The EMBO Journal vol.12 no.6 pp.2467–2476, 1993

A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*

Minna Pirhonen, Diana Flego[1], Riikka Heikinheimo and E.Tapio Palva[2]

Department of Molecular Genetics, Uppsala Genetic Center, Swedish University of Agricultural Sciences, Box 7003, S-750 07 Uppsala, Sweden
[1]Permanent address: Department of Plant Pathology, National University of Mar del Plata, Box 276, AR-7620 Balcarce, Argentina
[2]Corresponding author

Communicated by K.Nordström

Virulence of the plant pathogen *Erwinia carotovora* subsp. *carotovora* is dependent on the production and secretion of a complex arsenal of plant cell wall-degrading enzymes. Production of these exoenzymes is controlled by a global regulatory mechanism. Avirulent mutants in one of the regulatory loci, *expI*, show a pleiotropic defect in the growth phase-dependent transcriptional activation of exoenzyme gene expression. The *expI* gene encodes a 26 kDa polypeptide that is structurally and functionally related to the *luxI* gene product of *Vibrio fischeri*. Functional similarity of *expI* and *luxI* has been demonstrated by reciprocal genetic complementation experiments. LuxI controls bioluminescence in *V.fischeri* in a growth phase-dependent manner by directing the synthesis of the diffusible autoinducer, *N*-(3-oxohexanoyl) homoserine lactone. *E.c.* subsp. *carotovora expI*$^+$ strains or *Escherichia coli* harboring the cloned *expI* gene excrete a small diffusible signal molecule that complements the *expI* mutation of *Erwinia* as well as a *luxI* mutation of *V.fischeri*. This extracellular complementation can also be achieved by *E.coli* harboring the *luxI* gene from *V.fischeri* or by adding the synthetic *V.fischeri* autoinducer. Both the production of the plant tissue-macerating exoenzymes and the ability of the bacteria to propagate *in planta* are restored in *expI* mutants by autoinducer addition. These data suggest that the same signal molecule is employed in control of such diverse processes as virulence in a plant pathogen and bioluminescence in a marine bacterium, and may represent a general mechanism by which bacteria modulate gene expression in response to changing environmental conditions.

Key words: autoinducer/cell density signal/extracellular complementation/global regulation/pathogenicity

Introduction

The adaptation of bacteria to a particular environment requires that the organism is able to sense and respond to environmental stimuli and modulate its gene expression accordingly. For example, the virulence of a pathogen is dependent on the presence and controlled expression of specific virulence and pathogenicity genes. The pathogenicity of the major plant pathogenic enterobacterium *Erwinia carotovora* subsp. *carotovora* and related *Erwinia* species is correlated with their ability to produce and secrete plant cell wall-degrading enzymes (Collmer and Keen, 1986; Kotoujansky, 1987). The soft-rot disease caused by these broad host range pathogens is characterized by extensive maceration of the affected tissues by these enzymes which are instrumental for the ability of the bacteria to colonize the plant and propagate in the plant tissue. The importance of the exoenzymes is reflected by the impressive arsenal of different enzymes produced by *E.c.* subsp. *carotovora*. This includes isoforms of pectate lyase (Pel), pectin lyase (Pnl), polygalacturonase (Peh), cellulase (Cel) and protease (Prt) (Collmer and Keen, 1986). The crucial role of these enzymes, particularly the pectic enzymes, in the virulence of *E.c.* subsp. *carotovora* and the related soft-rot pathogens *E.c.* subsp. *atroseptica* and *E.chrysanthemi* is further demonstrated by isolation of avirulent mutants that are defective in production or secretion of the enzymes (Boccara *et al.*, 1988; Hinton *et al.*, 1989; Pirhonen *et al.*, 1991). Even inactivation of single exoenzyme-encoding genes can result in reduced virulence (Boccara *et al.*, 1988; Ried and Collmer, 1988; Saarilahti *et al.*, 1992).

In *E.c.* subsp. *carotovora* and other similar phytopathogens the production of plant cell wall-degrading enzymes is tightly regulated; in contrast, non-pathogenic bacteria produce similar enzymes but in a constitutive manner (Zucker *et al.*, 1972). The exoenzyme production by soft-rot *Erwinia* species responds to several environmental and metabolic stimuli involving both global and enzyme-specific factors and in *E.chrysanthemi* this response has been shown to include both positive and negative regulatory elements (Hugouvieux-Cotte-Pattat and Rober-Baudouy, 1989; Reverchon *et al.*, 1990; Nasser *et al.*, 1992). Clear examples of specific response to particular stimuli are (i) the activation of Pnl by DNA-damaging agents (Zink *et al.*, 1985; McEvoy *et al.*, 1992) and (ii) the induction of certain groups of enzymes by substrate availability e.g. *E.chrysanthemi* Prt is inducible by casein hydrolysate (Wandersman *et al.*, 1986), and the pectic enzymes are inducible by polygalacturonate and its degradation products (Kotoujansky, 1987; Nasser *et al.*, 1992). Globally the synthesis of most of the exoenzymes is subject to catabolite repression and is also controlled by environmental factors such as oxygen tension and temperature (Moran and Starr, 1969; Pérombelon and Kelman, 1980; Boyer *et al.*, 1984; Hugouvieux-Cotte-Pattat *et al.*, 1986; Aymeric *et al.*, 1988). However, the main stimulus for exoenzyme production appears to be the cessation of growth. Such a growth phase-dependent control of *Erwinia* exoenzymes has been observed in *E.chrysanthemi* for both pectic enzymes (Hugovieux-Cotte-Pattat *et al.*, 1986) and cellulase (Boyer *et al.*, 1984; Aymeric *et al.*, 1988). A similar induction was observed for Peh, Pel and Cel in *E.c.* subsp. *carotovora* at the onset of stationary phase (Saarilahti, 1990; D.Flego, M.Pirhonen, T.K.Palva, M.-B.Karlsson and E.T.Palva, in preparation).

Genetic analysis of exoenzyme gene regulation in *E.c.* subsp. *carotovora* suggests that both global and specific regulators are involved (Murata *et al.*, 1991; Pirhonen *et al.*, 1991; Saarilahti *et al.*, 1992). We have recently described *pehR* as a specific transcriptional activator of the polygalacturonase-encoding gene, *pehA* (Saarilahti *et al.*, 1992). Global control of exoenzyme production as well as virulence in *E.c.* subsp. *carotovora* was shown by isolation of avirulent mutants exhibiting a pleiotropic exoenzyme-negative phenotype (Murata *et al.*, 1991; Pirhonen *et al.*, 1991). We have characterized two separate loci that are involved in this regulation as shown by complementation analysis and by cloning the wild-type allele for one of the genes (Pirhonen *et al.*, 1991). These *exp* (exoenzyme production) loci, which control virulence in *E.c.* subsp. *carotovora*, may constitute key elements in the bacterial adaptation to specific environments, such as growth *in planta*. The *exp* loci could therefore be part of a sensory system that is required for this adaptation.

The best characterized of such sensory systems are the two-component regulators, which employ a membrane sensor protein for detection of the stimulus and transmission of the signal, usually through protein phosphorylation, to a response regulator that in turn activates transcription of specific genes resulting in the adaptive response (Stock *et al.*, 1989, 1990). Other sensory systems, however, do not employ the membrane sensors and phosphorylation for signal transduction but rely on small diffusible signal molecules. In particular, response to growth phase or cell density provides examples for such control in bacteria. In *Escherichia coli* accumulation of weak acids in stationary phase cultures serves as a signal to activate the expression of a stationary phase-specific sigma factor gene (the *katF* or *rpoS* gene) which is in turn required for the activation of other growth phase-specific sets of genes (Lange and Hengge-Aronis, 1991; Schellhorn and Stones, 1992).

A well known example of a regulatory system relying on small signal molecules is the cell density-dependent control of bioluminescence in *Vibrio fischeri* and related luminous bacteria (for review see Meighen, 1991). The activation of the structural *lux* genes required for light emission in *V.fischeri* is dependent on the accumulation of a small diffusible autoinducer molecule, *N*-(3-oxohexanoyl) homoserine lactone (HSL) (Eberhard *et al.*, 1981; Kaplan and Greenberg, 1985; Kaplan *et al.*, 1985). Accumulation of HSL over a threshold level is achieved at high cell densities e.g. with the bacteria either living as symbionts in fish light organs or in stationary phase *in vitro* cultures. Biosynthesis of the autoinducer is directed by the *luxI* gene product, and a defect in *luxI* can be complemented by extracellular addition of the autoinducer (Engebrecht and Silverman, 1984). HSL binds to a receptor, the *luxR* gene product, and this complex will in turn activate the transcription of *luxI* and the structural *lux* genes resulting in light emission (Engebrecht *et al.*, 1983). Although this environmentally controlled signalling system was originally thought to be confined to *V.fischeri* and a few related bacteria, recent work indicates that the same signal molecule is present in diverse bacteria (Bainton *et al.*, 1992a).

In this work we describe the genetic analysis of the growth phase-dependent regulation of *E.c.* subsp. *carotovora* virulence factors and show that these are controlled by the *expI* locus. Furthermore, we show that the ExpI protein is both structurally and functionally similar to *V.fischeri* LuxI and can direct the synthesis of an autoinducer molecule similar to *V.fischeri* HSL. These data demonstrate that *E.c.* subsp. *carotovora* employs the HSL-like signal molecule for global control of virulence and suggest that the autoinducer-response regulator system may be a general signalling mechanism employed by prokaryotes for activating genes in response to specific environmental stimuli.

Results

The expI locus is required for the growth phase-dependent transcriptional activation of exoenzyme genes

The production of extracellular plant cell wall-degrading enzymes of *E.c.* subsp. *carotovora* is induced when cells enter the stationary phase (Saarilahti, 1990; D.Flego *et al.*, in preparation). This exoenzyme production and hence virulence is globally controlled by *exp* loci (Pirhonen *et al.*, 1991). Mutations in one of these genes, designated *expI* (exoenzyme production inducer), lead to a pleiotropic avirulent phenotype deficient in the production of all the exoenzymes tested, namely Peh, Pel, Pnl, Cel and Prt. To assess the role of *expI* in the growth phase-dependent regulation of the virulence factors, we employed both fusions to reporter genes and Northern analysis, using cloned exoenzyme genes as probes. Transcriptional and translational fusions to the endopolygalacturonase-encoding gene, *pehA*, were introduced into both *expI* mutant and wild-type backgrounds. Assay of β-galactosidase activity from wild-type *E.c.* subsp. *carotovora* cells harboring a plasmid-borne transcriptional *pehA*−*lacZ* fusion demonstrates the growth phase-dependent expression of *pehA* with the activity starting to accumulate at the early stationary phase (Figure 1). This induction was essentially abolished in the *expI* mutant background where β-galactosidase activity stayed close to the level of the uninduced wild-type. Similar results were obtained when a plasmid-borne *pehA*−*bla* translational fusion (Saarilahti *et al.*, 1992) was introduced into these strains and the β-lactamase activity was assayed (M.Pirhonen, data not shown).

To verify the results obtained with plasmid-borne *pehA*−reporter gene fusions, and to assess the effect of the *expI* mutation on the transcriptional activity of other extracellular enzyme encoding genes, we performed a Northern analysis. In the wild-type, accumulation of the corresponding mRNAs was induced when cells were entering the stationary phase (Figure 2). In contrast, accumulation of *pehA* mRNA was drastically reduced in the *expI* mutant (Figure 2A). A similar lack of mRNA accumulation in the *expI* strain was observed for other exoenzyme genes such as *pelC* (Figure 2B) and *celV* (Figure 2C). Taken together these data demonstrate that the *expI* gene product is required for the transcriptional activation of several categories of exoenzyme genes at the early stationary phase.

The expI gene encodes a 26 kDa protein which is structurally and functionally related to Vibrio fischeri LuxI

To study the structure and the mode of action of the *expI* gene product, we set out to clone the corresponding gene. This was done by complementation of the pleiotropic avirulent and exoenzyme-negative phenotype of the original

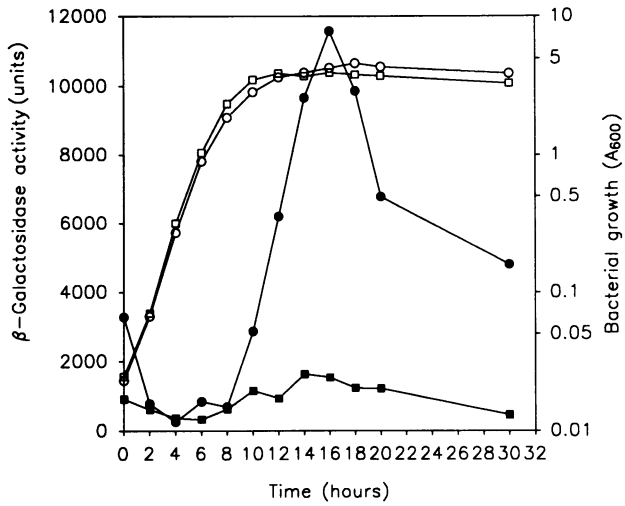

Fig. 1. Control of the growth phase-dependent expression of *pehA* by *expI*. The expression of the plasmid-borne transcriptional *pehA*−*lacZ* fusion (pDFL2) was determined in the wild-type *E.c.* subsp. *carotovora* strain SCC3193 and in the *expI* mutant strain SCC3065. The strains were grown in L medium supplemented with 0.4% glycerol and Ap, and the β-galactosidase activity was measured as a function of the time (in hours) after inoculation. The growth of the bacterial cultures was monitored by measuring the absorbance (A_{600}) and is indicated by open symbols. The corresponding β-galactosidase activities for strains SCC3193/pDFL2 (circles) and SCC3065/pDFL2 (squares) are displayed by filled symbols.

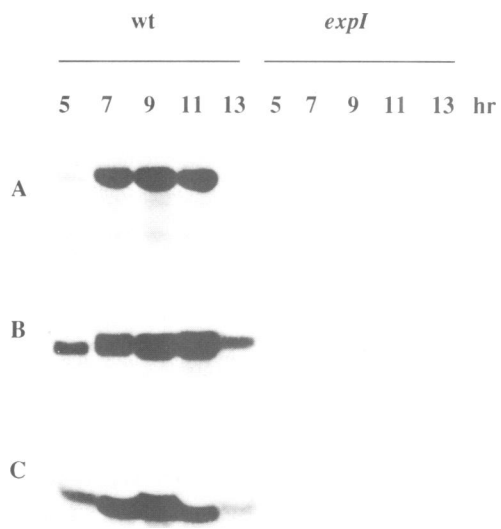

Fig. 2. Accumulation of *pehA* (**A**), *pelC* (**B**) and *celV* (**C**) mRNA is blocked in the *expI* mutant SCC3065 as compared with the wild-type (SCC3193). The strains were propagated in L medium supplemented with 0.5% sodium polypectate. Accumulation of mRNA was followed during the growth of the bacteria by Northern analysis of samples collected at the times indicated. Total RNA was isolated as described by Gilman (1987), resolved by formaldehyde denaturing gel electrophoresis and transferred on to nylon filters. The filters were hybridized to probes labelled with [^{32}P]dATP by random priming or nick translation. Internal fragments of the *pehA* (A) or *celV* (C) genes, or a 2.5 kb genomic fragment harboring the *pelC* gene (B) were used as probes.

expI transposon mutant (Pirhonen *et al.*, 1991). To localize the complementing region and to characterize the putative *expI* gene product the cloned fragment was subjected to

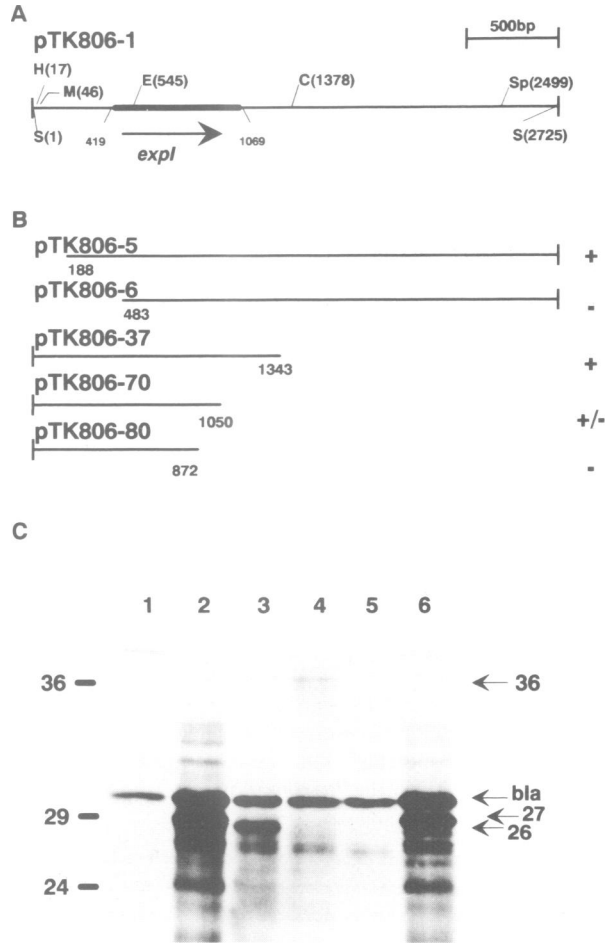

Fig. 3. Localization of the *expI* gene and characterization of the gene product. The chromosomal insert of the original complementing plasmid pTK806 was transferred to pBluescriptII as a *Sma*I−*Pst*I fragment. The localization of the *expI* gene in the insert of the resulting plasmid, pTK806-1 (**A**), was done by complementation of the corresponding *expI* mutant strain (SCC3065) by deletion derivatives of pTK806-1 (**B**). The *expI* ORF determined by sequencing (see Figure 4) is displayed by a thick bar with starting and ending base pairs indicated. The chromosomal regions present in the wild-type and deletion plasmids are indicated by solid lines; the numbers indicate the deletion endpoints in the sequence. Full, partial or no complementation of the *expI* mutation in SCC3065 by the clones was determined on enzyme indicator plates and is indicated by +, +/− or −, respectively. The cleavage sites for *Cla*I (C), *Eco*RI (E), *Hin*dII (H), *Mlu*I (M), *Sau*3A (S) and *Sph*I (Sp), and their locations in the pTK806-1 insert, are indicated in (A). Only the terminal *Sau*3A sites used in cloning of the chromosomal fragment are indicated. Analysis of the polypeptides encoded by the plasmid pTK806-1 and its deletion derivatives was by the maxicell technique (**C**). *E.coli* XL1 cells harboring the respective plasmids were converted to maxicells according to Sancar *et al.* (1979). After labelling with L-[^{35}S]methionine the polypeptides were characterized by 0.1% SDS−12% PAGE (Laemmli, 1970), and the labelled proteins were visualized by fluorography of the stained and dried gel. Plasmids carried by the cells were: lane 1, pBluescript; lane 2, pTK806-1; lane 3, pTK806-37; lane 4, pTK806-70; lane 5, pTK806-80; lane 6, pTK806-6. The sizes of the M_r markers are indicated in the left margin (in kDa). The following plasmid-encoded proteins are indicated in the right margin by arrows: β-lactamase (bla) encoded by the vector, the 26 and 27 kDa proteins encoded by the insert in pTK806-1, and the 36 kDa hybrid protein encoded by pTK806-70. The 26 kDa polypeptide is the *expI* gene product and the 36 kDa protein a truncated polypeptide resulting from the removal of the stop codon of the *expI* gene in pTK806-70. The lower molecular weight bands seen in some of the lanes are probably degradation products.

deletion analysis (Figure 3). The ability of the deletion derivatives to complement the *expI* mutant phenotype was assessed on enzyme indicator plates (Figure 3B) and resulted in the localization of the *expI* gene in the leftmost part of the 2.7 kb chromosomal fragment in pTK806-1 (Figure 3A).

The same deletion derivatives of pTK806-1 were employed to characterize the putative *expI* gene product from *E.coli* maxicells. Analysis of the plasmid-encoded proteins by SDS−PAGE suggested that *expI* directs the synthesis of a 26 kDa polypeptide (Figure 3C). To characterize the *expI* gene in more detail we sequenced the complementing region of pTK806-1 using a series of overlapping deletions. This DNA sequence analysis revealed the presence of a single 651 bp ORF between bases 419 and 1069 (Figure 4). The location of the ORF (solid bar in Figure 3A) was in agreement with the results from the complementation analysis. The deduced polypeptide of this *expI* ORF contained 217 amino acids with a calculated molecular weight of 25.3 kDa which correlates well with the apparent molecular weight (26 kDa) observed by SDS−PAGE (Figure 3C). The ExpI polypeptide contains many charged and polar residues with no obvious membrane spanning domains or signal sequence, suggesting that the protein is cytoplasmic.

A computer database search with the deduced amino acid sequence of *expI* indicated that the *expI* gene product has sequence similarity to the *luxI* gene product of *V.fischeri*.

```
TTGCGATTTTTTTTCGTGTGTGAACTCTTGCTGACAATGCAGGTTGCATCTGTACAACTA 298
TCGTGGAAAGCGACAGAATACAATAACAATAAGCCTAGGTTTTTGTTAAAAGGCGAAAGA 358
GTCTATTGGATGGTCTATCTGTTTTTTGATGTCTAGCTGCAAATTTTTAATGGAGGAAAA 418

ATGTTAGAAATATTCGATGTAAGCTACACACTACTGTCGGAAAAAAAAATCGGAAGAATTG 478
 M  L  E  I  F  D  V  S  Y  T  L  L  S  E  K  K  S  E  E  L
TTTACGCTTAGGAAAGAAACGTTCAAGGATAGGCTGAATTGGGCGGTAAAATGTATTAAC 538
 F  T  L  R  K  E  T  F  K  D  R  L  N  W  A  V  K  C  I  N
GGGATGGAATTCGATCAGTATGATGATGATAATGCGACTTATCTTTTCGGTGTAGAGGGT 598
 G  M  E  F  D  Q  Y  D  D  D  N  A  T  Y  L  F  G  V  E  G
GATCAGGTTATTTGCAGTTCTCGGCTAATTGAAACGAAATATCCTAATATGATTACTGGA 658
 D  Q  V  I  C  S  S  R  L  I  E  T  K  Y  P  N  M  I  T  G
ACGTTTTTCCCTTATTTTGAAAAAATAGATATTCCGGAAGGGAAGTATATCGAGTCGAGC 718
 T  F  F  P  Y  F  E  K  I  D  I  P  E  G  K  Y  I  E  S  S
CGGTTTTTTGTAGATAAAGCGCGGTCAAAAACTATTCTAGGAAATTCTTATCCCGTTAGT 778
 R  F  F  V  D  K  A  R  S  K  T  I  L  G  N  S  Y  P  V  S
ACGATGTTCTTCTTGGCAACGGTGAATTACTCAAAGAGTAAAGGATATGATGGTGTTTAT 838
 T  M  F  F  L  A  T  V  N  Y  S  K  S  K  G  Y  D  G  V  Y
ACGATTGTCAGTCATCCTATGCTCACAATACTAAAACGTTCTGGTTGGAAAATTTCGATT 898
 T  I  V  S  H  P  M  L  T  I  L  K  R  S  G  W  K  I  S  I
GTTGAACAGGGTATGTCAGAAAAACACGAAAGGGTTTATCTACTTTTTTTACCTGTCGAT 958
 V  E  Q  G  M  S  E  K  H  E  R  V  Y  L  L  F  L  P  V  D
AATGAAAGCCAGGATGTGCTAGTTCGTCGTATAAATCACAATCAGGAATTTGTTGAAAGT 1018
 N  E  S  Q  D  V  L  V  R  R  I  N  H  N  Q  E  F  V  E  S
AAGTTGCGAGAGTGGCCACTGTCTTTCGAGCCTATGACTGAACCGGTCGGATAAGTTGTA 1078
 K  L  R  E  W  P  L  S  F  E  P  M  T  E  P  V  G  *

GCTCGATGCCAAGTCTGATCGCATGTTTGGCATTTAATACGCCGAGTTTTTTCACAACAT 1138
TGCCGATGTGAAAATTTAACGGTGCCTGTTTTAATATCTAATATCAGTGCTATCTCCTGAT 1198
AGGTTTTACCCATACTGGCCCAATAAAGAATTTCATTTTCCCGTTGAGAAAAAATATCTT 1258
TATCATTCGATCTTTCATCTTCCTTGTTTCTAATCATCTCTCTGTAAAGAGAGATGGTTT 1318
```

Fig. 4. Nucleotide sequence of the *expI* gene and the amino acid sequence of the deduced translation product. The ribosome-binding site is indicated by underlining, the TAA termination codon by an asterisk and the putative *rho*-independent transcription terminator (von Heijne, 1987) by arrows. The numbering of the nucleotide bases is as in Figure 3.

These polypeptides are also similar in size (ExpI contains 217 and LuxI 193 amino acids) and in their high content of charged and polar residues. The lack of an obvious signal peptide and membrane-spanning domains indicates a cytoplasmic location for these proteins. Alignment of the ExpI and LuxI amino acid sequences reveals the presence of two conserved domains, one in the N-terminal part, the other in the central part of the polypeptides (Figure 5). The overall sequence contains 30% identical amino acids, suggesting that these proteins may have a similar function. In addition to this observed similarity to the *V.fischeri* LuxI protein ExpI appears even more closely related to the Eag1 polypeptide (Figure 5), a functional analogue of LuxI recently identified from *Enterobacter agglomerans* (S.Swift *et al.*, in preparation). In *V.fischeri* the LuxI protein is part of a regulatory circuit that controls bioluminescence (Meighen, 1991). LuxI directs the synthesis of a signal molecule, the autoinducer, which is required to activate the structural bioluminescence genes by the response regulator, the LuxR protein (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984; Choi and Greenberg, 1992).

To test the possible functional similarity of the ExpI and LuxI gene products suggested by the structural comparisons, we performed complementation analysis of both *expI* and *luxI* mutants. The wild-type *expI* and *luxI* alleles were introduced into an *expI* mutant of *E.c.* subsp. *carotovora* and the cellulase activity was measured (Table I). This analysis demonstrated clearly that the cellulase-negative phenotype of *expI* mutants could be complemented by both *expI*$^+$ and *luxI*$^+$ clones. The observed complementation

```
                 *   *       *          **  ** ** *     *  * *
LUXI   1 MTIMIKKSDFLAIPSEEYK  GILSLRYQVFKQRLEWDLVVENNLESD
EAG1   1    MLEIFDVSYNDLTERRSEDLYKLRKITFKDRLDWAVNCSNDMEFD
EXPI   1    MLEIFDVSYTLLSEKKSEELFTLRKETFKDRLNWAVKCINGMEFD

                *   *         *   *         *   *         *
LUXI  47 EYDNSNAEYIYACDDTENVSGCWRLLPTTGDYMLKSVFPELLGQQSAP
EAG1  46 EFDNSGTRYMLGIYDNQLV CSVRFIDLRLPNMITHTFQHLFGDVKLP
EXPI  46 QYDDDNATYLFGVEGDQ VICSSRLIETKYPNMITGTFFPYFEKIDIP

                   *   ***  * *   *              *           *
LUXI  95 KDPNIVELSRFAVGKNSSK INNSASEITMKLFEAIYKHAVSQGITEY
EAG1  93 EGDYI DSSRFFVDKNRAKALLGSRYPISYVLFLSMINYARHHGHTGI
EXPI  93 EGKYI ESSRFFVDKARSKTILGNSYPVSTMFFLATVNYSKSKGYDGV

               *  *       **                                *      *
LUXI 142 VTVTSTAIERFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKK
EAG1 140 YTIVSRAMLTIAKRSGWEIEVIKEGFVSENEPIYLLRLPIDCHNQHLL
EXPI 140 YTIVSHPMLTILKRSGWKISIVEQGMSEKHERVYLLFLPVDNESQDVL

LUXI 190 AVLN
EAG1 188 AKRIRDQSESNIAALCQCPMSLTVTPEQV
EXPI 188 VRRINHNQEFVESKLREWPLSFEPMTEPVG
```

Fig. 5. ExpI is similar to *Vibrio fischeri* LuxI. Alignment of amino acid sequences of ExpI, LuxI (Engebrecht and Silverman, 1987) and the *Enterobacter agglomerans* analogue of LuxI, Eag1 (S.Swift *et al.*, in preparation). Conserved residues in all three polypeptides are indicated by asterisks. The residues conserved between two of the three proteins are in bold face. The alignment was done according to the Needleman−Wunsch algorithm (Intelligenetics, Mountainview, CA, USA). Additional gaps have been introduced to improve the alignment.

was not specific to cellulase since the production of pectic enzymes and protease was also restored by these clones (data not shown). In addition, we performed the reverse experiment by introducing a plasmid carrying all the *lux* genes from *V.fischeri* but mutated for *luxI* (K.M.Gray and E.P.Greenberg, personal communication) into *E.c.* subsp. *carotovora* wild-type and *expI* mutant strains. Complementation of the *luxI* mutation was monitored by bioluminescence. The results show that wild-type but not *expI* strains of *E.c.* subsp. *carotovora* could complement the *luxI* mutation (Figure 6). The observed complementation of the *expI* mutation by the *luxI* gene and vice versa strongly suggest that the corresponding gene products indeed have similar functions.

The ExpI protein directs the synthesis of an excreted signal molecule similar to the V.fischeri autoinducer

The structural and functional similarity between ExpI and LuxI suggested that the ExpI polypeptide may be involved in the production of a small diffusible signal molecule similar to the autoinducer of *V.fischeri*. This autoinducer, HSL, is employed in communication between *V.fischeri* cells in culture. It is excreted by the cells and, when sufficient amounts of it have accumulated, the *lux* operon is activated (Meighen, 1991). We were interested in whether a similar diffusible signalling molecule is produced in *E.c.* subsp. *carotovora* which could complement the exoenzyme-negative phenotype of an *expI* mutant when applied from outside (Figure 7). Juxtaposition of the *expI* mutant bacteria near *expI*$^+$ strains of *E.c.* subsp. *carotovora*, or *E.coli* harboring *expI*$^+$ clones on enzyme indicator plates resulted in clear restoration of enzyme activity in the *expI* mutant near the complementing bacteria (Figure 7). Similar restoration was also obtained by application of spent culture supernatants from stationary phase cultures of *expI*$^+$ *E.coli* next to *expI* mutant bacteria. Ultrafiltration experiments suggested that a low molecular weight signalling compound is produced and released by cells carrying the *expI* gene and diffuses into the agar around the bacteria (data not shown). The relatedness of this signal molecule to the *V.fischeri* autoinducer was suggested by extracellular complementation of the *expI* mutation by *luxI*$^+$ cells. Growth of *E.coli* harboring *lux* genes from *V.fischeri* juxtaposed to the *expI* mutant resulted in partial restoration of the exoenzyme production in the mutant (Figure 7).

To determine the specificity of this extracellular complementation we tested whether we could restore the exoenzyme production of *exp* mutants belonging to the other complementation group. Application of spent culture supernatants from *expI*$^+$ or *luxI*$^+$ strains to the mutants or juxtaposition of the corresponding strains on enzyme indicator plates (Table II; Figure 7D) restored exoenzyme production to *expI* but not to *expA* mutants. In contrast, *expA* mutants were still able to excrete the signal molecule (Table II), indicating that the effect is specific to *expI* and that the other *exp* genes are not involved in the production of this signal.

To confirm the results of the plate assays, and to obtain quantitative data on the effect of this diffusible signal on exoenzyme gene expression we monitored the activation of a transcriptional *pehA*−*lacZ* fusion by crude autoinducer preparations. Spent culture supernatants from stationary phase cultures of *expI*$^+$ or *luxI*$^+$ *E.coli* cells were applied to wild-type and *expI* mutant strains of *E.c.* subsp. *carotovora* harboring a plasmid-borne *pehA*−*lacZ* fusion and the strains were assayed for β-galactosidase. The results of these experiments (Table III) demonstrate that the *expI* mutation could indeed be complemented extracellularly by both *expI*$^+$ and *luxI*$^+$ strains of *E.coli*. This is shown by the near wild-type levels of β-galactosidase observed in the mutant cultures supplemented with these spent culture supernatants. As found in the plate assay (Figure 7) the strain harboring an *expI*$^+$ clone appeared slightly more active than that harboring *luxI*$^+$ from *V.fischeri* (Table III). This is because the *expI*$^+$ strains produce more inducer molecules than strains harboring the *luxI*$^+$ clone (data not shown).

The demonstration that *luxI*$^+$ bacteria could also complement the *expI* mutant suggested that the same signalling molecule, HSL, was involved in both systems. Further evidence for this was provided by the restoration of bioluminescence to the *luxI* mutant by spent culture supernatants of *expI*$^+$ strains but not *expI*$^-$ strains of *E.c.* subsp. *carotovora* (Table II). To obtain direct proof for the involvement of HSL in *E.c.* subsp. *carotovora* exoenzyme gene regulation the effect of synthetic autoinducer was assessed. Application of the synthetic *V.fischeri* autoinducer to an *expI* mutant resulted in complementation of the mutant phenotype (Table III). The β-galactosidase activity of the mutant supplemented by 2 μg/ml HSL was even higher than that of the wild-type control. In conclusion the results demonstrate that ExpI directs the production of a small diffusible signal molecule required for exoenzyme gene expression and that this signal molecule is most likely identical to the HSL autoinducer of *V.fischeri*.

Table I. Complementation of the *expI* mutation in *E.c.* subsp. *carotovora* by the wild-type *expI* allele or by the *luxI* gene of *V.fischeri*

Strain	Genotype	Cellulase activity[a]
SCC3193	wild-type	0.38
SCC3065	*expI*	0.01
SCC3065/pTK806-1	*expI*/*expI*$^+$	0.27
SCC3065/pTK806-37	*expI*/*expI*$^+$	0.29
SCC3065/pJE202	*expI*/*luxI*$^+$	0.14

[a]The bacteria were grown to early stationary phase ($A_{600\ nm}$ = 4) in L medium with appropriate antibiotics prior to enzyme assay. The activity of cellulase in culture supernatant was assayed as described previously (Pirhonen *et al.*, 1991).

Restoration of virulence by autoinducer

One of the consequences of reduced exoenzyme production in *expI* mutants is the avirulent phenotype of these strains. The *expI* mutants are unable to macerate the inoculated plant tissue and cannot multiply *in planta* (Pirhonen *et al.*, 1991; Palva *et al.*, 1993). To test whether also this phenotype of *expI* strains could be complemented by autoinducer, we assayed the virulence of the strains using local inoculations into tobacco leaves (Figure 8). The disease symptoms are manifested as local lesions of macerated tissue (Figure 8) which subsequently spread throughout the plant. The *expI* mutant is completely avirulent in this test but wild-type *E.c.* subsp. *carotovora* can multiply and rapidly macerate the leaf tissue in accordance with our previous results (Palva *et al.*, 1993). To determine whether restoring the capacity to produce exoenzymes is sufficient to establish virulence *in*

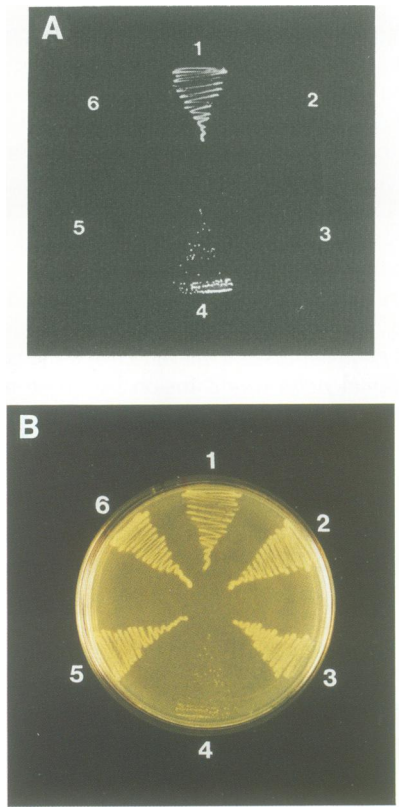

Fig. 6. Complementation of the *luxI* mutation by the *expI* wild-type allele. The plasmid pHV200-I carries all the *lux* genes from *V.fischeri* but has a frameshift mutation in *luxI* (K.M.Gray and E.P.Greenberg, personal communication). This plasmid was introduced into wild-type (1) and *expI* strains (3) of *E.c.* subsp. *carotovora* as well as to *E.coli* (5). As a positive control we used the plasmid pJE202, which carries the wild-type *lux* genes from *V.fischeri*, and introduced this plasmid into the *expI* mutant SCC3065 (4). The wild-type (6) and *expI* mutant (2) strains harboring pBR322 were used as negative controls. The image in A shows the bioluminescence documented by photography from the bacterial plate shown in B which was incubated overnight at 28°C. The impaired growth of the *lux*$^+$ pJE202 harboring strain (4) is most likely due to the high energy demand required for light production (Meighen, 1991).

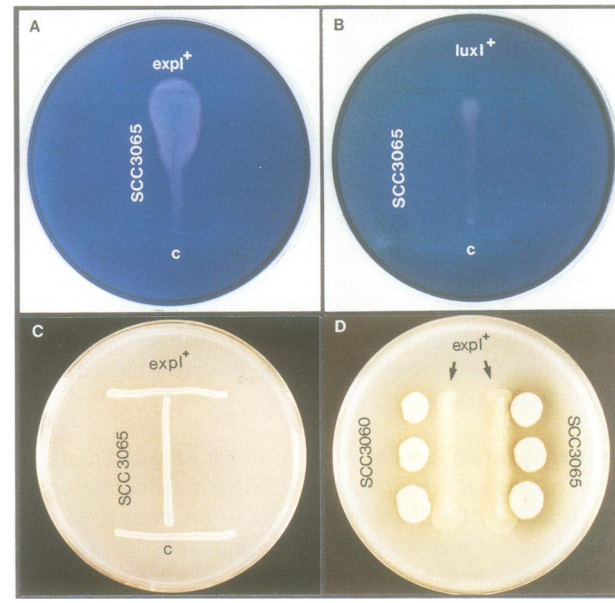

Fig. 7. Extracellular complementation of the *expI* mutation in *E.c.* subsp. *carotovora* SCC3065. Enzyme indicator plates showing complementation of the Cel$^-$ (**A** and **B**) and Prt$^-$ phenotype (**D**) of SCC3065 by nearby bacterial strains. The plate shown in **C** is the same as that in A but before staining for cellulase. The plate shown in B was inoculated in a manner analogous to the plate in A and C. The location of *E.coli* strains carrying the *expI*$^+$ clone pTK806-37 and the *V.fischeri luxI*$^+$ clone pJE202 is indicated by *expI*$^+$ and *luxI*$^+$, respectively. An *E.coli* strain carrying pBR322 was used as a negative control (indicated by c). The production of cellulase is seen as reddish haloes and that of protease as clear haloes around the bacterial growth. The strain SCC3060 (*expA*), which cannot be complemented extracellularly, is an *exp* mutant belonging to a different complementation group from *expI* (SCC3065).

planta, *expI* mutants were preincubated with acellular spent culture supernatants from *expI*$^+$ *E.coli* strains prior to inoculation of the tobacco leaves. As shown in Figure 8, this treatment resulted in restoration of virulence, apparent from the extensive maceration of the plant tissue and growth of the mutant bacteria (Figure 8A and D). The restoration of virulence was transient, however, and only relatively small lesions were caused by preincubated mutant bacteria. Further growth of the mutant bacteria and spreading of the infection required additional applications of the spent culture supernatants from *expI*$^+$ *E.coli*. This suggests that the supply of inducer was exhausted during bacterial growth and it was necessary to replenish it to allow the infection to continue. Similar restoration of virulence was obtained with spent supernatants from *luxI*$^+$ bacteria and, more importantly, by preincubation of the *expI* mutant culture with synthetic HSL (Figure 8F). The infection caused by the HSL-treated mutant was indistinguishable from that caused by wild-type bacteria. Taken together these data demonstrate that both the exoenzyme-negative and avirulent phenotypes of *expI* mutants are complemented by supplementing the cultures with autoinducer molecules. This strongly suggests that HSL is employed as a signal molecule during colonization of host plants by *E.c.* subsp. *carotovora*.

Discussion

Global control of exoenzyme production in E.c. subsp. carotovora by the expI gene product

The production of extracellular plant cell wall-degrading enzymes is central to the pathogenesis of soft-rot (Collmer and Keen, 1986). We now demonstrate that expression of the corresponding genes is coordinately induced at the onset of stationary growth phase, and that this global growth phase-dependent control is exerted by *expI*, one of the previously characterized *E.c.* subsp. *carotovora exp* loci (Pirhonen *et al.*, 1991). The *expI* mutants are not only deficient in exoenzyme production but appear to be completely avirulent. They can neither macerate the plant tissue nor multiply *in planta* (Pirhonen *et al.*, 1991; Palva *et al.*, 1993) demonstrating the central role of *expI* in the pathogenicity of *E.c.* subsp. *carotovora*. Our data suggest that *expI* may be part of a sensory system responsible for the environmental control of these virulence factors.

The first indication for a general signalling function of ExpI in exoenzyme gene regulation was provided by sequence analysis of the *expI* gene. The *expI* ORF encodes a protein with sequence similarity to the LuxI protein of *V.fischeri*. The amino acid alignment (Figure 5) revealed two clusters of conserved residues. The same pattern of conserved residues was found in the *Enterobacter*

Table II. Specificity of the extracellular complementation

Source of spent culture supernatant[a]		Cel phenotype		Bioluminescence[b]
Strain	Relevant genotype	*expI*	*expA*	
E.coli:				
DH5α		−	−	−
DH5α/pTK806-1	*expI*$^+$	+	−	+
DH5α/pTK806-37	*expI*$^+$	+	−	+
DH5α/pJE202	*luxI*$^+$	+	−	+
E.c. subsp. *carotovora*:				
SCC3193	wild-type	+	−	+
SCC3065	*expI*	−	−	−
SCC3060	*expA*	+	−	+

[a]The spent culture supernatants were from stationary phase cultures of the strains indicated and were filter sterilized before applying them near the *E.c.* subsp. *carotovora* mutant strains SCC3065 (*expI*) and SCC3060 (*expA*) growing on Cel indicator plates. Complementation or no complementation of the Cel phenotype is indicated by + and −, respectively.
[b]The effect of the same culture supernatants on the bioluminescence was assayed using an *E.coli* strain harboring the plasmid pHV200-I as an indicator. This plasmid carries the *lux* genes from *V.fischeri* but has the *luxI* gene inactivated by a mutation (K.M.Gray and E.P.Greenberg, personal communication).

Table III. Restoration of β-galactosidase activity to an *expI* mutant by autoinducer addition

Addition[a]	β-galactosidase activity[b]	
	wild-type control	*expI* mutant
Control	3060	720
expI$^+$ supernatant	3417	2718
luxI$^+$ supernatant	nt[c]	2115
Synthetic autoinducer (2 μg/ml)	4144	4647

[a]Spent culture supernatants from stationary phase cultures of *E.coli* harboring either the cloned *expI* gene of *E.c.* subsp. *carotovora* or the *luxI* gene from *V.fischeri*, or the synthetic autoinducer were added to logarithmically growing cultures of the *E.c.* subsp. *carotovora* wild-type strain SCC3193 or the *expI* mutant SCC3065. A spent culture supernatant from *E.coli* was used as a control.
[b]The *E.c.* subsp. *carotovora* wild-type and *expI* mutant strains harbor a transcriptional *pehA−lacZ* fusion. The β-galactosidase was assayed 8 h after addition of the supernatant or the autoinducer.
[c]nt, not tested.

agglomerans Eag1 polypeptide, a functional analogue of LuxI (S.Swift *et al.*, in preparation). The structural similarity of the ExpI and LuxI polypeptides and the fact that they are both located in the cytoplasm suggest that the proteins may have an analogous function.

We have demonstrated the functional similarity of the ExpI and LuxI proteins by genetic complementation of the respective mutations. The wild-type *expI* allele can complement the non-bioluminescent phenotype of *luxI* mutations. Similarly the *luxI*$^+$ allele can complement the exoenzyme-negative phenotype of *expI* mutations.

Autoinducer controls exoenzyme production and virulence in E.c. subsp. carotovora

In *V.fischeri* the LuxI protein directs the synthesis of a diffusible autoinducer, HSL, which is required for the cell density-dependent control of bioluminescence (Meighen, 1991). This kind of cell density-dependent control of gene expression is clearly reminiscent of the *expI*-controlled growth phase-dependent expression of exoenzyme genes in *E.c.* subsp. *carotovora*, and suggests that ExpI may direct the synthesis of a similar signal molecule. This was demonstrated by extracellular complementation of the pleiotropic defect in exoenzyme production in *expI* mutants. The results imply that a diffusible signal molecule is produced by *expI*$^+$ cells, is recognized by the mutant and is subsequently used to activate exoenzyme gene expression. The production of this inducer in *E.c.* subsp. *carotovora* is growth phase-dependent (D.Flego *et al.*, in preparation) with maximum inducer activity present at the onset of stationary phase. This is in agreement with the observed pattern of exoenzyme gene expression (see Figure 1) and is also similar to the pattern of HSL accumulation in *V.fischeri* (Meighen, 1991). The restoration of exoenzyme production was *expI*-specific (Table II): none of the other *exp* mutants could be complemented by addition of inducer-containing preparations. In contrast to *expI*, the other *exp* mutants (e.g. *expA*) produce a functional signal molecule, and can restore exoenzyme production to *expI* mutants. In agreement with this, the expression of exoenzyme genes in *expA* mutants is still responsive to growth phase (data not shown).

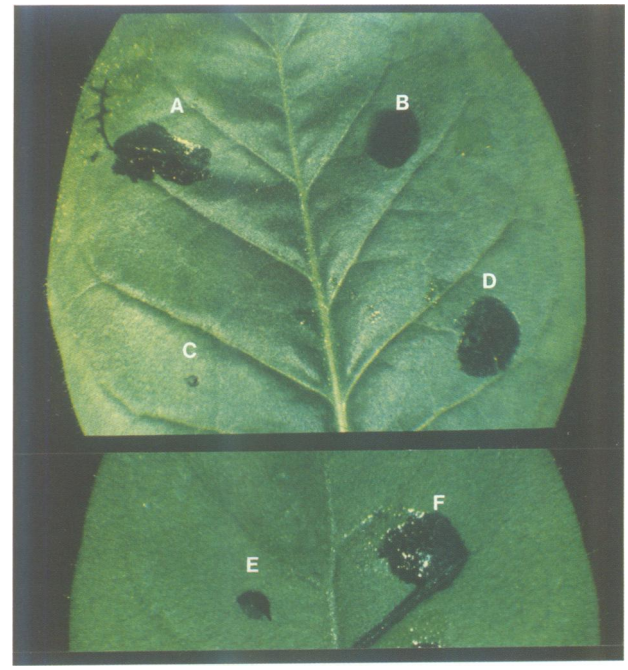

Fig. 8. Virulence of the *expI* mutant is restored by autoinducer addition. Virulence of the *E.c.* subsp. *carotovora* wild-type strain SCC3193 (**A** and **B**) and *expI* mutant strain SCC3065 (**C**−**F**) to tobacco leaves was assessed by local inoculation of 1 μl bacterial suspension (10^9 c.f.u./ml). The disease symptoms (i.e. maceration of the leaf tissue) were documented by photography after 6 h incubation with the bacteria. The wild-type inoculated in B and the *expI* mutant inoculated in D were preincubated with acellular spent culture supernatants from stationary phase cultures of *E.coli* carrying the *expI*$^+$ clone pTK806-1. The *expI* mutant inoculum in F was preincubated with synthetic HSL (2 μg/ml) before inoculation. Additional autoinducer was applied after inoculation to sustain the spreading of the bacteria. The wild-type inoculated in A and the *expI* mutant inoculated in C and E were not preincubated with culture supernatants or autoinducer and were used as positive (A) and negative (C and E) controls, respectively.

We propose that the signal molecule produced by *expI*$^+$ *E.c.* subsp. *carotovora* is either identical or functionally similar to *V.fischeri* HSL. This interpretation is supported by the following data. First, the *expI* mutant could be complemented extracellularly by *E.coli* carrying the *luxI* gene from *V.fischeri* in addition to *expI*$^+$ clones. Secondly, a *luxI* mutation could be complemented both intra- and extracellularly by *E.coli* as well as *E.c.* subsp. *carotovora* strains carrying the *expI*$^+$ allele. This was monitored by following light emission from a *luxI*$^-$ indicator plasmid. Thirdly, the *expI* mutant but not the other *exp* mutants could be complemented by introduction of clones carrying either *expI*$^+$ or *luxI*$^+$ into the mutant background. Fourthly, addition of synthetic HSL autoinducer to cultures of the *expI* mutant restored the exoenzyme production to wild-type levels. Furthermore, the effective concentration of the synthetic molecule (optimum at 2 μg/ml) was in the same range as found in the culture supernatant of the wild-type *E.c.* subsp. *carotovora* strain (D.Flego *et al.*, in preparation). Taken together the results strongly suggest that a signal molecule identical or functionally similar to the *V.fischeri* HSL is involved in control of exoenzyme gene expression and virulence in *E.c.* subsp. *carotovora*. Interestingly, recent work by Bainton *et al.* (1992a) has demonstrated the presence of HSL in *E.carotovora*, where it was shown to control production of carbapenem antibiotics (Bainton *et al.*, 1992a,b). Consequently, it is likely that the signal molecule employed for control of exoenzyme production is actually HSL.

Our results suggest that the accumulation of HSL-like signal molecules is required for the induction of the exoenzyme-encoding genes in *E.c.* subsp. *carotovora*. The production of the exoenzymes is in turn a prerequisite for the successful colonization of a plant by *E.c.* subsp. *carotovora*. This is indicated by restoration of virulence to the *expI* mutant by autoinducer addition (Figure 8). The data show that maceration of plant tissue and proliferation of the pathogen are absolutely dependent on the *expI* gene product, and that a defect in *expI* can be restored by HSL addition. The propagation of the *expI* mutant *in planta* seems to require the continuous presence of HSL molecules, as preincubation of the mutant bacteria with the HSL preparations only leads to transient restoration of virulence. In contrast, additional application of inducer allows the infection to spread throughout the plant, suggesting that coordinated high level production of exoenzymes is essential for successful *E.c.* subsp. *carotovora* infection.

A possible role of the autoinducer in plant pathogenicity

The data presented demonstrate that *E.c.* subsp. *carotovora* employs an HSL-like signal molecule to control its virulence gene expression in a growth phase-dependent manner. The production of plant cell wall-degrading exoenzymes is induced at high cell densities e.g. in stationary phase cultures, when enough autoinducer has accumulated. What is the biological relevance of this type of control of virulence factors; how does production of exoenzymes only at high cell density contribute to the success of *Erwinia* as a plant pathogen? The explanation could be as follows: under aerobic conditions, successful infection by *E.c.* subsp. *carotovora* requires a rather large inoculum load (10^6-10^7 c.f.u.) and the progress of the disease depends on the race between multiplication of the bacteria and development of plant resistance (Pérombelon and Kelman, 1980). The soft-rot *Erwinia* species can be regarded as brute-force pathogens which rely on their sheer numbers, rapid multiplication and massive production of the plant cell wall-degrading enzymes in order to overwhelm the host defenses. Consequently, production of the virulence factors, the exoenzymes, at low cell densities would not lead to a successful infection, but would only result in the induction of the plant defense response which in turn would hamper subsequent infections (Palva *et al.*, 1993). *E.c.* subsp. *carotovora* could employ the autoinducer as a signal for monitoring its numbers. Accumulation of this HSL-like molecule over a threshold value and the resulting induction of exoenzyme production would only take place at cell densities that are enough for a successful attack on a host plant.

A common signalling mechanism in bacteria?

The results presented in this paper as well as recent work from other laboratories (Gambello and Iglewski, 1991; Wang *et al.*, 1991; Bainton *et al.*, 1992a,b; Jones *et al.*, 1993) suggest that the autoinducer/response regulator system used to control bioluminescence in *V.fischeri* (Meighen, 1991) may represent a common signalling mechanism in bacteria. Evidence for this is provided by two types of data. Firstly, presence of the same or a similar autoinducer molecule has recently been observed in several genera of bacteria, including *Pseudomonas*, *Serratia*, *Erwinia*, *Citrobacter*, *Enterobacter* and *Proteus* (Bainton *et al.*, 1992a). The function of HSL in most of these bacteria has yet to be defined (Bainton *et al.*, 1992a) but in *Erwinia carotovora* it has been shown to control the growth phase-dependent biosynthesis of carbapenem antibiotics (Bainton *et al.*, 1992a,b). Secondly, regulatory proteins showing similarity to LuxI or LuxR of *V.fischeri* have recently been characterized in several bacterial species. Our results demonstrate that *E.c.* subsp. *carotovora* controls its virulence by ExpI, a functional analogue of LuxI. Recent work by Jones *et al.* (1993) indicate that a LuxI analogue is also present in antibiotic-producing strains of *Erwinia*. Related genes are involved in the control of virulence in *Pseudomonas aeruginosa* (Gambello and Iglewski, 1991; Cook and Iglewski, 1992), where homologues to both *luxI* and *luxR* have been described, the *lasI* and *lasR* genes, respectively. The transcriptional activator LasR controls expression of several virulence genes in *P.aeruginosa* in a growth phase-dependent manner (Gambello and Iglewski, 1991). The regulatory protein SdiA (Sharma *et al.*, 1986; Wang *et al.*, 1991), which controls cell division in *Escherichia coli*, also shows significant similarity to LuxR. In conclusion, processes as diverse as virulence in both plant and animal pathogens, antibiotic production, bioluminescence and maybe even cell division are controlled by HSL or HSL-like signals.

These results suggest that the autoinducer-response regulatory system may be a general signalling mechanism employed by prokaryotes for activating genes in response to specific environmental stimuli. Furthermore, structurally related lactones are involved in control of bioluminescence in *Vibrio harvey* (Meighen, 1991), and in regulation of antibiotic production and differentation in *Streptomyces* (Beppu, 1992). The weak acids that accumulate in stationary phase cultures and induce the stationary phase specific sigma

factor gene, *rpoS/katF* (Lange and Hengge-Aronis, 1991; Schellhorn and Stones, 1992), may represent yet another category of such signal molecules. Consequently, the HSL system is not the only environmental sensory system based on small signal molecules but may represent only one example of related signal systems ubiquitous in prokaryotes.

By analogy to *V.fischeri lux* gene regulation, *E.c.* subsp. *carotovora* should also contain HSL receptor protein(s) similar to LuxR. First indication for the presence of such response regulators was recently obtained by us by sequence analysis of a gene, *expR*, closely linked to *expI* (R.Heikinheimo, M.Pirhonen and E.T.Palva, in preparation). The gene product shows extensive similarity to LuxR and LasR, and we are currently characterizing its role in the exoenzyme production and pathogenicity of *E.c.* subsp. *carotovora*. This analysis should provide further insight into the virulence mechanisms of plant pathogens and clarify the role and mechanism of processes controlled by small signal molecules in environmental responses of prokaryotes.

Materials and methods

Bacterial strains and plasmids

The *E.coli* host strains used for plasmid maintenance and DNA preparation were DH5α (Hanahan, 1983) and XL1-blue. The latter was purchased from Stratagene (La Jolla, USA) together with a pBluescript II exonuclease III/mung bean DNA deletion kit. The *E.c.* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen *et al.*, 1988) and the *exp* mutant strains SCC3060 (*expA*) and SCC3065 (*expI*) (Pirhonen *et al.*, 1991) have been described. The plasmid pTK806 carrying the wild-type *expI* allele and complementing the mutation in SCC3065 has been described by Pirhonen *et al.* (1991). The plasmids pBR322, pJE202, pHSK24 and pHSK75-2 have been described (Bolivar *et al.*, 1977; Engebrecht *et al.*, 1983; Dunlap and Greenberg, 1988; Saarilahti *et al.*, 1990, 1992, respectively). The plasmid pHV200-I is a derivative of pHV200 (Gray and Greenberg, 1992) with a frameshift mutation introduced into the *luxI* gene at the *BglII* site (K.M.Gray and E.P.Greenberg, personal communication). The plasmid pHSK17 is pUC18 (Yanisch-Perron *et al.*, 1985) derivative carrying a *pelC* gene (R.Heikinheimo *et al.*, unpublished) on a 2.5 kb insert and plasmid pMUT61 is a pMUT201 (Karlsson *et al.*, 1991) derivative carrying the *celV* gene on a 6.1 kb fragment. This *celV* gene cloned from SCC3193 is closely related to the *celV* recently characterized from another *E.c.* subsp. *carotovora* strain (V.Cooper and G.P.C.Salmond, submitted). A transcriptional *pehA−lacZ* fusion is carried in the pDFL2 plasmid (D.Flego *et al.*, in preparation). Transfer of the plasmid was by standard transformation procedures or by T4GT7 transduction as described previously (Pirhonen *et al.*, 1991). The transducing bacteriophage T4GT7 has been described by Wilson *et al.* (1979).

Media, culture conditions and chemicals

E.coli was propagated in L medium (Miller, 1972) at 37°C, and *E.c.* subsp. *carotovora* at 28°C in L medium supplemented with either 0.4% glycerol or 0.5% sodium polypectate (PGA; Sigma P-1879; Sigma Chemical Co., USA). To obtain the spent culture supernatants used for extracellular complementation, the bacteria were propagated as described above to stationary phase ($A_{600\ nm}$ = 4), the cells removed by centrifugation and the supernatant sterilized by filtration. Chloramphenicol (Cm) and kanamycin (Km) were added to media at 25 μg/ml and ampicillin (Ap) at 150 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was from Bachem Inc. (USA), and added to media at 35 μg/ml, *o*-nitrophenyl-β-D-galactoside was from Merck (Germany), and Nitrocefin from Oxoid Ltd (UK). The restriction enzymes, T4 ligase and exonuclease III were from IBI (USA), mung bean nuclease from Stratagene (USA) and the sequencing kit from United States Biochemicals Corp. (USA). The multiprime DNA labelling kit, nick translation kit and the radiolabelled compounds were from Amersham International (UK). Samples of synthetic HSL were provided by H.B.Kaplan, A.Eberhard, C.Widrig and E.P.Greenberg (Kaplan *et al.*, 1985) and Paul Williams (Bainton *et al.*, 1992b).

Virulence tests

The virulence of the *E.c.* subsp. *carotovora* strains to tobacco (*Nicotiana tabacum* cv SR1) seedlings was tested by local inoculation. These seedlings were propagated axenically in MS-2 medium (Murashige and Skoog, 1962) at 25°C in a controlled environment (16 h light regime, 2 W/m^2) for 4−6 weeks before inoculation. Local inoculation was by pipetting 1 μl (adjusted to contain 10^6 c.f.u.) of bacterial suspension in early stationary phase to the leaves. The inoculated plants were incubated at 28°C under 100% humidity for the times indicated before assessment and documentation of the disease symptoms by photography. When applicable spent culture supernatants or synthetic HSL were added to the bacterial culture 6−8 h prior to the inoculation and also supplied after inoculation.

DNA manipulations, deletion analysis and determination of the DNA sequence

Unless otherwise stated, isolation of plasmid DNA, cloning, transformation and gel analyses of plasmids were by established procedures as described in Sambrook *et al.* (1989). Unidirectional deletions of pTK806-1 were generated by cleaving the plasmid with appropriate restriction enzymes within the multiple cloning site to create 3′ or 5′ overhangs, followed by digestion of the 3′ recessed strand with exonuclease III (Henikoff, 1984). Single-stranded DNA was removed with mung bean nuclease, the blunt ends were ligated and the plasmids transformed into *E.coli* strain XL1-blue. The sequencing was performed on both strands by the Sanger dideoxy-chain termination method (Sanger *et al.*, 1977) using the Sequenase™ (USB) DNA sequencing kit. The amino acid alignment was done according to the Needleman−Wunsch algorithm (Needleman and Wunsch, 1970) using the Intelligenetics program (Mountainview, CA, USA).

Assay procedures

For detection of exoenzymes on plates, indicator media were used as described by Andro *et al.* (1984) for cellulase and by Hankin and Anagnostakis (1975) for proteases. Alternatively, L-plates (Miller, 1972) supplemented with 1.5% skimmed milk (Oxoid Ltd, UK) were used for protease detection. The activity of Pel, Peh, Cel, Prt and β-lactamase was assayed and the units defined as previously described (Pirhonen *et al.*, 1991). The assay for β-galactosidase was as described by Miller (1972). The presence of autoinducer in spent culture supernatants was detected by a bioassay using as indicator an *E.coli* strain harboring the plasmid pHV200-I which carries the *lux* genes of *V.fischeri* but has a frameshift mutation in *luxI*. Serial dilutions of samples to be assayed were mixed with an overnight culture of the indicator in 24-well tissue culture plates, incubated for 2 h at 28°C and the plate was subsequently exposed to X-ray film. Known amounts of synthetic HSL were used as a standard.

Analysis of plasmid-encoded proteins

[^{35}S]methionine-labelled plasmid-encoded proteins were produced by the maxicell method of Sancar *et al.* (1979), and the proteins were separated by SDS−PAGE according to Laemmli (1970). Labelled proteins were visualized by fluorography of stained and dried gels.

RNA isolation and Northern analysis

RNA was isolated as previously described for Gram-negative bacteria (Gilman, 1987). 10 μg of total RNA was denatured in formamide and formaldehyde, separated by formaldehyde gel electrophoresis and blotted on to nylon filters (Hybond-N, Amersham International, UK) essentially as described by Sambrook *et al.* (1989). Hybridizations were performed overnight at 65°C in 5×Denhardt's, 5×SSP, 0.2% SDS, 500 μg/ml denatured herring sperm DNA. The *pehA* probe was prepared from a 770 bp internal *Bst*EII−*Eco*RV fragment from the *pehA* gene in pHSK24 (Saarilahti *et al.*, 1990); the *celV* probe was a 925 bp *Eco*RI−*Eco*RV gene specific fragment. The fragments were labelled with [^{32}P]dATP by random primer labelling. The *pelC* probe was a pUC18 plasmid carrying a 2.5 kb fragment harboring the *pelC* gene; it was labelled with [^{32}P]dATP by nick translation. After hybridization, membranes were washed in 2×SSC, 0.5% SDS at 65°C for 60 min and exposed to X-ray film.

Acknowledgements

We are indebted to Dr E.P.Greenberg for the various *lux* plasmid constructs used, and for helpful discussions. We thank Drs Kaplan, Eberhard, Widrig and Greenberg for an initial supply of the autoinducer. Dr Paul Williams is thanked for the supply of autoinducer used for the plant experiments. Dr Paul Williams as well as Dr Gordon Stewart and Dr George P.C.Salmond are also thanked for communicating unpublished data. We thank Dr Tuula Palva for helping with the illustrations, and Dr Jürgen Denecke and Kjell-Ove Holmström for help with the sequence comparisons, and Jürgen Denecke for critically reading the manuscript. Maj-Britt Karlsson is thanked for skilful technical assistance and Jill Thorngren for secretarial assistance. A grant from SAREC is gratefully acknowledged. This work was supported by the Swedish Council for Forestry and Agricultural Research.

References

Andro,T., Chambost,J.-P., Kotoujansky,A., Cattaneo,J., Bertheau,Y., Barras,F., van Gijsegem,F. and Coleno,A. (1984) *J. Bacteriol.*, **160**, 1199–1203.

Aymeric,J.-L., Guiseppi,A., Pascal,M.-C. and Chippaux,M. (1988) *Mol. Gen. Genet.*, **211**, 95–101.

Bainton,N.J., Bycroft,B.W., Chhabra,S.R., Stead,P., Gledhill,L., Hill,P.J., Rees,C.E.D., Winson,M.K., Salmond,G.P.C., Stewart,G.S.A.B. and Williams,P. (1992a) *Gene*, **116**, 87–91.

Bainton,N.J., Stead,P., Chhabra,S.R., Bycroft,B.W., Salmond,G.P.C., Stewart,G.S.A.B. and Williams,P. (1992b) *Biochem. J.*, **288**, 997–1004.

Beppu, T. (1992) *Gene*, **115**, 159–165.

Boccara,M., Diolez,A., Rouve,M. and Kotoujansky,A. (1988) *Physiol. Mol. Plant Pathol.*, **33**, 95–104.

Bolivar,F., Rodriques,R.L., Greene,P.J., Betlach,M.V. and Boyer,H.W. (1977) *Gene*, **2**, 75–93.

Boyer,M.H., Chambost,J.P., Magnan,M. and Cattanéo,J. (1984) *J. Biotechnol.*, **1**, 229–239.

Choi,S.H. and Greenberg.E.P. (1992) *J. Bacteriol.*, **174**, 4064–4069.

Collmer,A. and Keen,N.T. (1986) *Annu. Rev. Phytopathol.*, **24**, 383–409.

Cook,J.M. and Iglewski,B.H. (1992) Proceedings of the 92nd Annual Meeting of the American Society of Microbiology. ASM, New Orleans, USA, Abstract D-47, p. 103.

Dunlap,P.V. and Greenberg,E.P. (1988) *J. Bacteriol.*, **170**, 4040–4046.

Eberhard,A., Burlingame,A.L., Eberhard,C., Kenyon,G.L., Nealson,K.H. and Oppenheimer,N.J. (1981) *J. Biochem.*, **20**, 2444–2449.

Engebrecht,J.A. and Silverman, M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4154–4158.

Engebrecht,J.A. and Silverman,M. (1987) *Nucleic Acids Res.*, **15**, 10455–10467.

Engebrecht,J.-A., Nealson,K. and Silverman,M. (1983) *Cell*, **32**, 773–781.

Gambello,M.J. and Iglewski,B.H. (1991) *J. Bacteriol.*, **173**, 3000–3009.

Gilman,M. (1987) In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, units 4.4.1–4.4.4.

Gray,K.M. and Greenberg,E.P. (1992) *J. Bacteriol.*, **174**, 4384–4390.

Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–580.

Hankin,L. and Anagnostakis,S.L. (1975) *Mycologia*, **67**, 597–607.

Henikoff,S. (1984) *Gene*, **28**, 351–359.

Hinton,J.C.D., Sidebotham,J.M., Hyman,L.J., Pérombelon,M.C.M. and Salmond,G.P.C. (1989) *Mol. Gen. Genet.*, **217**, 141–148.

Hugouvieux-Cotte-Pattat,N. and Robert-Baudouy,J. (1989) *Mol. Microbiol.*, **3**, 1587–1597.

Hugouvieux-Cotte-Pattat,N., Reverchon,S., Condemine,G. and Robert-Baudouy,J. (1986) *J. Gen. Microbiol.*, **132**, 2099–2106.

Jones,S. *et al.* (1993) *EMBO J.*, **12**, 2477–2482.

Kaplan,H.B. and Greenberg,E.P. (1985) *J. Bacteriol.*, **163**, 1210–1214.

Kaplan,H.B., Eberhard,A., Widrig,C. and Greenberg,E.P. (1985) *Comp. Radiopharmacol.*, **22**, 387–395.

Karlsson,M.-B., Pirhonen,M., Saarilahti,H.T. and Palva,E.T. (1991) *Mol. Gen. Genet.*, **226**, 353–360.

Kotoujansky,A. (1987) *Annu. Rev. Phytopathol.*, **25**, 405–430.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lange,R. and Hengge-Aronis,R. (1991) *J. Bacteriol.*, **173**, 4474–4481.

McEvoy,J.L., Murata,H. and Chatterjee,A.K. (1992) *J. Bacteriol.*, **174**, 5471–5474.

Meighen,E.A. (1991) *Microbiol. Rev.*, **55**, 123–142.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 466.

Moran,F. and Starr,M.P. (1969) *Eur. J. Biochem.*, **11**, 291–295.

Murashige,T. and Skoog,F. (1962) *Physiol. Plant.*, **15**, 473–497.

Murata,H., McEvoy,J.L., Chatterjee,A., Collmer,A. and Chatterjee,A.K. (1991) *Mol. Plant–Microbe Interact.*, **4**, 239–246.

Nasser,W., Reverchon,S. and Robert-Baudouy,J. (1992) *Mol. Microbiol.*, **6**, 257–265.

Needleman,S.B. and Wunsch,C.D. (1970) *J. Mol. Biol.*, **48**, 443–453.

Palva,T.K., Holmström,K.-O., Heino,P. and Palva,E.T. (1993) *Mol. Plant–Microbe Interact.*, in press.

Pérombelon,M.C.M. and Kelman,A. (1980) *Annu. Rev. Phytopathol.*, **18**, 361–387.

Pirhonen,M., Heino,P., Helander,I., Harju,P. and Palva,E.T. (1988) *Microb. Pathogen.*, **4**, 359–367.

Pirhonen,M., Saarilahti,H., Karlsson,M.-B. and Palva,E.T. (1991) *Mol. Plant–Microbe Interact.*, **4**, 276–283.

Reverchon,S., Hugouvieux-Cotte-Pattat,N., Condemine,G., Bourson,C., Arpin,C. and Robert-Baudouy,J. (1990) In Klement,Z. (ed.), *Plant Pathogenic Bacteria*. Akademiai Kiado, Budapest, pp. 739–744.

Ried,J.L. and Collmer,A. (1988) *Mol. Plant–Microbe Interact.*, **1**, 32–38.

Saarilahti,H.T. (1990) Ph.D. thesis, Swedish University of Agricultural Sciences, Uppsala.

Saarilahti,H.T., Heino,P., Pakkanen,R., Kalkkinen,N., Palva,I. and Palva,E.T. (1990) *Mol. Microbiol.*, **4**, 1037–1044.

Saarilahti,H.T., Pirhonen,M., Karlsson,M.-B., Flego,D. and Palva,E.T. (1992) *Mol. Gen. Genet.*, **234**, 81–88.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sancar,A., Hack,A.M. and Rupp,W.D. (1979) *J. Bacteriol.*, **137**, 692–693.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Schellhorn,H.E. and Stones,V.L. (1992) *J. Bacteriol.*, **174**, 4769–4776.

Sharma,S., Stark,T.F., Beattie,W.G. and Moses,R.E. (1986) *Nucleic Acids Res.*, **14**, 2301–2318.

Stock,J.B., Ninfa,A.J. and Stock,A.M. (1989) *Microbiol. Rev.*, **53**, 450–490.

Stock,J.B., Stock,A.M. and Mottonen,J.M. (1990) *Nature*, **344**, 395–400.

von Heijne,G. (1987) *Gene*, **58**, 45–57.

Wandersman,C., Andro,T. and Bertheau,Y. (1986) *J. Gen. Microbiol.*, **132**, 899–906.

Wang,X., de Boer,P.A.J. and Rothfield,L.I. (1991) *EMBO J.*, **10**, 3363–3372.

Wilson,G.G., Young,K.K., Edlin,G.J. and Konigsberg,W. (1979) *Nature*, **280**, 80–82.

Yanisch-Perron,C., Vieira,J. and Messing,J.I. (1985) *Gene*, **33**, 103–119.

Zink,R.T., Engwall,J.K., McEvoy,J.L. and Chatterjee,A.K. (1985) *J. Bacteriol.*, **164**, 390–396.

Zucker,M., Hankin,L. and Sands,D. (1972) *Physiol. Plant Pathol.*, **2**, 59–67.

Received on December 22, 1992; revised on February 22, 1993

Note added in proof

The EMBL/GenBank/DDBJ accession number for the *E.carotovora expI* sequence presented in figure 4 is X72891.