Identification of a New Quorum-Sensing-Controlled Virulence Factor in *Erwinia carotovora* subsp. *atroseptica* Secreted via the Type II Targeting Pathway

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Two-dimensional polyacrylamide gel electrophoresis of the secreted proteins of *Erwinia carotovora* subsp. *atroseptica* revealed a low-abundance protein that was identified by mass spectrometry as a homologue of a *Xanthomonas campestris* avirulence protein with unknown function. The predicted Sv_x protein has an N-terminal signal sequence and zinc binding-region signature, and the mature protein is post-translationally modified. A 2D difference gel electrophoresis (DIGE) showed that the protein is secreted by the type II (out) secretion apparatus, which is also responsible for the secretion of the major known virulence factors, PelC and CelV. Transcription of the *svx* gene is under *N*-acyl-homoserine lactone-mediated quorum-sensing control. The *svx* gene was inactivated by transposon insertion. The mutant showed a decrease in virulence in potato plant assays, demonstrating a role for Sv_x in the pathogenicity of *E. carotovora* subsp. *atroseptica*. These results show that Sv_x is a previously unidentified virulence determinant which is secreted by the out machinery and is regulated by quorum sensing, two systems employed by several other virulence factors. Thus, the type II secretory machine is a conduit for virulence factors other than the main pectinases and cellulase in *E. carotovora* subsp. *atroseptica*.

Erwinia carotovora subspecies *atroseptica* is a plant-pathogenic member of the family *Enterobacteriaceae*. The host range of this pathogen is largely restricted to potato, on which it causes blackleg disease of stems and soft rot of tubers (Perombelon and Kelman 1980). The genome of *E. carotovora* subsp. *atroseptica*SCRI1043 recently has been sequenced and annotated (Bell et al. 2004).

Transposon mutagenesis has identified many genes contributing to pathogenicity in *E. carotovora*, including secreted virulence factors such as pectate lyases, cellulases, and proteases (Dahler et al. 1990; Hinton et al. 1989a and b, 1990; Stromberg et al. 1994; Walker et al. 1994). Of these exoenzymes, the pectate lyase PelC and the cellulase CelV have been shown to play important roles in degrading plant cell walls (Hinton et al. 1989a and b; Walker et al. 1994). However, there are other important known virulence factors in *E. carotovora* (Toth et al. 2003).

Exoenzymes act on plant cell walls outside the bacterial cell, and are secreted by various routes across the bacterial

membrane (Thanassi and Hultgren 2000). The protease PrtW is secreted by a type I protein secretion apparatus (Delepelaire and Wandersman 1991; Marits et al. 1999), whereas CelV and PelC are exported to the periplasm before being translocated across the outer membrane by the out apparatus, a type II secretion system (Reeves et al. 1993). The out genes in *Erwinia* spp. lie in a cluster of approximately 14 kb, encoding at least 13 out proteins, some of which are homologous to type IV pili proteins (Sandkvist 2001). In *E. carotovora*, the out genes have been shown to contribute to virulence in potato tuber assays (Reeves et al. 1993), although it is not known whether any virulence factors other than the known exoenzymes are secreted by the out machinery.

The regulation of pathogenicity in *E. carotovora* is coordinated by a complex network of interactions between various gene products (Whitehead et al. 2002). Of particular importance is the cell density-dependent quorum-sensing system. The signaling molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) is synthesized by the product of *luxI* homologue *carI* (*exl* and *hslI*) and is thought to mediate expression of exoenzymes through the action of a LuxR homologue regulator (Chatterjee et al. 1995; Jones et al. 1993; Pirhonen et al. 1993). In addition to causing reductions in exoenzyme production, mutations in the *carI* gene lead to decreased carbapenem antibiotic production and reductions in virulence (Jones et al. 1993; Pirhonen et al. 1993). This quorum-sensing system also is expected to regulate additional, as yet unidentified, virulence factors.

During infection, a phytopathogen may elicit a plant response that leads to resistance of the host to infection (Heath 2000a and b). These interactions usually are associated with an avirulence (*avr*) gene in the pathogen, the product of which leads to an interaction with a protein in the plant encoded by a resistance (*R*) gene. The plant resistance is often, though not always, accompanied by the hypersensitive response (HR), a form of programmed cell death occurring at the site of infection (Heath 2000a and b).

The gene *avrXca* of *Xanthomonas campestris* pv. *raphani* confers avirulence in most interactions with *Arabidopsis thaliana* (Parker et al. 1993). The nature of this plant resistance is not understood, and the interaction does not lead to characteristic HR, which appears to be characteristic of most, but not all, land races of *Arabidopsis* spp. The gene encodes a protein with a predicted mass of 67 kDa, with no homology to other known sequences, and a putative signal peptide in the N-terminal portion of the presumed protein. The avirulence phenotype is not Hrp dependent and mutation of the *avrXca* gene had no effect on virulence. Southern hybridization suggested

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that the *avrXca* gene is present in related *X. campestris* pathovars but not in *Erwinia* or *Pseudomonas* strains (Parker et al. 1993).

More recent work, however, has suggested the presence of an AvrXca homologue in *E. carotovora* subsp. *atroseptica*. The technique cDNA amplified fragment length polymorphism (cDNA-AFLP) was used to identify genes of *E. carotovora* that are differentially expressed in different growth conditions (Dellagi et al. 2000). One gene that was expressed at a higher level in Luria-Bertani broth (LB) compared with pectin minimal media in both *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* encoded a protein with some sequence similarity to AvrXca.

This work describes the identification and initial characterization of a previously unknown virulence factor in *E. carotovora* subsp. *atroseptica*. This protein, Sv_x, is a secreted, quorum-sensing-regulated, post-translationally modified homologue of AvrXca, with a clear role in virulence shown by plant tests on an Sv_x⁻ mutant. The function of this protein is not known, although the presence of AvrXca in *X. campestris* and the interaction of AvrXca with *Arabidopsis* spp. suggest that the *E. carotovora* subsp. *atroseptica* protein may play an important role in plant pathogenicity.

RESULTS

An AvrXca homologue is post-translationally modified and secreted in *E. carotovora* subsp. *atroseptica*.

Proteins secreted by *E. carotovora* subsp. *atroseptica* are known to play a role in the pathogenicity of this organism. We set out to define the complement of secreted proteins, and identify proteins not previously associated with *E. carotovora* subsp. *atroseptica* pathogenesis. In order to investigate the secreted proteins (the “secretome”), *E. carotovora* subsp. *atroseptica* MC2 was grown to stationary phase in pectin minimal media (PMM). This medium contains 0.5% polygalacturonic acid (PGA), a breakdown product of plant cell walls, and induces production of multiple exoenzymes. PMM was used on the presumption that virulence factors other than exoenzymes also may be induced. The secretome was collected from culture supernatant by trichloroacetic acid (TCA) precipitation.

The precipitated proteins were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) over a pH range of 3 to 10, and Coomassie stained. Several protein spots were picked from the gel, were trypsin digested, and were identified by liquid chromatography-tandem mass spectrometry. Two protein spots yielded peptides homologous to the predicted protein product of the *avrXca* gene of *X. campestris* pv. *raphani* (Fig. 1). Three peptides were identified in both spots, TTALEP VAGANASLR, STYGSYGAVQPNNADR, and FSVPFQDQ APQQLGYNIVR. The peptide LIPESGATK also was found in spot 2 (Fig. 1A). These peptides were used to search the *E. carotovora* subsp. *atroseptica* SCRI1043 annotated genome sequence. All four peptides were found once in the product of the same predicted coding sequence (CDS) ECA0931. This CDS encodes a protein of 622 amino acids, with a predicted size of 68,373 Da and pI 6.8. The ECA0931 gene product has a putative signal sequence, presumably targeting it to the periplasm, which is predicted to be cleaved after residue 28, and a neutral zinc metalloproteinase zinc binding-region signature in residues 137 to 146 (prosite PS00142). The protein is 54% identical to AvrXca at the amino acid level, and so was named Sv_x (secreted virulence factor from *Xanthomonas*).

The Sv_x protein spots were located in a position on the gel consistent with the protein's predicted mass and pI, and were observed in three independent experiments. The presence of two spots suggested that Sv_x is post translationally modified

and that two forms of the protein were secreted. Although other proteins in the supernatant migrate closely together, and may represent similar post-translational modification of different proteins, this is the first example we have identified. The resolved versions of the Sv_x proteins appeared to have approximately the same mass, but differ in pI by approximately 0.2 units, suggesting that the protein was not cleaved but was modified in a way that alters charge while not significantly changing mass. There are multiple potential post-translational modifications that have been seen on proteomic protein gels, including N-acetylation and adenylation (Hesketh et al. 2002). The secreted proteins were tested for phosphorylation using diamond Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR, U.S.A.), but no phosphorylated proteins were identified in the secretome, suggesting that this modification is not responsible for generating the two forms of Sv_x (data not shown). Work currently is under way to determine the exact nature of the modification.

In addition to Sv_x, the known virulence factors PelC, CelV, and PrtW were identified in the secretome. The flagellar proteins FliC, FliD, and FlgK also were present, presumably due to shearing from the cell surface during growth and sample preparation (Fig. 1).

Analysis of the genomic context of the *svx* gene.

The region of the genome around ECA0931 was examined to determine the predicted genes flanking the *svx* gene. A schematic of these genes is shown in Figure 2A, with the predicted homologues of the corresponding products (Fig. 2B). The *svx* gene is flanked on either side by predicted permease genes, transcribed in the same orientation as *svx*. Upstream of the permease gene 5' of the *svx* gene is a CDS, ECA0933, encoding a putative “LuxR family” regulator. The C terminal portion of the ECA0933 protein shows homology to the helix-turn-helix domain of known LuxR-type quorum-sensing regulator proteins, while the N-terminal half shows similarity to members of the more diverse LuxR family of regulators, including homologues in *Photobacterium luminescens* (Orf66) and *Vibrio cholerae* (Vca1078). However, this protein does not show similarity with the region required to bind the lactone molecule in known LuxR-type regulators (Whitehead et al. 2001), suggesting that the protein may not be a true lactone-binding regulator. Other CDSs found near the *svx* locus encode another putative transport protein, a putative acetyltransferase, and a protein with similarity to TonB. TonB couples cytoplasmic membrane electrochemical potential to active transport of iron-siderophore complexes and vitamin B12 through high-affinity outer membrane receptors of gram-negative bacteria (Postle and Kadner 2003).

Sv_x is secreted by the out machinery.

The out apparatus, a type II secretion system, has been shown to secrete the virulence factors PelC and CelV in *E. carotovora* (Reeves et al. 1993). The out machinery translocates proteins from the periplasm to the extracellular environment, and Sv_x is predicted to be targeted to the periplasm; therefore, this secretion pathway was a clear candidate for Sv_x transport. Difference gel electrophoresis (DIGE) was employed to investigate whether Sv_x is secreted by the out pathway. DIGE is a technique for comparing two protein samples, labeled with two different fluorescent dyes, Cy3 and Cy5, on the same two-dimensional (2D) gel. This eliminates gel variation issues and allows more accurate sample comparisons (Unlu et al. 1997). DIGE often is used for cellular protein comparison but has not been applied previously to secreted proteins. The secretomes of a wild-type *E. carotovora* subsp. *atroseptica* strain (MC2) and an OutD⁻ mutant (MC4) were compared. OutD is a secretin

and is predicted to multimerise and form a pore-like structure in the outer membrane using transmembrane β -strands (Sandkvist 2001) and, as for all *out* genes in *E. carotovora*, an *OutD*⁻ mutant is completely nonfunctional for type II secretion (Reeves et al. 1993).

The protein spots corresponding to Sv_x were present in the MC2 secretome sample but undetectable in MC4 (Fig. 3). This result was observed in three independent experiments. By reciprocally labeling both samples with both dyes, and running two gels, MC2 (Cy3)/MC4 (Cy5) and MC2 (Cy5)/MC4 (Cy3), the possibility of proteins being preferentially labeled by one of the dyes was controlled for (data not shown). The experiment revealed that the Sv_x protein was not secreted in an *OutD*⁻ mu-

tant, along with a subset of the other secreted proteins and, thus, is secreted by the Type II secretory apparatus. The gels also show that PelC, CelV, ECA0852, and ECA2220 are secreted by the type II apparatus, whereas PrtW is not.

Sv_x production is under quorum-sensing control.

To determine whether Sv_x production is regulated by the *expI* quorum-sensing system, as is the case for the exoenzyme virulence factors, the secreted proteins were compared in MC2 and an *ExpI*⁻ mutant strain (MC3), again using DIGE. The two spots corresponding to Sv_x were undetectable in the *ExpI*⁻ mutant (Fig. 4). This was repeated in three independent experiments, with reciprocal labeling of the two samples, and shows

Fig. 1. A, Coomassie stained two-dimensional polyacrylamide gel electrophoresis of 200 μ g of precipitated protein from MC2 (wild type) *Erwinia carotovora* subsp. *atroseptica* culture supernatant. **B**, Proteins identified from secretome. Numbers correspond to labels in A. Gene name or genome designation is given with a description of the gene.

that the quorum-sensing system is necessary for either Sv_x synthesis or secretion in *E. carotovora* subsp. *atroseptica*. PrtW, CelV, and PelC were not present in the ExpI⁻ sample. Quorum sensing is known to regulate the transcription of these exoen-

zymes, consistent with the DIGE result. In addition, the other exoenzymes identified, ECA0852 and ECA2220, were absent in the ExpI⁻ sample, suggesting that the synthesis of these novel putative enzymes also may be regulated by quorum sensing.

Fig. 2. A, Local geography surrounding the *svx* locus, extracted from annotated *Erwinia carotovora* subsp. *atroseptica* (Eca) genome (to scale). **B**, Homologues of predicted protein products for coding sequences ECA0928 to ECA0934.

Fig. 3. Difference gel electrophoresis analysis of wild-type (MC2) and Out⁻ mutant (MC4) secreted proteins; 50 µg of MC2 sample is labeled with Cy3, and 50 µg of MC4 sample is labeled with Cy5. The two images above show fluorescence scan images of the two Cy dyes. The two spots corresponding to the Sv_x protein (ringed) are present in the wild-type sample but absent in the mutant sample, as are enzymes 4 to 7. PrtW (3) is not Out-dependent, and is present on both samples. This result was confirmed on reciprocal gels, with reversed use of labels, and in three independent experiments.

The *Svx*⁻ mutant exhibits reduced virulence.

The discovery that *Svx* is regulated and secreted by systems associated with virulence, quorum sensing, and type II targeting led us to investigate the phenotype of an *Svx*⁻ mutant. A grid of approximately 4,600 *E. carotovora* subsp. *atroseptica* transposon mutants was screened by polymerase chain reaction (PCR) for an insertion in the *svx* gene. A mutant, P16C12, was identified and sequencing confirmed that the mutation was in the *svx* gene (data not shown). The secreted protein from this mutant was compared with MC2 using DIGE and the spots corresponding to *Svx* were undetectable (data not shown). This assay confirmed that mutant P16C12 was *Svx*⁻.

The in vitro growth rates of the *Svx*⁻ mutant and the wild-type strain in LB broth and minimal pectin medium were compared and found to be similar in each case ($P < 0.05$) (data not shown). The virulence of this mutant in planta was investigated on potato plant stems by inoculation with either 10^6 or 10^2 P16C12 cells per inoculation site, and compared with the equivalent level of the wild type over 9 days (Fig. 5). At both inoculum concentrations, lesion length was significantly ($P < 0.05$) reduced in stems inoculated with the mutant compared with the wild type over the same period, with reductions of approximately 35 and 65% at inoculum concentrations of 10^6 and 10^2 *E. carotovora* subsp. *atroseptica* cells per inoculation site, respectively (Fig. 5A–C). Comparable results were obtained in a repeat of the virulence assays.

The *svx* gene with the upstream and downstream intergenic sequence was cloned onto a plasmid vector and complementation was investigated in the tuber rot assay (Fig. 5D). The *Svx*⁻ mutant with vector shows a reduction in rot compared with the wild type with vector, as expected from the above stem assays. Interestingly, expressing *svx* in the wild type from the complementing plasmid pMC20 caused a reduction in rotting. This may represent a fitness cost of expressing the protein above physiological levels. As expected, expressing *svx* on the plasmid in the mutant increases the rotting relative to the mutant with vector. The rotting caused by the mutant with complementing plasmid is equal to that of the wild type with complementing plasmid; thus, the *svx* gene in trans complements the reduced

virulence phenotype. All differences were statistically significant, and observed in two independent experiments. This result confirms that *Svx* contributes to virulence in *E. carotovora* subsp. *atroseptica*, and that the reduction in virulence in P16C12 is not due to a polar effect on downstream genes or a secondary mutation.

Transcription of *svx* is under *ExpI* regulation.

The DIGE data above (Fig. 4) did not reveal the level of *ExpI* regulation of *Svx*. Therefore, the *svx* mutation was transduced into an *ExpI*⁻ mutant and the β -glucuronidase (GUS) activity of the *svx* transcriptional fusion was measured in both the *svx* mutant and *svx expI* double mutant. GUS activity was approximately five times higher in the *svx* mutant (0.190 ± 0.012 and 0.041 ± 0.008). This finding demonstrates that *ExpI* regulates synthesis of *Svx* at the transcriptional level.

DISCUSSION

We have shown that *Svx* is a virulence factor in *E. carotovora* subsp. *atroseptica*. In addition, it is a protein secreted by the type II secretion (out) machinery, a system associated with the translocation of other known virulence factors. Furthermore, transcription of *svx* is regulated by the *N*-acyl homoserine lactone (AHL) quorum-sensing system, which is linked to the regulation of virulence factors in many bacterial species (Whitehead et al. 2001).

The observation that *Svx* was present in the *E. carotovora* subsp. *atroseptica* culture supernatant led us to investigate the pathway by which this protein is secreted out of the bacterial cell. The *Svx* gene identified in the genome of *E. carotovora* subsp. *atroseptica* 1043 showed a predicted signal sequence. Thus, the protein was expected to be targeted to the periplasm. Because the signal sequence did not contain a double arginine motif, it is probable that the protein was exported by the Sec machinery rather than the twin arginine translocation (TAT) system, for which this motif is almost always necessary (Palmer and Berks 2003). The prediction that *Svx* was exported to the periplasm before being secreted across the outer membrane suggested

Fig. 4. Difference gel electrophoresis analysis of wild-type and *ExpI*⁻ mutant secreted proteins; 50 μ g of the wild-type sample (MC2) is labeled with Cy3, and 50 μ g of the *ExpI*⁻ mutant (MC3) sample is labeled with Cy5. The two images above show fluorescence scan images of the two Cy dyes. The two spots corresponding to the *Svx* protein (ringed) are present in the wild-type sample but absent in the *ExpI*⁻ mutant sample. Enzymes 3 to 7 are also *ExpI* dependent. This result was confirmed on reciprocal gels, with reversed use of labels, and in two independent experiments.

the type II secretion apparatus of the out proteins as an obvious candidate for this second membrane translocation step. This hypothesis was confirmed experimentally by showing that secretion of Sv_x is out dependent. In *E. carotovora* subsp. *atroseptica*, out secretion has been shown previously to be associated only with secretion of pectate lyases and cellulases. The large reduction in rotting caused by an inability to secrete these exoenzymes in an out mutant may have masked the loss of other, more phytopathologically subtle virulence factors secreted by the type II pathway. In other species, type II secretion has been shown to be involved in targeting of toxins and other virulence factors, as well as exoenzymes (Stathopoulos et al. 2000). The fact that Sv_x is also secreted by this system in *E. carotovora* subsp. *atroseptica* suggests that a larger range of substrates for the *Erwinia* out pathway may exist than has been considered so far. Consequently, we currently are investigating other out-system-dependent secreted proteins and their possible role in virulence.

The finding that *svx* transcription is regulated by the quorum-sensing system is also intriguing. Quorum sensing regulates synthesis of exoenzymes in *Erwinia* spp. and is assumed to delay the production of these virulence factors until the latter stages of bacterial growth within the plant. The discovery that quorum sensing also regulates *svx* transcription suggests that this protein may play a role in the disease process which, like exoenzyme activity, is delayed until the bacteria have reached their quorate population.

This is the first time that DIGE has been applied to bacterial secretome studies. The use of this labeling technique has clear advantages over comparisons between gels because the unavoidable gel variation is circumvented. When examining multiple adjacent protein spots, as for Sv_x, the ability to directly compare the samples is invaluable. The use of the Cy dyes allows quantification of signals received for each protein spot. In this case, the proteins are either present or absent, and the sensitivity of the labels gives increased confidence over visual examination of Coomassie or silver staining. We identified the major known exoenzymes, PelC, CelV, and PrtW. DIGE confirmed that all three are quorum-sensing dependent, and that PelC and CelV are out dependent. These results act as a validation of the technique. In addition, we identified two previously unidentified putative enzymes, ECA0852 and ECA2220, which are both quorum-sensing dependent and out dependent. Therefore, the latter two proteins are obvious candidates for further tests for possible roles in phytopathogenesis.

We have shown that Sv_x contributes to *E. carotovora* subsp. *atroseptica* virulence in planta. The mechanism of Sv_x action is not clear at present. Searches of Sv_x against the protein database reveal no clues as to a probable function of the protein. The presence of a zinc binding signature found in metallopeptidases and the similarities in regulation and secretion between Sv_x and the exoenzymes suggest a possible enzymatic activity. This could explain the contribution toward virulence, and perhaps also the role of Sv_x as an avirulence protein in *X. campestris* pv. *raphani*. A product of this putative enzyme reaction, for example a digestion product of the plant cell wall, may elicit the plant response, leading to avirulence. The fact that Sv_x goes through the type II secretion pathway rather than type III Hrp pilus, which also is present in *E. carotovora* subsp. *atroseptica* (present in the genome sequence) (Bell et al. 2002), suggests that the protein is targeted to the extracellular environment rather than the interior of the plant cell, consistent with the theory that Sv_x is an enzyme.

The low abundance of Sv_x in the secretome preparations of this study may be a result of the protocol used. The expression of the *svx* gene in another *E. carotovora* subsp. *atroseptica* strain (SCRI1039) was shown to be responsive to environ-

mental conditions (Dellagi et al. 2000). Expression of the gene was shown to be lower on pectin minimal media, which resembles the media used in the secretomic experiments here, compared with LB. Thus, the levels of Sv_x seen in this study may represent neither the maximum levels possible nor the levels seen during infection of the plant.

The genomic context of the *svx* locus makes it tempting to speculate that the LuxR family regulator Eca0933 may regulate

Fig. 5. Pathogenicity assay showing **A**, wild-type *Erwinia carotovora* subsp. *atroseptica* (SCRI1043) and **B**, Sv_x⁻ (P16C12) on potato (cv. Estima) stems following inoculation with 10⁶ cells/inoculation site and incubation at 22°C for 9 days. **C**, Comparison of lesion development between SCRI1043 (dark bars) and P16C12 (white bars) at two inoculation concentrations (10² and 10⁶ cells/inoculation site) together with their least significant difference (LSD) values ($P < 0.05$). Data presented shows average length of 12 replicates. **D**, Complementation of tuber rotting phenotype using plasmid pMC20 (pACYC184 with *svx* gene). The rot caused by SCRI1043 (pACYC184) (a), SCRI1043 (pMC20) (b), P16C12 (pACYC184) (c), and P16C12 (pMC20) (d) are shown with standard error from 20 replicates.

transcription of *svx* and respond to the acyl homoserine lactone quorum-sensing molecule. However, the low degree of homology to the known LuxR-type regulators makes this unlikely. If *Svx* has a role in enzymatic breakdown of the plant cell wall, perhaps the products of the permease genes flanking *svx* are required for uptake of the breakdown products. These genes may be located adjacent to *svx* to allow coordinate regulation for a linked physiological role, because all the genes are transcribed in the same orientation and, thus, may be part of an operon.

Svx corresponds to two protein spots on the 2D gels. We interpret this as evidence that *Svx* is post-translationally modified, and that two secreted forms exist in vivo. Both this work and previous experiments using very similar methods of secretome preparations in *Pseudomonas aeruginosa* (Nouwens et al. 2003) have shown most proteins corresponding to a single spot, while a small number of proteins were found in more than one spot, confirming that this is unlikely to be an artifact of the procedure. Taken together, these proteomic studies show that a small proportion of the secretome is post-translationally modified. The reason for secreting both forms of *Svx* is not clear. One of the two forms is seen consistently in higher abundance than the other; therefore, the presence of the second, less abundant spot could represent the unprocessed precursor, a small amount of which leaks through the secretion apparatus. Whether both forms of *Svx* are equally active in planta or have the same or different functions, or indeed whether other modified *Svx* proteins also are present, are questions yet to be answered, as is the reason that virulence of wild-type *E. carotovora* subsp. *atroseptica* decreases when *svx* is expressed on a plasmid.

In conclusion, using 2D-PAGE, DIGE, and analysis of selected mutants, we have identified a previously unseen quorum-sensing-regulated, type II secreted virulence factor in *Erwinia* spp. Discovering the phytopathological function and mechanism of action of this protein should lead to a greater understanding of the complex process of *E. carotovora* subsp. *atroseptica*-induced potato disease.

Moreover, this discovery highlights the fact that the type II secretory pathway of *E. carotovora* subsp. *atroseptica* is a conduit for virulence determinants other than the major plant cell wall-degrading enzymes. The possibility that the out machinery is involved in delivery of even more pathogenesis factors with "subtle" roles in disease induction is now under active investigation.

MATERIALS AND METHODS

Bacterial strains and mutagenesis.

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown in LB or M9

minimal medium except for secretome preparations (Sambrook et al. 1989). *Escherichia coli* strains were grown at 37°C and *Erwinia* strains were grown at 25 or 27°C. When required, the following antibiotics were added to these media: kanamycin (Km) at 50 µg ml⁻¹, ampicillin (Ap) at 100 µg ml⁻¹, streptomycin (Sm) at 100 µg ml⁻¹, and chloramphenicol (Cm) at 50 µg ml⁻¹.

Mutants were produced in one of three ways, including spontaneous mutagenesis and Tn5 mutagenesis followed by either phenotypic screening where such a screen was possible or PCR-based screening of a specific gene where no phenotypic assay was possible. First, MC2 was generated by selecting a spontaneously streptomycin-resistant strain of SCRI1043. MC2 then was mutagenized with mTn5*gusAgfp* on the pUT plasmid (Xi et al. 1999) by a triparental mating between MC2, cc118λ*pir* (pUT mTn5*gusAgfp*12), and HH26 (pNJ5000) (Grinter 1983; Herrero et al. 1990; Kaniga et al. 1991). Sm^r, Km^r, and Ap^s mutants which had incorporated the transposon and lost pUT were screened on pectate lyase indicator agar for phenotypes (Andro et al. 1984) and mutants were further phenotyped. Two mutants were identified which had an *ExpI*⁻ and *out*⁻ phenotype. The *ExpI*⁻ mutant did not produce AHL as shown by a CV026 bioassay (McClellan et al. 1997), and produced low levels of protease, cellulase, and pectate lyases on agar indicator plates (Gilkes 1984; Hinton et al. 1989a and b). The *out*⁻ mutant produced wild-type levels of AHL and protease and low levels of pectate lyase and cellulase, as shown by these assays. The transposon mutations were transduced into MC2 using phage ΦM1 (Toth et al. 1997) to show inheritance of the phenotype. These mutants were named MC3 (*ExpI*⁻) and MC4 (*out*⁻), respectively. PCR using primers annealing to the *expl* gene and to the transposon confirmed the location of the insertion in MC3. Single-specific-primer PCR using the transposon primer (Shyamala and Ames 1989) showed that the insertion in MC4 was in the *outD* gene.

The mutagenesis was repeated to generate a grid of insertion mutants. In all, 4,608 transconjugants were selected and grown in 12 384-well microtiter plates (M9 Km broth plus 4% glycerol). All transconjugants were tested for resistance to kanamycin and sensitivity to ampicillin. Cells from each microtiter plate were transferred to M9 Km agar and, following incubation at 27°C for 48 h, DNA was extracted from each plate of clones to give 12 pools of DNA for PCR screening using a transposon-specific primer (Tn5R: 5'-ATCCTCCTTAGCTAGTCAGG-3') and an *svx*-specific primer (*svx*R: 5'-CCTCTCGTGAAATAATAAGGCTGATTAGC-3'). Initially, all 12 plate pools were screened and, where a PCR product of the expected size was generated, individual wells from that plate were rescreened to identify the mutant clone. PCR products were purified and sequenced to confirm the location of the transposon.

Table 1. Bacterial strains and plasmids used in this study

Strain, plasmid	Description ^a	Source or reference
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>		
SCRI1043 (ATCC BAA-672)	Wild-type <i>E. carotovora</i> subsp. <i>atroseptica</i> strain	Hinton et al. 1989
MC2	Sm ^r spontaneous mutant of SCRI1043	This study
MC3	Sm ^r Km ^r MC2 with transposon insertion <i>expl::mTn5gusAgfp</i>	This study
MC4	Sm ^r Km ^r MC2 with transposon insertion <i>outD::mTn5gusAgfp</i>	This study
P16C12	Km ^r SCRI1043 with transposon insertion <i>svx::mTn5gusAgf</i>	This study
EMS6.1	Lac ⁻ SCRI1043	Hinton et al. 1989
TB6	EMS6.1, <i>expl::lacZ</i>	This study
MC6	TB6 with <i>svx::mTn5gusAgfp</i>	This study
<i>Escherichia coli</i>		
cc118λ <i>pir</i> (pUT)	Sp ^r donor with Ap ^r pUT plasmid carrying Km ^r mTn5 <i>gusAgfp</i>	Herrero et al. 1990; Xi et al. 1999
HH26 (pNJ5000)	Tc ^r mobilization strain	Grinter et al. 1983
Plasmids		
pACYC184	Tc ^r Cm ^r plasmid-cloning vector	Chang et al. 1978
pMC20	<i>svx</i> cloned onto pACYC184 plasmid, Cm ^r	This study

^a Sm = streptomycin, Km = kanamycin, Sp = , Ap = ampicillin, Tc = , and Cm = chloramphenicol.

Strain MC6 was generated by transducing the *svx* transposon mutation using phage Φ M1 from strain P16C12 into strain TB6, which carries an *exl::lacZ* fusion, from pBluescript, inserted in the *Xba*I site of the *exl* gene of EMS6.1, a SCRI1043 Lac⁻ derivative. The presence of the two mutations was confirmed by PCR and by plate assays as above. Complementation of the *svx* mutation was performed by cloning the *svx* gene and the upstream and downstream intergenic sequence into the Cla I (5'*svx*) and Sal I (3'*svx*) sites of pACYC184, using primers *svxCmpN* (5'-CCAATCGATACCTTTCTACAGAGGTCG-3') and *svxCmpC* (5'-CGGGTCGACATCAGCGAATAACCAATG-3'). Plasmids were transformed into *E. carotovora* subsp. *atroseptica* strains by electroporation.

Secretome preparations.

The cultures were grown at the same time with the same batch of media in the same incubator to maximize the comparability of the samples. Bacteria were grown in PMM, minimal media with the addition of PGA (Sigma-Aldrich, St. Louis). The 250-ml cultures were grown for 23 h at 25°C, and shaken at 250 rpm in 2-liter baffled flasks from an initial inoculum of overnight culture to an optical density at 600 nm (OD₆₀₀) of 0.05. The culture was pelleted by centrifugation for 15 min with an average relative centrifugal force (RCF) of 3,120 to remove most cells, and the supernatant then was pelleted by centrifugation for 30 min with an average RCF of 8,296 to remove remaining cells. β -Galactosidase activity of the supernatant was measured and compared with sonicated culture to estimate lysis (Miller 1972). Trichloroacetic acid was added to the supernatant to a concentration of 12.5% to precipitate the protein, and left on ice for 15 min. The protein was collected by centrifugation for 30 min with an RCF of 8,296. The pellet was washed three times in 80% acetone and resuspended in lysis buffer (8 M urea, 4% wt/wt 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 5 mM magnesium acetate, and 10 mM Tris, pH 8.0).

2D-PAGE and DIGE.

The pH of the protein samples was adjusted to 8.5 using 50 mM HCl and 50 mM NaOH, and quantified using the Bio-Rad DC protein assay (Bio-Rad, Hertfordshire, U.K.). DIGE experiments were carried out as by Karp and associates (2004). In brief, samples were labeled with cyanine (Cy) dyes (GE Healthcare, Amersham, Buckinghamshire, U.K.) according to manufacturer's protocols. Samples were labeled with either Cy3 or Cy5. A reciprocal experiment using the alternative labels also took place. Proteins were separated in the first dimension using 13 cm of immobilized linear pH gradient (IPG) strips, pH 3 to 10, on an IPGphor II (GE Healthcare). The sodium dodecyl sulfate PAGE second dimension was run onto 12% 13-cm acrylamide. The gels were run at 20 mV for 15 min and then at 40 mV, at 20°C. DIGE gels were scanned at appropriate wavelengths on a Typhoon 9410 imager (GE Healthcare). The Cy3 images were scanned using a 532-nm laser and a 580-nm band pass (BP) 30 emission filter. Cy5 images were scanned using a 633 nm laser and a 670 nm BP30 emission filter. Images were obtained and compared using ImageQuant software (GE Healthcare). Gels were Coomassie stained and protein spots were cut out of the gel, trypsin digested, and peptides identified on a Q-TOF mass spectrometer (Waters, Manchester, U.K.). MASCOT (Matrix Science, London) was used to identify proteins from peptide identifications using the NCBI database.

Bioinformatic tools.

The *E. carotovora* subsp. *atroseptica* SCRI1043 (ATCC BAA-672) genome sequence can be obtained from The Wellcome Trust Sanger Institute. Protein similarities were identified

by BLAST searching of the NCBI database (Altschul et al. 1990). Molecular mass and pI predictions were made by the ExPASy Compute pI/MW tool (Bjellqvist et al. 1993). Signal sequence and Prosite signature identifications were made as part of the annotation of the *E. carotovora* subsp. *atroseptica* genome sequencing project.

Plant pathogenicity tests.

Microplants of the potato cv. Estima (*E. carotovora* subsp. *atroseptica* susceptible) were obtained from a commercial source (Gentech, Dundee, Scotland), replanted in 15-cm pots containing compost and grown to approximately 20 cm in height in a controlled glasshouse environment of 22°C with a 16-h light regime. Bacterial strains were grown overnight in LB broth, supplemented with kanamycin for mutants, at 27°C and cells were harvested by centrifugation with an RCF of 4,000 for 10 min, washed in phosphate buffered saline (PBS, pH 7.4), and resuspended in the same volume of PBS. Two dilutions (10⁸ and 10⁴ cells/ml) were prepared in PBS from these suspensions, and potato plant stems were inoculated under the second fully expanded leaf with 10 μ l of bacterial suspension (equivalent to either 10⁶ or 10² cells per inoculation site) using a micropipette tip. Inoculation sites then were covered with parafilm to prevent desiccation. At least 12 replicate plants were used for each strain and at each inoculum. The inoculated plants were kept at 22°C with a 16-h light regime and high humidity, and scored daily over a 9-day period for symptom development, which was recorded as length of rot (millimeters). Virulence assays were repeated and comparable data obtained (data not shown). Genstat for Windows (version 6.1.0.200) (Genstat, 6th ed.; Lawes Agricultural Trust, Rothamsted, U.K.) was used for all statistical analyses (analysis of variance; $P < 0.05$).

In tuber-rotting assays, the virulence of *E. carotovora* subsp. *atroseptica* strains was determined by local inoculation of intact Estima potato tubers as described by Walker and associates (1994). Starting inocula of 10² cells/inoculation site were used. Two independent experiments using 20 replicate tubers were performed. After 96 h, the level of potato tissue maceration was determined by weighing the necrotic tissue. Average rotted mass then was converted to a percentage of the wild-type and vector average rotted mass.

GUS assays.

Cultures were grown in an identical way to the secretome preparations. Samples were permeabilized with toluene, and incubated in GUS assay buffer (50 mM NaPO₄, pH 7, 1 mM EDTA, and 5 mM dithiothreitol) at 37°C with 1 mM *p*-nitrophenyl β -D-glucuronide. Reactions were stopped with 0.4 M Na₂CO₃. Values are expressed as change in OD₄₀₅ min⁻¹ (milliliters of culture)⁻¹ OD₆₀₀⁻¹.

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LITERATURE CITED

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.

- Andro, T., Chambost, J. P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., van Gijsegem, F., and Coleno, A. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *J. Bacteriol.* 160:1199-1203.
- Bell, K. S., Avrova, A. O., Holeva, M. C., Cardle, L., Morris, W., De Jong, W., Toth, I. K., Waugh, R., Bryan, G. J., and Birch, P. R. 2002. Sample sequencing of a selected region of the genome of *Erwinia carotovora* subsp. *atroseptica* reveals candidate phytopathogenicity genes and allows comparison with *Escherichia coli*. *Microbiology* 148(Pt 5):1367-1378.
- Bell, K. S., Sebaihia, M., Pritchard, L., Holden, M. T., Hyman, L. J., Holeva, M. C., Thomson, N. R., Bentley, S. D., Churcher, L. J., Mungall, K., Atkin, R., Bason, N., Brooks, K., Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail, M. A., Sanders, M., Walker, D., Whitehead, S., Salmond, G. P., Birch, P. R., Parkhill, J., and Toth, I. K. 2004. Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* 101(30):11105-11110.
- Bjellqvist, B., Hughes, G. J., Pasquali, C., Paquet, N., Ravier, F., Sanchez, J.-C., Frutiger, S., and Hochstrasser, D. F. 1993. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14:1023-1031.
- Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation/cell density-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. *Appl. Environ. Microbiol.* 61(5):1959-1967.
- Dahler, G. S., Barras, F., and Keen, N. T. 1990. Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. *J. Bacteriol.* 172(10):5803-5815.
- Delepelaire, P., and Wandersman, C. 1991. Characterization, localization and transmembrane organization of the three proteins PrtD, PrtE and PrtF necessary for protease secretion by the gram-negative bacterium *Erwinia chrysanthemi*. *Mol. Microbiol.* 5(10):2427-2434.
- Dellagi, A., Birch, P. R., Heilbronn, J., Lyon, G. D., and Toth, I. K. 2000. cDNA-AFLP analysis of differential gene expression in the prokaryotic plant pathogen *Erwinia carotovora*. *Microbiology* 146 (Pt 1):165-171.
- Gilkes, N. R., Nilburn, D. G., Miller, R. C., and Warren, R. A. J. 1984. A mutant of *Escherichia coli* that leaks cellulase activity encoded by cloned cellulase genes from *Cellulomonas fimi*. *Biotechnology* 2:259-263.
- Grinter, N. J. 1983. A broad-host-range cloning vector transposable to various replicons. *Gene* 21:133-143.
- Heath, M. C. 2000a. Hypersensitive response-related death. *Plant. Mol. Biol.* 44(3):321-334.
- Heath, M. C. 2000b. Nonhost resistance and nonspecific plant defenses. *Curr. Opin. Plant Biol.* 3:315-319.
- Herrero, M., de Lorenzo, V., and Timmis, K. N. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 172(11):6557-6567.
- Hesketh, A. R., Chandra, G., Shaw, A. D., Rowland, J. J., Kell, D. B., Bibb, M. J., and Chater, K. F. 2002. Primary and secondary metabolism, and post-translational protein modifications, as portrayed by proteomic analysis of *Streptomyces coelicolor*. *Mol. Microbiol.* 46(4):917-932.
- Hinton, J. C., Gill, D. R., Lalo, D., Plastow, G. S., and Salmond, G. P. 1990. Sequence of the *peh* gene of *Erwinia carotovora*: Homology between *Erwinia* and plant enzymes. *Mol. Microbiol.* 4(6):1029-1036.
- Hinton, J. C., Sidebotham, J., Gill, D., and Salmond, G. 1989a. Extracellular and periplasmic isoenzymes of pectate lyase from *Erwinia carotovora* belong to different families. *Mol. Microbiol.* 3:1785-1795.
- Hinton, J. C., Sidebotham, J. M., Hyman, L. J., Perombelon, M. C., and Salmond, G. P. 1989b. Isolation and characterisation of transposon-induced mutants of *Erwinia carotovora* subsp. *atroseptica* exhibiting reduced virulence. *Mol. Gen. Genet.* 217(1):141-148.
- Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G., and Williams, P. 1993. The Lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12(6):2477-2482.
- Kaniga, K., Delor, I., and Cornelis, G. R. 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: Inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* 109:137-141.
- Karp, N. A., Kreil, D. P., and Lilley, K. 2004. Determining a significant change in protein expression with DeCyder during a pair-wise comparison using two-dimensional difference gel electrophoresis. *Proteomics* 4(5):1421-1432.
- Marits, R., Koiv, V., Laasik, E., and Mae, A. 1999. Isolation of an extracellular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity. *Microbiology* 145 (Pt 8):1959-1966.
- McClellan, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. A. B., and Williams, P. 1997. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143(12):3703-3711.
- Miller, J. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Nouwens, A. S., Beaton, S. A., Whitchurch, C. B., Walsh, B. J., Schweizer, H. P., Mattick, J. S., and Cordwell, S. J. 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* 149:1311-1322.
- Palmer, T., and Berks, B. C. 2003. Moving folded proteins across the bacterial cell membrane. *Microbiology* 149(Pt 3):547-556.
- Parker, J. E., Barber, C. E., Fan, M. J., and Daniels, M. J. 1993. Interaction of *Xanthomonas campestris* with *Arabidopsis thaliana*: Characterization of a gene from *X. c. pv. raphani* that confers avirulence to most *A. thaliana* accessions. *Mol. Plant-Microbe Interact.* 6(2):216-224.
- Perombelon, M., and Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annu. Rev. Phytopathol.* 18:361-387.
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12(6):2467-2476.
- Postle, K., and Kadner, R. J. 2003. Touch and go: Tying TonB to transport. *Mol. Microbiol.* 49(4):869-882.
- Reeves, P. J., Whitcombe, D., Wharam, S., Gibson, M., Allison, G., Bunce, N., Barallon, R., Douglas, P., Mulholland, V., Stevens, S., Walker, D., and Salmond, G. P. C. 1993. Molecular cloning and characterization of 13 out genes from *Erwinia carotovora* subspecies *carotovora*: Genes encoding members of a general secretion pathway (GSP) widespread in gram-negative bacteria. *Mol. Microbiol.* 8(3):443-456.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Sandkvist, M. 2001. Biology of type II secretion. *Mol. Microbiol.* 40(2):271-283.
- Shyamala, V., and Ames, G. F. 1989. Genome walking by single-specific-primer polymerase chain reaction: SSP-PCR. *Gene* 84(1):1-8.
- Stathopoulos, C., Hendrixson, D. R., Thanassi, D. G., Hultgren, S. J., St. Geme, J. W., 3rd, and Curtiss, R., III. 2000. Secretion of virulence determinants by the general secretory pathway in gram-negative pathogens: An evolving story. *Microbes Infect.* 2(9):1061-1072.
- Stromberg, V., Lacy, G., and Kyöstiö, S. 1994. *Erwinia carotovora* subsp. *carotovora*: Protease in soft rot. (Abstr.) *Phytopathology* 84:1109.
- Thanassi, D. G., and Hultgren, S. C. 2000. Multiple pathways allow protein secretion across the bacterial outer membrane. *Curr. Opin. Cell Biol.* 12:420-430.
- Toth, I., Mulholland, V., Cooper, V., Bentley, S., Shih, Y.-L., Perombelon, M., and Salmond, G. 1997. Generalised transduction in the potato blackleg pathogen *Erwinia carotovora* subsp. *atroseptica* by bacteriophage ΦM1. *Microbiology* 143:2433-2438.
- Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. 2003. Soft rot *Erwiniae*: From genes to genomes. *Mol. Plant Pathol.* 4(1):17-30.
- Unlu, M., Morgan, M. E., and Minden, J. S. 1997. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis* 18(11):2071-2077.
- Walker, D., Reeves, P., and Salmond, G. 1994. The major secreted cellulase, CelV, of *Erwinia carotovora* ssp. *carotovora* is an important soft rot virulence factor. *Mol. Plant-Microbe Interact.* 7:425-431.
- Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L. S., and Salmond, G. P. C. S. 2001. Quorum-sensing in gram-negative bacteria. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 25:365-404.
- Whitehead, N. A., Byers, J. T., Commander, P., Corbett, M. J., Coulthurst, S. J., Everson, L., Harris, A. K., Pemberton, C. L., Simpson, N. J., Slater, H., Smith, D. S., Welch, M., Williamson, N., and Salmond, G. P. 2002. The regulation of virulence in phytopathogenic *Erwinia* species: Quorum sensing, antibiotics and ecological considerations. *Antonie Van Leeuwenhoek* 81(1-4):223-231.
- Xi, C., Lambrecht, M., Vanderleyden, J., and Michiels, J. 1999. Bi-functional *gfp*- and *gusA*-containing mini-Tn5 transposon derivatives for combined gene expression and bacterial localisation studies. *J. Microbiol. Methods* 35:85-92.