cDNA-AFLP analysis of differential gene expression in the prokaryotic plant pathogen *Erwinia carotovora*

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For studies of differential gene expression in prokaryotes, methods for synthesizing representative cDNA populations are required. Here, a technique is described for the synthesis of cDNA from the potato pathogens *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Erwinia carotovora* subsp. *carotovora* (*Ecc*) using a combination of short oligonucleotide (11-mer) primers that were known to anneal to conserved sequences in the 3' regions of enterobacterial genes. Specific PCR amplifications with primers designed to anneal to 14 known genes from either *Eca* or *Ecc* revealed the presence of the corresponding transcripts in cDNA, suggesting that the cDNA represented a broad genomic coverage. cDNA-amplified fragment length polymorphism (cDNA-AFLP) was used to identify differentially expressed genes in *Eca*, including one that shows significant similarity, at the protein level, to an avirulence gene from *Xanthomonas campestris* pv. *raphani*. Northern analysis was used to confirm that differentially amplified cDNA fragments were derived from differentially expressed genes. This is the first report of the use of cDNA-AFLP to study differential gene expression in prokaryotes.

Keywords: prokaryotic differential gene expression, *Erwinia*, plant pathogen, cDNA-AFLP

INTRODUCTION

The enterobacterial plant pathogens *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Erwinia carotovora* subsp. *carotovora* (*Ecc*) are responsible for soft rot diseases in a number of crops worldwide. *Eca* has a host range limited to potato, causing soft rot of tubers and blackleg of stems. In contrast, *Ecc* has a wide host range that includes potato, but fails to cause blackleg disease (Pérombelon, 1992). Although our knowledge of the molecular processes involved in pathogenicity is increasing (Pérombelon & Salmond, 1995), little is known about mechanisms that control the onset of infection or host specificity in these pathogens. Only through a comprehensive understanding of the nature of their

interactions with host plants, and the identification of genes involved in such interactions, will we be able to develop novel control strategies to protect potato production.

Traditionally, transposon mutagenesis has been used as a method to identify genes involved in bacterial processes such as pathogenicity (reviewed by Mills, 1985). This technique has yielded important information about a number of genes involved in disease development in *Er. carotovora* (e.g. genes encoding pectolytic enzymes or involved in secretory and regulatory processes; Pérombelon & Salmond, 1995). However, 'hot-spots' for transposon insertion, secondary transposition with possible genome rearrangements, low throughput and the generation of lethal mutations limit the use of this method (Mills, 1985). In essence, many subtle interactions between the pathogen and its host may be lost when the pathogen is disabled by mutagenesis. An alternative approach to studying plant-pathogen interactions is to investigate changes in gene expression during the interaction.

Abbreviations: *Eca*, *Erwinia carotovora* subsp. *atroseptica*; *Ecc*, *Erwinia carotovora* subsp. *carotovora*; AFLP, amplified fragment length polymorphism.

The GenBank accession numbers for the EL1, EL2, EL3, EP5, EP22, EP26, EP11 and EP21 sequences determined in this work are AJ274641–AJ274648, respectively.

Methods for profiling gene expression, such as differential display (DD) RT-PCR (Liang & Pardee, 1992) and cDNA-amplified fragment length polymorphism (cDNA-AFLP; Bachem *et al.*, 1996) have yielded important differentially expressed genes from eukaryotes. Both the purification of mRNA and synthesis of cDNA for these techniques involves an oligo(dT) primer that anneals to the poly(A) tail at the 3' end of the transcript. The low levels of polyadenylation in prokaryotic mRNAs mean that efficient methods for cDNA synthesis that exclude rRNA are lacking. Nevertheless, DD RT-PCR techniques have been reported for prokaryotes (Wong & McClelland, 1994; Abu Kwaik & Pederson 1996; Akins *et al.*, 1998), although insufficient amplification products were generated to allow an entire genome to be screened for differentially expressed genes. Recently, a method has been developed for profiling gene expression in members of the *Enterobacteriaceae* that provides a broad genome coverage (Fislage *et al.*, 1997). This method uses combinations of short (10-mer and 11-mer) oligonucleotide primers that anneal, respectively, to conserved sequences in the 5' and 3' regions of *Escherichia coli* genes. We report the use of these primers for representative cDNA synthesis from *Eca* and *Ecc* and the subsequent use of cDNA-AFLP to profile gene expression for the first time in bacteria.

METHODS

Bacterial strains, media and infiltration of potato leaves. The bacterial strains used in this study were *Ecc* SCRI 193 (Salmond *et al.*, 1986), *Eca* SCRI 1039 (Hyman *et al.*, 1997) and *Es. coli* DH1. The media used were Luria-Bertani Broth (LB) (Miller, 1972) and minimal medium containing 1% polygalacturonate and 1% pectin (MM-pectin) (McMillan *et al.*, 1994). Overnight cultures of *Er. carotovora* and *Es. coli* were diluted 20-fold in the same medium and left to grow at 27 °C for *Er. carotovora* and at 37 °C for *Es. coli* to OD₆₀₀ ~ 0.4 before RNA extraction. Bacterial suspensions were prepared by centrifuging overnight cultures of *Eca* or *Ecc* and resuspending them in 10 mM MgSO₄ to the required con-

centration. Detached potato leaves were vacuum-infiltrated with a suspension of either *Eca* or *Ecc* at 10⁸ c.f.u. ml⁻¹ for 15 min, and incubated for 1 h at 18 °C before freezing in liquid nitrogen and storing at -80 °C until RNA was extracted.

RNA extraction and cDNA synthesis. Total RNA was prepared from bacterial cells as described by Aiba *et al.* (1981). Prior to cDNA synthesis, RNA was treated with RNase-free DNase I (Pharmacia Biotech) (1 U DNase I: 20 µg RNA) and incubated for 20 min at 37 °C. The RNA was then heat-denatured at 65 °C for 10 min, simultaneously inactivating DNase I. A mixture of all 11-mer oligonucleotide primers (Ea1-Ea10) at 100 ng µl⁻¹ (Fislage *et al.*, 1997) was used to generate first-strand cDNA from 10 µg DNase-I-treated RNA following the procedure described in the First-strand cDNA Synthesis Kit (Pharmacia Biotech). The second strand of cDNA was synthesized using the Universal RiboClone cDNA Synthesis System (Promega).

PCR amplification. Approximately 200 ng genomic DNA and 1 µl of a 100-fold dilution of first-strand cDNA were subjected to 35 cycles of amplification by PCR. In addition to template DNA, the amplification mixture contained, in a final volume of 50 µl, 2.5 U *Taq* polymerase (Gibco-BRL), 10 mM Tris/HCl (pH 8.4), 50 mM KCl, optimized amounts of MgCl₂ within the range 1.5–2.5 mM, 100 µmol of each deoxynucleotide triphosphate and 50 ng of each primer for a given gene (Table 1).

cDNA-AFLP. Double-stranded cDNA (50 ng) was incubated with 0.5 U each of *Eco*RI and *Mse*I in the presence of OnePhorAll (OPA) buffer (Pharmacia Biotech) and 10 µg BSA ml⁻¹ for 3 h at 37 °C, according to Vos *et al.* (1995). The digestion products (10 µl) were incubated with 5 pmol each of an *Mse*I-adaptor and an *Eco*RI-adaptor (which were annealed as described by Vos *et al.*, 1995), 1 mM ATP and 1 U T4 DNA ligase (Gibco) in OPA buffer at 37 °C for 3 h. The adaptors were: 5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACG-CATGGTTAA-5' (*Eco*RI), and 5'-GACGATGAGTCCTG-AG-3' and 3'-TACTCAGGACTCAT-5' (*Mse*I). The *Eco*RI (*Eco*) primer was radiolabelled at 37 °C for 1 h. The labelling reaction contained 3.5 ng primer, 0.125 U T4 polynucleotide kinase, 0.1 µl 5× Forward Reaction Buffer provided with the enzyme (Gibco), and 0.5 µCi (18.5 kBq) [γ -³²P]ATP, and the

Table 1. Sequences of oligonucleotide primers designed to anneal to gene sequences derived from *Eca* and *Ecc*

Gene	Forward primer	Reverse primer	Product length (bp)	GenBank accession no. or reference
<i>pelB</i> ^a	Y1: 5'-TTACCGGACGCCGAGCTGTGGCGT-3'	Y2: 5'-CAGGAAGATGTCGTTATCGCGAGT-3'	440	X16397
<i>pelB</i> ^a	Y45: 5'-TCACCGGACGCCGAACGTGGGCGT-3'	Y46: 5'-TCGCCAACGTTACGACAGAACAGT-3'	438	A62563
<i>mopB</i> ^a	Mop F: 5'-GCCGACAACGGAAGGCATTTT-3'	Mop R: 5'-TCGTTGGTTGGTTGTGCTGGAA-3'	656	X72969
Matching formate acetyltransferase from <i>Es. coli</i> ^a	Eca1: 5'-CGGCATCATATAAACACG-3'	Eca2: 5'-GCACATTCATCCAGGA-3'	690	De Boer & Ward (1995)
<i>hexA</i> ^a	HexA F: 5'-GGCTGCAGTCAATCGAACTCAA-3'	HexA R: 5'-ACCCTTCTCTCGCTAACAC-3'	671	AF057064
<i>ogl</i> ^a	Ogl F: 5'-GGCCAAAGGTAACAAGATCCCC-3'	Ogl R: 5'-AATTCGTGGGTACAGCTTTCGC-3'	728	X77391
<i>celN</i> ^a	CelN F: 5'-GCAGCTCACCAGAACGTGATTTA-3'	CelN R: 5'-ATTACATTGCTCCAGTCACCG-3'	961	L39788
<i>rffDG</i> ^a	RffDG F: 5'-GTTTATCGTGCGCAGAAATCCT-3'	RffDG R: 5'-TTGTACGTCTCACCGACTTCGC-3'	1193	AF044332
<i>pnl</i>	Pnl F: 5'-CGGGAGGTAAGTCGTTACGG-3'	Pnl R: 5'-TCCCTTTATTTCCGCGCCGG-3'	680	M59909
<i>out</i>	Out F: 5'-CGGTACGCTGTGGCAGGGG-3'	Out R: 5'-CGGGTAAGTCATTCCGCTGC-3'	530	X70049
<i>rdg</i>	Rdg F: 5'-GATTGGCGTATCTCAGGCCG-3'	Rdg R: 5'-CGACCAGAAGCTCGGGACC-3'	1160	L32173
<i>rpf</i>	Rpf F: 5'-GCCGGACGATCTGCATCGC-3'	Rpf R: 5'-TGATGTCATCATCCGTTTGCCC-3'	620	U62023
<i>nucA</i>	NucA F: 5'-GTGCTGGATCAAAAAATACCCC-3'	NucA R: 5'-CAGTCAAGTGCCCCAACCG-3'	720	AF057063
<i>hrpN</i>	HrpN F: 5'-GAGTTGAACAACATCAGTACCG-3'	HrpN R: 5'-ATCGTGTCATCAATACCCAGC-3'	344	L78834

^a These primers were designed from *Eca* sequences. All other primers are from *Ecc*.

Fig. 1. The cDNA-AFLP procedure for studying *Eca* gene expression, adapted from Bachem *et al.* (1996). Using total RNA extracted from *Er. carotovora*, first-strand cDNA was synthesized using a mixture of the ten 11-mer primers described by Fislage *et al.* (1997) (a). Second-strand cDNA was synthesized using standard protocols (b). The double-stranded cDNA was restriction-digested using a combination of a 6-bp-cutting enzyme, *EcoRI* (left) and a 4-bp-cutting enzyme, *MseI* (right) (c). Adaptors (Ec = *EcoRI*; M = *MseI*) were ligated to the ends of the molecules (d). Non-selective primers which anneal to the adaptor sequences (E00 = *EcoRI*; M00 = *MseI*) were used to PCR-pre-amplify the cDNA molecules (e). Specific primers which anneal to the adaptors and which possess 2 nucleotide extensions were used to PCR-amplify subsets of the cDNA fragments. The *EcoRI* primer was end-labelled with [32 P]dATP (f). PCR amplification products were electrophoresed on polyacrylamide gel and visualized by autoradiography.

total volume was adjusted to 0.5 μ l with sterile distilled water. Following digestion of the cDNA and ligation of *EcoRI* and *MseI* adaptors, 2 μ l of the ligation mixture was used as a template for primary PCR amplification with the non-selective *MseI* (M00) (5'-GATGAGTCCTGAGTAA-3') and *EcoRI* (E00) (5'-GACTGCGTACCAATTC-3') primers of Vos *et al.* (1995). The PCR contained 2.5 μ l AmpliTaq LD buffer and 1 U AmpliTaq LD (Perkin Elmer), 200 μ M of all four dNTPs and 50 ng of each adaptor primer, and the total volume was adjusted to 25 μ l with sterile distilled water. The PCR was performed under the following conditions: 32 cycles of 30 s denaturing at 94 $^{\circ}$ C, 30 s annealing at 60 $^{\circ}$ C and 1 min extension at 72 $^{\circ}$ C. The amplification products were diluted 300-fold and 2 μ l used as a template in a second PCR with selective primers with 2-base extensions. The *EcoRI* primers were E11 (extension AA) and E19 (GA), and the *MseI* primers were M14 (AT), M15 (CA), M16 (CC) and M17 (CG). The reaction contained 1 μ l Perkin Elmer AmpliTaq LD buffer,

200 μ M of all four dNTPs, 12.5 ng unlabelled *EcoRI* primer, 3.5 ng labelled primer (contained in the 0.5 μ l of labelling reaction), 15 ng *MseI* primer and 1 U *Taq* polymerase (Gibco), and the total volume was adjusted to 11 μ l with sterile distilled water. The selective PCR was performed under the following conditions: (i) 1 cycle of 30 s denaturing at 94 $^{\circ}$ C, 30 s annealing at 65 $^{\circ}$ C, 1 min extension at 72 $^{\circ}$ C; (ii) 11 cycles over which the annealing temperature was reduced from 72 $^{\circ}$ C by 0.7 $^{\circ}$ C each cycle; (iii) 23 cycles of 30 s denaturing at 94 $^{\circ}$ C, 30 s annealing at 56 $^{\circ}$ C and 1 min extension at 72 $^{\circ}$ C. All amplifications were performed in a PE-9600 thermocycler (Perkin Elmer). AFLP products were electrophoresed through a 6% polyacrylamide denaturing gel; the gel was then dried on Whatman paper and exposed to autoradiographic film (Kodak) to visualize results. A flow diagram of the cDNA-AFLP procedure for studying *Erwinia* gene expression is given in Fig. 1.

Isolation of amplified cDNA products. Following development, autoradiographic films were repositioned on polyacrylamide gels and the segments corresponding to differentially amplified cDNAs were excised. The gel fragments were then heated in 100 μ l sterile distilled water at 65 $^{\circ}$ C for 10 min and a PCR performed with 10 μ l of the eluted DNA as template and the same primers as those used to generate the cDNA-AFLP profile. Depending on the efficiency of elution, one or two rounds of PCR were necessary to obtain sufficient DNA for sequencing (using an ABI Cycle Sequencer; Perkin Elmer) and for generating radiolabelled probes for Northern analysis (see below).

Northern hybridization. Total RNA (10 μ g) was denatured, separated by gel electrophoresis and blotted onto Amersham Hybond-N+ (Pharmacia Biotech) membranes following the procedure of Fourney *et al.* (1988). Hybridization was performed with DNA probes in 5 $\times$ SSPE, 0.2% SDS, 500 μ g denatured herring sperm DNA (Boehringer Mannheim) ml $^{-1}$ and 5 $\times$ Denhardt's solution (Sambrook *et al.*, 1989). After hybridization, filters were washed twice with 5 $\times$ SSC, 0.5% SDS at 65 $^{\circ}$ C for 20 min and twice with 1 $\times$ SSC, 0.5% SDS at 65 $^{\circ}$ C for 20 min. Probes were generated using, as template, PCR products purified through Wizard columns (Promega) by a random-primed reaction with High Prime (Boehringer Mannheim). Unincorporated nucleotides were eliminated from the probe by purification through Sephadex Nick-Columns (Pharmacia Biotech).

RESULTS AND DISCUSSION

Synthesis of representative cDNA from *Er. carotovora*

An alternative approach to transposon mutagenesis for studying the role of genes in physiological, morphological and biochemical processes in bacteria is to investigate changes in gene expression. For any study of gene expression it is desirable to synthesize cDNA representative of all the genes that are expressed in a particular condition. RNA prepared from *Eca*, *Ecc* and *Es. coli* after growth to mid-exponential phase in LB medium (Miller, 1972) or MM-pectin medium (McMillan *et al.*, 1994), was converted to first-strand cDNA using a combination of all ten 11-mer primers that anneal to conserved 3' regions in enterobacterial genes (Fislage *et al.*, 1997). The media, LB and MM-pectin, were chosen because they represent different conditions for growth of *Erwinia* spp. and thus potentially stimulate differential gene expression. LB is a

Fig. 3. AFLP fingerprints of genomic DNA from *Eca* (lanes 1), cDNA from *Eca* after growth in MM-pectin medium (lanes 2) and cDNA from *Eca* after growth in LB medium (lanes 3). (a), (b) and (c) show amplifications with the E11 primer in combination with M16, M14 and M17, respectively (see Methods). The arrow points to a differentially amplified cDNA fragment, the sequence of which contains an ORF that shows significant similarity, at the protein level, to an avirulence gene from *X. campestris* pv. *raphani* (Parker *et al.*, 1993).

First-strand cDNAs from *Eca* were converted to double-stranded cDNA and used to prepare cDNA-AFLP templates. An AFLP template from *Eca* genomic DNA was prepared for comparison. Following PCR amplifications with eight independent *MseI/EcoRI* primer combinations, radiolabelled PCR products were visualized by PAGE. Amplification products specific to a template, as well as many common to all templates, were observed (Fig. 3). A mean of 34 ± 9 amplification products were detected from the genomic DNA template. In contrast, means of 26 ± 10 and 17 ± 3 amplification products were obtained from cDNA templates derived from LB and MM-pectin, respectively (Fig. 3). The larger number of amplification products generated from genomic DNA may have been due to either of two factors: (i) additional DNA fragments present in this AFLP template were derived from non-transcribed portions of the genome, or (ii) gene sequences were detected that were not expressed on LB or MM-pectin media. Similarly, the larger number of amplification products generated from LB-derived template than from MM-pectin-derived template (Fig. 3) may be representative of more complex gene expression on a complete medium than on a minimal medium. On three separate occasions, fresh cDNA-AFLP templates generated from independent RNA extractions, after growth of *Eca* on LB and MM-pectin media, yielded reproducible ampli-

fication profiles when PCR amplified with the same primer combinations (results not shown). In all cases, PCR amplification was performed using *MseI* and *EcoRI* primers with two nucleotide extensions. A total of 256 combinations of such primers can be made, suggesting that as many as 6656 amplification products may be generated from LB-derived template and 4352 from MM-pectin-derived template. Given that there are approximately 4300 expressed genes in the closely related enterobacterium *Es. coli*, cDNA-AFLP appears to provide a broad coverage of the *Eca* transcriptome. Nevertheless, it is unlikely that all amplification products are derived from independent genes or that all genes contain an *EcoRI* site. It is therefore advisable to employ more than one restriction enzyme combination, and to include 6-bp-cutting enzymes such as *PstI*, which recognizes sites with a greater G + C content and may thus, in some cases, increase the chances of digesting G + C-rich cDNA sequences.

Eleven differentially amplified PCR products specific to either LB or MM-pectin-derived cDNA-AFLP templates, and five commonly amplified products, were excised from polyacrylamide gels, reamplified and directly sequenced following purification. In all cases, unambiguous sequences were obtained, suggesting that only a single species of cDNA molecule was present in each excised band. Of the eleven differentially amplified cDNA fragments, five showed significant similarity to known bacterial genes (EL1, EL2, EL3, EP5 and EP22; Table 3), one matched a previously sequenced bacterial gene of unknown function (EP26) and five made no matches in international databases (results not shown) using BLAST searches (NCBI web server; Altschul *et al.*, 1990). Of the cDNA fragments amplified under all conditions, four showed significant similarity to known bacterial sequences (EP11, EP21, EP12 and EP2), including two of 188 bp (EP12) and 130 bp (EP2) that showed homology to portions of *Erwinia rhapontici* and *Er. carotovora* 16S rRNA, respectively.

Northern analysis reveals that differentially amplified cDNA fragments are derived from differentially expressed genes

Three differentially amplified cDNA fragments larger than 200 bp (EL1, EL2, EL3; Table 3) and one cDNA-AFLP fragment of 118 bp (EP26) were used as radiolabelled probes to screen Northern filters of RNA extracted from *Eca* and *Ecc* grown on either LB or MM-pectin media. As a control, the 188 bp fragment of 16S rRNA sequence (a commonly amplified cDNA-AFLP band; see Table 3) was also used as a radiolabelled probe (Fig. 4). In the case of two of the larger fragments and the 118 bp fragment, clear differential hybridization was observed, which was in agreement between *Eca* and *Ecc* and which confirmed the differential amplification from *Eca*. The other cDNA fragment over 200 bp in size gave no differential hybridization. The Northern result for one of the cDNA fragments (EL2; Table 3), which was expressed in both *Eca* and *Ecc* specifically in LB medium but not MM-pectin medium, is shown in Fig. 4.

Table 3. Similarities at the protein level between cDNA-AFLP fragment sequences and sequences in databases

cDNA fragment	Length (bp)	Matching sequence from the database	Origin of matching sequence and accession nos	% Similarity (BLAST)
EL1	312	PmbA, antibiotic maturation factor	<i>Escherichia coli</i> , P24231	88 (9.2e-52)*
EL2	292	AvrXca, avirulence protein	<i>Xanthomonas campestris</i> pv. <i>raphani</i> , M99059	75 (6.4e-25)*
EL3	261	LacE, lactose-binding protein	<i>Agrobacterium radiobacter</i> , P29822	66 (2.9e-16)*
EP5	151	Nox-1, NADH oxidase	<i>Streptococcus mutans</i> , D21803	72 (6.4e-03)*
EP22	161	GlnH, glutamine-binding protein	<i>Salmonella typhimurium</i> , U73111	92 (9.5e-24)*
EP26	118	Unknown protein	<i>Salmonella typhimurium</i> , AF036677	87 (2.2e-02)*
EP11	266	MalE, putative maltose-binding protein	<i>Streptococcus coelicolor</i> , Y07706	55 (1.8e-08)*
EP21	237	Tsx, nucleoside-specific channel-forming protein	<i>Klebsiella pneumoniae</i> , P40786	85 (1.0e-30)*
EP12	188	16S rRNA gene	<i>Erwinia rhapsontici</i> , U80206	94 (3.0e-18)†
EP2	130	16S rRNA gene	<i>Erwinia carotovora</i> , Z96090	98 (3.0e-42)†

* Expect values of best match for protein alignment (BLASTX) given in parentheses.

† Expect values of best match for DNA alignment (BLASTN) given in parentheses.

Fig. 4. Northern blot hybridization of RNA isolated from *Eca* (lanes A) and *Ecc* (lanes B) after growth in LB medium (lanes 1) or MM-pectin medium (lanes 2). The two ³²P-radiolabelled probes used were: (upper panel) the cDNA-AFLP fragment that was differentially amplified from template prepared after growth on LB medium but not from template prepared after growth on MM-pectin medium (indicated in Fig. 3), and which shows similarity to the *X. campestris* pv. *raphani* avirulence gene; and (lower panel) a cDNA-AFLP fragment that was amplified from templates prepared after growth on both LB and MM-pectin media and which shows strong homology to a portion of *Es. coli* 16S rRNA.

An ORF in this sequence showed significant similarity at the protein level (6.4 e-25) with an avirulence gene from *Xanthomonas campestris* pv. *raphani* (Parker *et al.*, 1993). In *X. campestris* pv. *raphani*, the product from this gene confers an avirulence phenotype in most interactions with *Arabidopsis thaliana* accessions. On the basis of Southern hybridization, related sequences were previously thought to be absent from *Ecc* (Parker *et al.*, 1993). This sequence is being further investigated to see whether it also elicits an avirulence response and may thus play a role in either pathogenicity or host specificity in *Er. carotovora*.

In this work, we present a method for synthesizing cDNA from the bacterial plant pathogen *Er. carotovora*,

which gives a broad genome coverage. Such a method will allow cDNA libraries to be constructed and differential gene expression to be analysed, providing an alternative route to transposon mutagenesis for gene isolation and characterization. For example, included in the battery of genes required for plant infection by *Er. carotovora* are some encoding pectic enzymes that are induced only when grown in plant extract or *in planta* (McMillan *et al.*, 1994). Differential cDNA screening methods, such as the suppression subtractive hybridization technique (Diatchenko *et al.*, 1996; Birch *et al.*, 1999) may allow such previously unidentified genes to be isolated.

Adaptation of bacteria to their environment can be highly efficient, involving many metabolic and physiological changes. This work shows that it is possible to reproducibly profile gene expression in a bacterial plant pathogen under different environmental conditions, and to isolate differentially regulated sequences using a modification of the cDNA-AFLP protocol of Bachem *et al.* (1996).

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