DsbA plays a critical and multi-faceted role in the production of secreted virulence factors by the phytopathogen, *Erwinia carotovora* subsp. *atroseptica*

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**Erwinia carotovora** subsp. *atroseptica* (*Eca*) is an enterobacterial phytopathogen causing economically-significant soft rot disease. Pathogenesis is mediated by multiple secreted virulence factors, many of which are secreted by the Type II (Out) secretion system. DsbA catalyses the introduction of disulphide bonds into periplasmic and secreted proteins. In this study, the extracellular proteome (secretome) of wild type *Eca* SCRI1043, and *dsbA* and *out* mutants, was analysed by spectral counting mass spectrometry. This revealed that *dsbA* inactivation had a huge impact on the secretome and identified diverse DsbA- and Out-dependent secreted proteins, representing known, predicted and novel candidate virulence factors. Further characterisation of the *dsbA* mutant showed that secreted enzyme activities, motility, production of the quorum sensing signal and virulence were absent or substantially reduced. The impact of DsbA on secreted virulence factor production was mediated at multiple levels, including impacting on the Out secretion system and the virulence gene regulatory network. Transcriptome analyses revealed that the abundance of a broad, but defined, set of transcripts, including many virulence factors, was altered in the *dsbA* mutant, identifying a new virulence regulon responsive to extracytoplasmic conditions. In conclusion, DsbA plays a crucial, multi-faceted role in the pathogenesis of *Eca*.

The soft-rot erwinias are Gram-negative, enteric bacteria and economically-important plant pathogens. *Erwinia carotovora* subsp. *atroseptica* (*Eca*, also called *Pectobacterium atrosepticum*), *Erwinia carotovora* subsp. *carotovora* (*Ecc*, or *Pectobacterium carotovorum*) and *Erwinia chrysanthemi* (*Ech*, or *Dickeya* sp.) cause soft rot disease of crop plants. *Eca* is exclusively a pathogen of potato, causing blackleg disease of stems and rotting of tubers (1,2). Production of secreted virulence factors is key to the pathogenesis of the soft-rot erwinias. Their primary virulence characteristic is the co-ordinated production of large amounts of multiple secreted plant cell wall degrading enzymes (PCWDEs), which leads to the breakdown of plant tissue and soft rot disease (2). The most important PCWDEs are the pectinases: primarily multiple isoforms of pectate lyase (*Pel*), together with isoforms of polygalacturonase (*Peh*), pectin methylesterase and pectin lyase. Soft-rot erwinias also produce at least one secreted endoglucanase (cellulase, *Cel*) (2,3). In addition to these primary 'brute-force' secreted virulence factors, it is now clear that *E. carotovora* also produces other secreted virulence factors involved in more subtle interactions with the plant host, such as proteases, Type III effectors and the recently-identified virulence factors, Nip (Necrosis Inducing Protein) and Svx (3-6). Consistent with this central role of protein secretion, the genome of *Eca* SCRI1043 encodes all known major protein secretion systems (7). The Type II Secretion System (T2SS) of *Erwinia*, the Out system, is required for the secretion of the major PCWDEs, *Pel*, *Cel* and *Peh*, and is thus required for virulence (3,8). The T2SS is a key conduit for secretion of virulence factors in many Gram-negative pathogens (9). Type II secretion is a two-step pathway in which proteins are exported to the periplasm via the Sec or Tat export machinery and are then secreted to the exterior of the cell by the T2SS apparatus, a complex multiprotein complex which spans the cell
envelope (10). The secretion signal for T2SS substrates is poorly defined but is believed to involve recognition of folded structure motifs. In *Eca*, the Out T2SS is required not only for secretion of Pel and Cel, but also for that of a putative proteoglycan hydrolase, ECA0852, and Svx (6). However, its full spectrum of substrates remains to be defined. *Erwinia* secreted proteases are secreted by a simple, one-step, Type I Secretion System (T1SS), well-studied in *Ech*, in which the substrate is moved directly from the cytoplasm to the cell exterior, bypassing the periplasm (11). In *E. carotovora*, production of PCWDEs and other secreted virulence factors is tightly regulated by N-acyl homoserine lactone quorum sensing (AHL QS) and other environmental cues (12). QS is a mechanism of cell-cell communication in which a bacterial population co-ordinately regulates gene expression in response to cell density by the production and detection of chemical signals (13). In *Eca* SCR11043, QS is required for expression of PCWDEs and other secreted proteins and for virulence in potatoes (14). The AHL synthase, ExpI, produces the major AHL signal, N-3-oxohexanoyl-homoserine lactone (OHHL), which is freely diffusible. At high cell density, either in stationary phase in culture or at high population levels within a plant host, a threshold concentration of OHHL is achieved and production of PCWDEs is activated. This activation is mediated by the LuxR-family transcriptional regulator, VirR, which, directly or indirectly, represses expression of target genes at low OHHL levels (14).

Several studies have analysed the extracellular proteome (secretome) of *Eca* SCR11043 (6,15) and *Ech* 3937 (16). These studies used 2D gel-based methods to identify secreted proteins produced by the wild type under different conditions. In addition, a limited number of Out-dependent proteins (PelC, CelV, Svx, ECA0852, ECA2220) and Expl-dependent proteins were identified in *Eca* by comparison of the secretomes of Out and Expl mutants with that of the wild type (6). However, examination of the genome sequence suggests that not all components of the *Eca* secretome have been identified by these studies. Flagellar motility is also a virulence factor in *Erwinia* (1). Flagellar assembly involves movement of proteins to the exterior of the cell in a manner analogous to Type III secretion and flagellar proteins are detected at significant levels in the external milieu (17). Therefore, for the purposes of the current study, flagellar proteins are also considered part of the secretome.

DsbA is a thiol-disulphide oxidoreductase which catalyses disulphide bond formation in the periplasm. As reviewed (18,19), DsbA catalyses the formation of disulphide bonds between pairs of cysteine residues in target proteins, allowing them to fold correctly. Once DsbA has donated a disulphide bond to its substrate protein, it is reoxidised by the membrane protein, DsbB, which in turn donates electrons to the electron transport chain. Incorrect disulphide bonds (which can occur in proteins with more than two Cys residues) are reshuffled by the disulphide isomerase, DsbC. DsbA is responsible for disulphide bonding in both resident periplasmic proteins and secreted proteins which access the periplasm, including some T2SS substrates. DsbA is required for the production of secreted virulence factors and/or for virulence in diverse pathogens. For example, it is required for cholera toxin assembly in *Vibrio cholerae*, secreted elastase production in *Pseudomonas aeruginosa*, Type III secretion in *Yersinia pestis* and adhesion (Type IV pilus formation) in *Escherichia coli* (20,21). DsbA has also been implicated in virulence in *Erwinia*. In *Ech*, DsbA is required for the stability and secretion of CelZ and several Pels, and a *dsbA* mutant is reduced in motility and tuber rotting (22). In *Ecc* SCR1193, DsbA was shown to be required for secreted Pel and Peh activity and for virulence in potato tubers; in *Eca* SCR11043, a *dsbA* mutant was reduced in tuber rotting and motility, but was not investigated further (23). However, these studies only examined the impact of DsbA on a small selection of known virulence determinants.

In this study, we undertook a global investigation into the role of DsbA in secreted protein production in *Eca* SCR11043, including the use of state-of-the-art proteomic analyses to identify novel DsbA-dependent secreted proteins. Since DsbA and T2SSs can act in concert in the post-translational processing of secreted virulence factors, we also examined further the Out-dependent secretome and the relationship between DsbA and Out. We found that DsbA is required for the proper production of almost all secreted
virulence factors in Eca SCR1043 and that its effect on virulence factor production is mediated at multiple levels, from transcript to protein secretion. Moreover, we identified novel Out- and DsbA-dependent secreted proteins, representing known, predicted and novel candidate virulence factors.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and culture conditions** - Bacterial strains and plasmids used in this study are detailed in Supplementary Table 4. Erwinia strains were cultured with good aeration in Pel Minimal Medium (PMM) or Luria Broth (LB), at 25°C (Eca) or 30°C (Ecc) as described (24); overnight cultures were grown in LB. Growth was measured as optical density at 600 nm (OD600). When required, media were supplemented with antibiotics: kanamycin (Kn) 50 μg/ml, ampicillin (Ap) 100 μg/ml, streptomycin (Sm) 50 μg/ml, tetracycline (Tc) 10 μg/ml and chloramphenicol (Cm) 25 μg/ml; or with synthetic OHHL (Sigma) 5 μM in dimethylsulphoxide. 

**Construction of strains and plasmids** - Eca chromosomal mutants defective in dsbA and celV were constructed by marker (allelic) exchange using the suicide vector pKNG101 (25) as described (24). Briefly, the target gene was cloned and disrupted in pBluescript, the disrupted allele cloned into pKNG101 and the resulting plasmid introduced into Eca by conjugation. Selection on streptomycin and then high sucrose allowed isolation of mutants in which the disrupted allele had replaced the wild type copy. The primers used to amplify dsbA and celV were SC80+SC81 and SC94+SC95, respectively, and plasmid details are given in Supplementary Table 4. The integrity of mutants was confirmed by PCR and sequencing (data not shown). Strains SCC32 and SCC33 were made by introduction of the dsbA-uidA, CmR allele from LS1A into MH1000, and strain SCC21 was generated by transducing the carLU:TnphoA-2, TcR allele from M17 into SCC20, both using the Ecc generalised transducing phage, fKP (26).

**2D gel-based secretome analysis** - Secreted proteins were prepared from 150 ml cultures grown in PMM for 24 h at 25°C. Secreted proteins were isolated as described (6). Briefly, following centrifugation to remove the cells, protein was precipitated from the supernatant using trichloroacetic acid to a concentration of 12.5% and collected by centrifugation. Following washing with 80% acetone, the pellet was air-dried and resuspended in 0.25 ml CHAPS lysis buffer (8 M urea, 4% CHAPS (3-(3-cholamidopropyl) dimethylammonio)-1-propane-sulphonate), 5 mM magnesium acetate, 10 mM Tris-HCl pH 8.0). Insoluble material was removed by centrifugation and the pH of the soluble protein sample adjusted to pH 8.5. Secreted protein samples from each strain (wild type, SCC22 and MC4) were compared using 2D-DiGE as described (24). 50 μg of each protein sample was labelled with a different Cy dye (Cy2, Cy3 or Cy5), the samples were pooled and separated on a 2D gel, and the fluorescent image from each channel was visualised. Three replicate gels varying the dye used for each sample gave identical results. 2D-DiGE separation was performed using a linear pH 3-10 separation in the first dimension and 12% SDS-PAGE in the second. The DiGE comparison of wild type and SCC22 was performed on three independent samples with essentially identical results and the MC4 secretome included for the three-way comparison was as reported previously (6). Proteins were identified by comparison with previous data and mass spectrometry (data not shown, 6).

**Secretome analysis by mass spectrometry (spectral counting)** - Secreted protein samples were prepared from triplicate independent cultures of wild type, SCC22 and MC4 grown in PMM as above. Secreted protein samples were prepared as above, except that 50 μg of phosphorylase B was added to each sample and total secreted proteins were resuspended in 0.5 ml 2x gel sample buffer (100 mM Tris-HCl pH 6.8, 3.2% SDS, 2.5 mM EDTA, 8% glycerol, 0.1 mg/ml bromophenol blue, 4% β-mercaptoethanol). 10 μl of each protein sample was loaded on to a 3% stacking gel and a 10% resolving gel. SDS-PAGE was run and gels were stained with Coomassie blue. Gels were imaged and bands were excised and digested with trypsin for mass spectrometry.
sample was separated on a 12% Tris-glycine SDS-PAGE mini-gel. Hence, since all cultures were harvested at the same optical density, total secreted protein from an equivalent number of cells was loaded for each strain (i.e. not normalised for amount of total protein). The gel was stained with colloidal Coomassie stain (34% methanol, 17% ammonium sulphate, 0.5% acetic acid, 0.1% Coomassie G-250) and a continuous vertical series of 20 2x2 mm gel pieces was cut from the middle of each lane (i.e. fractionating the protein sample by electrophoretic mobility). In-gel digestion was performed using a MassPrep station (Perkin Elmer). Proteins within gel slices were reduced and alkylated using dithiothreitol and iodoacetamide respectively and then digested to peptides using trypsin. Resultant peptides were eluted from the gel pieces in 15 μl of 0.1% formic acid. 5 μl of this was injected onto a reverse phase column (15 cm, 75 μm internal diameter C18 PepMap column) attached to a 1200 liquid chromatography system (Agilent). The column eluate was sprayed into an LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA). This LTQ was operated in triple play mode (for each ion of sufficient intensity, three scans were performed: a precursor scan, then a zoom scan to assign charge state and finally an MSMS scan). Resulting data files from all gel fractions for each sample were converted to .dta format using Bioworks version 3.2 (Thermo) and the .dta files merged to form a single .mgf file, using an in-house script. The .mgf files were searched against a protein database derived from the Eca SCRI1043 genome sequence (www.sanger.ac.uk; 7) using MASCOT version 2.1.6 (Matrix Science, London, UK) with the following parameter settings: 2 miscleavages, variable methionine oxidation, carboxymethyl cysteine fixed modification, peptide tolerance of 1.0 Da. Results were exported from MASCOT to Excel (with significance threshold 0.05, ions score cut-off 10, standard protein scoring and require bold red) and all peptides not ranked 1 were excluded. The cut-off for genuine protein identifications, as opposed to false positives, was set by allowing 25% of identifications to score below the top Mowse score obtained when searching the output against a reversed database. For each protein in each sample, the total number of spectra acquired (including redundant parent ions) for peptides belonging to that protein was summed to give the spectral count, a measure of relative protein abundance (27). Where the same peptide sequence was present in > 1 protein, e.g. in the PeLA and PeLB isoforms, spectra from that peptide were excluded from the count. For each strain, the data from triplicate samples was combined; any proteins detected in only one of the three samples were excluded; and, for each protein, the mean, standard error and rank within that sample (according to abundance) was calculated.

*Spectrophotometric enzyme activity assays* - Supernatant samples were prepared by centrifugation to remove cells followed by careful removal of supernatant; samples were snap-frozen in liquid nitrogen if not assayed immediately. Cell-associated samples were prepared by isolating the cells from 1 ml culture by centrifugation, resuspending the cells in 1 ml fresh medium, sonicating for 30s and then snap-freezing. Protease, cellulase and pectate lyase were determined by measurement of azocasein, Ostazin Brilliant Red-cellulose sodium polygalacturonate breakdown, respectively, as described (24).

*Potato virulence assays* - Stem infection assays were performed as described (28). The stems of microplants of potato (cv. Estima) were stab inoculated with 10^2 bacterial cells in 10 μl phosphate-buffered saline (PBS) and the plants maintained at 22°C. Infected plants were scored over a 12 day period for symptom development, which was recorded as length of rot (mm). At least 12 replica plants were used for each strain and GENSTAT for Windows version 6.1.0.200 (Lawes Agricultural Trust) was used for statistical analysis (Anova), to determine the least significant difference (LSD, p < 0.05). Tuber rotting assays were performed using Maris Piper potatoes, surface-sterilised using 5% bleach and stab inoculated with 10^7 cells using a sterile pipette tip. Following inoculation, the tubers were incubated at 19°C in a dark and moist environment for seven days, and then the mass of rotted tissue was measured.

*Other phenotypic assays* - To measure OHHL levels, the pSB401-based, luminometric assay (29) was used. Cell-free supernatant samples were diluted 1/100 in LB and 100 μl of the diluted samples aliquotted into the wells of a black microtitre plate. The sensor strain *E. coli* JM109
(pSB401) was grown to an OD<sub>600</sub> of 1, and 100 μl of this sensor culture was then added to each sample in the plate. Following incubation at 37°C for 3 h, light production was measured using an Anthos LUCY1 luminometer. Synthetic OHHL and medium alone were used as positive and negative controls, respectively. Swimming motility was measured in motility agar (minimal medium {40 mM K<sub>2</sub>HPO<sub>4</sub>, 15 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 mM MgSO<sub>4</sub>, 0.2% glucose} plus 0.1% casamino acids, 0.3% agar). Plates were inoculated with 4 μl of a 1/10 dilution (in PBS) of an overnight culture, incubated at 25°C for 18 h and the area of the swim haloes observed. Where required, sterile copper II chloride was added to the stated final concentration. For measurement of β-glucuronidase (Gus) activity, cells were permeabilised with toluene, added to 50 μl 10 mM p-Nitrophenyl β-D-glucuronide in a total volume of 500 μl Gus buffer, and the rate of increase of A<sub>405</sub> (absorbance at 405 nm, ΔA<sub>405</sub>/min) at 37°C measured immediately. Gus buffer contained 50 mM sodium phosphate pH 7, 1 mM EDTA and 5 mM DTT, and Gus activity is reported as ΔA<sub>405</sub>/min/ml/OD<sub>600</sub>.

**Anti-CelV western blot** - Cultures were grown for 24 h in PMM at 25°C. Cell-associated protein samples were prepared as follows. Cells from 1 ml of culture were isolated by centrifugation, resuspended in 500 μl 2x gel sample buffer (as above) and boiled for 10 min. Secreted proteins were precipitated from 1 ml of culture supernatant by addition of 1 ml 1:1 chloroform methanol, washed with 1 ml methanol, air-dried, resuspended in 500 μl 2x gel sample buffer and boiled for 10 min. Protein samples (0.5 μl of cell-associated and 2 μl of secreted samples) were separated by 12% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF, Millipore). CelV was detected by hybridisation of the primary antibody, polyclonal rabbit anti-CelV (23), followed by the secondary antibody, HRP-conjugated goat anti-rabbit (Sigma), and then the use of an enhanced chemiluminescent detection kit (Millipore). The same results were obtained using replicate samples prepared from independent cultures.

**qRT-PCR analysis** - RNA was prepared from four cultures of each strain grown to an OD<sub>600</sub> of ~3.4 in PMM at 25°C using the Qiagen RNeasy kit and quantified using a NanoDrop spectrophotometer. cDNA synthesis and qRT-PCR analysis was performed as described (14). Data were normalised to 16S rRNA as an internal control. All genes significantly altered were also significantly altered when dnaA (dsbA/WT in array = 1.0) was used as an alternative internal control (data not shown). Primers not described previously are in Supplementary Table 3.

**Microarray experiment** - The above RNA samples were also used for genome-wide transcriptional profiling using microarrays. RNA was hybridised to four Eca genomic arrays, with each array hybridising one wild type and one dsbA mutant sample (from four independent cultures of each strain) and incorporating a dye-swap (i.e. wild type labelled with Cy3 in 2/4 and with Cy5 in 2/4). For each array, two total RNA samples (3 μg each) were independently labelled by reverse transcription using an 11mer oligonucleotide mixture in the presence of amino-allyl dUTP, as described (30). Labelled cDNA samples were then coupled with Cy3 or Cy5 dye esters (GE Healthcare) and purified (30). Cy-labelled wild type and mutant cDNAs were co-hybridised to Agilent Technologies Inc. custom ECA_11K Genome Arrays according to the manufacturer's instructions and (30). The probes (60mers) include all 4521 predicted protein coding genes and 23 Lucidea Universal ScoreCard Controls (GE Healthcare), with each probe printed twice. Arrays were scanned using an ArrayWoRx Auto scanner (Applied Precision Inc.) with optimal exposure settings for each dye wavelength. Images were imported and aligned with probe position (Agilent GAL file) using GenePix Pro (v. 6.1, Molecular Devices) and spot intensity data were imported into GeneSpring (v. 7.3.1, Agilent Technologies Inc.). Microarray data was transformed to account for the dye-swap and normalised using default settings (LOWESS algorithm). Unreliable data (signal-background < 50 in 50% replicates) was removed by filtering. Transcripts were considered to be significantly altered in abundance in the mutant if the mean normalised signal from their probes was increased or decreased by more than 2x in the mutant compared with the wild type, with a Student's t-test P value < 0.05 (the experiment contained four biological replicates). Microarray data has been deposited in the GEO database with accession number GSE10647.
RESULTS AND DISCUSSION

Inactivation of dsbA has a very large impact on the secretome of Eca SCR11043 as visualised using 2D-DiGE - A chromosomal insertion mutant of dsbA in Eca SCR11043, strain SCC22, was constructed by marker exchange. As expected, this mutant showed negligible secreted Pel activity on indicator plates (data not shown) and was also impaired in motility (see below). The mutant showed no difference in growth rate compared with the wild type in several media (data not shown and see below). In order to assess the impact of dsbA inactivation on the extracellular protein profile (secretome) of Eca, an initial 2D-Difference in Gel Electrophoresis (2D-DiGE)-based comparison of the secreted proteins of the wild type, dsbA and out mutants was performed. MC4 is an out (T2SS) mutant of Eca SCR11043 (6). As shown in Fig. 1, all of the well known proteins in the Eca secretome were absent or greatly reduced in the dsbA mutant, e.g. PelABC, CelV, PrtW, Svx, ECA0852 and FliCD. Many of these proteins were also present at negligible levels in the out mutant e.g. PelC and Svx, but others were unaffected by Out inactivation, e.g. FliC and PrtW (as seen previously, 6). It should be noted that many other spots only appear, or seem more abundant, in the dsbA mutant; this is because DiGE involves equal amounts of protein being labelled for each sample.

In the dsbA mutant, the major secreted proteins were missing and the total amount of secreted protein recovered was less than in the wild type, hence loading the same amount of protein in each channel artificially amplified the signal from contaminating cellular proteins. Since the secretomes of the three strains (wild type, SCC22, MC4) were radically different, quantitative DiGE and quantitative mass spectrometry (MS) techniques which rely on normalisation over the whole sample were judged to be inappropriate for a quantitative comparison of the three strains. In addition, secreted proteins of Eca tend to be resolved poorly by 2D gels (e.g. the basic Pels streak across the gels, Fig 1). Hence we decided to utilise a semi-quantitative MS approach, spectral counting (27), a technique distinct from the 2D gel-based methods used previously to study the secretome of Erwinia (6,15,16), to gain a more detailed description of the proteins present in the secretome of wild type Eca, and in the dsbA and out mutants.

Spectral counting mass spectrometry reveals multiple DsbA-dependent and Out-dependent proteins in the secretome of Eca - In a spectral counting approach, a complex mixture of proteins (here secretome samples) is analysed by LC-MSMS, following an initial 1D gel fractionation step. For each protein, the total number of spectra acquired (total number of parent ions detected) by the mass spectrometer from peptides in that protein is counted. (Parent ions with the same peptide sequence and charge state can be counted multiple times and the numbers of spectra from all the detectable peptides from a given protein are added together). The total number of spectra observed for a given protein has been shown to be dependent on the relative abundance of that protein in a complex mixture (27). In this study, three independent secretome samples from each of the wild type, MC4 (out) and SCC22 (dsbA) were analysed by spectral counting. Secreted protein samples derived from equivalent amounts of culture were compared (i.e. samples were not normalised for total protein amount). Almost three hundred proteins were detected in the secretome of one or more of these strains (according to our quality criteria, see Experimental Procedures) The results for all of these proteins are given in Supplementary Table 1. Selected proteins, including the most abundant proteins in each strain, are detailed in Table 1.

In the wild type secretome, many known or predicted secreted PCWDEs and other virulence factors were identified, including several PCWDEs detected for the first time in Eca (Table 1). The majority of the top-scoring identifications (most abundant proteins) were known or predicted secreted proteins and flagellar proteins. Several abundant periplasmic and cytoplasmic proteins were also in the top twenty identifications, assumed to be present in the 'secretome' due to normal cellular lysis or leakage. Other, less abundant, known or candidate secreted proteins were also observed (Table 1). Proteins missing or greatly reduced in abundance in the out mutant revealed both new and previously-described Out-dependent secreted proteins (see below). In the dsbA mutant, as suggested by the 2D-gel experiment (Fig. 1), all the major secreted and flagellar proteins were missing or greatly reduced;
almost all (including the top-scoring) proteins identified were outer membrane, periplasmic or cytoplasmic proteins. In general, cellular proteins were more abundant (or only seen) in the dsbA mutant compared with the other strains (Table 1). This is most likely due either to artificial amplification of these proteins during preparation (in the absence of large amounts of genuinely secreted proteins) and/or to the dsbA mutant cells being more 'leaky' than the wild type. At least a partial contribution of the former effect seems likely as, in general, the out mutant (missing some but not all of the major secreted proteins) showed an intermediate level of cellular proteins between the dsbA mutant and the wild type, e.g. for RpsA and OppA.

Many secreted proteins were found to be both Out- and DsbA-dependent. Six secreted pectate lyase isoforms (PelABCZ, Pel-3, ECA2553) were identified in the wild type secretome, and all were missing, or greatly reduced, in the out mutant and missing, or negligible, in the dsbA mutant (Table 1). This confirms previous reports of secreted Pel enzymes being Out-dependent and having a requirement for DsbA-dependent disulphide bond formation for stable folding and secretion (6,22). The virulence factors, Svx and Nip, and the polygalacturonases, PehA and PehX (all with multiple Cys residues) were also shown to be Out and DsbA-dependent. Interestingly, PehA levels were not reduced as dramatically in the dsbA mutant as the Pels and Svx (30% of wild type levels compared with < 5%), suggesting that it may not be destabilised to the same extent by loss of disulphide bonding. In addition, three known or predicted cellulases, CelV, CelB and ECA2220, were identified in the secretome of the wild type but not that of the out and dsbA mutants, and the predicted proteoglycan hydrolase, ECA0852, was significantly decreased in the absence of Out or DsbA. This approach therefore identified multiple new substrates of the Out T2SS (Nip, PelABZ, PehAX, Pel-3, ECA2553, CelB, ECA2134, ECA3580 and ECA3496), including several which are not PCWDEs. Nip is an important virulence determinant in planta and we hypothesised previously that its targeting might be Out-dependent (5). The cellular location of PehX has been controversial in Ech (16), but it was clearly secreted in an Out-dependent manner in Eca.

The major protease, PrtW, was not Out-dependent (as expected, 6), yet was reduced to 23% of wild type levels in the dsbA mutant. Similarly, pectin lyase (Pnl) levels were unaffected in the out mutant but were reduced (~ 50%) in the dsbA mutant. Pnl is not expected to be secreted in an Out-dependent manner since it does not have a classical N-terminal signal sequence. However, in Ecc, it has been suggested to be efficiently released by stress-induced cellular lysis and thus to be present in the supernatant at reasonable abundance (31), consistent with the observations here. The HecA2 protein (see below) was also observed in the secretome, in a DsbA- but not an Out-dependent manner. There were also a few candidate PCWDEs identified only, or more strongly, in the dsbA samples and which are thus DsbA-independent: PelX, PehN, PelB2, PemA. These are known or likely to be periplasmic enzymes.

Importantly, several proteins of unknown function, ECA2134, ECA3580 and ECA3946, were shown to be present in the secretome in an Out- and DsbA-dependent manner, similar to many known secreted virulence factors. Thus these proteins represent novel candidate secreted virulence factors. Although ECA2134 is annotated as a putative ABC transporter periplasmic iron binding protein, its Out-dependence and the lack of any genes encoding other ABC transporter components nearby suggest otherwise (7). Its similarity to iron binding proteins raises the possibility that it may represent a novel iron binding secreted protein involved in host interaction. Four other novel DsbA-dependent exported proteins, possibly secreted, were observed: ECA0467, ECA1185, ECA1186, ECA3090. Interestingly, the latter three were recently reported to be induced or repressed by plant extracts in the wild type secretome of Eca SCRI1043 (15), whereas the former (designated a hypothetical protein) has been seen for the first time in this study.

Overall, the spectral counting analysis identified dsbA- and out-dependent secreted proteins, and dsbA-dependent, out-independent secreted proteins, and confirmed that dsbA inactivation affects essentially the entire complement of major secreted proteins in Eca, a much wider-ranging impact than was initially anticipated. This study represents the first report,
to our knowledge, of a proteomic analysis of the extracellular proteins (secretome) of a DsbA mutant. However, proteomic studies to identify periplasmic DsbA-dependent proteins in E. coli (32,33) and Salmonella (34) have been described. These studies saw a FliC defect in the dsbA mutant similar to that observed in Eca, and also reported increased OsmY in an E. coli dsbA mutant, consistent with our observation of increased osmY expression in Eca (see below).

Production of secreted enzyme activities, production of OHHL and motility are reduced or eliminated by loss of DsbA - In order to confirm the proteomic results and to investigate further the massive impact of the dsbA mutation on the extracellular proteome, production of secreted enzyme activities and motility were examined. Measurement of secreted Pel and endoglucanase (Cel) activity throughout growth showed that both activities were expressed in a typical growth phase dependent manner in the wild type but were negligible in the dsbA mutant, consistent with the (virtual) absence of multiple Pels and Cels from the secretome (Fig. 2A). Secreted protease (Prt) activity in the dsbA mutant was reduced to one third that of the wild type, consistent with the reduced levels of the major protease, PrtW, observed in the secretome (Fig. 2A). Production of all three secreted enzyme activities could be restored in the dsbA mutant by expression of DsbA in trans, confirming the requirement for DsbA (Fig 2B).

As part of the phenotypic characterisation of the dsbA mutant, and since QS is central to secreted enzyme production in Eca (14), the production of the QS signal molecule, OHHL, was also measured in the dsbA mutant. Surprisingly, since the AHL synthase, ExpI, is a cytoplasmic enzyme, levels of OHHL were reduced in the dsbA mutant to approximately two thirds that of wild type (Fig. 3A), and this decrease could be complemented by expression of dsbA in trans (Fig. 3B). Loss of swimming motility is a well known phenotype of dsbA mutants. As shown in Fig. 4, strain SCC22 failed to exhibit normal swimming motility in 0.3% agar, but motility was restored by expression of DsbA in trans. Chemical suppression of a dsbA mutation by the oxidant Cu²⁺ has been reported in E. coli (35). The dsbA mutation in Eca could also be chemically suppressed for the motility phenotype in a dose-dependent fashion by Cu²⁺ (Fig. 4C).

The virulence of the dsbA mutant is severely reduced in planta - The virulence in planta of strain SCC22 was compared with that of the wild type using a potato stem infection assay, designed to mimic Blackleg disease (28). The dsbA mutant was found to be greatly reduced in lesion formation compared with the wild type. The overall wild type score was 27 mm whereas that of SCC22 was 7.7 mm (the difference between the strains is statistically significant: least significant difference 5.7, p < 0.05). This is consistent with the impaired secretion of PCWDEs and other virulence proteins and lack of motility observed for this mutant in vitro. Interestingly, the dsbA mutant was not totally avirulent. The residual lesions could be due to slight environmental suppression of the dsbA phenotype in an oxidative host environment, dsbA-independent virulence factors such as the product of the upregulated cfa gene cluster (see below), and/or small amounts of Pnl and other 'leaking' cellular enzymes. The in planta virulence defect of the dsbA mutant was similarly observed in potato tubers and could be complemented by the expression of dsbA in trans (Fig. 4D).

The Out secretion system is impaired in the DsbA mutant of Eca - Next we investigated the cause(s) of the wide-ranging impact of dsbA mutation on the levels of multiple extracellular proteins. The simplest reason for DsbA dependence is that the protein is a direct target of DsbA: it requires disulphide bond formation in the periplasm for stability and/or activity. These include: five secreted Pels, PehA, PehX, Svx, Nip and ECA2220, all of which have multiple Cys residues and are Out-dependent (therefore will exist as periplasmic intermediates). Consistent with this, in addition to the loss of secreted Pel proteins and secreted Pel activity, negligible Pel activity was observed in the cell-associated fraction of the dsbA mutant (data not shown). However, many proteins reduced or absent in the secretome of the dsbA mutant do not possess any cysteine residues and so cannot require disulphide bonding for stability and/or activity. These include CelIV, CelB, ECA2134 and ECA0852, all of which were also Out-dependent. In fact it was apparent from the secretomic data that all secreted proteins
that were Out-dependent were also DsbA dependent (Table 1). This strongly suggested that the Out secretion machinery was itself DsbA-dependent. To confirm this hypothesis, the major endoglucanase, CelV, was used as an example of a DsbA-dependent secreted protein lacking Cys residues.

Western blotting with an anti-CelV antibody (36) was used to detect CelV in the supernatant and cell-associated (including cytoplasm and periplasm) fractions of wild type, dsbA, and out cultures grown to stationary phase in PMM. CelV was detectable in the supernatant of the wild type but not the dsbA or out mutants, confirming the proteomic findings. In contrast, CelV protein was detectable in the cell-associated fractions of all three strains (Fig. 5A). CelV was present in the cell-associated fraction at higher levels in the out mutant than the wild type, as expected from a mutant unable to secrete the protein. Similarly, in the dsbA mutant, CelV was present in the cell-associated fraction at higher levels than in the wild type, but was not present in the supernatant, demonstrating a secretion defect in this mutant. Spectrophotometric Cel activity assays were performed on supernatant and cell-associated samples of wild type, dsbA, out and celV cultures. A defined celV mutant, SCC29 (see Experimental Procedures for construction), showed undetectable Cel activity, confirming that CelV was responsible for all detectable Cel activity in Eca under these conditions. Similar to the western blot, Cel activity was detected in the supernatant only in the wild type strain but was detectable in the cell-associated fraction of the wild type, out and dsbA strains; also more Cel activity was detectable in the cellular fractions of the out and dsbA mutants than in the wild type (Fig. 5B). Thus the cell-associated CelV in the dsbA and out mutants showed enzymatic activity, again consistent with a secretion defect. The reason why less cellular Cel/CelV was observed in the dsbA mutant than in the out mutant is not clear, but it could be due to slightly reduced transcription (see below) and/or to greater activity of proteases in the 'stressed' periplasm of the dsbA mutant.

The Pul T2SS of Klebsiella oxytoca is also DsbA dependent (37) and, similarly, the Out system of Ech is unable to secrete the Cys-free substrate, RhiE, in a dsbA mutant (38). In contrast, we concluded previously that the Out system in

Ecc SCRI193 was not DsbA-dependent, since some CelV, also lacking Cys residues, could be secreted in the dsbA mutant (23). However, in this earlier study, less than half the total CelV produced was secreted, in contrast with 80% secretion in the wild type, suggesting that Out function was impaired, although not eliminated. The reason why Out function was not eliminated in the earlier study is likely to be because it was performed in media containing yeast extract, whereas in the current study a minimal medium was used. It has been reported that oxidising compounds (e.g. cystine) in rich media can at least partially suppress some phenotypes of dsbA mutants by nonspecific oxidation (32). Indeed we observed that the motility defect of the Eca dsbA mutant was much less severe when the swim agar contained tryptone (data not shown). However, we are unable to rule out the possibility of a difference in behaviour between the subspecies. Examination of the predicted Out protein sequences in Eca SCRI1043 revealed that only two Out proteins, the minor pseudopilin, OutK, and the secretin 'pilot' protein, OutS, have Cys residues predicted to be suitably located in the periplasm. Thus these two proteins are candidates for DsbA-dependent disulphide bonding. Consistent with this prediction, both PulK and PulS, the corresponding T2SS components in K. oxytoca, are disulphide bonded and at least the disulphide bond in PulK is required for T2SS function (39).

Reduced PrtW and OHHL are due to a transcriptional effect of dsbA mutation - The impairment of Out secretion in the dsbA mutant is able to explain the absence of certain Cys-free proteins from the secretome of the dsbA mutant, e.g. CelV. However other Cys-free, Out-independent proteins were also affected in the dsbA mutant. For example, levels of the major secreted protease, PrtW, and secreted Prt activity were significantly reduced in the dsbA mutant (Fig. 1, 2; Table 1). PrtW contains no cysteines, is Out independent and, by analogy with Ech, is predicted to be secreted by the adjacent T1SS (whose periplasmic-exposed components, PrtEF, also lack Cys residues) and hence would not access the periplasm. In order to determine whether the PrtW defect was at the level of transcript abundance, quantitative RT-PCR (qRT-PCR) was used to compare the level of prtW
mRNA between the wild type and the dsbA mutant. The abundance of prtW mRNA was indeed reduced 4x in the dsbA mutant compared with the wild type (Table 2), consistent with the observed 3-4x fold reduction in secreted PrtW/Prt activity (Fig 2A, Table 1). The cytoplasmic AHL synthase, ExpI, would also not depend on DsbA for activity, yet levels of OHHL were reduced in the dsbA mutant to 66% of the wild type. qRT-PCR analysis of the expl transcript showed that it was reduced to 64% of wild type levels in the dsbA mutant (Table 2), providing an explanation for the observed decrease in OHHL levels. Given these results, and since altered transcription of three secreted enzyme genes in a dsbA mutant had been suggested previously (23), we examined other related genes by qRT-PCR, including those encoding dsbA-dependent secreted proteins. As shown in Table 2, the transcript levels of pelC, pehA, svx and celB, were decreased 2-5x in the dsbA mutant compared with the wild type. The transcripts encoding the transcriptional regulator, Hor, and the dsbA-dependent conserved hypothetical protein, ECA2134, were also significantly decreased in the dsbA mutant compared with the wild type. In addition, nip and celW were decreased to 50% and 75% of wild type levels, respectively, although the results did not meet the p < 0.05 significance threshold. Control genes, including rpoS, recA, and dnaA were not significantly affected in the dsbA mutant (data not shown), although rpoA was found to be > 2x increased in the dsbA mutant (Table 2).

Most of the above genes found to have reduced transcript levels in the dsbA mutant are known to be regulated by QS in Eca (6,14). It was therefore possible that the reduced transcript levels of these genes (and hence also the reduced secreted PrtW) was due simply to the decreased OHHL levels observed in the dsbA mutant (Fig. 3). However addition of 5 μM OHHL to the culture medium had no effect on either the reduced levels of secreted Prt or the reduced transcript levels in the dsbA mutant (data not shown). We noted that not only did the set of genes influenced by dsbA and expl in Erwinia carotovora appear to overlap (this study; (14,23), but also that the QS-dependence of dsbA expression had never been tested in the model Ecc strain, SCRI193. In order to examine further a possible relationship between dsbA and expl in Erwinia, the QS-dependence of dsbA expression was determined in both Ecc SCR1193 and Eca SCR11043 using dsbA-uidA transcriptional fusions. In both Ecc and Eca, expression of dsbA-uidA throughout growth was modestly reduced in the QS mutant and could be restored by the addition of OHHL (Fig. 6). Interestingly, expression of an expl-lacZ fusion was unaffected in the dsbA mutant of either strain (data not shown), suggesting that the dsbA impact on expl transcript levels (Table 2) might be post-transcriptional. Although the transcriptional impact of dsbA inactivation was independent of QS, modulation of OHHL levels in the presence of functional DsbA might be expected to 'fine tune' the regulatory system in vivo. Similarly, the modulation of dsbA expression by QS might provide a means of 'topping-up' DsbA protein levels to cope with the simultaneous increase in substrate expression.

Microarray analysis confirms a pleiotropic transcriptional impact of DsbA inactivation – The qRT-PCR experiments, targeting specific genes of interest, showed that absence of DsbA produced a transcriptional effect on multiple genes. This suggested that DsbA might influence the transcription of an even larger set of genes. To test this hypothesis, a genome-wide transcriptional profiling experiment to compare the dsbA mutant with the wild type was performed using an Eca SCR11043 microarray. This confirmed that DsbA does indeed exert a wide-ranging but defined transcriptional influence. In total, 184 genes showed significantly altered transcript levels (> 2x change, p < 0.05) in the dsbA mutant compared with the wild type, with 91 decreased and 93 increased in the mutant (Supplementary Table 2). Details of selected genes altered in the dsbA mutant are given in Table 3.

The expression of very many known or predicted virulence-related genes was reduced in the dsbA mutant (Table 3). Consistent with the qRT-PCR results, transcript levels of expl, svx, pelA, prtW and celB were significantly decreased in the mutant (and rpoA was significantly increased). The pehA and nip transcripts were also decreased in the dsbA mutant (p < 0.05) but missed the stringent 2x cut-off (relative abundance values of 0.77 and 0.57 respectively; data not shown). Many other secreted virulence factor-related genes were also identified as being decreased in expression in the dsbA mutant, e.g.
four other predicted Pels, genes involved in pectin breakdown product uptake (kdgT, kdgM, togM), a predicted virulence-related outer membrane protein and several putative Type VI secretion-related genes, including the putative secretion substrate, HepA (40). Pnl was decreased by ~50% in the secretome of the dsbA mutant (Table 2), and the corresponding transcript was decreased in the dsbA mutant (p < 0.05) but just missed the 2x cut-off (relative abundance 0.56; data not shown).

Motility is an important virulence determinant in bacterial pathogens. The microarray experiment revealed that expression of 25 genes involved in motility and chemotaxis, or predicted to encode methyl-accepting chemotaxis proteins, was significantly decreased in the dsbA mutant (Table 3). For example, the major flagellin (flIC), the flagellar hook-associated protein-2 (flID) and the anti-sigma factor (flgM) were reduced at least 10x in the dsbA mutant. To verify this result, qRT-PCR analysis of flIC was performed and gave a significant, 25x decrease in flIC transcript levels in the dsbA mutant (Table 2). These transcriptomic data are consistent with the virtual absence in the dsbA mutant of all the flagellar proteins observed in the secretome of the wild type (Table 1) and with the lack of motility of this mutant (Fig. 4). Flagellar gene expression is sequential and hierarchical, in concert with the ordered assembly of the organelle (41-43). In a dsbA mutant, the P-ring, and thus the flagellar basal body, cannot be assembled properly due to a lack of disulfide bonding in FlgI (44). Since late gene expression is dependent on successful completion of hook-basal body assembly, it is unsurprising that late/FliA-dependent genes (e.g. flIC, motB, cheA and flID) were reduced in expression. However all types of flagellar transcripts, early (flhC) and middle (e.g. flgC, flgM, flgK and flID), as well as late, were reduced in the dsbA mutant. Thus the entire flagellar regulon is down-regulated in response to DsbA inactivation. This response is likely to be mediated via the master transcriptional regulators, FlhDC, which are required for the expression of all the other flagellar genes and are regulated by multiple other regulatory systems (43). (As well as flhC, the flhD transcript was also decreased in the dsbA mutant by 1.7x (p < 0.05), data not shown).

The co-ordinated downregulation of many virulence-related genes in the dsbA mutant suggests the existence of an important pleiotropic virulence regulator(s) which is responsive to periplasmic redox status and/or the presence of misfolded proteins and can influence the expression of multiple genes in response to this cue. Presumably such a system allows the expression of virulence determinants to be co-ordinately ‘dampened down’ if their post-translational processing cannot proceed efficiently. The predicted regulatory protein(s) responsible for sensing the absence of DsbA activity in the periplasm and relaying this signal back to gene expression in the cytoplasm is not yet known. The regulatory system may involve previously-identified regulators of virulence factor production (e.g. PecSM, AepA) or of virulence and motility (e.g. HexA) in Erwinia (3,7,45), and/or components of known periplasmic stress response pathways, e.g. Cpx and σE (46,47). Alternatively it may involve novel regulatory proteins, such as the predicted GntR-family transcriptional regulator, ECA0348, whose expression was decreased 5x in the dsbA mutant. A 3x reduction was also seen in the slyA family virulence regulator, hor. In order to detect the presence/absence of DsbA activity, we predict that the primary sensor in such a signal cascade will be a protein that is structurally disulphide-bonded, redox regulated or sensitive to misfolded proteins.

The microarray also identified a set of transcripts increased in abundance in the dsbA mutant (Table 3). Many of these represented known stress-response genes, consistent with a cellular response to protein misfolding in the periplasm, including cytoplasmic heat shock proteins (e.g. groLS and dnaK) and periplasmic stress response proteins: degP, pspA and osmY (47). In addition, 25 genes involved in protein synthesis were upregulated. This is also likely to be a response to protein misfolding and/or mistranslation. Unexpectedly, three genes in the coronafacic acid synthesis cluster (cfl, cfa6 and cfa8A) were also significantly upregulated in the dsbA mutant. The increase in cfl transcript levels was verified by qRT-PCR analysis, where the an increase of almost 20x was seen (Table 2). The cfa gene cluster is predicted to direct the biosynthesis of coronafacic acid, the polyketide precursor of the phytotoxin coronatine, and cfa has been implicated as an important virulence determinant (7). The increased expression of cfl/cfa may be simply due
to the misfolding and inactivation of a periplasmic negative regulator of cfa in the absence of DsbA. Alternatively, this finding may provide the first evidence that cfa expression is stress-induced. Other transcripts increased in abundance in the mutant include those of genes involved in anaerobic respiration and a putative exported protein (ECA0658), increased 7x in the dsbA mutant (Table 3).

DsbA has been shown to exert an effect on specific virulence gene expression at the transcript level in other organisms. For example, in P. aeruginosa, transcription of the Type III secretion regulator, exsA, and the downstream effector, exoT, were greatly decreased in a dsbA mutant (48). However, in the current study we have demonstrated a genome-wide transcriptional response to dsbA inactivation, including diverse virulence genes. This impact of DsbA on transcript levels could be via altered transcription rates and/or via altered mRNA stability.

Since the regulon of genes requiring dsbA for full expression is clearly populated by key virulence factors, novel genes identified as being part of this regulon are therefore good candidates for new virulence factors, for example, ECA3580, ECA3581, ECA2134 and the predicted transcriptional regulator ECA0348. This is clearly even more true for those which represent both DsbA-dependent transcripts and also Out/DsbA-dependent secreted proteins, for example ECA2134 (see above) and ECA3580. HecA, secreted by a Type V two-partner mechanism, has a role in virulence in Ech (49). In Eca, hecA2 is one of two adjacent open reading frames which show homology to Ech HecA but were judged likely to be pseudogenes (7). Surprisingly, this study revealed that HecA2 protein is expressed and that expression of hecB (encoding its putative transporter) is DsbA-dependent, in common with other virulence factors. The role of HecAB in Eca clearly merits further investigation. The transcript of another candidate virulence factor, the putative Type VI secretion system component, ECA3444, was also found to be decreased > 10x in the dsbA mutant. During the preparation of this manuscript, ECA3444 was confirmed to indeed be required for full virulence in planta in Eca SCRI1043 (50).

Conclusions – DsbA is required for the proper expression of very many, if not all, secreted virulence factors in the phytopathogen, Eca SCRI1043. The Out T2SS is responsible for the secretion of a wider range of virulence determinants than just PCWDEs. DsbA affects the production of multiple virulence factors at one or more levels: transcript abundance, protein stability and/or protein secretion. The transcriptional impact of dsbA mutation implies that a feedback regulatory system controls expression of a broad spectrum of virulence genes in response to extracytoplasmic conditions. Novel candidate virulence factors have been identified through their presence in the Out- and DsbA-dependent secretome and/or membership of the DsbA-dependent regulon.

REFERENCES
1. Perombelon, M. C. M. (2002) Plant Pathology 51, 1-12
2. Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. (2003) Molecular Plant Pathology 4, 17-30
3. Thomson, N. R., Thomas, J. D., and Salmond, G. P. (1999) Methods in Microbiology 29, 347-426
4. Toth, I. K., and Birch, P. R. (2005) Curr Opin Plant Biol 8, 424-429
5. Pemberton, C. L., Whitehead, N. A., Sebaihia, M., Bell, K. S., Hyman, L. J., Harris, S. J., Matlin, A. J., Robson, N. D., Birch, P. R., Carr, J. P., Toth, I. K., and Salmond, G. P. (2005) Mol Plant Microbe Interact 18, 343-353
6. Corbett, M., Virtue, S., Bell, K., Birch, P., Burr, T., Hyman, L., Lilley, K., Poock, S., Toth, I., and Salmond, G. (2005) Mol Plant Microbe Interact 18, 334-342
7. 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.,
8. Reeves, P. J., Whitcombe, D., Wharam, S., Gibson, M., Allison, G., Bunce, N., Barallon, R.,
Douglas, P., Mulholland, V., Stevens, S., and et al. (1993) Mol Microbiol 8, 443-456
9. Sandkvist, M. (2001) Infect Immun 69, 3523-3535
10. Filloux, A. (2004) Biochim Biophys Acta 1694, 163-179
11. Delepelaire, P. (2004) Biochim Biophys Acta 1694, 149-161
12. 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) Antonie Van Leeuwenhoek 81, 223-231
13. Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J., and Salmond, G. P. (2001) FEMS
Microbiol Rev 25, 365-404
14. Burr, T., Barnard, A. M., Corbett, M. J., Pemberton, C. L., Simpson, N. J., and Salmond, G. P.
(2006) Mol Microbiol 59, 113-125
15. Mattinen, L., Nissinen, R., Riipi, T., Kalkkinen, N., and Pirhonen, M. (2007) Proteomics 7, 3527-
3537
16. Kazemi-Pour, N., Condemine, G., and Hugouvieux-Cotte-Pattat, N. (2004) Proteomics 4, 3177-
3186
17. Macnab, R. M. (2004) Biochim Biophys Acta 1694, 207-217
18. Kadokura, H., Katzen, F., and Beckwith, J. (2003) Annu Rev Biochem 72, 111-135
19. Nakamoto, H., and Bardwell, J. C. (2004) Biochim Biophys Acta 1694, 111-119
20. Yu, J., and Kroll, J. S. (1999) Microbes Infect 1, 1221-1228
21. Lasica, A. M., and Jagusztyn-Krynicka, E. K. (2007) FEMS Microbiol Rev 31, 626-636
22. Shevchik, V. E., Bortoli-German, I., Robert-Baudouy, J., Robinet, S., Barras, F., and Condemine,
G. (1995) Mol Microbiol 16, 745-753
23. Vincent-Sealy, L. V., Thomas, J. D., Commander, P., and Salmond, G. P. (1999) Microbiology
145, 1945-1958
24. Coulthurst, S. J., Lilley, K. S., and Salmond, G. P. (2006) Molecular Plant Pathology 7, 31-35
25. Kaniga, K., Delor, I., and Cornelis, G. R. (1991) Gene 109, 137-141
26. Toth, I., Perombelon, M., Salmond, G. (1993) Journal of General Microbiology 139, 2705-2709
27. Liu, H., Sadygov, R. G., and Yates, J. R., 3rd. (2004) Anal Chem 76, 4193-4201
28. Holeva, M. C., Bell, K. S., Hyman, L. J., Avrova, A. O., Whisson, S. C., Birch, P. R., and Toth, I.
K. (2004) Mol Plant Microbe Interact 17, 943-950
29. Winson, M. K., Swift, S., Fish, L., Throup, J. P., Jorgensen, F., Chhabra, S. R., Bycroft, B. W.,
Williams, P., and Stewart, G. S. (1998) FEMS Microbiol Lett 163, 185-192
30. Venkatesh, B., Babujee, L., Liu, H., Hedley, P., Fujikawa, T., Birch, P., Toth, I., and Tsuyumu, S.
(2006) J Bacteriol 188, 3088-3098
31. Chatterjee, A., McEvoy, J. L., Chambost, J. P., Blasco, F., and Chatterjee, A. K. (1991) J
Bacteriol 173, 1765-1769
32. Hiniker, A., and Bardwell, J. C. (2004) J Biol Chem 279, 12967-12973
33. Vertommen, D., Depuydt, M., Pan, J., Leverrier, P., Knoops, L., Szikora, J. P., Messens, J.,
Bardwell, J. C., and Collet, J. F. (2008) Mol Microbiol 67, 336-349
34. Agudo, D., Mendoza, M. T., Castanares, C., Nombela, C., and Rotger, R. (2004) Proteomics 4,
355-363
35. Hiniker, A., Collet, J. F., and Bardwell, J. C. (2005) J Biol Chem 280, 33785-33791
36. Walker, D. S., Reeves, P. J., and Salmond, G. P. (1994) Mol Plant Microbe Interact 7, 425-431
37. Sauvonnet, N., and Pugsley, A. P. (1998) Mol Microbiol 27, 661-667
38. Laatu, M., and Condemine, G. (2003) J Bacteriol 185, 1642-1649
39. Pugsley, A. P., Bayan, N., and Sauvonnet, N. (2001) J Bacteriol 183, 1312-1319
40. Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L.,
Joachimiak, G., Ordonez, C. L., Lory, S., Walz, T., Joachimiak, A., and Mekalanos, J. J. (2006)
Science 312, 1526-1530
41. Berg, H. C. (2003) *Annu Rev Biochem* **72**, 19-54
42. Aldridge, P., and Hughes, K. T. (2002) *Curr Opin Microbiol* **5**, 160-165
43. McCarter, L. L. (2006) *Curr Opin Microbiol* **9**, 180-186
44. Dailey, F. E., and Berg, H. C. (1993) *Proc Natl Acad Sci U S A* **90**, 1043-1047
45. Harris, S. J., Shih, Y. L., Bentley, S. D., and Salmond, G. P. (1998) *Mol Microbiol* **28**, 705-717
46. Raivio, T. L. (2005) *Mol Microbiol* **56**, 1119-1128
47. Duguay, A. R., and Silhavy, T. J. (2004) *Biochim Biophys Acta* **1694**, 121-134
48. Ha, U. H., Wang, Y., and Jin, S. (2003) *Infect Immun* **71**, 1590-1595
49. Rojas, C. M., Ham, J. H., Deng, W. L., Doyle, J. J., and Collmer, A. (2002) *Proc Natl Acad Sci U S A* **99**, 13142-13147
50. Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P., Ravensdale, M., Burr, T., Takle, G., Brurberg, M. B., Birch, P. R., Salmond, G. P., and Toth, I. K. (2008) *PLoS Pathogens* In press

**FOOTNOTES**

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**FIGURE LEGENDS**

**FIGURE 1.** Inactivation of *dsbA* has a large impact on the secretome of *Eca* SCRI1043 as shown by 2D DiGE. Secreted proteins were prepared from wild type *Eca* SCRI1043 (left), SCC22 (*dsbA*, middle) and MC4 (*out*, right) and labelled with different Cy dyes as indicated. The pooled sample was then separated by 2D SDS-PAGE (over pH 3-10) and the fluorescent images visualised. Proteins absent or at greatly reduced levels in both the *dsbA* and the *out* mutants are labelled in red, whilst those absent or greatly reduced in the *dsbA* mutant but present in the *out* mutant are labelled in blue. Note that equal amounts of protein were labelled in each channel, although the total amount of secreted protein recovered was much lower in the *dsbA* mutant than the wild type.

**FIGURE 2.** The effect of DsbA inactivation on the levels of secreted pectate lyase (Pel), endoglucanase (Cel) and protease (Prt) enzyme activity. *A*, Levels of Pel (top), Cel (middle) and Prt (bottom) activity in the culture supernatant were determined at intervals throughout growth in PMM for wild type *Eca* SCRI1043 (WT, dark grey bars) and SCC22 (*dsbA* mutant, light grey bars). *B*, To demonstrate complementation of the *dsbA* phenotypes, levels of secreted Pel (top), Cel (middle) and Prt (bottom) were measured for WT (pQE80) [vector control, white bars], WT (pSJC58) [*dsbA in trans*, darker grey bars], SCC22 (pQE80) [lighter grey bars] and SCC22 (pSJC58) [black bars]. Secreted enzyme activities were measured as described in Experimental Procedures. Growth is reported as OD$_{600}$. A full key to symbols and shading is given at the base of each panel. Bars show mean +/- sem (n ≥ 3).

**FIGURE 3.** Production of OHHL is reduced in the *dsbA* mutant. *A*, Levels of OHHL activity in the culture supernatant were determined at intervals throughout growth in PMM for wild type *Eca* SCRI1043 (WT, dark grey bars) and SCC22 (*dsbA* mutant, light grey bars). *B*, To demonstrate complementation, levels of OHHL were measured for WT (pQE80) [vector control, white bars], WT (pSJC58) [*dsbA in trans*, darker grey bars], SCC22 (pQE80) [lighter grey bars] and SCC22 (pSJC58) [black bars]. OHHL levels were measured using the biosensor *E. coli* JM109 (pSB401) and are reported as light units (lu) per OD$_{600}$, relative to (A) WT at 20 h or (B) WT (pQE80) at 25 h (with these values set to 100). Growth is reported as OD$_{600}$. A full key to symbols and shading is given at the base of each panel. Bars show mean +/- sem (n ≥ 3).
FIGURE 4. The impact of DsbA inactivation on motility and virulence in potato tubers. A, Motility plate (0.3% agar) showing the swim haloes formed by wild type Eca SCR1043 (WT) and SCC22 (dsbA) after 18 h incubation. B, Motility of WT (pQE80) [vector control], SCC22 (pQE80) and SCC22 (pSJC58). C, Motility of wild type (dark grey bars) and SCC22 (light grey bars) in varying concentrations of Cu^{2+}. For panels B and C, motility is reported as halo area after 18 h incubation; bars show mean +/- sem (n ≥ 3). D, Potato tuber virulence assay measuring the amount of rotted tissue generated by WT (pQE80), SCC22 (pQE80) and SCC22 (pSJC58) after seven days incubation (inoculum of 10^7 cells); bars show mean +/- sem (n = 20).

FIGURE 5. CelV is not secreted to the medium in the dsbA mutant. A, Anti-CelV western blot of proteins in the supernatant (S) and the cell-associated (C) fractions of wild type Eca SCR1043 (WT), SCC22 (dsbA) and MC4 (out). Protein samples were prepared from equal volumes of culture (and resuspended in equal volumes of sample buffer) for each fraction and each strain (see Experimental Procedures). Protein samples were separated by 12% SDS-PAGE and immunoblotted with anti-CelV antibody; four times the amount of protein sample was loaded for the S than the C samples. B, Endoglucanase (Cel) activity was determined for supernatant and cell-associated culture samples prepared from wild type Eca SCR1043, SCC22, MC4 and SCC29 (celV), after growth for 24 h in PMM. Bars show mean +/- sem (n = 3).

FIGURE 6. Expression of a dsbA-uidA fusion is modulated by quorum sensing. A, Expression of dsbA-uidA in Ecc SCR193 was measured during growth in LB in a wild type background (SCC20, dsbA-uidA, black bars), a carI (expI) background (SCC21, dsbA-uidA, carI, light grey bars) and in SCC21 with exogenous 5 μM OHHL (SCC21 + OHHL, dark grey bars). B, Expression of dsbA-uidA in Eca SCR11043 was measured in PMM in a wild type background (SCC33, dsbA-uidA, black bars), an expI background (SCC32, dsbA-uidA, expI, light grey bars) and in SCC32 with 5 μM OHHL added (SCC21 + OHHL, dark grey bars). Expression of dsbA-uidA was measured as β-glucuronidase activity per cell and growth reported as OD_600. A full key to symbols and shading is given at the base of the figure. Bars show mean +/- sem (n = 3).
TABLE 1. Selected proteins present in the secretome of the wild type, *out* and *dsbA* mutants of *Eca* SCRI1043 as determined by mass spectrometry (spectral counting)*a*  

| Genomic no. | Name          | Wild Type Abundance* | out (MC4) Abundance | dsbA (SCC22) Abundance | Description                                      |
|------------|---------------|----------------------|---------------------|------------------------|--------------------------------------------------|
|            |               | [rank] | [sem] | [rank] | [sem] | [rank] | [sem] |                         |
|            | *Secreted virulence factors and plant cell wall degrading enzymes* |             |                     |                        |                                                  |
| ECA1094    | Pel-3         | 39.7 [18] | 10.7 | | | | | Pectate lyase |
| ECA2553    | dsbA          | 8.3 | 0.9 | | | | | Pectate lyase |
| ECA4067    | PelA          | 184.0 [6] | 21.5 | 43.3 [18] | 10.9 | 7.0 | * | Pectate lyase I |
| ECA4068    | PelB          | 199.3 [5] | 39.8 | 28.7 | 5.9 | 8.5 | | Pectate lyase II |
| ECA4069    | PelC          | 35.7 | 4.6 | 13.7 | 3.8 | | | Pectate lyase III |
| ECA4070    | PelZ          | 7.5 | * | | | | | Pectate lyase |
| ECA0931    | Slv            | 47.0 [13] | 8.5 | | | | | Secreted virulence protein |
| ECA3087    | Nip           | 9.0 | | | | | | Necrosis Inducing Protein |
| ECA1095    | PehA          | 236.0 [4] | 31.6 | 51.3 [14] | 18.3 | 70.7 [17] | 14.1 | Endo-polygalacturonase |
| ECA3111    | PehX          | 19.7 | 3.9 | | | | | Exo-polygalacturonase |
| ECA1981    | CelIV         | 28.3 | 4.3 | | | | | Endoglucanase V |
| ECA2220    |               | 12.0 | 3.6 | | | | | Putative cellulase |
| ECA2827    | CelB          | 10.7 | 3.3 | | | | | Beta(1,4)-glucan glucanohydrolase |
| ECA0852    |               | 63.7 [10] | 2.3 | 10.5 | * | 35.7 | 11.8 | Putative exported plant proteoglycan hydrodrolase |
| ECA2785    | PrtW          | 463.7 [2] | 41.4 | 426.0 [1] | 71.4 | 107.0 [7] | 7.6 | Metalloprotease |
| ECA1499    | Pnl           | 43.7 [15] | 13.8 | 40.3 [19] | 1.2 | 20.3 | 4.5 | Pectin lyase |
| ECA2118    | HecA          | 25.0 | 3.5 | 26.5 | * | | | Hemolysin/hemagglutinin-like protein |
| ECA4510    | PelX          | 18.0 | 2.6 | 25.0 | 3.5 | 52.0 | 21.5 | Periplasmic exopolygalacturonate lyase |
| ECA1190    | PehN          | | | | | | | Putative polygalacturonase |
| ECA2135    | PelB2         | | | | | | | Periplasmic pectate lyase |
| ECA3253    | PemA          | | | | | | | Pectinesterase A |
|            | *Flagellar motility* |             |                     |                        |                                                  |
| ECA1704    | FlgD          | 13.7 | 3.7 | | | | | Basal-body rod modification protein |
| ECA1705    | FlgE          | 27.0 | 8.5 | 32.7 | 2.3 | | | Flagellar hook protein |
| ECA1707    | FlgG          | 37.3 | 7.7 | 20.7 | 6.2 | | | Flagellar basal-body rod protein |
| ECA1710    | FlgJ          | 11.0 | | | | | | Peptidoglycan hydrolase |
| ECA1711    | FlgK          | 127.3 [7] | 18.8 | 146.7 [4] | 21.7 | | | Flagellar hook-associated protein 1 |
| ECA1712    | FlgL          | 101.7 [8] | 14.3 | 119.3 [5] | 18.9 | | | Flagellar hook-associated protein 3 |
| ECA1721    | FlkB          | 33.0 | * | 31.0 | * | | | Flagellar hook-length control protein |
| ECA1730    | FlID          | 356.0 [3] | 20.8 | 337.7 [3] | 6.3 | | | Flagellar hook-associated protein 2 |
| ECA1731    | FlIC          | 632.0 [1] | 137 | 382.3 [2] | 13.6 | 47.3 | 5.5 | Flagellin |
|            | *Novel out and/or dsbA-dependent secreted proteins* |             |                     |                        |                                                  |
| ECA2134    |               | 16.7 | 3.3 | | | | | Predicted ABC transporter substrate binding protein |
| ECA3580    |               | 7.7 | 1.7 | | | | | Conserved hypothetical protein |
| ECA3946    |               | 14.0 | 3.5 | | | | | Putative exported protein |
| ECA0467    |               | 30.0 | 4.4 | 17.7 | 3.2 | | | Hypothetical protein |
| ECA1185    |               | 23.0 | 5.9 | 18.0 | 1.5 | | | Hypothetical protein |
| ECA1186    |               | 14.7 | 2.7 | 20.3 | 0.3 | | | Hypothetical protein |
| ECA3090    |               | 19.5 | * | 12.0 | 0.6 | | | Hypothetical protein |
|            | *Other* | | | | | | | | |
| ECA0216    | TufA          | 53.0 [11] | 3.6 | 72.3 [7] | 10.5 | 152.7 [1] | 29.8 | Elongation factor Tu |
| ECA0303    | DegQ          | | | | | | | Exported protease |
| ECA0312    | GltB          | 26.0 | 4.2 | 24.3 | 7.3 | 94.3 [8] | 44.9 | Glutamate synthase large chain |
| ECA0625    | GroL          | 79.3 [9] | 9.7 | 108.3 [6] | 3.2 | 130.3 [3] | 24.8 | 60 kDa chaperonin |
| ECA0685    | Mdh           | 37.7 [20] | 2.7 | 53.7 [13] | 9.3 | 71.0 [16] | 8.1 | Malate dehydrogenase |
| ECA0894    | CysK          | 36.0 | 3.5 | 36.3 | 6.4 | 59.3 [20] | 17.3 | Cysteine synthase A |
| ECA1444    | Gnd           | 24.7 | 2.9 | 29.3 | 2.6 | 73.3 [14] | 31.1 | Phosphogluconate dehydrogenase |

*Table entries indicate the abundance values determined by mass spectrometry (spectral counting). The rank column indicates the order of abundance from highest to lowest. The sem column represents the standard error of the mean. The asterisk (*) indicates a significant abundance difference compared to the wild type. Additional descriptions are provided for each protein.*
| Protein ID | Protein Name               | Abundance | Error | Rank | Remarks                           |
|-----------|----------------------------|-----------|-------|------|-----------------------------------|
| ECA1751   | OmpA                       | 19.3      | 2.7   | 24.7 | Outer-membrane protein A          |
| ECA2324   | OppA                       | 23.3      | 1.9   | 34.0 | Periplasmic oligopeptide-binding  |
| ECA2344   | GapA                       | 33.3      | 3.2   | 36.7 | Glyceraldehyde 3-phosphate        |
| ECA2406   | TogB                       | 26.0      | 3.5   | 58.0 | Periplasmic pectic oligomers      |
| ECA2407   | KdgM                       | 24.7      | 2.7   | 38.0 | Oligogalacturonate-specific porin |
| ECA2542   | OmpN                       | 40.3      | 5.7   | 59.0 | Isocitrate dehydrogenase [NADP]   |
| ECA2591   | RpsA                       | 27.3      | 3.4   | 38.3 | Outer membrane protein            |
| ECA2771   | MalE                       | 11.0      | *     | 10.0 | 3OS ribosomal protein S1          |
| ECA3175   | Icd                        | 23.0      | 1.7   | 30.3 | Putative peptidase                |
| ECA3258   | PurL                       | 12.7      | 1.2   | 18.0 | Maltose-binding periplasmic protein |
| ECA3789   | AceE                       | 38.3      | 4.8   | 51.3 | Predicted ABC transporter, substrate binding protein |
| ECA3882   | DnaK                       | 46.3      | 1.2   | 59.0 | Phosphoribosylformylglycin-amidine synthase |
| ECA4036   | FusA                       | 26.7      | 2.7   | 43.7 | Pyruvate dehydrogenase e1 subunit |
| ECA4161   | ECA3175                    | 47.7      | 12.7  | 53.7 | Chaperone protein                 |
| ECA4161   | ECA3258                    | 38.3      | 12.7  | 53.7 | Elongation factor G               |

a. The table includes all proteins which were in the top twenty identifications in any strain, together with selected other proteins of interest and examples. A complete list of all proteins detected and analysed in this experiment is given in Supplementary Table 1. b. Abundance was measured as the total number of spectra detected for each protein (see text) and is expressed as the mean of two or three replicates. A number in square brackets indicates the rank of that protein in terms of abundance within that strain (for the top twenty most abundant proteins in each strain). Where no value is given, the protein was not detectable in that strain at the stringency used (see Experimental Procedures). c. Sem (standard error of the mean) is given where three replicate values were obtained. An asterisk represents that the protein was detected in only two of three replicates. d. Note that there are several peptides identical between the PelA, PelB and PelC isoforms. All spectra corresponding to these peptides were excluded from the analysis, hence the total number of ions detected (and thus the abundance of these proteins) is actually higher than suggested by the values given.
| Gene     | Genome identifier | Relative Abundance (dsbA/WT) | P value $^b$ |
|----------|-------------------|-----------------------------|--------------|
| prtW     | ECA2785           | 0.25                        | 0.0003       |
| celB     | ECA2827           | 0.22                        | 0.0006       |
| pelC     | ECA4069           | 0.47                        | 0.0163       |
| pehA     | ECA1095           | 0.39                        | 0.0410       |
| svx      | ECA0931           | 0.35                        | 0.0004       |
| expI     | ECA0105           | 0.64                        | 0.0354       |
| hor      | ECA1931           | 0.36                        | 0.0150       |
| ECA2134  | ECA2134           | 0.37                        | 0.0006       |
| fliC     | ECA1731           | 0.039                       | 0.0038       |
| rpoA     | ECA4006           | 2.3                         | 0.0024       |
| cfl      | ECA0609           | 19.9                        | 0.0009       |
| nip      | ECA3087           | 0.50                        | 0.0790       |
| celV     | ECA1981           | 0.75                        | 0.0684       |
| dnaA     | ECA4441           | 1.1                         | 0.1480       |

$^a$. Relative abundance of transcripts is expressed as dsbA/wild type; hence a value of 0.2 represents a 5x decrease in abundance in the dsbA mutant compared with the wild type and a value of 5 represents a 5x increase.  

$^b$. Transcripts were considered to be significantly altered if p < 0.05 (n ≥ 3); dnaA, celV and nip are not significantly altered but are referred to in the text.
TABLE 3. Selected genes with transcripts altered in abundance in the *dsbA* mutant of *Eca* SCRI1043 compared with the wild type by microarray analysis.

| Gene   | Name      | Abundance (dsbA/WT)* | P value   | Description                                      |
|--------|-----------|----------------------|-----------|--------------------------------------------------|
| I) Decreased in *dsbA* mutant (SCC22)                      |
| Virulence factor-related                                   |
| ECA0105  | expl      | 0.4402               | 0.001723  | N-acylhomoserine lactone synthase                 |
| ECA0931  | svx       | 0.3298               | 0.000004  | Secreted protein required for virulence          |
| ECA4067  | pelA      | 0.2277               | 0.000001  | Pectate lyase I                                  |
| ECA1094  | pel-3     | 0.4054               | 0.000255  | Pectate lyase                                    |
| ECA2553  |           | 0.3502               | 0.000104  | Pectate lyase                                    |
| ECA3112  |           | 0.4409               | 0.035056  | Pectate lyase                                    |
| ECA4510  | pelX      | 0.3574               | 0.009923  | Periplasmic exopolysaccharide lyase               |
| ECA2785  | prtW      | 0.3682               | 0.000652  | Secreted metalloprotease                          |
| ECA2827  | celB      | 0.3397               | 0.001322  | Secreted β(1,4)-glucan glucanohydrolase          |
| ECA4124  | kdgT      | 0.4884               | 0.004670  | 2-keto-3-deoxygluconate permease                 |
| ECA2302  | kdgM      | 0.4477               | 0.000457  | Oligogalacturonate-specific porin                 |
| ECA2403  | togM      | 0.4740               | 0.000178  | Pectic oligomers transporter, inner membrane protein |
| ECA0045  |           | 0.3401               | 0.001937  | Putative virulence-associated OMP (Rck-like)      |
| ECA2116  | hecB      | 0.3740               | 0.000012  | HecA transport protein                           |
| ECA3431  |           | 0.4027               | 0.000383  | Putative Type VI secretion system component      |
| ECA3444  |           | 0.0959               | 0.000081  | Putative Type VI secretion system component      |
| ECA3428/ | hcpA      | 0.1062               | 0.000703  | Putative Type VI secretion system substrate       |
| ECA4275b |           |                      |           |                                                  |
| Motility/chemotaxis                                       |
| ECA1686  | flhC      | 0.4824               | 0.000234  | Flagellar transcriptional activator              |
| ECA1688  | motB      | 0.2355               | 0.000002  | Motor protein                                    |
| ECA1689  | cheA      | 0.1219               | 0.000003  | Chemotaxis protein                               |
| ECA1690  | cheW      | 0.1302               | 0.000021  | Chemotaxis protein                               |
| ECA1691  | cheD      | 0.0869               | 0.000003  | Methyl-accepting chemotaxis protein              |
| ECA1692  | cheR      | 0.4934               | 0.001700  | Chemotaxis protein methyltransferase             |
| ECA1694  | cheY      | 0.4940               | 0.000948  | Chemotaxis protein                               |
| ECA1699  | flgN      | 0.1087               | 0.000000  | Flagella synthesis protein                       |
| ECA1700  | flgM      | 0.1098               | 0.000055  | Regulator of flagellin synthesis                  |
| ECA1703  | flgC      | 0.4639               | 0.004149  | Flagellar basal-body rod protein                 |
| ECA1707  | flgG      | 0.4984               | 0.001621  | Flagellar basal-body rod protein                 |
| ECA1711  | flgK      | 0.1232               | 0.000000  | Flagellar hook-associated protein 1              |
| ECA1712  | flgL      | 0.2077               | 0.000001  | Flagellar hook-associated protein 2              |
| ECA1729  | flIS      | 0.1455               | 0.000002  | Flagellar protein                                |
| ECA1730  | flID      | 0.0973               | 0.000005  | Flagellar hook-associated protein 2              |
| ECA1731  | flIC      | 0.0756               | 0.000000  | Flagellin                                        |
| ECA1740  | fliTZ     | 0.2444               | 0.000142  | Putative regulatory protein                      |
| ECA0183  |           | 0.3142               | 0.000031  | Putative methyl-accepting chemotaxis protein     |
| ECA0346  |           | 0.1102               | 0.000001  | Putative methyl-accepting chemotaxis protein     |
| ECA1105  |           | 0.1438               | 0.000004  | Putative methyl-accepting chemotaxis protein     |
| ECA1509  |           | 0.2827               | 0.000022  | Putative methyl-accepting chemotaxis protein     |
| ECA3902  |           | 0.2648               | 0.000000  | Putative methyl-accepting chemotaxis protein     |
| ECA4120  |           | 0.4789               | 0.002317  | Putative methyl-accepting chemotaxis protein     |
| ECA4333  |           | 0.1056               | 0.000001  | Putative methyl-accepting chemotaxis protein     |
| ECA1368  |           | 0.2058               | 0.000010  | Putative chemotaxis signal transduction protein  |

**Other**
| Gene   | Log2 Fold Change | p-value | Description                                      |
|--------|-----------------|---------|-------------------------------------------------|
| ECA0348 | 0.2071          | 0.000000| GntR-family transcriptional regulator          |
| ECA3580 | 0.1155          | 0.000198| Conserved hypothetical protein                   |
| ECA3581 | 0.1748          | 0.000033| Conserved hypothetical protein                   |

II) Increased in dsbA mutant (SCC22)

**Virulence-related**

| Gene   | Log2 Fold Change | p-value | Description                                      |
|--------|-----------------|---------|-------------------------------------------------|
| ECA0609 | 6.045           | 0.001210| Coronafacate ligase                              |
| ECA0603 | 3.495           | 0.000649| Type I polyketide synthase                       |
| ECA0601 | 2.457           | 0.016803| Putative oxidoreductase                          |

**Stress response**

| Gene   | Log2 Fold Change | p-value | Description                                      |
|--------|-----------------|---------|-------------------------------------------------|
| ECA0624 | 3.601           | 0.000000| 10 kDa Chaperonin                               |
| ECA0625 | 2.525           | 0.000957| 60 kDa Chaperonin                               |
| ECA3882 | 3.395           | 0.001244| Chaperone protein                               |
| ECA2427 | 2.779           | 0.004344| Heat shock protein                              |
| ECA3301 | 2.542           | 0.000001| Protease D₀                                     |
| ECA4261 | 2.841           | 0.001464| Heat shock protein (ATP-dependent protease)     |
| ECA4262 | 2.911           | 0.002044| Heat shock protein (ATP-binding subunit)        |
| ECA4403 | 3.820           | 0.000022| Heat shock protein A                             |
| ECA1983 | 3.597           | 0.005503| Phage shock protein A                           |
| ECA1984 | 2.091           | 0.038622| Phage shock protein C                           |
| ECA0469 | 2.468           | 0.003059| Osmotically-inducible protein Y                 |

**Protein synthesis**

| Gene   | Log2 Fold Change | p-value | Description                                      |
|--------|-----------------|---------|-------------------------------------------------|
| ECA2591 | 2.404           | 0.00076 | 30S ribosomal protein S1                        |
| ECA4012 | 2.372           | 0.00319 | 50S ribosomal protein L15                       |
| ECA4013 | 2.476           | 0.00156 | 50S ribosomal protein L30                       |
| ECA4014 | 2.668           | 0.00079 | 30S ribosomal protein S5                        |
| ECA4016 | 2.452           | 0.00002 | 50S ribosomal protein L6                        |
| ECA4018 | 2.087           | 0.01287 | 30S ribosomal protein S14                       |
| ECA4023 | 2.592           | 0.00073 | 50S ribosomal protein L29                       |
| ECA4026 | 6.073           | 0.00020 | 50S ribosomal protein L22                       |
| ECA4027 | 6.668           | 0.00000 | 30S ribosomal protein S19                       |
| ECA4029 | 5.412           | 0.00016 | 50S ribosomal protein L23                       |
| ECA4036 | 2.460           | 0.00450 | Elongation factor G                             |
| ECA3357 | 2.380           | 0.00215 | tRNA (guanine-N1) methyltransferase             |
| ECA4011 | 2.505           | 0.00048 | Preprotein translocase subunit                  |
| ECA3875 | 2.007           | 0.01805 | Lipoprotein signal peptidase                    |

**Anaerobic respiration**

| Gene   | Log2 Fold Change | p-value | Description                                      |
|--------|-----------------|---------|-------------------------------------------------|
| ECA1407 | 3.116           | 0.00088 | Formate dehydrogenase, nitrate-inducible, Fe-S subunit |
| ECA1408 | 3.121           | 0.00032 | Formate dehydrogenase, nitrate-inducible, major subunit |
| ECA2033 | 3.174           | 0.00067 | Respiratory nitrate reductase 1, delta chain     |
| ECA2034 | 3.950           | 0.00037 | Respiratory nitrate reductase 1, gamma chain     |
| ECA1228 | 2.425           | 0.00762 | Hydrogenase 2, large subunit                    |

**Other**

| Gene   | Log2 Fold Change | p-value | Description                                      |
|--------|-----------------|---------|-------------------------------------------------|
| ECA0658 | 7.250           | 0.00093 | Putative exported protein                        |
| ECA4006 | 2.043           | 0.01278 | DNA-directed RNA polymerase alpha chain          |

**a.** Relative abundance of transcripts is expressed as dsbA/wild type; hence a value of 0.2 represents a 5x decrease in abundance in the dsbA mutant compared with the wild type and a value of 5 represents a 5x increase. **b.** The two HcpA homologues ECA3428 and ECA4275 are indistinguishable in the microarray; the values given are from a probe bound by both genes. **c.** Only a representative selection of the altered genes involved in protein synthesis is given. All the genes significantly altered in the dsbA mutant are detailed in Supplementary Table 2.
Figure 1

Wild type
Cy2-labelled

SCC22 (dsbA)
Cy3-labelled

MC4 (out)
Cy5-labelled
Figure 4

A

B

C

D
Figure 5

A

\[ \text{CelV} \rightarrow \]

\begin{array}{cccc}
C & S & C & S & C & S \\
\text{WT} & \text{dsbA} & \text{out} \\
\end{array}

B

\begin{tikzpicture}
\begin{axis}[
    title = {Cell activity (100\text{U}A_550/\text{min}\text{OD}_{600})},
    ybar, 
    enlarge x limits = 0.5, 
    symbolic x coords = {WT, dsbA, out, celV}, 
    xtick = data, 
    nodes near coords, 
]
\addplot[ybar, fill=gray!30] coordinates {
    (WT, 1.8) 
    (dsbA, 0) 
    (out, 0) 
    (celV, 0) 
};
\addplot[ybar, fill=black] coordinates {
    (WT, 0) 
    (dsbA, 0.6) 
    (out, 1.2) 
    (celV, 1.8) 
};
\legend{supernatant, cell-associated}
\end{axis}
\end{tikzpicture}
Figure 6

A

\[\text{\(\mu\)-glucuronidase (\(\Delta A_{405}\) min/ml/OD\(_{600}\))}\]

\[
\begin{array}{c}
0 & 2 & 4 & 6 & 8 & 10 & 12 \\
\text{Time (h)} & \\
\end{array}
\]

\[
\begin{array}{c}
0 & 1 & 2 & 3 & 4 \\
\text{OD\(_{600}\)} & \\
\end{array}
\]

- SCC20 activity
- SCC20 growth
- SCC21 activity
- SCC21 growth
- SCC21 + OHHL activity
- SCC21 + OHHL growth

B

\[\text{\(\mu\)-glucuronidase (\(\Delta A_{405}\) min/ml/OD\(_{600}\))}\]

\[
\begin{array}{c}
0 & 6 & 9 & 12 & 15 & 18 \\
\text{Time (h)} & \\
\end{array}
\]

\[
\begin{array}{c}
0 & 1 & 2 & 3 & 4 \\
\text{OD\(_{600}\)} & \\
\end{array}
\]

- SCC33 activity
- SCC33 growth
- SCC32 activity
- SCC32 growth
- SCC32 + OHHL activity
- SCC32 + OHHL growth
DsbA plays a critical and multi-faceted role in the production of secreted virulence factors by the phytopathogen, Erwinia carotovora subsp. atroseptica
Sarah J. Coulthurst, Kathryn S. Lilley, Peter E. Hedley, Hui Liu, Ian K. Toth and George P. C. Salmond

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