Microarray profiling of host-extract-induced genes and characterization of the type VI secretion cluster in the potato pathogen *Pectobacterium atrosepticum*

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Pectobacterium atrosepticum is a Gram-negative plant-pathogenic bacterium that rots potato stems and tubers. Microarray analysis was used to identify genes that were differentially expressed when host extracts were added to the growth medium. Potato extracts downregulated the expression of ribosomal genes and genes related to uptake and metabolism of nutrients, and upregulated genes needed for nitrate or phosphonate use. Some of the observed changes in gene expression in host-extract-induced cultures are similar to those during attachment of the bacterium to host tissues. Other responses indicated defence against toxic metabolites in the extract. Tuber extract induced a large gene cluster having homology to type VI secretion genes shown to be virulence determinants in many, but not all, animal and human pathogens. Two of the genes in the type VI cluster were found to be expressed during infection in potato tubers and stems, and mutants with knockouts of the corresponding genes had increased virulence on potato. One of the type VI secretion mutants was further characterized and found to grow to higher cell density in culture in the presence of host extract and to produce slightly more extracellular tissue-macerating enzymes than the wild-type strain. Analysis of secreted proteins showed that this type VI mutant was affected in the production of haemolysin-coregulated proteins (Hcps), which have been suggested to be secreted by the type VI pathway in other bacteria. The results suggest that the type VI secretion system of *P. atrosepticum* is needed for secretion of Hcps but not for virulence on its host plant, potato.

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INTRODUCTION

Pectobacterium atrosepticum (formerly known as *Erwinia carotovora* subsp. *atroseptica*) is a Gram-negative enterobacterium that causes economically important diseases in potato. It causes rotting of the stems (blackleg) in the field and soft rot of tubers during storage (Pérombelon, 2002). It is very host specific and infects almost only potato. The bacteria may, however, live in the rhizosphere of cultivated plants and weeds without causing disease (McCarter-Zorner *et al.*, 1985). Latent periods are typical of this

pathogen. The disease spreads via contaminated seed tubers and once the conditions are favourable, the bacteria start to replicate and finally rot the plant tissue. *P. atrosepticum* uses quorum sensing for cell-density-dependent regulation of virulence genes (Whitehead *et al.*, 2002). When the population is large enough, the bacteria start to produce virulence proteins, such as pectinases and cellulases, which degrade the host macromolecules into smaller fragments that the bacteria use as a source of energy.

A majority of the known virulence determinants of *P. atrosepticum* are secreted to the exterior of the bacterium. There are at least eight secretion systems present in the genome of *P. atrosepticum*: the type I–VI systems, the fimbrial usher system, and the twin arginine pathway (Bell *et al.*, 2004; Preston *et al.*, 2005). Type VI is the most

Abbreviation: FDR, false discovery rate.

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A supplementary table of microarray data is available with the online version of this paper.

recently described secretion pathway in bacteria (Bingle *et al.*, 2008; Filloux *et al.*, 2008). It was first reported in *Rhizobium leguminosarum* (Bladergroen *et al.*, 2003) and subsequently in several proteobacteria (Parsons & Heffron, 2005; Pukatzki *et al.*, 2006; Mougous *et al.*, 2006; Schell *et al.*, 2007; de Bruin *et al.*, 2007). The conserved system is present in numerous bacterial species including animal and plant pathogens and symbiotic bacteria. Most bacteria have one or two type VI loci. However, some species, such as *Burkholderia pseudomallei*, harbour as many as six type VI clusters in their genome (Shalom *et al.*, 2007).

Very little is known about the detailed function of the type VI secretion system. It is evidently used to transport proteins lacking a signal sequence. To date, haemolysin-coregulated proteins (Hcps) and VgrG (valine-glycine repeat) proteins have been identified as the major proteins secreted through this system (Pukatzki *et al.*, 2006; Schell *et al.*, 2007). Hcps have been shown to form hexameric ring structures outside the bacterial cell, and have been speculated to form a channel for macromolecule transport (Mougous *et al.*, 2006). VgrG-related proteins may assemble into a trimeric complex that is analogous to the host cell membrane puncturing base plate, 'tail spike', of *Escherichia coli* bacteriophage T4. Because of the structural characteristics of the secreted proteins, the type VI secretion system has been postulated to form a host cell membrane penetrating secretion channel, but the hypothesis has not been verified experimentally (Mougous *et al.*, 2006; Pukatzki *et al.*, 2007).

The contribution of type VI secretion to virulence seems to vary depending on the bacterial species. In many species the system appears to be a major virulence determinant (Pukatzki *et al.*, 2006; Schell *et al.*, 2007; Zheng & Leung, 2007), but in others it has been noted to impair or decrease the infection (Bladergroen *et al.*, 2003; Parsons & Heffron, 2005; Wu *et al.*, 2008). In this study, the aim was to use microarray analysis to study the responses of *P. atrosepticum* to host extracts. Induced expression of the genes coding for proteins in the type VI secretion system was detected in the presence of host plant extracts, and further experiments showed that the type VI system has a role in secretion of Hcps but not in virulence of *P. atrosepticum*.

METHODS

Strains, plasmids and basic techniques. *P. atrosepticum* strain SCRI1043 (Hinton *et al.*, 1985) was used to make the *vasK* and *vasH* knockout mutant strains. Competent cells of *E. coli* TOP10F' were obtained from Invitrogen and plasmid pBluescript II KS⁺ from Stratagene. The plasmids pKD3 and pKD46 have been described (Datsenko & Wanner, 2000). Plasmids were transformed into competent *E. coli* cells by heat shock. The previously described T4GT7 transduction method (Pirhonen *et al.*, 1991) and electroporation were used to introduce plasmids into strain SCRI1043. PCR products were transferred to competent *P. atrosepticum* cells by electroporation. Plasmid and chromosomal DNA were purified with Qiagen kits. RNA from *in vitro*-cultured bacteria was extracted as previously described (Summers, 1970). The Trizol method was used

to extract RNA from plant material (Caldo *et al.*, 2004). The assays were performed three times unless otherwise noted.

Growth conditions and media. The *P. atrosepticum* and *E. coli* strains were cultured on Luria-Bertani medium (LB) at 28 °C or 37 °C respectively. Ampicillin (Amp, 150 µg ml⁻¹) and chloramphenicol (Cm, 25 µg ml⁻¹) were added to the culture medium when needed. For the secretome analysis and microarray sample preparation the bacteria were cultured in inducing conditions, at 15 °C in Huynh's minimal medium (Huynh *et al.*, 1989) supplemented with 10 mM sucrose and 10% (v/v) potato tuber or stem extract. Potato extracts were made by grinding potato tissues in water, followed by removal of plant debris and proteins larger than 5 kDa by centrifugation and filtering (Mattinen *et al.*, 2007).

Protein and RNA sample preparation. For secretome analysis the bacteria were cultured overnight in LB, diluted 1:30 and cultured for a further 7 h to exponential phase. The cells were washed three times with minimal medium and suspended to a final volume of 200 ml at OD₆₀₀ 0.1. The bacteria were grown for 36 h at 15 °C with shaking (150 r.p.m.), pelleted and the supernatant filter-sterilized. Protease inhibitor (Roche Diagnostics) was added and the proteins were concentrated with Ultra-15 centrifugal filter devices (cut off 5 kDa, Millipore) to final volume of 2 ml. The sample was phenol-extracted, methanol-precipitated and acetone-precipitated as previously described (Mattinen *et al.*, 2007). Proteins were separated by two-dimensional PAGE (2-DE PAGE) (Mattinen *et al.*, 2007) and silver-stained (O'Connell & Stults, 1997). The gels were run from three biological replicates with similar results and proteins were analysed from two gels.

RNA samples were prepared from bacteria grown for 24 h *in vitro* in minimal medium with or without potato extracts. Samples were also prepared from potato tubers and stems inoculated with bacteria. Inoculation was done with a sterile toothpick and the wound was sealed with Parafilm. The plants were kept in moist conditions in a plastic container for 24, 48, 72 and 96 h at 20 °C and the tissue around the inoculation point was collected. The sample was frozen in liquid nitrogen and RNA was extracted by the Trizol method.

Protein identification. The protein spots obtained from the 2-DE gels were reduced with DTT (Duchefa Biochemie) and alkylated with iodoacetamide. The proteins were then in-gel digested with trypsin (Sequencing Grade Modified Trypsin, Promega) overnight at 37 °C (Shevchenko *et al.*, 1996). Peptides were extracted from the gel pieces using two washes in 150 µl 50% acetonitrile/5% formic acid at 37 °C, pooled and dried. Next, peptides were reconstituted in 2 µl 1% trifluoroacetic acid (TFA), after which 0.5 µl was spotted onto a prespotted AnchorChip MALDI target (Bruker Daltonik). The plate contains 384 prespotted MALDI matrix spots and 96 calibrant spots (mass range 700–4000 Da; Product information version 05-01-20, Bruker Daltonik). Prior to MS analysis the deposited samples were desalted by incubating the spots on-target with 10 µl 0.5% TFA for a few seconds.

MS analysis and database search criteria. MS analysis was performed on a Bruker Ultraflex II equipped with a 200 Hz Smartbeam laser system. Data were acquired using FlexControl version 3.0 (build 99) and analysed using FlexAnalysis version 3.0 (build 54) and BioTools version 3.0 (build 1.88). MASCOT version 2.1 (Matrix Science) was used to search the MSDB database (<ftp://ftp.ncbi.nih.gov/repository/MSDB/>; release 20050227). The following search settings were used: mass error tolerance, 100 p.p.m.; fixed modification, cysteine carbamidomethylation; variable modification, methionine oxidation; enzyme, trypsin; number of missed cleavages, one.

Microarray hybridizations. Hybridizations were carried out using Agilent 8*15K custom microarrays. Probes of the array covered 4434

ORFs of *P. atrosepticum* SCRI1043. RNA samples (10 µg) were treated with DNase (Promega) according to the manufacturer's instructions. Random hexamers (2.5 µg) were added and the sample was incubated for 10 min at 70 °C. cDNA synthesis was carried out at 37 °C for 4 h in 40 µl volumes with the following ingredients: 400 U reverse transcriptase (Promega); 7.5 mM DTT; 1.25 mM dATP/dTTP/dGTP; 0.5 mM dCTP and 2.5 mM Cy3 or Cy5 (GE Healthcare). The synthesis was stopped by adding 2 µl 25 mM EDTA. RNA was hydrolysed by adding 20 µl 0.1 M NaOH and incubating at 70 °C for 15 min. Finally the samples were neutralized with 20 µl 0.1 M HCl. The Cy3-Cy5 sample pairs were combined and purified with the Qiagen PCR purification kit. Samples were dried in a speed-vacuum centrifuge and dissolved in 16 µl water. Hybridization solution was added and samples were loaded onto coverslips (Agilent). The array slide was laid upside down on top of the coverslip and tightened with a slide holder. Hybridization was performed at 65 °C with 20 r.p.m. rotation for 16 h. Prior to scanning, the slide was washed three times with washing buffer, following the instructions of the manufacturer (Agilent).

Microarray data analysis. Microarray slides were scanned with an Axon 4200 AL scanner (Axon Instruments) using 5 µm pixel resolution. PMT gains were manually adjusted in order to avoid saturated pixel values. Image analysis was done using Axon GenePix 6.0 software. Data normalization and statistical analysis were performed using the statistical software tool R with package 'limma' (Smyth, 2005). Local background intensity was subtracted from each spot foreground signal intensity and the ratio between Cy5 and Cy3 channels was computed and converted into a logarithmic domain. Intensity-dependent mean of log-ratio was corrected using lowess normalization within each array. Different spreads of log-ratios between replicate arrays were normalized by equalizing the median absolute deviations. For tuber-extract-induced expression data there were technical replicate hybridizations available with dye-swaps. By means of a statistical test, the technical replicates were utilized to validate that there was no dye effect present in the data. In order to find differentially expressed genes, a moderated *t*-test was applied to each gene. The empirical Bayes method was used to smooth the estimates of standard deviations of expression values in order to make the results more reliable. Technical replicates were not used as independent observations, but a mixed-model approach was used, where the correlation between the technical replicates was first calculated and utilized before merging all data. *P*-values obtained for each gene were converted into false discovery rate (FDR) values. There were several hundred up- and downregulated genes with FDR

values below 0.05 within the stem-extract-induced genes. In tuber-extract induction data, there were several up- and down-regulated genes with original *P*-values below 0.05 but none of them remained below 0.05 after FDR conversion. The total number of gene-wise tests affects the FDR values, so that equal *P*-values may result in different FDR values depending on the number of other genes tested. The purpose of using FDR values is to reduce the number of false positives in the results. Since the number of genes with a *P*-value below 0.05 was small, all of them were considered as differentially expressed without FDR correction in tuber-extract data. The stem-extract-induced genes were selected based on the FDR values.

Real-time PCR. PrimerExpress software (Applied Biosystems) was used to design primers for *vasK* (ECA3232), *vasH* (ECA3435) and *proC* (ECA3628) (Table 1). cDNA was produced from the RNA samples (1 µg) using M-MLV reverse transcriptase (Promega), following the instructions of the manufacturer. The samples were adjusted to 100 µl with water and 5 µl was used as a template in real-time PCR. For the real-time assays, the Dynamo SYBR green qPCR kit (Finnzymes) was used. PCR was done with the ABI 7700 sequence detection system (Applied Biosystems) using the system's default cycling settings. The normalized sample threshold cycle (*C_t*) values were calculated comparing to the housekeeping gene *proC* (Savli *et al.*, 2003; Takle *et al.*, 2007). Expression ratios were calculated with the 2^{-*C_t*} method, using a sample grown in minimal medium at 15 °C as a control to which the actual samples were compared. Melting curve analysis, sequencing and negative controls were used to verify the specificity of the PCR.

Construction of *vasK* and *vasH* knockout mutants. A knockout mutant of the *vasK* gene (ECA3432) was made by marker-exchange mutagenesis. Fragments (~3 kb) upstream and downstream of *vasK* were amplified with PCR primers (Table 1) and cloned in pBluescript. A Cm-resistance gene fragment was cloned between the inserts, yielding a construct in which the Cm-resistance gene replaced *vasK*. The plasmid was introduced into SCRI1043 by T4GT7 transduction (Pirhonen *et al.*, 1991). For the homologous recombination, the strain carrying the deletion plasmid was grown overnight in LB with Cm and kept at room temperature for 1 week. The culture was plated to yield single colonies, which were tested for Amp sensitivity. The Amp^r Cm^r strain was analysed by Southern blotting to verify the loss of *vasK* and the presence of the Cm^r marker (Sambrook *et al.*, 1989).

The *vasH* mutant was created with the Red recombinase system (Datsenko & Wanner, 2000). The Cm-resistance gene was multiplied by PCR using plasmid pKD3 as a template. The primers (Table 1)

Table 1. Primers used in this study

Primer		Sequence (F, forward; R, reverse)	
Cloning			
vasK (ECA3432) upstream	F: AAATACTCGAGATCGTTACGCGCATGAAT		R: AAAAAGGATCCCGATGTGGATTGGATGCTG
vasK (ECA3432) downstream	F: ATAAAAGGATCCAGTAACAACATCGCCG		R: ATAAATCTAGATTAAGGATGAAGGGCAGA
vasH (ECA3435) forward	ATGCAACATGCCCTCAAACCTGGCGCTAGCACTACCCGACAGCATGATGAAAGTGTAGGCTGGAGCTGCTTC		
vasH (ECA3435) reverse	CTTCTGGCATTTGTGGTCAAGAGTGCCTTTAGGGATATTGAGGCTATCGGCTCATATGAATATCCTCCTTAG		
Real-time PCR			
vasK	F: TTGAAGGCACTTATCTGCAAATG		R: CTTCCAACAGCCCCTGCAT
vasH	F: TCACCCGACAGCATGATGA		R: GCGGGCTGCATCGT
proC	F: AATGTCGCGGGCAAGCT		R: TGAAAACGGGCGATGTTGA

were designed to have 50 nt homology regions to ECA3435 at their 5' end and FRT priming sites at their 3' end. The product was gel-purified and 200 ng was electroporated into competent, arabinose-induced *P. atrosepticum* cells carrying plasmid pKD46. Transformants were incubated at 28 °C for 3 h and plated on Cm plates. The colonies were tested with PCR to confirm the correct orientation of the Cm-resistance gene. The absence of the *vasH* gene was confirmed by Southern analysis.

The pathogenicity of the *vasK* and *vasH* mutants was tested on potato (cultivars Bintje and Rosamunda) as described by Mattinen *et al.* (2007). The virulence assays were repeated three times, except for the tuber maceration test on Rosamunda, which was performed twice with similar results.

Enzyme and biofilm assays and motility test. Pectate lyase activity was analysed by mixing 16 µl supernatant with 80 µl substrate (1% polygalacturonic acid, 0.25 mM CaCl₂, 100 mM Tris pH 8.5) and incubating at 37 °C for 1 h. The reaction was stopped by adding 400 µl 0.5 M HCl and 800 µl 10 mM thiobarbituric acid (TBA). The samples were boiled for 1 h and A₅₄₈ was measured.

The ability of the *vasK* mutant to produce biofilm was tested with a biofilm assay (O'Toole & Kolter, 1998). Four media (LB, minimal medium and minimal medium supplemented with potato stem or tuber extract) and two temperatures (28 °C and 15 °C) were used. The bacteria were cultivated in LB overnight and diluted 1:50. For the minimal medium samples, the bacteria were washed three times with minimal medium and finally suspended to the same cell density as the LB sample. The cultures were plated on microtitre plates and incubated for 24 and 48 h. The biofilms were stained by adding 1% crystal violet to the well and incubating for 15 min. The wells were emptied and washed twice with PBS. Finally 100 µl ethanol was added, the samples were transferred to a clean plate and A₅₉₅ was measured.

RESULTS

Microarray analysis of host-extract-induced genes

The influence of host extracts on gene expression in *P. atrosepticum* SCRI1043 was studied by microarray analysis. Two RNA samples were compared, one prepared from cells grown in minimal medium and the other from cells grown in minimal medium supplemented with either tuber or stem extract (Supplementary Table S1, all data). The data revealed that tuber extract induced 18 genes that were also previously identified as tuber-extract-induced by proteomics (Mattinen *et al.*, 2007). Among them were genes coding for known virulence proteins, such as pectic enzymes (ECA1095, ECA4067, ECA4068 and ECA1499), protease PrtW (ECA2785) and virulence protein Svx (ECA0931), but also several genes coding for poorly characterized proteins. However, there were differences in the induction levels between the independent experiments, and therefore only a few genes had statistically significant *P*-values indicating differential expression (Table S1). About 34% of the tuber-extract-induced genes with *P*-values below 0.05 were named hypothetical in the annotation of *P. atrosepticum* genome. Potato stem extract caused statistically significant (FDR value <0.05) induction of more than 200 genes, among which 42% were

hypothetical (Table S1). Furthermore, many of the extract-induced genes were not classified in the conserved orthologous groups (COGs) (Fig. 1).

Visual analysis with the MeV program (Saeed *et al.*, 2003) revealed several up- or downregulated clusters of genes (Fig. 2). Stem extract induced a cluster of genes needed for nitrate transport and metabolism (ECA2989–2995). Tuber extract induced a group of four genes (ECA0421–0425), including a putative lipase gene, and genes for ABC multidrug transporters and efflux pumps, possibly having a protective function. Furthermore, three large tuber-extract-induced gene clusters were identified, one of them (ECA0487–0498) involved in phosphonate metabolism, and the second one (ECA0789–0801) containing homologues of genes coding for long, bundled Flp pili, associated with tight adherence and biofilm formation by bacteria (Kachlany *et al.*, 2001; Planet *et al.*, 2003). The third tuber-extract-induced gene cluster (ECA3420–3445) contained homologues of the components in the newly identified type VI secretion system (Table S1).

Fig. 1. Genes showing statistically significant changes in expression 24 h after addition of potato stem extract to the growth medium. The genes are shown as a percentage of all expressed genes placed in different conserved ontology groups (COGs) in the annotation of the *P. atrosepticum* genomic sequence.

Fig. 2. Differentially expressed gene clusters containing at least four genes, plotted according to log-ratios of individual genes in the clusters. Top left, genes downregulated by tuber extract and upregulated by stem extract; top right, genes upregulated by both tuber and stem extracts; lower left, genes downregulated by both tuber and stem extracts; lower right, genes upregulated by tuber extract and downregulated by stem extract. Log-ratios are computed on a $\log_2$ scale using the expression levels in minimal medium as reference.

Several genes in the type VI secretion cluster of *P. atrosepticum* were upregulated with tuber extract (Table S1). The induction of individual genes varied between log-ratios 0.79 and 9.11 (fold change from 1.7 to 554.0), the mean being 3.00, which corresponds to a fold change of 31. The type VI cluster included genes coding for Hcp and VgrG proteins (ECA3427 and ECA3428) that were identified as tuber-extract-induced proteins in secretome analysis (Mattinen *et al.*, 2007). Homologues of these two genes appear together in two additional positions in the genome. These genes, ECA2866–2867 and ECA4275–4277, were also upregulated by tuber extract and so were the additional *hcp* homologues ECA0456, ECA0176 and ECA3672 (Table S1). Of the genes in type VI cluster, only a few were expressed in stem-extract-induced cultures, and only *hcp* and *vgrG* genes clearly upregulated, as also were two additional *hcp* genes (ECA2866 and ECA4275), but not their upstream *vgrG* genes.

Large numbers of individual genes and gene clusters were downregulated by host extracts (Table S1). Many of the downregulated genes were involved in translation, motility, secretion and transport of various molecules. There were

fewer hypothetical genes or genes not having COGs among the downregulated genes when compared to the upregulated genes (Fig. 1). The downregulated clusters contained genes coding for uncharacterized transporters (ECA1548–1552), proteins for high-affinity zinc uptake (ECA2483–2486) and a gene cluster involved in nitrate transport and assimilation (ECA2989–2995). Stem extract reduced the expression level of many gene clusters, among them genes coding for Flp pilus and flagellar proteins (ECA1702–1727) as well as genes involved in iron and zinc uptake, histidine biosynthesis, sulphate assimilation and macromolecule import and cell wall integrity (Fig. 2).

Type VI genes of *P. atrosepticum* and their expression

Because several genes in a putative type VI secretion cluster of *P. atrosepticum* were induced by host extracts and because some of the secreted Hcp and VgrG proteins had been identified earlier by proteomics (Mattinen *et al.*, 2007), the type VI genes of *P. atrosepticum* were characterized further. Analysis of the genes with BLAST revealed that many of the type VI genes of *P. atrosepticum*

had highest similarity to genes in the type VI clusters of *Aeromonas* (Fig. 3) and *Yersinia* species. The highest resemblance, 68 % identity and 81 % similarity, was found between VasK (virulence associated protein K) of *A. hydrophila* and ECA3432, named as VasK in the annotation of the *P. atrosepticum* genome.

Expression levels of two genes in the type VI cluster, *vasK* and *vasH* (ECA3435, a putative sigma-54 dependent transcriptional regulator), were analysed by quantitative real-time RT-PCR from samples prepared from potato stems and tubers after inoculation with *P. atrosepticum*. The expression of *vasK* and *vasH* was induced in all samples, and was highest in the stem sample 48 h after inoculation (Fig. 4). The expression of the genes followed the same pattern, which suggests that *vasH* might be involved in the regulation of *vasK* transcription, or alternatively, they are present in the same operon under the same regulation and are transcribed simultaneously.

Phenotypic characterization of type VI mutants

Knockout mutants of *vasK* and *vasH* were constructed to study the involvement of type VI secretion in the virulence of *P. atrosepticum*. The *vasK* mutant was found to be significantly more efficient in rotting potato tubers when compared to the wild-type strain in two potato cultivars having low (Bintje) or high (Rosamunda) glycoalkaloid levels. In the tuber maceration assay with the cultivar Bintje the mutant rotted 5.2 g (SD 3.4) of potato tissue as compared to 1.9 g (SD 1.0) rotted by the wild-type (*P*-value 0.0001). Even in cultivar Rosamunda the mutant caused more rotting, 9.6 g (SD 3.5), when compared to the wild-type 1.9 (SD 0.5, *P*-value 0.0002). Also in the potato stem test with cultivar Bintje the VasK mutant was more effective in maceration than the wild-type strain (data not shown). The mutant grew to a higher cell density in minimal medium supplemented with tuber extract (Fig. 5a) and produced slightly more pectolytic enzymes in LB medium and in minimal medium supplemented with tuber extract than the wild-type strain. However, it had the same phenotype as the wild-type strain in standard motility and biofilm assays (data not shown). The *vasH* knockout mutant also produced more rot than the wild-type strain in a tuber maceration test, but the difference was not statistically significant (data not shown).

It has been previously reported that VasK is involved in the secretion of Hcp and VgrG proteins in *Vibrio cholerae* (Pukatzki *et al.*, 2006). To study whether this is the case with *P. atrosepticum* VasK, the mutant was compared with the wild-type strain in 2-DE analysis of the secreted proteins from tuber-extract-induced samples (Fig. 5b, Table S2). Two dense protein spots missing from the VasK gel were identified as Hcps. Both of the spots were identified as either ECA4275 or ECA3428 (because these two proteins contain only one amino acid difference, it is not possible to distinguish them by mass fingerprinting).

The VgrG protein, whose secretion has been reported to be VasK dependent, was not detected in this analysis.

DISCUSSION

In this study, microarray analysis of host-extract-induced genes was used as a tool to identify putative new virulence genes of *P. atrosepticum*. The results of the analysis suggested that tuber and stem extracts affected different genes. Many of the changes in gene expression in the cultures exposed to extracts may be related to presence of nutrients in the host extracts, as downregulation of genes needed for uptake and metabolism of iron, zinc, sulphate and amino acids was evident, especially in the samples containing stem extract. Stem extract also reduced the expression of ribosomal genes and upregulated genes needed for nitrate transport and assimilation. Some of the observed changes in tuber-extract-induced cultures were possibly related to attachment of the bacterium to host tissues or defence against toxic metabolites present in the extract. Tuber extracts also induced the expression of genes involved in utilization of phosphonate. These results may suggest the use of phosphonates as a source of phosphorus (Fox & Mendz, 2006) or the contamination of the potato extracts by phosphonate-containing herbicides and pesticides widely used in agriculture (Singh & Walker, 2006).

Many of the host-extract-induced genes were annotated as hypothetical or did not have characterized COGs, whereas a larger proportion of the downregulated genes had been assigned a known function in the annotation of *P. atrosepticum*. This may indicate that the host extracts induced poorly characterized genes with a possible function during virulence, whereas the downregulated genes are active during *in vitro* growth in laboratory media and have therefore been studied in many bacteria.

In a previous study, we observed induced production of Hcp and VgrG proteins when *P. atrosepticum* was grown in the presence of host extracts (Mattinen *et al.*, 2007). Microarray profiling of host-extract-induced genes, presented in this report, showed that the host extracts also induced genes coding for the proteins in the type VI secretion system suggested to mediate the secretion of Hcp and VgrG. The type VI secretion cluster is present in the genome of at least 85 Gram-negative bacteria and many of them harbour several copies of the cluster (Schell *et al.*, 2007). In the *P. atrosepticum* genome the cluster is composed of more than 20 genes and is present in one locus (ECA3420–3445). Two other loci (ECA2866–2869 and ECA4275–4278) contain genes that show homology to type VI secretion genes. Both of these sites contain, in the same order, *hcp* and *vgrG* genes, followed by a hypothetical gene and an *Rhs* element. Some homologous genes are scattered in the genome, for example two *vgrG* genes (ECA2104 and ECA4142) and one *hcp* (ECA0456) do not have any type VI secretion genes close to them. Our

Fig. 3. Comparison of the type VI secretion cluster of *P. atrosepticum* (a) with the clusters of *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (b) and *Vibrio cholerae* O1 biovar Eltor strain N16961 (c). The locus numbers and names of some cluster members are marked under each cluster, and the deletions present in *P. atrosepticum* *vasK* and *vasH* mutants are marked with black bars. Names of the homologous proteins in different bacteria are shown in (d).

Fig. 4. Real-time RT-PCR results showing the expression of *P. atrosepticum* *vask* and *vash* genes 24–96 h after inoculation of potato tuber or stem tissues. The expression of the bacterial genes *in planta* was compared to expression in minimal medium, which was set to 1. For comparison, the activities present in the inoculum, bacterial cells grown in Luria broth (LB), are shown.

microarray and real-time RT-PCR data, as well as our previous results, imply that almost the entire type VI cluster and four *hcp* and three *vgrG* genes are upregulated in the presence of potato tuber extract. This suggests that the type VI cluster and the scattered homologous genes may have a function during interaction of *P. atrosepticum* with its host plant.

Pukatzki *et al.* (2006) suggested that VasK is a structural element of the type VI secretion mechanism and required for Hcp secretion, and that VasH is a sigma 54-dependent positive regulator of Hcp production. The corresponding *P. atrosepticum* homologues were expressed *in planta*, and the secretome of the *vask* mutant lacked Hcps, suggesting that

also in *P. atrosepticum* VasK is involved in secreting Hcps from the cell. The phenotype of the *vask* and *vash* mutants suggested that the type VI secretion system is not involved in the maceration of the host plant by *P. atrosepticum*. On the contrary, the mutants had increased virulence when compared to the wild-type strain. This is most likely caused by the observed increased growth of the mutant and improved production of pectic enzymes due to higher cell density.

The type VI secretion mutants or *hcp* deletion mutants seem to have distinctly different phenotypes in different bacteria. In *P. atrosepticum*, an Hcp overproducing mutant had increased virulence and a knockout mutant lacking one of the seven *hcp* homologues had slightly reduced virulence, which supported the hypothesis that Hcps are virulence proteins even in this bacterium (Mattinen *et al.*, 2007). However, a *P. atrosepticum* type VI secretion mutant unable to secrete Hcp had increased virulence. A similar discrepancy has been observed in *Agrobacterium tumefaciens*, where the production of Hcp, but not its secretion by the type VI system, was needed for full virulence, which led to the suggestion that Hcp may function inside the bacterial cell (Wu *et al.*, 2008). Mutation in the *R. leguminosarum* type VI locus enabled a strain that is normally not able to form functional nodules on pea to infect pea plants and to fix nitrogen (Bladergroen *et al.*, 2003). In *Salmonella typhimurium*, deleting the *vask* orthologue *sciS* rendered the mutant hypervirulent in mice (Parsons & Heffron, 2005). In *Burkholderia mallei* the results were completely opposite and a type VI secretion mutant was found to be nonvirulent in hamsters (Schell *et al.*, 2007). *vask* as well as *hcp* deletion mutants of *V. cholerae* were attenuated in a *Dictyostelium* predation assay (Pukatzki *et al.*, 2006); however, an *hcp* knockout mutant did not have altered virulence in mice (Williams *et al.*, 1996). Deletion of *hcp* in *Edwardsiella tarda* resulted in attenuation of the pathogenicity (Rao *et al.*, 2004). Hcps

Fig. 5. Characterization of the *P. atrosepticum* *vask* mutant. (a) Growth of the wild-type *P. atrosepticum* strain SCRI1043 (△) and its *vask* knockout mutant (●) at 15 °C in minimal medium supplemented with 10% tuber extract. (b) Analysis of the secreted proteins of wild-type strain SCRI1043 and the *vask* mutant.

have also been reported to be expressed during chronic infections, such as glanders caused by *B. mallei* in horses and *Pseudomonas aeruginosa* infections in patients with cystic fibrosis, which indicates a role in virulence (Mougous *et al.*, 2006; Schell *et al.*, 2007).

Mutations in *vasK* and *hcp* seem to affect the replication rates of bacteria. As with virulence, also the effect on replication depends on the bacterial species. An *Ed. tarda* *hcp* deletion mutant had lower replication rates (Rao *et al.*, 2004) and in *Legionella pneumophila*, a *vasK* homologue (*icmF*) was required for intracellular growth (Zusman *et al.*, 2004). In *S. typhimurium*, however, the *vasK* mutant had increased intracellular replication (Parsons & Heffron, 2005). Also in *P. atrosepticum*, the *vasK* mutant grew to about 30 % higher cell density *in vitro* when compared to the wild-type strain. This result suggests that *VasK* downregulates growth and virulence in wild-type *P. atrosepticum*. This might be advantageous for the pathogen during latent infection, when the bacterial population may be too small to initiate successful infection. This hypothesis is in agreement with the real-time RT-PCR data, which showed that *vasK* was expressed at early time points in infected tissue, whereas its expression level decreased later when rotting was visible. Production of Hcp has been reported to be induced in *Ps. aeruginosa* during biofilm formation (Southey-Pillig *et al.*, 2005). However, no difference was found between the *vasK* mutant and wild-type *P. atrosepticum* in the ability to form biofilm *in vitro*.

In spite of the fact that the type VI secretion genes were induced by plant extracts and *in planta* during infection, type VI secretion by *P. atrosepticum* did not seem to be involved in promoting virulence during infection in potato stems or tubers. More research is needed to understand the function of type VI secretion and the secreted proteins during host–pathogen interaction.

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