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ORIGINAL PAPER

AepA of *Pectobacterium* is not involved in the regulation of extracellular plant cell wall degrading enzymes production

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Abstract Plant cell wall degrading enzymes (PCWDE) are the major virulence determinants in phytopathogenic *Pectobacterium*, and their production is controlled by many regulatory factors. In this study, we focus on the role of the AepA protein, which was previously described to be a global regulator of PCWDE production in *Pectobacterium carotovorum* (Murata et al. in Mol Plant Microbe Interact 4:239–246, 1991). Our results show that neither inactivation nor overexpression of *aepA* affects PCWDE production in either *Pectobacterium atrosepticum* SCRI1043 or *Pectobacterium carotovorum* subsp. *carotovorum* SCC3193. The previously published observation based on the overexpression of *aepA* could be explained by the presence of the adjacent regulatory *rsmB* gene in the constructs used. Our database searches indicated that AepA belongs to the YtcJ subfamily of amidohydrolases. YtcJ-like amidohydrolases are present in bacteria, archaea, plants and some fungi. Although AepA has 28% identity with the formamide deformylase NfdA in *Arthrobacter pascens* F164, AepA was unable to catalyze the degradation of NdfA-specific N-substituted formamides. We conclude that AepA is a putative aminohydrolase not involved in regulation of PCWDE production.

Keywords Phytopathogenic bacteria · PCWDE · Amidohydrolases · Substrate specificity

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Introduction

Pectobacterium species cause soft rotting or tissue macerating diseases in a wide variety of plants (Pérombelon and Kelman 1980; Pérombelon 2002). *Pectobacterium* cause tissue maceration by producing extracellular plant cell wall degrading enzymes (PCWDE) including pectinases (Pel), polygalacturonases (Peh), cellulases (Cel) and proteases (Prt) (Pirhonen et al. 1991; Heikinheimo et al. 1995; Mäe et al. 1995; Marits et al. 1999; Mattinen et al. 2004). Tissue maceration is a complex process that is tightly controlled by different regulators, among which the RsmA/RsmB system is the most thoroughly studied (Chatterjee et al. 1995; Liu et al. 1998; Cui et al. 2001; Kõiv and Mäe 2001; Burr et al. 2006; Sjöblom et al. 2006). In 1991, Murata et al. described a new gene, *aepA*, which regulates PCWDE synthesis in *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) strain 71. *AepA* overexpression reportedly enhanced PCWDE production in different *Pectobacterium* strains (Liu et al. 1993). On the other hand, later studies identified *rsmB* gene next to *aepA* (Murata et al. 1994; Liu et al. 1998). *rsmB* codes for regulatory RsmB RNA which acts by neutralizing the effect of global repressor of virulence genes, RsmA (Ma et al. 2001). Thus, the overexpression of *rsmB* might have been responsible for the reported effect of *aepA* overexpression on virulence genes expression in *Pcc*.

Using genomic sequencing and bioinformatics, we classified AepA as a member of the YtcJ-like subfamily in the amidohydrolase superfamily. This superfamily includes enzymes that hydrolyze a wide range of substrates with amide or ester functional groups attached to carbon or phosphorus centers (Seibert and Raushel 2005). To date, investigations of YtcJ-like subfamily members have been limited to a functional study of the N-substituted

formamide deformylase (NfdA) in *Arthrobacter pascens* F164 (Fukatsu et al. 2004) and elucidation of the involvement of LAF3-1 in phytochrome A signaling pathway in *Arabidopsis thaliana* (Hare et al. 2003).

The possible relation between the role of AepA in regulation of PCDWE and its potential amidohydrolase activity has remained obscure. Solving this issue was the aim of the current study.

Materials and methods

Bacterial strains, vectors and growth conditions

The strains and plasmids used in this study are listed in Table 1. *Pcc* strains were grown at 30°C, *Pectobacterium atrosepticum* (*Pa*) strains at 25°C and *Escherichia coli* at 37°C. Bacteria were grown in Luria–Bertani (LB) medium (Miller 1972) or in M9 minimal medium (Sambrook and Russell 2001) containing 0.4% glycerol (w/v) with or without 0.4% (w/v) polygalacturonic acid (PGA; Sigma), 10% potato extract or 10% celery extract. Potato extracts were prepared as described by Marits et al. (1999), and the celery extract was prepared according to Murata et al. (1991).

The nitrogen sources, *N*-benzylformamide (NFBA), acetonitrile, formamide and urea, were applied at concentrations between 0.05 and 0.025% (w/vol). When necessary, the growth medium was supplemented with 100 μg/ml ampicillin (Amp), 20 μg/ml chloramphenicol (Cm) or 50 μg/ml kanamycin (Km).

Enzyme assays

The semiquantitative agar plate assays for extracellular Peh, Cel and Prt were performed as described by Chatterjee et al. (1995). The enzyme activity on agar plates was evaluated according to the size of the halo around each colony, which is proportional to the amount of the secreted enzyme. The assays were repeated ten times, and no significant variations were observed.

Table 1 Strains and plasmids

Strains and plasmids		Source or reference
Strains		
P. carotovorum subsp. *carotovorum*		
SCC3193	Wild type	Pirhonen et al. (1988)
SCC3193aepA	SCC3193 *aepA* knockout derivative (Km^R)	This study
SCC3193rsmB	SCC3193 *rsmB* knockout derivative (Cm^R)	This study
P. atrosepticum		
SCRI1043	Wild type	Hinton et al. (1985)
SCRI1043aepA	SCRI1043 *aepA* knockout derivative (Km^R)	This study
SCRI1043aepA1	SCRI1043 *aepA1* knockout derivative (Cm^R)	This study
SCRI1043aepA-aepA1	SCRI1043aepA *aepA1* knockout derivative (Cm^R)	This study
E. coli		
DH5α	*supE4, ΔlacU169, (lacZΔM15), hsdR17, recA1, endA1, gyrA 96, thi-1, relA1*	BRL (San Diego, CA)
Plasmids		
pBluescript SK	Cloning vector (Amp^R)	Stratagene
pTZ57R/T	High-copy cloning vector; (Amp^R)	Fermentas
pBAD33	Plasmid vector (Cm^R); araC-P_{BAD}	Guzman et al. (1995)
pADrsmaep	Vector pAD33 containing *Pcc* SCC3193 *rsmB* and *aepA* genes in *Sac*I site	This study
pADaep	Vector pAD33 containing *Pcc* SCC3193 *aepA* gene in *Sac*I site	This study
pADrsm	Vector pADrsmaep with *Sph*I and *Hin*dIII excised 1 kb DNA out of *aepA* gene	This study
pUTmini-Tn5km	Delivery plasmid for mini-Tn5km (Km^R; Amp^R)	de Lorenzo et al. (1990)
pKD46	Derivative of pINT-t containing araC-P_{araB} and γ β exo (without or with tL3) DNA fragments	Datsenko and Wanner (2000)
pKD3	Derivative of pANTSγ containing an FRT (FLP recognition target)-flanked Cm^R gene from pSC140	Datsenko and Wanner (2000)
pKD4	Derivative of pANTSγ containing an FRT (FLP recognition target)-flanked Km^R gene from pCP15	Datsenko and Wanner (2000)

Potato tuber assay

Solanum tuberosum potato tubers (varieties Irga and Ando) were inoculated with 10^7 colony forming units (CFU) of *Pcc* or *Pa* cells. The macerated tissue was weighed after 48 and 72 h of incubation at 24°C and 100% humidity. Differences between the macerated tissue weights were analyzed with the Student's *t* test.

RNA isolation and real-time RT-PCR

Pcc cells were grown either in 30 ml liquid glycerol minimal medium with or without PGA or on solid glycerol minimal agar plates using PGA, 10% potato extract or 10% celery extract for induction. In the liquid medium experiments, 1 ml of cells was collected 4, 6, 8, and 10 h after inoculation. Bacterial cells grown on the agar plates were harvested after 24 h of incubation. Total RNA was isolated using a Macherey–Nagel RNA extraction kit. An additional DNase I (Fermentas) treatment was performed according to the supplier's protocol. Real-time PCR was performed using SYBR Green Kit (Thermo Scientific). The following primer pair was designed using PrimerExpress 3.0 software (Applied Biosystems): aepRT1 (CGAACGCGAACTATT TGTTG) and aepRT2 (ATGCCGCTGTCATAAACCA). The reaction mixture contained 1 pg–1 ng total RNA, 100 nM of the forward and reverse primers, QPCR SYBR ROX Mix containing all the nucleotides, reaction buffer and ROX Dye, Verso Enzyme Mix and RT enhancer. The reaction was performed at 50°C for 15 min, 95°C for 15 min, and 40 cycles 95°C for, 60°C for 30 s and 72°C for 30 s.

The amount of *aepA* mRNA was normalized with *ffh* (encoding a signal recognition particle involved in targeting and integration of inner membrane proteins) mRNA. The amount of *ffh* mRNA was determined using the same reverse transcription PCR protocol described above, replacing the primers with ffhFw (CGCCATATTACTG GCAAGCCTATT) and ffhRev (ACCGAGAATGCGTG ATGCAA). The expression ratio was calculated by comparing the threshold cycle (C_t) value of the induced sample with the C_t value of non-induced sample, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All of the RNA extractions and reverse transcriptase real-time PCR reactions were performed in triplicate.

Construction of plasmids and strains

The *Pcc* SCC3193 *aepA*-deficient mutant was constructed as follows. The aepAUP (GATAGCTATAAAACGA ACCG) and aepAD (CTAATTCATCTAATCGATGCTA) primer pair was designed using the *aepA*/aepH sequence of *Pcc* 71 (Murata et al. 1994) and the genomic sequence of *Pa* SCRI1043, respectively (http://www.sanger.ac.uk/Projects/E_carotovora; Bell et al. 2004). Following PCR, the obtained DNA fragment was ligated into a *Sma*I-digested Bluescript SK plasmid to yield pBaepA, and then sequenced. The kanamycin (Km) resistance gene was obtained from the pUTmini-Tn5km plasmid (de Lorenzo et al. 1990) as a *Bam*HI fragment and cloned into *Van*91-digested pBaepA to create the pBLaepAKm plasmid. The *aepA*-negative mutant, SCC6032, was made using the λ Red system (Datsenko and Wanner 2000) using the aepAA (CGCAAAAGCGTGTAAAATCG) and aepAY (CATG CATGAAAGTGCGATGC) primers and the pBaepAKm plasmid as the template.

The *Pcc rsmB*-negative mutant was created as described by Datsenko and Wanner in 2000, using the rsmBP1 (GCAGGAGCCTAAAGGGATTGAATCACGGAAGAT ACAGGATGGAAACGTGTAGGCTGGAGCTGCTTC) and rsmBP2 (ACTTTTCCTTTTTGGTCATCCTGACC CACAATCCCTGCTGGCGTCCATATGAATATCCTC CTTAG) primers and the pKD3 plasmid as the template.

The *Pa* SCRI1043 *aepA*- and *aepA1*-negative mutants were also constructed as described by Datsenko and Wanner in 2000. The SCRI1043aepA mutant was generated using the AepECAP1/AepECAP2 primer pair (TTG CCACCAAACACTTCTTTCAGGCTCGCAGCACTTGC TGTCACCGTCGTGTAGGCTGGAGCTGCTTC; GGAG TAAATATCTTCATAGCCACCGTCTAACGGATGAGC GTGGACATCCATATGAATATCCTCCTTAG) and pKD4 plasmid as the template. The SCRI1043aepA1 mutant was constructed using the HYPaepP1/HYPaepP2 primer pair (ATGAAATTGAATGTAAAAATGCTGTCAGTCACAC TTGGCCTGTTCACCGTGTAGGCTGGAGCTGCTTG; CA TAAACCACGATCGGAATACTCGTGGAGACGGTATC TAAATCAGCAGCATATGAATATCCTCCTTAG) and pKD3 plasmid as the template. The *aepA/aepA1* double mutant was generated in SCRI1043aepA mutant background using the same method and primers.

Overexpression of *aepA* and *rsmB*

To express *aepA* under the *araBAD* promoter, the *aepA* gene in *Pcc* SCC3193 was PCR-amplified from the wild-type strain using the primers aepAlg (TATTTATCGG ACTATTTAG) and aepLopp (ATTACCTACCCAACA TAT). The PCR product was ligated into the *Sac*I site in a pBAD33 vector. Here, the PCR product was under the control of the *araBAD* promoter to yield pADaep, and it was sequenced. As a negative control, the PCR fragment was cloned into the same plasmid in the opposite direction to yield the $pADaep_{rev}$ plasmid. The production of AepA protein was verified by SDS-PAGE electrophoresis.

To express both the *Pcc* SCC3193 *rsmB* and *aepA* genes under the control of the *araBAD* promoter, PCR was

performed using the rsmB2 (AATACATCATCTTA TTACTTAAG) and aepLopp (ATTACCTACCCAACA TAT) primers. The PCR product was cloned into the *Sac*I site of pBAD33 under the control of the *araBAD* promoter to yield the pADrsmaep vector. *Sph*I and *Hin*dIII were used to excise a 1 kb DNA fragment (from nucleotides 490 to 1418 with respect to the translation start codon of *aepA*) out of the *aepA* coding region, to make the pADrsm plasmid. The pADrsm plasmid contains the full functional *rsmB* gene (Fig. 1b).

The GenBank accession number for the *Pcc* SCC3193 nucleotide sequence of *rsmB* and *aepA* is GQ344499.

Results and discussion

Chromosomal context of the *aepA* gene in *Pa* and *Pcc*

Analysis of the full genomic sequence in *Pa* strain SCRI1043 demonstrated that there were two alleles of the *aepA* gene (*aepA* and *aepA1*) present in the chromosome. The *aepA* gene, located at nucleotides (nt) 1144917–1146593, is flanked by the *rsmB* gene, which encodes a regulatory RNA, and a gene hypothesized to be a regulator in the MarR family (Fig. 1a). The product of the *aepA1* gene showed only 28% amino acid (aa) identity when compared with AepA. The *aepA1* gene, located at nt 4141061–4142812, is flanked by the single-strand binding protein (*ssp*) gene and a gene hypothesized to encode for a GCN5-related N-acetyltransferase (GNAT) family protein.

To isolate and sequence the *aepA* allele from *Pcc* strain SCC3193 (Pirhonen et al. 1988), we designed PCR primers based on the *Pa* strain SCRI1043 sequence (Bell et al. 2004). The *aepA* allele from *Pcc* strain SCC3193 encoded a protein with high levels of homology to the *aepA* alleles in *Pa* strain SCRI1043 (92% identity), *Pectobacterium wasabiae* WPP163 (100% identity), *Pectobacterium carotovorum* subsp. *brasiliensis* PBR1692 (93% identity), and *Pcc* WPP14 (92% identity). Additionally, the *aepA* protein from *Pcc* strain SCC3193 was homologous to an N-terminal, 453-aa fragment of AepA from *Pcc* 71, which may have been used in experiments by Liu et al. in 1993 (the sequence of the 105 aa on the C-terminal end of this protein was missing from the database). The genomic context of *aepA* is very similar in all of these *Pectobacterium* strains (Fig. 1a). *AepA1* is present in *P. wasabiae* WPP163 (96% identity to *aepA1* from *Pa*) and in *Pcc* strain PC1 (96% identity to *aepA1* from *Pa*) and has the same location as in *Pa*, but the allele is absent in *P. carotovorum* subsp. *brasiliensis* PBR1692 and *Pcc* WPP14. Currently, we do not know if *aepA1* is present in *Pcc* strain SCC3193.

Role of AepA in virulence

Next, we investigated whether disrupting AepA function in *Pcc* SCC3193 affected PCWDE synthesis by evaluating protease production on milk plates. Subsequently, we measured cellulase and pectinase production on carboxymethylcellulose and PGA containing indicator plates, respectively. In the AepA-negative mutant, the PCWDE production was similar to the wild-type strain, whereas we measured significantly lower PCWDE production in the *rsmB*-negative mutant (Fig. 2a). PCWDE production was also unaffected in the *Pa aepA* knockout (Fig. 2b). Additionally, inactivation of the *Pa aepA1* gene did not alter PCWDE production in comparison to the wild-type strain (data not shown).

Several studies have shown that parallel pathways may control PCWDE expression. For example, in both *Pcc* strains SCC3193 and 71, the response to quorum sensing

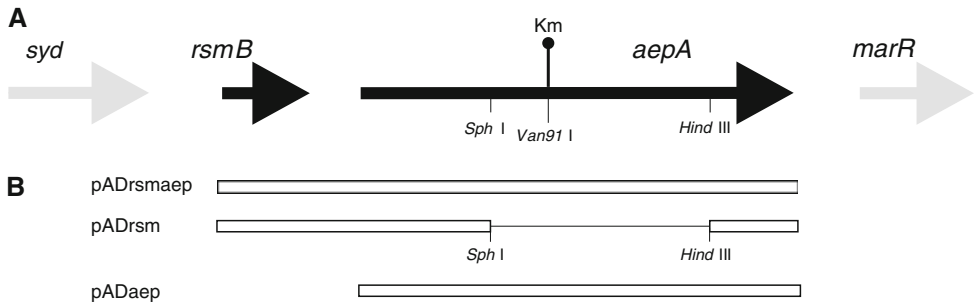

Fig. 1 Genetic context of *aepA* in *Pcc* SCC3193 and *aepA* and *rsmB* overexpression constructs. **a** The *aepA* locus in *Pectobacterium carotovorum* subsp. *carotovorum* SCC3193. The *aepA* gene (*black*) is shown with its flanking genes. *rsmB* (*black arrow*) is located upstream of *aepA*, and the proposed Syd protein gene (*gray arrow*) is located upstream of *rsmB*. A putative MarR family regulator gene (*gray arrow*) is located downstream of *aepA*. The location of the kanamycin resistance cassette in *aepA* is indicated by a *lollypop-shaped symbol*. **b** Construction of the *aepA* and *rsmB* overexpressing plasmids. The *aepA* gene and *rsmB/aepA* genes were PCR-amplified from *Pcc* wild-type strain SCC3193 and ligated into the pBAD33 vector under the *araBAD* promoter to yield pADaep and pADrsmaep, respectively. Using the *Sph*I and *Hin*dIII enzymes, the *aepA* gene was cut out of pADrsmaep to yield pADrsm

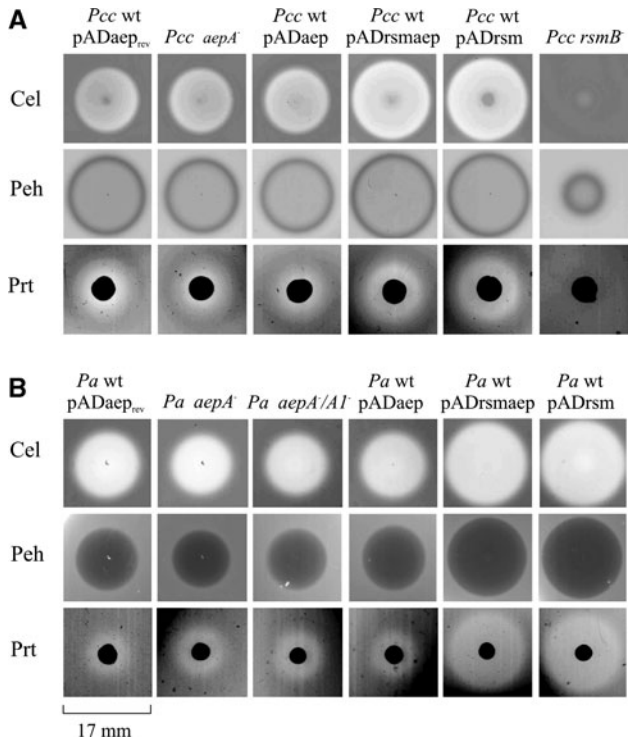

Fig. 2 The effect of *aepA* and *rsmB* overexpression on PCWDE production. **a** Exoenzyme indicator plates showing the production of cellulases (*Cel*), polygalacturonases (*Peh*) and proteases (*Prt*) from the following strains: the *Pcc aepA*-deficient strain (*Pcc aepA*$^-$), the *Pcc rsmB*-deficient strain (*Pcc rsmB*$^-$), and the *Pcc* wild-type SCC3193 strain containing plasmids pADaep$_{rev}$, pADaep, pADrsmaep, or pADrsm. The cells were incubated at 30°C for 24 h on cellulose or PGA plates and for 48 h on milk plates containing 10 mM arabinose. **b** Exoenzyme indicator plates showing the production of Cel, Peh and Prt in the following strains: the *Pa aepA*-deficient strain (*Pa aepA*$^-$), *Pa aepA/aepA1*-deficient strain (*Pa aepA*$^-$*/aepA1*$^-$) and the *Pa* wild-type SCRI1043 strain containing plasmids pADaep$_{rev}$, pADaep, pADrsmaep or pADrsm. The cells were incubated at 25°C for 48 h on cellulose or PGA plates and for 72 h on milk plates containing 10 mM arabinose

signals is cooperatively controlled by two distinct ExpR regulators (Sjöblom et al. 2006; Cui et al. 2006). To detect whether AepA and AepA1 are components of parallel pathways that regulate PCWDE expression, we constructed an *aepA*$^-$*/aepA1*$^-$ double knockout in *Pa* SCRI1043. The PCWDE production in the *aepA*$^-$*/aepA1*$^-$ strain was similar to the production in the wild-type and single knockout strains (Fig. 2b).

To exclude the possibility that AepA has an effect on PCWDE production only when overexpressed, we cloned *Pcc* SCC3193 *aepA* into the highly efficient expression vector pAD33, placing it under control of the inducible *araBAD* promoter. As shown in Fig. 2, *aepA* overexpression from pADaep in the *Pcc* wild-type strain SCC3193, as well as in the *Pa* wild-type strain SCRI1043, had no effect on the degradation of milk proteins, cellulose and PGA in comparison to a strain containing a control pADaep$_{rev}$ vector. We conclude that the regulation of PCWDE production is not attributable to *aepA* inactivation or overexpression in the above strains.

A potato tuber assay was used to examine whether AepA regulates PCWDE synthesis during pathogenesis in the plant. The tubers (cv. Irga and Ando) were inoculated with the *Pcc* or *Pa* wild-type strains or the *Pcc aepA, Pa aepA* or *Pa aepA/aepA1* knockouts strains. There were no significant differences in the maceration capacities of the wild-type versus knockout strains, irrespective of the potato variety used (*Pcc* SCC3193 vs. *Pcc* SCC3193aepA, $P = 0.84$; *Pa* SCRI1043 vs. *Pa* SCRI1043aepA, $P = 0.3$; *Pa* SCRI1043 vs. *Pa* SCRI1043aepA-aepA1, $P = 0.62$). Taken together, our results demonstrate that functional *aepA* is not required for *Pcc* strain SCC3193 or *Pa* strain SCRI1043 to show their full virulence.

RsmB overexpression results in increased PCWDE production

In 1993, Liu et al. described that the overexpression of *aepA* on the pAKC264 plasmid increased production PCWDE in *Pcc* strain 71. Sequence analysis of the region upstream of the *aepA* showed that there was significant identity with *rsmB*, a small RNA that has been characterized as a regulatory component of the Rsm system (Liu et al. 1998). These findings suggest that the reported PCWDE overproduction in *Pcc* strain 71 containing pAKC264 may have been caused by overproduction of *rsmB*, since both *aepA* and *rsmB* genes were present in this plasmid.

To verify our hypothesis, the genomic region of *Pcc* SCC3193 that contained both *rsmB* and *aepA* was cloned into the pBAD33 plasmid to construct pADrsmaep. The *aepA* gene was deleted from pADrsmaep to produce pADrsm, a vector that expresses RsmB RNA at the same level as pADrsmaep. When either pADrsmaep or pADrsm was introduced into the wild-type *Pcc* SCC3193 or *Pa* SCRI1043, PCWDE production was considerably increased in contrast to the corresponding strains carrying the control pADaep$_{rev}$ vector (Fig. 2). As noted above, overexpression of *aepA* alone did not influence PCWDE production. Therefore, *Pcc* SCC3193 cells carrying pADrsmaep show increased PCWDE production due to *rsmB* overproduction. Based on these results, we conclude that *aepA* has no role in regulating PCWDE production. Our results suggest that the previously reported PCWDE overproduction due to the presence of pAKC264 in strain 71 and other *Pcc* strains (Liu et al. 1993) resulted not from *aepA* overexpression but from overexpression of *rsmB*, which is present in the same plasmid.

Regulation of *aepA* expression

In 1993, Liu et al. showed that *aepA-lacZ* fusion operon could be induced with pectate and celery extract in *Pcc* AC5006. Although AepA has no effect on PCWDE production in *Pcc* SCC3193, this does not rule out the possibility that *aepA* is induced when the bacterium infects the plant. Therefore, we analyzed the level of *aepA* mRNA using quantitative RT-PCR to measure whether *aepA* expression is enhanced in the presence of PGA like the expression of PCWDE genes. Although *aepA* mRNA was detectable in the cells grown in liquid minimal glycerol medium, the ratio between mRNA levels in PGA-induced and non-induced cells did not increase considerably during the growth curve (Fig. 3). In *Pcc* SCC3193, necrosis-inducing protein (Nip) is induced only on solid minimal medium supplemented with potato extract (our unpublished data). We considered the possibility that AepA production was also favored on solid surfaces. Therefore, we compared the *aepA* mRNA levels in cells grown on minimal glycerol solid medium with those of cells grown on minimal glycerol solid medium supplemented with different plant extracts, including PGA, potato extract and celery extract. While PGA and potato extract had no effect on *aepA* expression, celery extract slightly increased *aepA* expression (ratio 1.49 ± 0.06) (Fig. 3). This indicates that celery may contain compound(s) that induce *aepA* expression. Taken together, we failed to detect any large induction of *aepA* by plant components.

The possible function of AepA

While our data showed that AepA does not regulate PCWDE production, the question remains: what is the function of the AepA protein? A BLAST analysis revealed

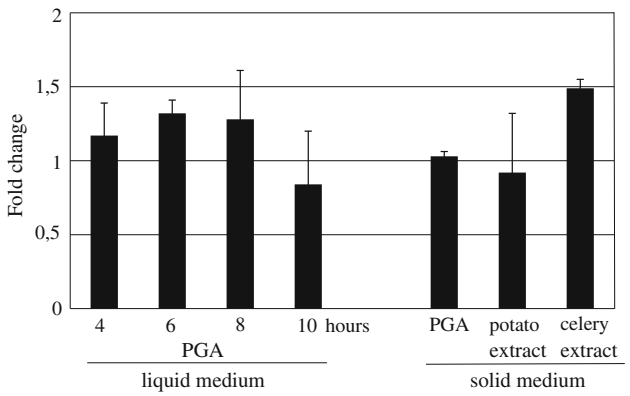

Fig. 3 Real-time RT-PCR analysis of the expression of *Pcc aepA* gene in response to plant extracts. The ratio between *aepA* mRNA in cells grown in minimal glycerol medium to the cells grown in the presence of 0.4% PGA, 10% potato or 10% celery extract in liquid and solid medium

that AepA has similarities to YtcJ-like metal-dependent hydrolases, a subfamily of the diverse amidohydrolase superfamily, whose members catalyze the hydrolysis of amide or ester bonds in different substrates (Seibert and Raushel 2005). Homologues of AepA were found in all three domains of life: Archaea, Bacteria and Eukaryota (plants and some fungi). Although the similarities between *Pectobacterium* AepA and other YtcJ proteins is equipoise over the entire polypeptide, the pairwise amino acid identity was generally less than 30%. The highly conserved portion of the polypeptide included four histidines and an aspartic acid that are known to bind a divalent metal ion in the active center of the amidohydrolases (Fig. 4). Several three-dimensional structures of amidohydrolases from other subfamilies have been published (Buchbinder et al. 1998; Benini et al. 1999; Thoden et al. 2001; Abendroth et al. 2002; Vincent et al. 2004), but to date, no X-ray structures of YtcJ subfamily members have been reported.

In the literature, there are two proposed functions for YtcJ subfamily members. Firstly, LAF3-1 of *Arabidopsis thaliana* (28% identity with AepA) has been suggested to regulate the phytochrome A signaling pathway, which is involved in plant growth and development. The transcription of LAF3-1 and its shorter isoform, $LAF3_{ISF2}$, is induced during germination (Hare et al. 2003). Unfortunately, the protein is not yet biochemically characterized, and its possible substrate(s) are unknown. Secondly, a functional analysis of N-substituted formamide deformylase (NfdA) from *Arthrobacter pascens* F164 (28% identity with AepA) showed that it catalyzes the deformylation of N-substituted formamide and produces the corresponding amine and formate. NfdA can also use other nitrogen-containing compounds as substrates, but with lower efficiencies (Fukatsu et al. 2005). This indicates that NfdA may be responsible for detoxifying cells of a relatively wide variety of chemicals and providing cells with nitrogen (Fukatsu et al. 2005).

Since N-substituted formamides are the only compounds that have been shown to be substrates for YtcJ-like metal-dependent hydrolases, we tested whether AepA of *Pcc* is involved in the degradation of known NfdA substrates, such as *N*-benzylformamide (NFBA), acetonitrile, formamide and urea (Fukatsu et al. 2005). To do this, we incubated *Pcc* strains SCC3193, SCC3193*aepA*, and SCC3193, containing the *aepA* overexpression plasmid pADaep, and SCC3193, containing the control vector $pADaep_{rev}$, for 24 h in minimal medium containing 0.4% glycerol as carbon source and NBFA, acetonitrile, formamide, urea or NH_4^+ as the sole nitrogen source. The strains containing pADaep or $pADaep_{rev}$ were induced by the addition of 10 mM arabinose. No strain could use NBFA, acetonitrile or formamide as its sole nitrogen source (data not shown). The consumption of urea,

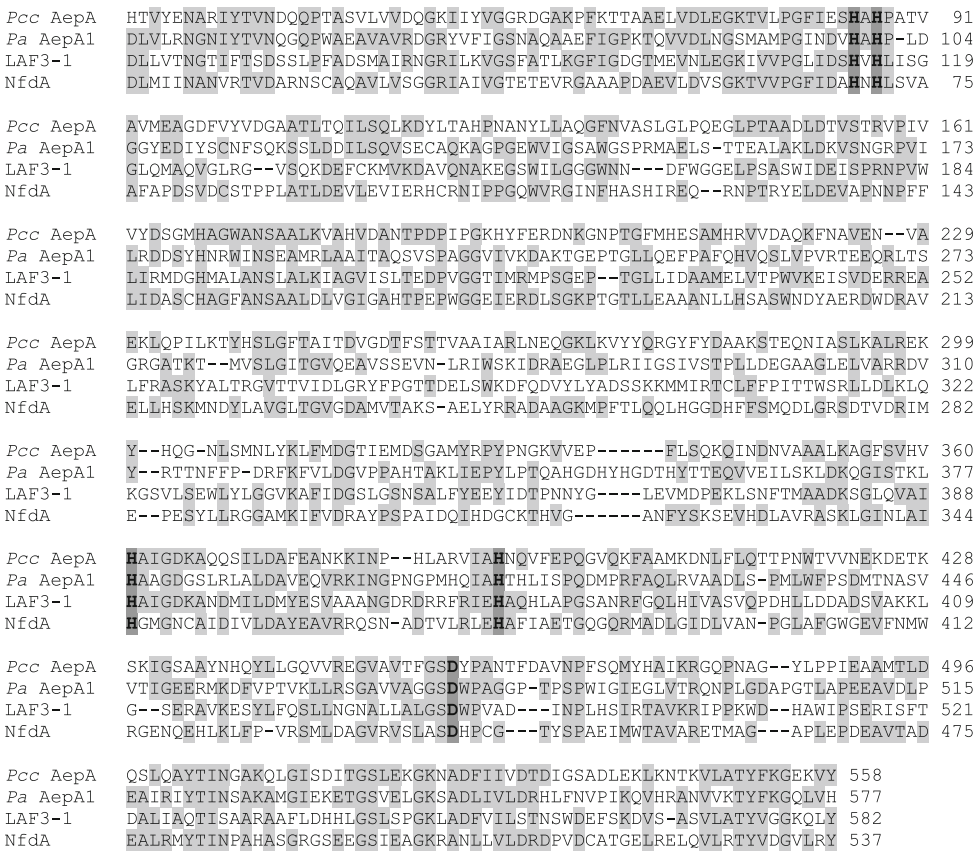

Fig. 4 Sequence alignment of *Pcc* AepA with YtcJ-like amidohydrolases. The sequence alignment includes the amino acid sequences of *Pcc* SCC3193 AepA (*Pcc* AepA), *Pa* SCRI1043 AepA1 (*Pa* AepA1), *Arthrobacter pascens* F164 NfdA (NfdA), and *Arabidopsis thaliana* LAF3-1 (LAF3-1). The conserved amino acids and functional residues are *shaded in gray*. The conserved residues, which are known to bind to a divalent metal ion in the active center of each amidohydrolase, are bold and shaded in dark gray. The numbers specify the length of the sequences. The alignments were performed using the CLUSTALW program (Thompson et al. 1997)

however, was equal in all of the strains, with the growth rate being 50% that observed in media with NH_4^+ as the nitrogen source (data not shown). In order to test the possibility that AepA or AepA1 could use some of these substrates as nitrogen sources in *Pa* cells, wild-type *Pa* SCRI1043, and the *Pa aepA*, *aepA1* and *aepA/aepA1* knockout strains were incubated in minimal media containing one of the nitrogen sources mentioned above. None of the knockout strains differed from the wild type in their ability to use the nitrogen sources tested.

There remained the possibility that the strains were unable to use the above-mentioned nitrogen sources not due to a lack of AepA-catalyzed initial degradation, but rather due to deficiencies in enzymes responsible for downstream conversions. Therefore, we tested whether AepA or AepA1 could enhance cellular resistance to toxic NBFA concentrations. We incubated the wild-type *Pcc* and the *aepA/aepA1* knockout strains in media containing 0.1, 0.3 or 0.6% NBFA and observed that the strains had similar sensitivities to NBFA. Our results indicate that neither AepA nor AepA1 have the substrate specificity described for NfdA. Although AepA and AepA1 did not hydrolyze NBFA, formamide or acetonitrile, this does not rule out the possibility that these enzymes metabolize other N-substituted formamides.

Arthrobacter pascens F164 was isolated from soil through enrichment culturing using NBFA as a nitrogen source (Fukatsu et al. 2004). NBFA and other N-substituted formamides are produced by the action of isonitrile hydratase on isonitriles (Fukatsu et al. 2004). A wide array of isonitriles (isocyanides) and formamides are naturally synthesized by microorganisms, fungi, phytoplankton, plants, and marine sponges (Garson and Simpson 2004). These toxic compounds are generally nonessential for basic metabolic processes, and they have been proposed to be involved with self-defense, since many possess antibiotic activity or have an ecological significance in establishing and maintaining host–microbe equilibriums.

The presence of AepA may enable *Pcc* to use atypical nitrogen sources, which are secreted by the host plant or microorganisms that colonize the host plant's rhizosphere. The high number of possible candidates makes it difficult to identify AepA substrates.

Conclusion

Our data indicate that AepA is not involved in the regulation of PCWDE in *Pcc* SCC3193 or *Pa* SCRI1043. Sequence analysis strongly suggests that AepA proteins are amidohydrolases, although their substrate remains to be determined.

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