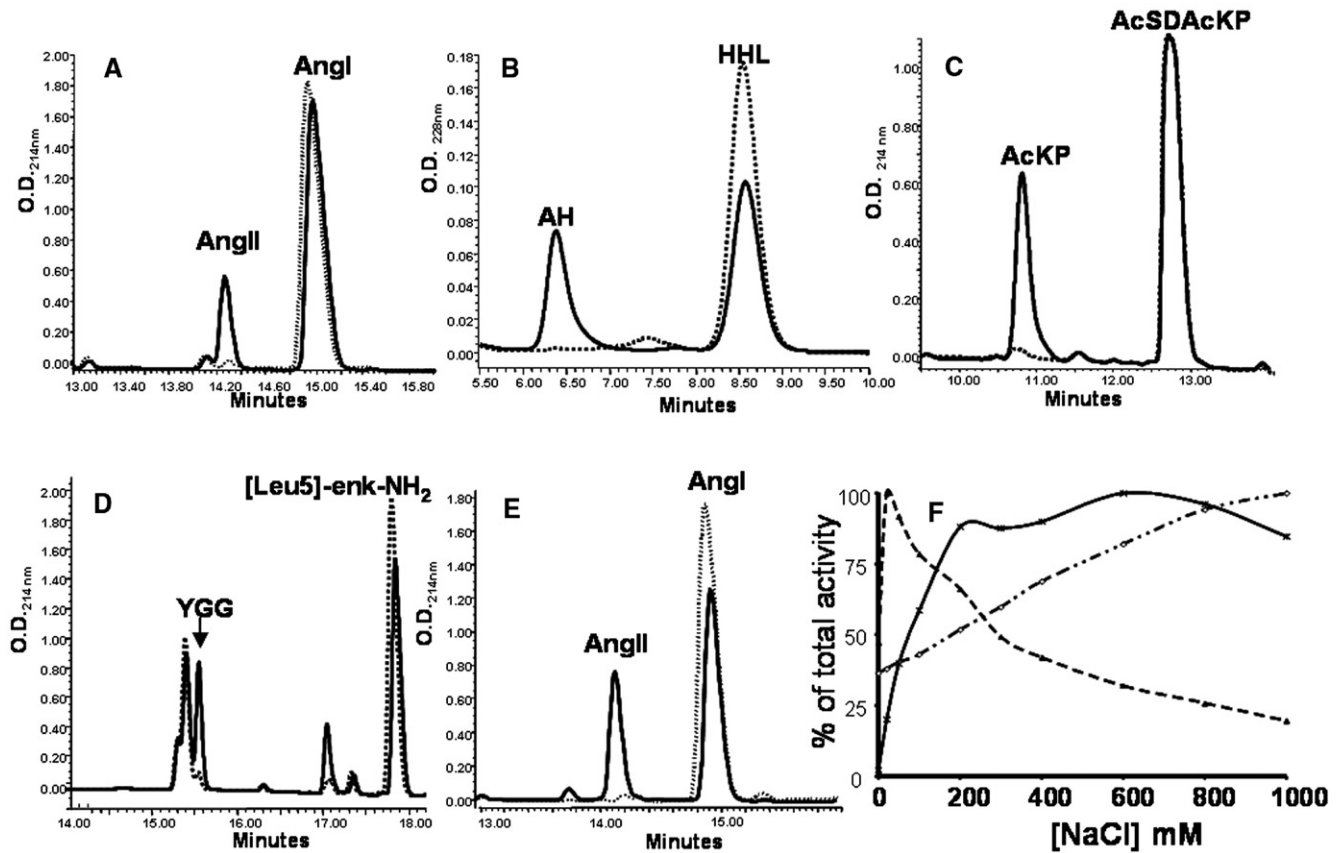
Fig. 4. Biochemical characterization of *Xc*ACE. Hydrolysis of various ACE substrates by native (n*Xc*ACE) (A–D) and recombinant (r*Xc*ACE) (E). Hydrolysis of angiotensin I (A), hydrolysis of HHL (B), AcSDAcKP (C), [Leu5]-enkephalin (D) and [Leu5]-enkephalinamide (E) by native *Xc*ACE. Hydrolysis of angiotensin I by recombinant XcACE (F). The specificity of the reactions was assessed in the presence of 10 µM captopril (dashed line). AngI, angiotensin I; AngII, angiotensin II; AH, hippuric acid; YGG, tyrosyl-glycyl-glycine. Chloride sensitivity of *Xc*ACE (F). Effect of chloride ions concentration on HHL hydrolysis by native (◇) *Xc*ACE, C-(ж) and N-domain (▲) of human somatic ACE.
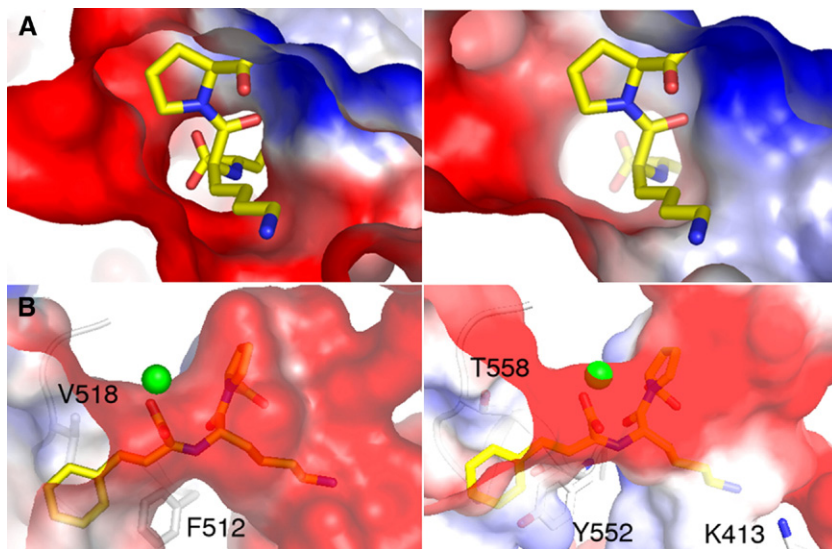
Fig. 5. Comparison of the charge of the active site between tACE and *Xc*ACE. ACE is represented as a surface with the positive charge indicated in blue and the negative in red. The zinc atom is represented (green), and some of the residues are indicated. Lisinopril (in yellow) is shown to orientate the active site. A, The active site viewed as a surface from the lysyl pocket for tACE (left) and XcACE (right). B, The active site cavity viewed from the outside for tACE (left) and XcACE (right). tACE is shown to have a higher negative charge in the lysyl binding pocket.

3.3.2. Immunological characterization

Both native and recombinant XcACE were analysed by Western blotting and migrated with an apparent molecular weight of 72 kDa, matching theoretical predictions (Fig. 3B), and have a similar mobility on SDS-PAGE to deglycosylated human tACE (Yu et al., 1997).

3.3.3. Enzymatic activity

XcACE shows dipeptidyl-carboxypeptidase activity, cleaving a carboxyterminal dipeptide from peptidic substrates. It generates AngII from AngI (Fig. 4A), hippuric acid from HHL (Fig. 4B), AcKP from AcSDAcKP (Fig. 4C), and Tyrosyl-glycyl-glycine (YGG) from Leu-enkephalin (data not shown). XcACE also displays an endopeptidase activity, as shown by its ability to hydrolyse the amidated substrate, Leu-enk-NH2 (Fig. 4D). As for AngI hydrolysis (Fig. 4E), the same results were obtained with recombinant XcACE (data not shown). XcACE activity depends on zinc binding. Indeed, hydrolysis of Mca-SDKP-DpaOH was abolished after zinc chelation with EDTA and dialysis but was restored by zinc addition (data not shown).

3.3.4. Effect of chloride concentration

Native XcACE is already active in the absence of chloride ions. However, its activity in the presence of increasing chloride ion concentrations increases 3-fold. In this respect, XcACE Cl$^-$ activation presents intermediate characteristics when compared to both N- and C-domain human ACE (Riviere et al., 2004). (Fig. 4F).

3.3.5. Effect of ACE inhibitors

Fosinoprilat is the most potent inhibitor of XcACE activity (IC$_{50}$: $3.0 \cdot 10^{-8} \pm 1.0 \cdot 10^{-8}$ M), whereas captopril is less efficient ($1.6 \cdot 10^{-7} \pm 0.1 \cdot 10^{-7}$ M). RXP 407 ($1.9 \cdot 10^{-6} \pm 1.5 \cdot 10^{-6}$ M) and Enalaprilat ($3.8 \cdot 10^{-6} \pm 2.8 \cdot 10^{-6}$ M) displayed IC$_{50}$ values in the micromolar range. Perindoprilat ($1.3 \cdot 10^{-4} \pm 1.3 \cdot 10^{-4}$ M), delaprilat ($3.7 \cdot 10^{-5} \pm 1.7 \cdot 10^{-5}$ M) and lisinopril ($4.7 \cdot 10^{-5} \pm 4.3 \cdot 10^{-5}$ M), all containing carboxylate zinc-binding groups, were the least effective inhibitors of XcACE. Values are means ± standard error from three independent experiments.

3.4. Predicted structure of XcACE

As the crystal structures of human ACE (Corradi et al., 2006; Natesh et al., 2003), ACE2 (Towler et al., 2004) and the Drosophila AnCE (Kim et al., 2003) all have the same fold, the 34% sequence identity of human tACE and bacterial XcACE allowed us to model with confidence the overall fold of XcACE based on the tACE structure (pdb code **1O8A**). We identified active site residues that could account for the differences in inhibitor affinities (see Section 4.2) (Fig. 5).

3.5. XcACE expression during bacterial growth and localization

Only background ACE activity was detected in the culture medium, which remains constant over time. In contrast, ACE activity in bacterial extract correlates bacterial growth indicat-
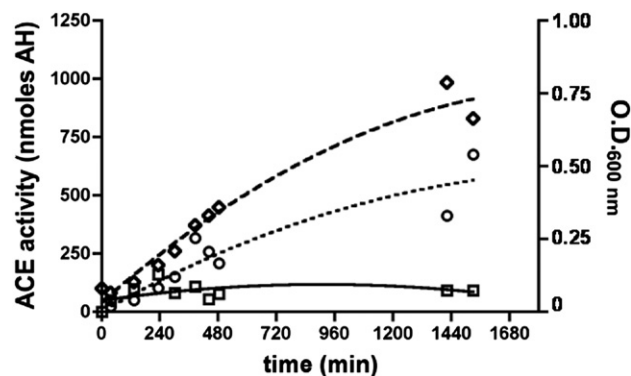

Fig. 6. XcACE activity during bacterial growth. Follow-up of bacterial growth by O.D. at 600 nm (◇) and ACE activity (nmoles hippuric acid generated) in culture medium (□) and within bacteria (○). Error bars were omitted for clarity.

ing that XcACE is constituvely expressed and is likely to be secreted and further localised in the periplasmic space (Fig. 6).

4. Discussion

4.1. ACE is an ancestral enzyme

Databank analysis revealed the presence of putative ACE-encoding genomic DNA sequences in a very wide range of bacterial species, corresponding to a broad range of ecological lifestyles and terminal electron acceptor for growth (DeLong and Pace, 2001), suggesting that ACE is an ancestral enzyme. Characterization of XcACE constitutes the first evidence of the presence of an ACE-like enzyme in more distant organisms than cellular metazoans. Indeed, in contrast to XcACE, the amino-acid sequence of L. donovani dicarboxypeptidase (LdDCP) (Goyal et al., 2005) does not show any significant alignment with all other known ACEs, was not reported to hydrolyse Ang I to Ang II, and lacks conserved consensus motifs found in almost all ACE primary sequences (for references see the MEROPS database available at http://merops.sanger.ac.uk/). However, in contrast with Leishmania, none of the bacterial species putatively possessing an ACE mentioned in this study is known to be a human pathogen, whereas xanthomonadacaea are phytopathogens among which X. citri was chosen for technical reasons.

4.2. Structure–activity relationship of XcACE, a functional angiotensin-converting enzyme in vivo

The XcACE possesses a single M2 catalytic site and a signal peptide but no membrane anchor, like the ACE characterized in invertebrates. Despite the fact that the recombinant enzyme was expressed in a heterologous system, both native and recombinant forms display almost identical features, indicating a satisfactory purity of XcACE and strongly suggesting that the ACE in X. citri is a functional ACE enzyme in vivo. XcACE, like Drosophila AnCE (data not shown), is active in the absence of chloride ions and less sensitive to variations in chloride ion concentration than human C-domain ACE (Houard et al., 1998). This characteristic is likely to be due to the absence in the primary sequence of XcACE, as in AnCE, of two key amino

acids observed to bind ClI in human tACE (*Xc*ACE : R186H and R489K; AnCE : R1867Y and W485F, when compared to tACE). *Xc*ACE hydrolyses a broad range of substrates, including human N- and C-domain ACE specific substrates (AcSDAcKP and HHL, respectively). In addition to this dicarboxypeptidase activity, *Xc*ACE also acts as an endopeptidase, hydrolysing the C-terminal blocked amidated substrate [Leu5]-enkephalina-mide. Western blot results using polyclonal antibody raised against the full-length human sACE protein show a unique band corresponding to *Xc*ACE, indicating that *Xc*ACE shares common epitopic features with the human enzyme. Molecular modelling allowed us to visualise the structural differences in the catalytic site. Key active site residues such as the zinc-binding motif, His^{513} and Tyr^{526} (tACE numbering) are conserved in *Xc*ACE and many other ACE-like enzymes. However, in line with the assay results, the non-conserved active site residues are neither identical to the C- or N-domain of human ACE, and are the presumed cause of the different substrate selectivity and inhibition profile of *Xc*ACE. Comparison of the *Xc*ACE model with either the human tACE or N-domain shows that lisinopril fits in the *Xc*ACE active site and retains most of the same binding interactions. The exception is in the lysyl pocket, which has features in common with AnCE or the human N-domain including a positively charged residue, lysine (equivalent to Arg^{356} in AnCE and Arg^{350} in the human N-domain) that appears to affect the overall charge of the pocket (Fig. 5). Indeed, similar to *Xc*ACE, neither AnCE nor the N-domain have as high affinity for lisinopril as tACE (Michaud et al., 1997; Williams et al., 1996). Furthermore, the difference in affinity of *Xc*ACE and tACE for inhibitors with hydrophobic P1 groups (*i.e.* lisinopril, delaprilat and perindoprilat) could be attributed to the difference in hydrophobicity of the S1 subsite. *Xc*ACE has a threonine and tyrosine in contrast to Phe^{512} and Val^{518} in tACE (Fig. 5). The N-terminal aspartate, *N*-acetyl groups and the C-terminal amide of RXP407 are the reported determinants required for its remarkable N-domain specificity (Dive et al., 1999). Of the four possible residues suggested for N-domain selective recognition of RXP 407 (Corradi et al., 2006; Tzakos and Gerothanassis, 2005), *Xc*ACE only retains the threonine (T^{496} in the N-domain) and has a lysine which replaces Arg^{500} of the N-domain. This, combined with the hydrophobic P1 group, may be the reason that RXP407 is not a strong inhibitor for *Xc*ACE. The affinity of *Xc*ACE for fosinoprilat is harder to rationalise as it has been modelled in different conformations for the N- and C-domains of human ACE (Tzakos and Gerotha-nassis, 2005). However, *Xc*ACE retains all the residues suggested to be involved in inhibitor binding with both domains of human ACE.

4.3. Putative physiological roles

The biological functions of *Xc*ACE remain unclear but could constitute the original, ancestral function of ACE. *Xc*ACE is soluble and its sequence exhibits a signal peptide indicating, along with the absence of ACE activity in the culture medium, that *Xc*ACE is a secreted enzyme which is trapped into the periplasmic space. The constitutive expression could indicate an important function for ACE in *X. citri*, as a possible role for the enzyme in peptidoglycan remodelling, a critical parameter in environmental adaptation. This is in line with the characteriza-tion in the periplasm of other gram negative bacteria of VanX D-alanyl-D-alanine dipeptidase, a regulated carboxypeptidase that is responsible for Vancomycin resistance (Lessard and Walsh, 1999). *Xc*ACE could also participate in *X.citri* pathogenicity within the cocktail of degradative enzymes required during plant infection. Interestingly, in this regard, ACE inhibitors are found in hydrolysates of plants sensitive to the "black rot" caused by *Xanthomonas*, including rice and soybean (He et al., 2005; Wu and Ding, 2001). The large number of substrates of *Xc*ACE does not exclude a possible function of protein degradation that might generate dipeptides for protein biosynthe-sis. To this extent, it is interesting to note that ACE is highly expressed in the digestive tract of all metazoan species presenting an active ACE.

4.4. Evolutionary and phylogenetic aspects

One of the most relevant questions raised by this study deals with phylogenetic considerations and evolution of ACE. Its presence in eukarya as well as in bacteria indicates that the enzyme could have appeared before the divergence between these two groups. This strongly suggests that *Xc*ACE is the most ancestral present-day representative of ACE character-ized to date. However, as all completely sequenced genomes of plants, algaes and mycetes lack ACE, caution should be exercised with this hypothesis. The presence of an ACE-like enzyme in bacteria could also be the result of a horizontal gene transfer from eucaryotic to bacterial species. However, conservation in sequences surrounding the *ACE* gene, suggestive of mobile genetic elements, is not observed within 30 kb of the gene in seven distinct bacterial strains (data not shown). Furthermore, the parasitic bacterial species with an ACE-like gene are all hosted by organisms lacking the enzyme. In addition, not all bacterial species with a putative ACE gene are parasitic. Thus, the presence of ACE in bacteria, like the situation encountered in annelids, is unlikely to be related to parasitism, suggesting that bacterial ACE, represented herein by *Xc*ACE, corresponds to the ancestral, original enzyme.

4.5. Ancestral ACE is neither related to the N- nor the C-domain of mammalian ACE

*Xc*ACE exhibits a similar primary sequence identity with both N- and C-domains and, although *Xc*ACE cleaves the N-domain substrate AcSDAcKP, the N-domain specific inhibitor RXP407 is not a potent inhibitor of *Xc*ACE activity. In contrast, lisinopril, a C-domain-related inhibitor, is less efficient than captopril, related to the N-domain, though most of the residues for binding of both are conserved. Thus, from the biochemical and the structural properties of *Xc*ACE it is not possible to ascribe this bacterial enzyme to either the mammalian N-or C-domain of human ACE. The primitive ACE would then not be closer to either domain of the mammalian enzyme, in contrast to what was previously

suggested (Coates et al., 2000; Hubert et al., 1991; Lattion et al., 1989).

5. Conclusion

This work describes the cloning, expression, purification, biochemical characterization, molecular modelling and localization of *Xc*ACE, the first ACE-like enzyme in a bacterium, *X. citri*. Taken together, our results bring the evidence of the presence of a functional ACE in prokaryotes, in contrast to the previous hypothesis about the latter appearance of ACE during phylogeny. It also appears that sequence/structure conservation of ACE enabled preservation of dipeptidase activity during evolutionary specialisation. *Xc*ACE is the most ancestral ACE present-day representative and has features in common to both the N- and the C-domain of mammalian somatic ACE. It is possible that this ancestral ACE was a poorly specialised protease which has acquired its present specialisation over a long evolutionary period. However, the biological function(s) as well as the endogenous substrates and/or inhibitors of bacterial ACE remain to be characterized. To clarify these issues, the use of ACE inhibitors *in vivo* and the generation of a mutant bacterial strain lacking ACE may be helpful. Some of these functions of ACE might also be conserved in humans, and their characterization could lead to a better understanding of the evolution of this crucial enzyme.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2007.05.010.

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