Erwinia carotovora DsbA mutants: evidence for a periplasmic-stress signal transduction system affecting transcription of genes encoding secreted proteins

Lois V. Vincent-Sealy, Joanna D. Thomas, Paul Commander and George P. C. Salmond

The dsbA genes, which encode major periplasmic disulfide-bond-forming proteins, were isolated from Erwinia carotovora subsp. carotovora (Ecc) and Erwinia carotovora subsp. atroseptica (Eca), and the dsbC gene, encoding another periplasmic disulfide oxidoreductase was isolated from Ecc. All three genes were sequenced and mutants deficient in these genes were created by marker exchange mutagenesis. The Ecc mutants were severely affected in activity and secretion of pectate lyase, probably due to the absence of functional PelC, which is predicted to require disulfide bond formation to achieve its correct conformation prior to secretion across the outer membrane. Similarly, endopolygalacturonase, also predicted to possess disulfide bonds, displayed reduced activity. The major Ecc cellulase (CelV) does not contain cysteine residues and was still secreted in dsbA-deficient strains. This observation demonstrated unequivocally that the localization and activity of the individual components of the Out apparatus are independent of disulfide bond formation. Surprisingly, cellulase activity was shown to be increased - two- to threefold in the DsbA mutant. This phenomenon resulted from transcriptional up-regulation of celV gene expression. In contrast, transcription of both pelC and peh were down-regulated in dsbA-deficient strains when compared to the wild-type. Protease (Prt) activity and secretion were unaffected in the Ecc dsbA mutant. Prt activity was considerably reduced in the double dsbA dsbC mutant. However Prt was secreted normally in this strain. The Eca dsbA mutant was found to be non-motile, suggesting that disulfide bond formation is essential for motility in this strain. All of the dsb mutants showed reduced tissue maceration in planta. These results suggest that a feedback regulation system operates in Ecc. In this system, defects in periplasmic disulfide bond formation act as a signal which is relayed to the transcription machinery regulating gene expression in diverse ways.

Keywords: Erwinia carotovora, general secretory pathway, secretion, exoenzymes, motility
INTRODUCTION

Erwinia carotovora subsp. carotovora (Ecc) and Erwinia carotovora subsp. atroseptica (Eca) are Gram-negative phytopathogens which cause soft rotting of vegetable crops via a battery of exoenzymes, including cellulases (Cel), pectate lyases (Pel), endopolygalacturonase (Peh), pectin lyase and pectin methylesterase. Several of these exoenzymes are secreted via the sec-dependent type II or general secretory pathway (GSP) in a two-step manner. The first step is analogous to sec-dependent export in Escherichia coli (Pugsley, 1993), and in the second step proteins are secreted from the periplasm to the extracellular milieu by a complex of at least 13 proteins comprising the Ecc Out apparatus (Reeves et al., 1993; unpublished data). This secretion pathway is highly conserved among Gram-negative bacteria and homologues of the Out proteins are involved in targeting virulence determinants in other plant and animal pathogens (Pugsley, 1993; Salmond & Reeves, 1993; Salmond 1994; Wharam et al., 1995).

Several Erwinia spp. produce protease (Prt) activity. The role of Prt in the virulence of Erwinia spp. is not clear (Andro et al., 1984). The normal secretion of Prt in Ecc Out mutants (Murata et al., 1990; Reeves et al., 1993) indicates that these enzymes are not secreted via the type II system. Evidence from work carried out in the closely related phytopathogen Erwinia chrysanthemi (Ech) strongly suggests that the type I secretory pathway is used instead (Wandersman & Letoffe, 1993). Proteins that go through this pathway do so in a sec-independent, one-step manner which does not appear to involve a free periplasmic intermediate (Pugsley, 1993).

Many of the proteins targeted via the type II pathway require the formation of disulfide bonds in the periplasm for activity (Peek & Taylor, 1992; Yu et al., 1992, 1993; Bortolli-German et al., 1994; Shevchik et al., 1994, 1995). Achieving a particular conformation might be necessary for periplasmic intermediates to present a signal that can be recognized by the secretion apparatus. However, attempts to identify such a signal have been inconclusive (Wong & Buckley, 1991; Py et al., 1993; McVay & Hamood, 1995; Lu & Lory, 1996; Sauvonnet & Pugsley, 1996; Palomaki & Saarilaiti, 1997).

In E. coli periplasmic disulfide bond formation is achieved by the Dsb system comprising a family of disulfide oxidoreductases which possess the -C-X-X-C-motif characteristic of thioredoxin and protein disulfide-isomerase (Missiakas & Raina, 1997). The periplasmic DsbA appears to be the most important, its role being to transfer disulfide bonds to folding proteins in the periplasm – a process that results in their oxidation (Bardwell, 1994). DsbC acts as a periplasmic isomerase involved in disulfide rearrangement, especially in proteins with several disulfide bonds (Rietsch et al., 1996).

Homologous members of the Dsb family have been found in other Gram-negative bacteria, and several are important in periplasmic folding of virulence factors prior to secretion (Peek & Taylor, 1992; Tomb, 1992; Yu et al., 1992, 1993; Yamanaka et al., 1994; Shevchik et al., 1994, 1995; Ishihara et al., 1995; Okamoto et al., 1995; Watari et al., 1995; Foreman et al., 1995; Abe & Nakazawa, 1996; Rodriguez-Pena et al., 1997). Absence of the Dsb system generally results in inactive proteins, a lack of secretion competence and retention of the unfolded proteins in the periplasm, where they are usually degraded by resident Prt (Peek & Taylor, 1992; Yu et al., 1992, 1993; Bortolli-German et al., 1994; Shevchik et al., 1994, 1995). However, the question as to why DsbA mutations reduce secretion via the type II pathway remains open.

Pullulanase (PulA) of Klebsiella oxytoca contains at least one DsbA-catalysed intramolecular disulfide bond. The absence of DsbA diminishes the rate of pullulanase secretion but the disulfide bonds in this enzyme are not needed for its secretion (Pugsley, 1992; Sauvonnet & Pugsley, 1998). By contrast, the major cellulase, CelZ, of Ech requires disulfide bond formation and hence DsbA for both enzyme stability and secretion (Bortolli-German et al., 1994). The role of DsbA in the secretion of lipase and aerolysin by Aeromonas has not been studied, but these proteins contain disulfide bonds whose removal has no effect on their secretion (Hardie et al., 1995; Brumlík et al., 1997). Thus, three different phenomena occur in related secretion systems.

It is presumed that a direct effect on the assembly or function of the secretion apparatus (Pugsley, 1992; Peek & Taylor, 1992; Yu et al., 1992; Bortolli-German et al., 1994; Savonnet et al., 1994, 1995; Sauvonnet & Pugsley, 1998) despite most of the components comprising the secretion apparatus being inner-membrane proteins protruding into the periplasm (Reeves et al., 1994; Bleves et al., 1996; Thomas et al., 1997). However, the potential for these periplasmic domains to form disulfide bonds is equivocal. Pugsley (1992) stated that although several of the K. oxytoca type II apparatus (Pul) proteins have one or more cysteine residues, almost all of these are in putative transmembrane regions. We noted that the Ecc OutK and OutN proteins (both of which have large periplasmic domains; Reeves et al., 1994) each contain two cysteines (residues 103 and 209 in OutK, and residues 145 and 177 in OutN) with the potential to form an intramolecular disulfide bond. Moreover, an alignment of the OutK and OutN homologues showed that these residues are highly conserved.

We decided to investigate the role of periplasmic disulfide bond formation in Ecc. This bacterium produces enzymes which are secreted via two different pathways – type I (Prt) and type II (pectinases and Cel) – and it was predicted that, due to the absence of a periplasmic intermediate, the Ecc Prt isozymes should achieve a Dsb-independent secretion-competent state. Most of the type-II-targeted proteins investigated have disulfide bonds (Hardy et al., 1988; Pugsley, 1992; Peek & Taylor, 1992; Bortoll-German et al., 1994; Shevchik et al., 1994, 1995) and, hence, present difficulties when used to assess the effect of disulfide bond formation on the secretion apparatus. Not all the proteins which traverse the Ecc type II pathway contain cysteine...
residues; unlike Echr CelZ (Guiseppi et al., 1988), the major Cel isozyme of Ecc, CelV, does not contain cysteines (Cooper & Salmond, 1993). However, Peh, and the major Pcl isozyme (PclC) of Ecc each contain four cysteines with potential to form two intramolecular disulfide bonds (Hinton et al., 1989, 1990). Therefore, we were in the position to test unequivocally if disulfide bond formation has any effect on the type II system. In addition, we investigated the role of disulfide bond formation in virulence in Ecc and Eca.

**METHODS**

Bacterial strains, DNA constructs, bacteriophages and media. Bacterial strains, DNA constructs and bacteriophages used are listed in Table 1. Bacterial strains were routinely grown with shaking at 250 r.p.m. in Luria broth (LB), or on Luria broth agar (LBA) or nutrient broth agar (NBA), containing appropriate antibiotics where necessary, at 37, 30 and 27 °C for *E. coli*, Ecc and Eca, respectively. Media supplements were added at the following final concentrations: ampicillin (Ap), kanamycin (Kn), spectinomycin (Sp) and chloramphenicol (Cm), 50 μg ml⁻¹; tetracycline (Tc) 10 μg ml⁻¹; streptomycin (Str), 50 μg ml⁻¹; IPTG, 30 μg ml⁻¹; 5-bromo-4-chloro-3-indolyl β-D-galactoside, 30 μg ml⁻¹; 5-bromo-4-chloro-3-indolyl β-D-glucurondime, 50 μg ml⁻¹. All general recombinant DNA techniques were performed as described by Sambrook et al. (1989). Plasmid DNA was introduced into *E. coli*, Ecc and Eca by electroporation (Solioz & Bentely, unpublished).

**DNA sequence analysis.** Nucleotide sequence analysis was performed by the method of Sanger et al. (1977) using the Sequenase (USB) DNA sequencing kit. The sequences were analysed using the Wisconsin Genetics Computer Group, version 8 (1994) computer program (GCG) and BLAST.

**Cell fractionation.** Ecc strains were grown in 5 ml Pcl minimal broth (PMB; Reeves et al., 1993) at 30 °C to stationary phase (OD₆₅₀ 4.0). An aliquot (0.5 ml) of 0.5 M Tris/HCl (pH 7.8) was added and after incubation for 10 min at room temperature the cultures were centrifuged (Labor 50-M, 3630 g, 5 min, 4 °C) and the supernatant harvested (extracellular fraction). The pellet was resuspended in 5 ml 50 mM Tris (pH 7.8) and sonicated (MSE sonicator) on ice for 3 × 30 s at an amplitude of 6 with a 0.7 inch probe at 4 °C, with 30 s rest between each sonication, to produce the cytoplasmic fraction. Cell debris was removed by centrifugation as before. Fractions were stored at −20 °C. The fidelity of cell fractionation was assessed by assaying for the marker enzymes β-lactamase (periplasmic) and β-galactosidase (BGal; cytoplasmic) as described previously (Reeves et al., 1993).

**Exoenzyme activity.** Exoenzyme activity was assessed using indicator media (Reeves et al., 1993) and quantified using spectrophotometric assays of cellular fractions (Hinton & Salmond, 1987; Reeves et al., 1993). BGal activity of strains carrying transcriptional lacZ fusions was assessed as follows. Overnight cultures were diluted in 50 ml PMB to an initial OD₆₅₀ of ~ 0.06. Three replicates were sampled at 30 °C and 250 r.p.m. to stationary phase ( ~ 10 h). Samples were removed, sonicated and assayed in triplicate as described previously (Reeves et al., 1993). Where necessary, OD₆₅₀ values were corrected for light scattering by cell debris using the formula OD₆₅₀ = (1/75 × OD₅₅₀) (Miller, 1992). Protein concentrations were determined using the Bio-Rad Protein Assay.

**Cosmid complementation of *E. coli* strain JCB571.** Three cosmid libraries were used to transduce the non-motile *E. coli* strain JCB571 following standard procedures (Sambrook et al., 1989). pSF6- and pHCT79-based cosmid libraries of Ecc SCR1193 packaged into phage λ (Sp⁸ and Ap⁸ selection, respectively; P. Reeves, unpublished) were used to isolate the Ecc dsbA and dsbC genes, respectively. A pSF6-based cosmid library of Eca SCR11043 packaged into phage λ (Sp⁸ selection; S. Bentely, unpublished) was used to isolate the Eca dsbA gene. Transductants were screened for restoration of motility as described below.

**Motility assay.** *E. coli* and Eca colonies were screened for complementation by stabbing into tryptone swam agar (TSA) plates (Wolfe & Berg, 1989) and incubating at 37 °C for 16 h, or at 27 °C for 48 h, respectively. The motility of Eca mutants was assessed via halo diameter after spotting 5 μl aliquots of cultures, grown overnight in PMB at 27 °C, onto TSA plates and incubating at 27 °C for 48 h.

**In planta potato tuber virulence assays.** Virulence assays were done essentially as described previously (Walker et al., 1994). Potatoes of the cultivars Colmo Tops, Pentland Javelin or Maris Piper were inoculated with 10⁶ bacterial cells and incubated for 96 h at 25 °C. Macerated tissue was removed and weighed every 24 h. Results were expressed as the mean value of six replicates of each strain for each time point. Each assay was carried out at least twice and using at least two different cultivars.

**Immunoblot analysis.** SDS-PAGE and immunoblot analysis were performed as described by Silhavy et al. (1984) and Sambrook et al. (1989), respectively. Proteins were transferred to a nitrocellulose filter (Hybond-C, Amersham) and incubated with primary antibody followed by a secondary anti-rabbit horseradish peroxidase antibody (Amersham). 4-Chloro-1-naphthol (Sigma) was used for signal detection.

**Marker exchange mutagenesis.** To generate pCM3, a gusA/Cm⁸ cassette (from pUD1C1; Bardonnent & Blanco, 1992) was excised on an EcoRI/HindIII fragment, end-filled and cloned into the Ecc dsbA gene (carried in pBH) at the XbaI site (which had also been end-filled). The ddBA—gusA allele was excised from pCM3 on a CiaI/SalI fragment and end-filled, then cloned into the Smal site of the suicide vector pKNG101 (Kaniga et al., 1991), generating pACS. The construction of marker exchange plasmids for the Ecc dsbC and Eca dsbA genes was carried out in a similar manner. A gusA/Kn⁸ cassette (from pUIDK1; Bardonnent & Blanco, 1992) was excised on a HindIII/HpaI fragment and end-filled. This fragment was inserted at the HpaI site of the Ecc dsbC gene carried in pHH, and at the NcoI (end-filled) site of the Eca dsbA gene carried in pBAT, to generate pCAK and pAM, respectively. A HindIII fragment from pCAK and an EcoRI/HindIII fragment from pAM were cloned separately into the Smal site of pKNG101 to generate the marker exchange constructs pCSK and pAMK, respectively.

To generate pVicL, a promoterless lacZ cassette (from pSH24; S. Harris, unpublished) was excised on a BamHI/HindIII fragment, end-filled and cloned into the EcoRV site of celV (carried on pVic626; Cooper & Salmond, 1993). The mutated gene was excised on a SphI/EcoRI fragment, end-filled and cloned into the Smal site of pKNG101, generating pVicL. lacZ transcriptional fusions were created in the Ecc pelC and
<table>
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<th>Strain/construct/phage</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<td><strong>Strains</strong>&lt;br&gt;Escherichia coli</td>
<td>CC118 (λpir) (ara-λce) araD lacZ4 galE galK phoA20 thi-1 rpsE rpoB argE(AM) recA1, lysogenized with λpir phage</td>
<td>Herrero et al. (1990)</td>
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<td></td>
<td>JCB570 phoR zih2::Tn10</td>
<td>Bardwell et al. (1991)</td>
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<td>JCB571 JCB570, dsbA::kan1</td>
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<td>HH26 pNJS000, TeH</td>
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<td>E. coli subsp. atroseptica (Eca)</td>
<td>SCR11043 Wild-type, motile</td>
<td>Mulholland et al. (1993)</td>
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<td>LS4AA SCR11043, dsbA::gusA/KnH, non-motile</td>
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<td>Harris et al. (1998)</td>
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<td>LS2C SCR1193, dsbC::gusA/KnH</td>
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<td>LS3AC SCR1193, dsbA::gusA/CmH</td>
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<td>SCRI1043 Wild-type, non-motile</td>
<td>Forbes &amp; Perembelon (1985)</td>
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<tr>
<td><strong>Plasmids/cosmids</strong></td>
<td>pBR322 Cloning vector, ApH TeH</td>
<td>Bolivar et al. (1977)</td>
</tr>
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<td>pSH24 Promotorless lacZ gene, ApH</td>
<td>S. Harris, University of Cambridge, UK</td>
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<td>pKNG101 Suicide vector, StrH SacB+</td>
<td>Kaniga et al. (1991)</td>
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<tr>
<td></td>
<td>pSF6COSM promotorless vector, Mob+ SpH StrH</td>
<td>Selvaraj et al. (1984)</td>
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<td>pNJS000 IncP, TeH Tetr</td>
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<td>cM1 pSF6, Ecc dsbA yihE yihD mobA, SpH</td>
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<td>cM2 pHC79, Ecc dsbC, ApH</td>
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<td>cAT1 pSF6, Ecc dsbA, SpH</td>
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<td>pMS16D pBR322, Ecc dsbA yihE yihD mobA, ApH</td>
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<td>pBAT pBR322, Ecc dsbA yihE, ApH</td>
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<td>pCM3 pBR322, Ecc dsbA::gusA/CmH, ApH</td>
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<td>pACS pKNG101, Ecc dsbA::gusA/CmH, StrH</td>
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<td>pACG pHH, Ecc dsbC::gusA/KnH, ApH</td>
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<td>pCSK pKNG101, Ecc dsbC::gusA/KnH, StrH</td>
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<td>pAM pBAT, Ecc dsbA::gusA/KnH, ApH</td>
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<td>pE4S pUC19, Ecc pelC, ApH</td>
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<tr>
<td><strong>Phage</strong></td>
<td>M13mp8 Sequencing coliphage vector</td>
<td>Messing &amp; Vieira (1982)</td>
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<td>pKP Ecc generalized transducing phage</td>
<td>Toth et al. (1993)</td>
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RESULTS AND DISCUSSION

Isolating dsb genes of Ecc SCR1193 and Ecc SCR11043

Cosmid complementation of JCB571 was used to isolate dsb genes of Ecc SCR1193 and Ecc SCR11043. \( \lambda \)-based cosmid libraries of Ecc and Eca genomic DNA were used to transduce JCB571 and transductants were screened for restoration of motility on TSA plates, compared to the wild-type strain, JCB570 (data not shown). Two types of complementing clones were isolated from the Ecc SCR1193 libraries. The first type, represented by cMS1 and isolated from a pSF6-based library, allowed full restoration of motility at an incubation temperature of 37 °C, and was subsequently found to contain the Ecc dsbA gene. The second type, represented by cM2 and isolated from a pHCSF-based library, consistently provided full complementation at 30 °C but only partial complementation at 37 °C, and was later found to contain the Ecc dsbC gene. The lower temperature was used to increase the possibility of isolating dsbC (Shevchik et al., 1994). The cosmid cAT1, carrying the Ecc dsbA gene, was isolated similarly at 30 °C using a pSF6-based cosmid library of Eca SCR11043.

These cosmids carried \( \sim 30 \) kb fragments of genomic DNA and were shown to display different enzyme restriction patterns (data not shown). Random cloning was used to subclone each cosmid into pBR322. JCB571 transformants were scored for restoration of motility on TSA plates (data not shown). In the case of cMS1, a subclone containing an 8 kb EcoRI fragment, pMS1E, was isolated. pMS1E was restriction-mapped and pBR322 subclones were tested for complementation of JCB571 to generate pJSLK and pESLK, respectively. All genetic manipulations using the recombinant pKNG101 plasmids were carried out in the E. coli strain CC118 (pair; Herrero et al., 1990).

Marker exchange mutagenesis was done essentially as described by Thomson et al. (1997). Strains that had undergone resolution of the integrated plasmid were selected in the absence of antibiotic \((\text{lacZ})\), or the presence of Cm \((\text{gusA/Cm}^\beta)\) or Kn \((\text{gusA/Kn}^\beta)\) as appropriate. Southern blotting confirmed the genotype of putative mutants. The Ecc-specific transducing phage \(\phi\text{KP}^\prime\) was used to transduce chromosomal mutations into different Ecc genetic backgrounds, essentially as described previously (Toth et al., 1993).

DNA sequence analysis of the Ecc and Eca dsbA genes

The genomic DNA inserts of pBH, pH and pBAT were sequenced. pBH contained an Ecc DNA insert of 1126 bp (GenBank accession no. AF146615) with a single ORF of 621 nucleotides, beginning at residue 264 and ending at residue 884. This ORF encoded a predicted protein of 207 amino acids, with an 81% and 70% identity to the Echr and E. coli DsbA proteins, respectively.

pBAT had an Eca DNA insert of 1058 bp (GenBank accession no. AF146613) with a single ORF of 621 nucleotides, beginning at residue 155 and ending at residue 775. This ORF encoded a predicted protein of 207 amino acids, with an 83% and 70% identity to the Echr and E. coli DsbA proteins, respectively. The Ecc and Eca DsbA proteins displayed 96% identity.
DNA sequence analysis of the Ecc *yihE* gene and the *yihE*/*dsbA* distal promoter

The sequence data from pBH and pBAT revealed the presence of incomplete ORFs 236 and 127 bp in length, only 27 bp upstream of, and transcribed in the same orientation as, the Ecc and Eca *dsbA* genes, respectively. These ORFs encoded two virtually identical putative C-terminal truncated proteins with strong homology to the C-terminal region of the *E. coli* YihE protein (Blattner et al., 1997). Thus, the organization of the *dsb/yihE* region is similar in all three organisms (see Fig. 1).

In *E. coli*, the *dsbA* gene is expressed via two promoters, a proximal promoter (P1) directly upstream of the *dsbA* gene and a distal promoter (P2) upstream of *yihE* (Belin & Boquet, 1994), and the latter of these is positively regulated by the CpxA/R two-component regulator (Pogliano et al., 1997). CpxR regulates several genes whose protein products are associated with folding and misfolding of proteins in the periplasm. A CpxR consensus binding site has been identified in the promoter regions of several of these genes, including the *E. coli* *dsbA* P2 promoter (Pogliano et al., 1997; Nakayama & Watanabe, 1998). The Ecc *dsbA* gene 5' region did not contain a putative CpxR-binding site. The subclone pHIN was also sequenced. As expected, pHIN contained sequence encoding the 5' region of the Ecc *yihE* gene (GenBank accession no. AF146615), but the promoter region of Ecc *yihE* showed no consensus CpxR-binding site (data not shown).
DNA sequence analysis of the Ecc dsbC gene

Preliminary sequence data analysis of the fragment from pHH revealed that it did not carry the complete Ecc dsbC gene so the sequence was extended using the original cosmid, cM2. The data were compiled into a contiguous sequence of 1186 bp (GenBank accession no. AF146614) containing an ORF of 714 nucleotides, beginning at residue 315 and ending at residue 1028. This ORF encoded a predicted protein of 238 amino acids, with an M, of 257 kDa, and exhibiting 70% and 58% identity to the Echr and E. coli DsbC proteins, respectively.

Like the DsbA and DsbC proteins previously reported, the Ecc equivalents are predicted to have a signal sequence indicating export to the periplasm as well as the characteristic -C-X-X-C- motif, confirming them as members of the disulfide oxidoreductase protein family. Since the C-terminal truncated protein encoded by pHH (incomplete dsbC gene) fully complemented the DsbC mutant, the six amino acids at the carboxy terminus of Ecc dsbC (-S-K-K-T-G-G-) are not essential for function.

The sequence data also revealed the presence of an incomplete ORF (186 bp), upstream of, and transcribed in the same orientation as, DsbC, which encoded a C-terminal truncated protein with significant homology to XerD of E. coli and Haemophilus influenzae (44% and 50% identity, and 55% and 55% similarity, respectively). Downstream of DsbC, a second incomplete ORF was identified (143 bp), which encoded the N-terminal region of a protein with significant homology to the RecJ proteins of Echr and E. coli (67% and 76% identity, and 82% and 61% similarity, respectively). All three genes are thought to form part of the same operon in E. coli and Echr (Blakely & Sherratt, 1994; Missiakas et al., 1994).

Marker exchange mutagenesis of the Ecc dsbA and dsbC genes and the Eca dsbA gene

In order to investigate the role of the Dsb proteins in exoenzyme activity and secretion in Ecc, and in motility in Eca, marker exchange mutagenesis was done for all three Erwinia dsb genes (see Methods). The genotype of the resulting mutants (LS1A, Ecc dsbA::gusA/CmR; LS2C, Ecc dsbC::gusA/KnR; LS4AA, Eca dsbA::gusA/CmR) was confirmed by Southern blot analysis (data not shown). A double dsbA dsbC mutant of Ecc (LS3AC) was created using the Ecc-specific transducing phage, qKP (see Methods), and Southern blot analysis confirmed the presence of both mutated genes in this strain (data not shown). None of the dsb mutants was impaired in growth rate or total protein content in comparison to the wild-type strains (data not shown).

Virulence of Ecc and Eca dsb mutants

Potato tuber virulence assays were performed on Ecc and Eca Dsb mutants using the cultivars Colmo Tops and Pentland Javelin, respectively, and the results are shown in Fig. 2. A 52%, 28%, 86% and 63% reduction in the ability to cause rotting was observed for LS1A, LS2C, LS3AC and LS4AA, respectively when compared to the wild-type strain. Each assay was also performed on the cultivar Maris Piper with similar results. The reduced rotting capacity of these strains implied that the Dsb system is essential for the activity of virulence factors, probably Pel and Peh, in Ecc and Eca. However, the fact that all dsb mutant strains were able to produce some degree of maceration indicated that other DsbA-independent virulence factors were operating.

The Ecc dsbA gene is essential for motility

Earlier experiments in this laboratory have shown motility to be an important virulence determinant in Eca (Harris et al., 1998; Mulholland et al., 1993). Therefore, we assessed the motility of the Ecc strain LS4AA (Fig. 3). The mean diameter of LS4AA colonies was 8 mm whilst that of the wild-type, SCRI1043, was 45 mm. Since halo size represents an exponential function, the reduced halo observed for LS4AA represents a >82% reduction in motility and suggests that disulfide bond formation is a prerequisite for motility of Eca. The residual motility in this strain was due to the activity of the chromosomal copy of the dsbC gene (Pugsley, 1992), as shown by restoration of full motility when the Ecc dsbC gene was expressed in trans from pHH in LS4AA (data not shown).

Enzymic activity in Ecc dsb mutants

Cellular fractions (supernatant, periplasm and cytoplasm) were prepared from LS1A, LS2C, LS3AC and wild-type Ecc SCRI193, and analysed for the secretion and activity of Prt, Pel, Peh and Cel (Table 2). More than 90% of the total Prt activity of each Ecc dsb mutant was...
Table 2. Enzyme localizations of Ecc Dsb mutants

Samples were fractionated into supernatant (S), periplasm (P) and cytoplasm (C), and all enzyme assays were carried out in triplicate and performed at least twice. Total enzyme activities (T) were calculated as the sum of the absolute enzyme activity in each fraction: Prt, $\Delta A_{250}$ units h$^{-1}$ ml$^{-1}$; Pel, $\Delta A_{235}$ units min$^{-1}$ ml$^{-1}$; Peh, $\Delta A_{340}$ units min$^{-1}$ ml$^{-1}$; Cel, $\Delta A_{450}$ units min$^{-1}$ ml$^{-1}$. The activities of each fraction are expressed as a percentage of the total enzyme activity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Prt</th>
<th>Pel</th>
<th>Peh</th>
<th>Cel</th>
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<tbody>
<tr>
<td></td>
<td>T</td>
<td>S</td>
<td>P</td>
<td>C</td>
</tr>
<tr>
<td>SCR1193</td>
<td>11:29</td>
<td>97</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>10:84</td>
<td>93</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
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<td>11:40</td>
<td>92</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>LS3AC</td>
<td>3:95</td>
<td>95</td>
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</table>

**Fig. 4.** Western analysis of CelV expression in LS1A and LS3AC. Cells were fractionated into supernatant (S), periplasm (P) and cytoplasm (C) and immunoblotted using a $\beta$-66 rabbit anti-CelV polyclonal antibody. The wild-type Ecc strain SCR1193 and the celV mutant GS7000 were used as controls. The positions of molecular mass markers are shown. A 53 kDa protein corresponding to CelV is shown by an arrow.

Immunoblot analysis with an antibody specific to a 53 kDa metalloprotease of Ecc SCR1177 (Heilbronn et al., 1995) was performed on cell fractions to determine if the reduced level of total Prt activity in LS3AC was due to catalytically inactive enzyme. However, a 46 kDa protein was detected in all supernatant fractions except LS3AC (data not shown), but not the other fractions, implying that this protein was either degraded or not produced in LS3AC.

At least three Prt have been purified from various strains of Ecc (Smith et al., 1987; Kyostio et al., 1991; Heilbronn et al., 1995), but the number and secretability of isozymes in Ecc SCR1193 is not known. However, reduction in Prt secretion/production due to Dsb effects is not without precedent among bacterial pathogens, and Prt deficiency of $\Delta db$ mutants has been reported for several strains, including Ecc (Peek & Taylor, 1992; Shevchik et al., 1994; Abe & Nakazawa, 1996). Interestingly, PrtI of Ecc strain EC14 (Kyostio et al., 1991) has a typical sec-dependent signal sequence, implying it may be a type-II-targeted protein, and contains three cysteine residues. Therefore, the protein detected here might be the PrtI equivalent in Ecc SCR1193. Nonetheless, the contribution of this protein to total Prt activity in Ecc is likely to be minor since Prt secretion appears to be normal in Ecc Out" mutants (Reeves et al., 1993).

Total Pel activity of LS1A and LS3AC was reduced to 4% and 6% of the wild-type level, respectively, whilst the level of Pel activity in LS2C was essentially that of the wild-type (Table 2). In addition, almost 100% of the Pel activity of the wild-type and LS2C were secreted to the supernatant. It is likely that the reduced activity observed in the DsbA- strains is due to improper folding of one of the major Pel isozymes, PelC. Mis- or unfolded proteins in the periplasm are often degraded by periplasmic proteases (Shevchik et al., 1995; Yu et al., 1992); therefore the reduced Pel activity in the DsbA$^-$ strains might result from periplasmic PelC intermediates being locked in an inactive misfolded form, which is degraded.
Disulfide bond formation in Erwinia carotovora

The residual Pel activity in these strains may be attributed to other Pel that fold in a Dsb-independent fashion. The wild-type levels of Pel activity recorded for LS2C imply that mutation in dsbC alone is insufficient to prevent disulfide bond formation in PelC and PelD and supports the notion that DsbA is the major contributor to disulfide bond formation in the bacterial periplasm. It is also consistent with the view that DsbC is of greater importance to those proteins that possess several disulfide bonds.

The total Peh activity of LS1A and LS3AC was 36% and 32%, respectively, that of the wild-type, whilst LS2C had essentially wild-type activity. In both LS2C and the wild-type, Peh activity was found only in the supernatant. Therefore, the mutation in dsbC had no effect on Peh secretion. However, the location of 88% and 86% of the Peh activity in LS1A and LS3AC, respectively, in the cytoplasmic fractions (Table 2) was surprising since, in the absence of DsbA, Peh was predicted to remain export-competent, and any mis- or unfolded species in the periplasm were predicted to be degraded. Cross-contamination of the cell fractions was unlikely since ~90% of the activity of the periplasmic and cytoplasmic enzyme markers was correctly located (data not shown). In the absence of DsbA, Peh, disulfide bonds might form in the cytoplasm. The reduced environment of the cytoplasm means that this is an unusual, although not unprecedented (Nilsson et al., 1991), phenomenon. A recent report on disulfide bond formation in bacterial proteins suggests that we may need to reassess our views on folding of cytoplasmic proteins containing cysteine residues (Stewart et al., 1998).

CelV contains no cysteines (Cooper & Salmond, 1993) and hence no disulfide bonds, and was originally intended as a control in examining the effect of disulfide bond deficiency on the Ecc type II apparatus. If a Dsb- strain was unable to secrete CelV this would indicate an effect on the type II apparatus itself. Therefore, we were surprised to find that the total Cel activity of the Dsb- strains was ~two- to threefold greater than that of the wild-type (Table 2). LS2C displayed a wild-type phenotype, and in both LS2C and SCRI193 75–84% of total Cel activity was found in the supernatant. By contrast, in LS1A and LS3AC there was an almost equal distribution of Cel activity among the supernatant and the periplasm, and the three cellular fractions, respectively. These data provide conclusive evidence that the type II proteins are not affected by mutation in dsbA or dsbC since wild-type amounts of Cel were secreted normally.

To verify that the observed Cel activity was due to the presence of CelV, immunoblot analysis of cell fractions was carried out using a rabbit polyclonal antibody raised against the purified CelV protein (Walker et al., 1994; Fig. 4). The wild-type strain SCRI193, and

**Fig. 5.** The expression of CelV, PelC and Peh±DsbA. Strains were grown in PMB at 30°C and growth and BGal activity were monitored at hourly intervals. Cell growth (black triangles and squares) is expressed as log OD_{600}. BGal activity (white and hatched bars) is expressed as change in OD_{420} min⁻¹ ml⁻¹ and the mean value of three replicas is shown. (a) LS5V, celV::lacZ; LS6VA, celV::lacZ dsbA::gusA/CmR. (b) LS7P, pelC::lacZ; LS8PA, pelC::lacZ dsbA::gusA/CmR. (c) LS9H, peh::lacZ; LS10HA, peh::lacZ dsbA::gusA/CmR.
GS7000, a CelV-derivative, were used as positive and negative controls, respectively. CelV was observed as a 53 kDa protein exclusively in the extracellular fraction of SCR1193. However, CelV was observed in all fractions of the DsbA- mutants LS1A and LS3AC. More than 50% of CelV was cell-associated in these strains, confirming that the increased levels of Cel activity previously observed in the periplasm and cytoplasm of LS1A and LS3AC was due to overproduction of the CelV protein.

We have previously observed that CelV accumulates in the periplasm and cytoplasm of Ecc when it is expressed at high levels (Cooper & Salmond, 1993; Walker et al., 1994). Assuming folded Cel molecules require recognition by the Out apparatus prior to secretion, accumulation of CelV in the periplasm might be explained by saturation of the apparatus. However, this might also be expected to result in an indirect effect on Pel secretion, yet none was observed. Walker et al. (1994) suggested that accumulation of CelV in the cytoplasmic fraction might be due to the formation of Cel aggregates that render the enzyme non-secretable. The apparently high level in the ‘cytoplasm’ would then be artefactual, due to the inability of Cel aggregates to dissociate from the inner membrane during removal of the periplasmic fraction, but which are released into the cytoplasm during sonication.

**Analysis of an Ecc celV::lacZ transcriptional fusion**

The ~ two- to threefold increase in Cel activity caused by the mutation in dsbA was surprising and warranted further investigation. Two hypotheses were tested: firstly, that in the absence of functional DsbA a cryptic Cel is induced, and secondly, that an increase in the transcription of celV in the dsbA background might result in greater production of CelV and its accumulation in the cell. To test the latter, a lacZ transcriptional fusion was created in celV which, in addition to causing a mutation so that any novel cryptic Cel might become apparent, allowed for monitoring the transcription of celV.

The construct pVicLK, carrying a celV-lacZ transcriptional fusion, was created and used to replace the wild-type celV gene of MH1000 (a Lac- derivative of SCR1193). The genotype of the resulting Cel- strain, LS5V, was confirmed by Southern blot analysis (data not shown). A double celV dsbA mutant of Ecc, LS6VA, was created using ΦKP. Cell fractions from both strains were assessed on Cel detection media and neither strain produced detectable Cel activity (data not shown). This showed that no cryptic Cel is induced in the DsbA-deficient strain, and implied that some other phenomenon must be responsible for the increased Cel activity observed in LS1A and LS3AC.

To investigate whether the transcription of celV is up-regulated in a dsbA background, lacZ expression was monitored for the LS5V and LS6VA mutants (Fig. 5a). There was no difference in the growth rates, nor in the total protein content (data not shown), of the strains.

However, the difference in the level of expression of the celV-lacZ transcriptional fusion in the wild-type and DsbA-deficient backgrounds was quite marked. Throughout growth the expression of celV was greater in LS6VA than in LS5V. This ranged from a 15% (4 h) to a 162% (9 h) increase. The maximum level of celV expression in the wild-type background was observed at 8 h.

The regulation of exoenzymes in Erwinia species is extremely complex, being affected by both intrinsic and environmental factors (Barras et al., 1994). Exoenzyme production is induced during late exponential growth (Hugouvieux-Cotte-Pattat et al., 1986, 1992; Boyer et al., 1984; Aymeric et al., 1988). The molecular mechanism for this cell-density-dependent gene regulation in Ecc centres on the production of a small diffusible molecule, N-(3-oxohexanoyl)-1-homoserine lactone (OHHL; Jones et al., 1993). The dramatic increase in the transcription of celV in the DsbA-deficient strain raised the question as to whether similar transcriptional up-regulation was taking place with the other exoenzymes. These displayed reduced activity in the dsbA background and were thought to be degraded, hence exoenzyme assays could not indicate if there was an increased transcription. It was therefore necessary to compare the transcription of both pelC and peh in a wild-type and dsbA background.

**Analysis of Ecc pelC::lacZ and peh::lacZ transcriptional fusions**

The constructs pJSLK and pESLK were produced and used to create chromosomal lacZ transcriptional fusions in the pelC (LS7P) and peh (LS9H) genes of MH1000. The genotypes of the resulting mutants were confirmed by Southern blot analysis (data not shown) and fusions were transduced into a dsbA background to produce LS8PA and LS10HA. The transcription of pelC and peh was analysed in the wild-type and dsbA background as described for celV. None of the strains showed an appreciable difference in either growth rate (Fig. 5b and c) or protein content (data not shown).

A difference in the level of transcription of pelC in the wild-type and dsbA backgrounds was evident (Fig. 5b). Transcription appeared to be slightly higher in LS8PA than in LS7P at 1 and 4 h. After 5 h, the level of transcription in LS7P was consistently higher than that in LS8PA. Between 6 and 10 h there was ~ 50% decrease in the transcriptional level of pelC in LS8PA compared to LS7P. This is unlike the transcription of celV, indicating that genes encoding two enzymes secreted by the Out pathway are differentially affected by the dsbA mutation.

Although the expression of peh in the wild-type background appeared to lag slightly behind that in the DsbA-deficient strain during the early stages of growth, a dramatic induction of peh expression in the wild-type took place after 8 h. At this point the BGal expression in the wild-type background was ~ 64% greater than that
in the dsbA background. This trend continued into the stationary phase although the difference was less marked (~ 12%). This indicates that the dsbA mutation causes a down-regulation of peh transcription.

Regulation of the Ecc exoenzymes is not mediated by a single agent

Several factors which appear to co-ordinately regulate exoenzyme production during the late post-exponential-growth phase have been identified in Ecc. These include: (i) OHH1 (Jones et al., 1993; Pirhonen et al., 1993); (ii) pectate and other plant signals (Liu et al., 1993); (iii) activation genes, variously designated aep (Liu et al., 1993), exp (Pirhonen et al., 1991) and rex (Jones et al., 1993), thought to encode regulatory proteins; and (iv) negative regulators, such as the Ecc rsmA gene product, which represses extracellular enzyme production and other metabolic functions in Erwinia (Mukherjee et al., 1996).

However, the results presented here indicate a differential expression of exoenzymes in the DsbA-deficient strain: celV increased by ~ 200%, pelC decreased by ~ 50% and peh decreased by ~ 60 to ~ 12% from late exponential to stationary phase. This implies that the regulatory effect on transcription of these genes is unlikely to be mediated via a single agent. It is interesting that transcription of pelC and peh, both predicted to be disulfide-bonded, appear to respond in a similar manner. A dsbA mutation depletes the periplasm of DsbA, whose role is to catalyse disulfide bond formation in this compartment. Our results therefore indicate that what is essentially a periplasmic event is having an effect (presumably indirect) on gene transcription in the cytoplasm. This implies the operation of a signal transduction feedback mechanism linking periplasmic protein stability with transcription.

Recent reports have identified protein misfolding in the periplasm (as caused by dsb mutation) as a stimulus that can trigger up-regulation of other genes in E. coli. For example, Missiakas & Raina (1997) reported that protein misfolding in the periplasm leads to up-regulation of degP (which encodes a periplasmic Prt that degrades misfolded proteins in the periplasm) via σE, the second heat shock-inducible sigma factor. In E. coli, degP expression is regulated by the CpxA/R two-component regulator (Danese et al., 1995), which also regulates proteins involved in periplasmic folding (e.g. DsbA; Danese & Silhavy, 1997). Intriguingly, in an independent study we have identified a role for CpxA in the regulation of Pel in Ecc (unpublished data); evidently, a regulatory network similar to the one in E. coli is in effect in Ecc.

Concluding remarks

The results of this study are consistent with the notion that the ability of Erwinia spp. to cause virulence results from a series of tightly co-ordinated events, including exoenzyme synthesis and secretion in Ecc, and motility in Eca. Two inferences can be made. Firstly, the Dsb system has no effect on the type II secretion system. Secondly, the Dsb system plays a role in feedback signal transduction on transcription, resulting in differential expression of exoenzymes. Interestingly, the DsbA effect can be mimicked by DTT treatment (data not presented). We also observed that mutation in dsbC alone does not affect the phenotypes assessed, and, except in the case of Prt production and tissue maceration, the deficient phenotypes seem to be due to mutation in dsbA alone. This is consistent with the findings of Shevchik et al. (1994) for Ech. Future work will focus on the molecular nature of the feedback signal transduction and its ability to differentially affect exoenzyme synthesis.

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Disulfide bond formation in *Erwinia carotovora*


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