Flavohaemoglobin HmpX: a new pathogenicity determinant in *Erwinia chrysanthemi* strain 3937

S. Favey,¹ G. Labesse,^{1,2} V. Vouille¹ and M. Boccara¹

Author for correspondence: M. Boccara. Tel: +33 1 44 08 16 98. Fax: +33 1 44 08 17 00.
e-mail: Boccara@inapv.inapg.inra.fr

¹ Laboratoire de pathologie végétale, INRA, INA P/G, 16 rue Claude Bernard, 75005 Paris, France

² Département des macromolécules biologiques, Laboratoire de Minéralogie-Cristallographie, Universités Paris VI and VII, CNRS URA 09, 4 place Jussieu, 75252 Paris cedex 05, France

Unlike wild-type *Erwinia chrysanthemi* strain 3937, which fully macerates inoculated Saintpaulia plants, HmpX⁻ mutants produce necrotic lesions or no symptoms. The *hmpX* gene was sequenced and the corresponding protein sequence analysed. We show that HmpX belongs to a family of flavohaemoproteins (HMP), previously identified in two yeasts and in *Escherichia coli*. Comparisons of protein sequences at the secondary structure level by hydrophobic cluster analysis have shown that HmpX possesses two functional regions, a haemoglobin domain in its N-terminal part and a flavin reductase domain in its C-terminal part. In an HmpX⁻ strain, the synthesis of pectate lyases, which are pathogenicity determinants in *E. chrysanthemi*, was reduced in conditions of low oxygen tension. Using *gus* fusion in *hmpX*, it was shown that *hmpX* transcription was induced in coculture with tobacco cells. A putative function for HmpX is discussed.

Keywords: anaerobiosis, bacterial haemoglobin, *Erwinia chrysanthemi*, HMP, pectate lyase

INTRODUCTION

Erwinia chrysanthemi is an enterobacterium which causes soft rot disease on a wide range of plants. This pathogen causes maceration of parenchymatous tissues by degrading the middle lamella and the primary cell wall through the action of depolymerizing enzymes, pectinases, endoglucanases and proteases. Among the pectinases produced *in vitro*, *E. chrysanthemi* strain 3937 secretes five pectate lyase (PL) isozymes (PLa to PLe; Bertheau *et al.*, 1984). The five *pel* genes are organized in two clusters: *pelB*, C encoding the neutral PL and *pelA, D, E* encoding the acidic and basic PL (Kotoujansky *et al.*, 1985). Strains with single mutations in genes encoding pectinases have been constructed and their pathogenicity on axenically grown plants (*Saintpaulia ionantha*, the South African violet) has been studied. The results have shown that several pectinolytic enzymes are involved in systemic infection of the host plant (Boccara *et al.*, 1988). For example, a mutant unable to produce its five PLs is still

able to produce localized maceration but the infection does not progress to destroy the whole plant (Beaulieu *et al.*, 1993). Therefore the PLs of *E. chrysanthemi* 3937 detected *in vitro* do not appear to be involved in the early events of infection.

In the search for bacterial functions involved in early steps of plant infection, several groups have isolated mutants unable to multiply in the host plant and unable to induce the hypersensitive response on non-host plants (for a review, see Willis *et al.*, 1991). In phytopathogenic bacteria, including enterobacteria such as *E. chrysanthemi* (Bauer *et al.*, 1994), a locus composed of a large number of genes named *hrp* (hypersensitive response and pathogenicity) has been defined. The Hrp proteins probably encode a secretion apparatus for pathogenicity factors (Van Gijsegem *et al.*, 1993). The function of these factors could be to cause release from the plant of the carbon and/or nitrogen sources necessary for bacterial growth within intercellular spaces.

Other environmental factors such as the availability of iron (Expert & Gill, 1992) or oxygen tension (Billing, 1987) have been shown to be of great importance in the early steps of infection by plant pathogenic bacteria. For example, conditions that reduce oxygen tension in potato tubers, such as a film of surface water, make potatoes

Abbreviations: a.u., arbitrary unit; DHPR, dihydropteridine reductase; Gus, β -glucuronidase; HCA, hydrophobic cluster analysis; HMP, flavohaemoprotein.

The EMBL accession number for the sequence reported in this paper is X75893.

more susceptible to *Erwinia carotovora* (De Boer & Kelman, 1978), suggesting that oxygen availability may be an important determinant in soft rot pathogenesis.

To find new pathogenicity factors we systematically mutated the region located in the vicinity of the *pel* genes. We found a cluster of mutations close to the *pelA,D,E* region of strain 3937 (Reverchon *et al.*, 1986), affecting the pathogenicity of *E. chrysanthemi* on Saintpaulia plants. In this paper, we present the sequence of the gene corresponding to the mutated region, which we have named *bmpX*, and we discuss the structure-function relationship according to the homologies shared by the encoded protein and families of related flavoenzymes. We show that this protein is important for PL production in a microaerobic environment.

METHODS

Bacterial strains, plasmids, media and growth conditions.

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown in Luria broth medium (Miller, 1972) at 37 °C. *E. chrysanthemi* strains were grown with vigorous shaking at 30 °C in M9 synthetic medium (Miller, 1972), plus 0.5% glycerol. Aerobic cultures were grown in Erlenmeyer flasks ten times larger than the volume of the culture medium. Microaerobic conditions were obtained by covering the cultures grown in tubes (18 × 160 mm) in the same medium with 2 ml

vaseline and maintaining them without shaking at 30 °C for 3 d. Antibiotics were used at the following concentrations: ampicillin (Ap), 50 µg ml⁻¹; chloramphenicol (Cm), 15 µg ml⁻¹; and kanamycin (Km), 25 µg ml⁻¹.

Genetic manipulations. Plasmid pB413 was introduced into POII1734 or POIIPR13 dilyogenic strains to obtain mini-Mu (MudII1734 and dMudPR13) insertions (Catilho *et al.*, 1984). The Mu prophages were thermo-induced. The resulting lysate was used to infect strain M8820, from which Cm^r or Km^r transductants were selected.

To marker exchange the mutated *bmpX* gene with the *E. chrysanthemi* chromosomal allele, *E. chrysanthemi* was first transformed with plasmid harbouring a mini-Mu insertion in *bmpX* by the conventional calcium chloride method (Maniatis *et al.*, 1982). Plasmid destabilization was obtained in low-phosphate medium (Roeder & Collmer, 1985), and mutants growing on chloramphenicol or kanamycin but not on ampicillin were selected.

DNA manipulations. Standard molecular techniques were used, according to Ausubel *et al.* (1987) and Maniatis *et al.* (1982).

DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et al.* (1977) using the Sequenase kit (United States Biochemical Corporation). The 1.5 kb *DraI* fragment containing *bmpX* (Fig. 1) was hydrolysed by *Sau3A* or *TaqI* and subcloned into pUBS3. Sequence was obtained from primer sites in the vector and using synthetic oligonucleotides (Unité de Chimie organique, Institut Pasteur, Paris). Computer-

Table 1. Bacterial strains, bacteriophages and plasmids

Strain, bacteriophage or plasmid	Relevant characteristics	Reference
<i>Escherichia coli</i>		
POII1734	F <i>araD139 ara::</i> (Mucts62)3 Δ (<i>lac</i>)X74 <i>galU galK rpsL</i> with MudII1734	Castilho <i>et al.</i> (1984)
POIIPR13	<i>recA1 endA1 gyr-196 Thi⁻ hsdR17 supE44 relA1</i> Δ (<i>lac-proAB</i>) with dMudPR13 and Mucts62	Reyes <i>et al.</i> (1987)
M8820	F ⁻ <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>rpsL</i> Δ (<i>proAB-argF-lacIPOZYA</i>)XIII	Castilho <i>et al.</i> (1984)
AR 1062	Strain which generates minicells	
DH5 α	Strain for propagating plasmids	
<i>Erwinia chrysanthemi</i>		
3937	Wild-type, isolated from Saintpaulia	Our collection
PMV4125, PMV4127	<i>bmpX::</i> dMudPR13 derivatives of 3937	This study
PMV4128	<i>bmpX::</i> MudII1734 derivative of 3937	This study
X8	<i>bmpX::gus</i> Km (transcriptional fusion)	This study
X2	<i>bmpX::gus</i> Km (cassette in the reverse orientation)	This study
Bacteriophages		
MudII1734	Km ^r Mu derivative	Castilho <i>et al.</i> (1984)
dMudPR13	Cm ^r Mu derivative	Reyes <i>et al.</i> (1987)
Plasmids		
pB413	Ap ^r / <i>pelA</i> ⁺ <i>bmpX</i> ⁺ derivative of pUC9	Reverchon <i>et al.</i> (1986)
p0165	pB413 hydrolysed by <i>EcoRI</i> and religated	This study
pUBS3	pUC derivative containing pBLUESCRIPT polylinker	Murphy & Kavanagh (1988)
p19014	Plasmid harbouring <i>uidA</i> -Km cassettes	Bardonnnet & Bianco (1992)

assisted analysis of the sequences was carried out using programs developed by the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Minicell isolation and protein analysis. Minicells were isolated, labelled with [³⁵S]methionine (Amersham) as described by Rambach & Hogness (1977), and their protein content analysed on a 12% (w/v) SDS-polyacrylamide gel. Proteins were stained with Coomassie blue, and labelling was visualized by autoradiography, using Kodak X-OMAT AR films.

Protein sequence analysis. The deduced amino acid sequence of HmpX was compared to sequences from the SWISSPROT data bank (February 1994) with the FASTA program (Pearson & Lipman, 1988), using the matrices of Dayhoff *et al.* (1978) and Risler *et al.* (1988). BLAST searches were performed to screen the GenPept databank (release no. 83) using the network service of the NCBI (Altschul *et al.*, 1990). Pairwise and multiple alignments were determined by hydrophobic cluster analysis (HCA) as described in the original papers (Gaboriaud *et al.*, 1987; Woodcock *et al.*, 1992).

Enzyme activities in vitro. PL assays were performed on supernatants of cells grown to late exponential phase in M9 minimal medium plus 0.5% glycerol. PL activity was determined by monitoring the appearance of unsaturated products from polygalacturonate (Sigma), absorbing at 235 nm (Moran *et al.*, 1968), in 0.1 M Tris/HCl pH 8.6, 5×10^{-4} M CaCl₂. PL specific activity was expressed as μmol unsaturated products liberated min^{-1} (mg bacterial dry weight)⁻¹.

Dihydropteridine reductase activity was assayed as described by Vasudevan *et al.* (1988).

β -Glucuronidase (Gus) activity was measured by following the degradation of PNPU (*p*-nitrophenyl β -D-glucuronide) into *p*-nitrophenol, which absorbs at 405 nm (Bardonnet & Blanco, 1992). Specific activity is expressed as nmol product liberated min^{-1} per 10^9 bacteria.

Construction of the *hmpX::gus* fusion. We used the *gus*-Km cassettes described by Bardonnet & Blanco (1992), in which the *gus* gene is deleted of its own promoter region but conserves its Shine-Dalgarno sequence. The cassette obtained by *Sma*I digestion was ligated to the Klenow filled-in *Xma*III site of *hmpX*. This gives rise to *gus* insertion in the orientation of transcription (pX8) and in the reverse orientation (pX2). The *gus* insertions were then introduced into the *E. chrysanthemi* chromosome by marker exchange recombination, generating strains X2 and X8, respectively.

Pathogenicity test. Pathogenicity tests were performed on *Saintpaulia ionantha* cultivar 'Blue Rhapsody'. Leaf parenchyma was inoculated with $100 \mu\text{l}$ bacterial suspension in 0.9% NaCl (10^8 c.f.u. ml^{-1}). After inoculation the plants were placed at 28 °C under saturating humidity. The results were recorded daily for 15 d to give sufficient time for the development of symptoms. The inoculations were repeated three times.

Measure of enzyme activities in planta. To study PL activity in *planta*, leaf intercellular space was infiltrated *in vacuo* with bacteria (10^8 c.f.u. ml^{-1}). Infiltrated leaves were kept for 24 h at 30 °C and then centrifuged as described by De Witt & Spikman (1982). The intercellular fluid was used for PL assay and for bacterial enumeration by dilution plating.

Cell suspension cultures of *Nicotiana tabacum* cultivar Wisconsin clone 19-3 were grown in a slightly modified Murashige and Skoog medium, as described by Gregorini & Laloue (1980), on a rotary shaker (150 r.p.m.) in a growth chamber at 25 °C under constant white light (1500 lx). Exponentially growing tobacco cells (10 ml, 4 d after subculture) were mixed with 3×10^8

bacteria in the late exponential phase. After 24 h the plant cells were separated by filtration and bacteria collected by centrifugation for Gus activity assays and bacterial cell counting.

RESULTS AND DISCUSSION

Pathogenicity of *hmpX* mutant strains

Plasmid pB413, which contains a 5.4 kbp insert, was mutated in *Escherichia coli* by insertion of the mini-Mu transposable elements dMudPR13 and MudII1734. Three insertions (0163, 238 and 239, Fig. 1) were mapped 5' to the *pelA* gene and were shown by restriction enzyme analysis to be clustered on a 500 bp region. These insertions were exchanged with the *E. chrysanthemi* 3937 chromosomal allele, generating strains PMV4125, PMV4127 and PMV4128, respectively. The pathogenicity of these strains on *Saintpaulia* plants was compared with that of the wild-type strain 3937. When the wild-type strain was inoculated to *Saintpaulia* plants, a translucent spot was visible at the inoculation site after 24 h. A week later, maceration had spread to the whole leaf and after two weeks the whole plant was diseased. In contrast, plants inoculated with strain PMV4125, PMV4127 or PMV4128 exhibited no symptoms, or only necrotic spots at the inoculation site, and necrosis did not spread over the two weeks of observation. This result, which is different from that observed with a *pelA* mutant (localized maceration; Boccara *et al.*, 1988) prompted us to sequence the mutated region.

Nucleotide sequence of *hmpX*

The nucleotide sequence of the *hmpX* gene was determined (Fig. 2). We identified a 1185 bp open reading frame, coding for a protein of 395 residues with a calculated molecular mass of 44 230 Da. Analysis of the region upstream of the coding sequence revealed the presence of a putative promoter similar to the consensus sequence promoter of *E. coli*. Furthermore, the presumed ATG translational start codon located at base 242 is favourably positioned behind a Shine-Dalgarno sequence. Downstream of the putative *hmpX* stop codon TAA, a region similar to a bidirectional rho-independent transcription terminator in *E. coli* was detected. No

Fig. 1. Restriction map of the fragment in the pUC9 vector (pB413) containing the *hmpX* gene. The locations of the insertions are indicated by flags. Restriction enzymes: A, *AccI*; B, *Bgl*II/*Bam*HI; C, *Clai*; D, *Dra*I; E, *Eco*RI; H, *Hpa*I; K, *Kpn*I; S, *Sal*I.

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1  TGGGCTGACAGAGGTTTTTACGCAACGATTAGCTACAGGCTACGCAAGGCTTTACGTCTTTCCGGGATTAGCCATTCGGGCTGATTTTT
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91  TCTCATCTCAACCTGCATTTTTTTCATACGCCCTTCCCGCTGATTGCTGCCCCGCCCTACCCAGACCATCACCGGGGATTACAGG
-----
181 CAACATCCTTTTTAATAAGATGCATTTAAATGCAACTTATAGACAACAATGAGGTTACCATGCTGGATCAACAAACCATCGCCACCAT
      SD      MetLeuAspGlnGlnThrIleAlaThrIl
      M L D Q Q T I A T I

271 CAAATCCACCATTCCTCTGCTGGCCGAAACCGGTCCGGCGTTGACCGCTCACTTTTATCAGCGGATGTTCCACCATAACCGGAACGTAA
eLysSerThrIleProLeuLeuAlaGluThrGlyProAlaLeuThrAlaHisPheTyrGlnArgMetPheHisHisAsnProGluLeuLy
K S T I P L L A E T G P A L T A H F Y Q R M F H H N P E L K

361 AGATATCTTCAATATGAGTAATCAGCGTAATGGCGACCGCGAAGCGGTGTTCAATGCCATTGCGCCTACGCGACGCATATCGAAAA
sAspIlePheAsnMetSerAsnGlnArgAsnGlyAspGlnArgGluAlaValPheAsnAlaIleCysAlaTyrAlaThrHisIleGluAs
D I F N M S N Q R N G D Q R E A V F N A I C A Y A T H I E N

451 CCTGCCGCGTGTGCTGCCCGGTAGAACGTATCGCGCAAAAGCATGCCAGCTTCAATATTCAGCCGGAACAGTATCAGATAGTGGGTAC
nLeuProAlaLeuLeuProAlaValGluArgIleAlaGlnLysHisAlaSerPheAsnIleGlnProGluGlnTyrGlnIleValGlyTh
L P A L L P A V E R I A Q K H A S F N I Q P E Q Y Q I V G T

541 TCACCTGCTGGCAACGCTGGAAGAAATGTTCCAGCCCGGTGAGGCGGTGCTGGATGCCCTGGGGCAACGCTACGCGGTGCTGGCAATGT
rHisLeuLeuAlaThrLeuGluGluMetPheGlnProGlyGlnAlaValLeuAspAlaTrpGlyLysArgTyrGlyValLeuAlaAsnVa
H L L A T L E E M F Q P G Q A V L D A W G K R Y G V L A N V

631 GTTTATCCAGCGGAAAGCGACATTTATCAGCAAGCGCGGACAAAACGGCGGTGGCATGGCATCCGGCCGTTTCGCATTGTGGCGAA
lPheIleGlnArgGluSerAspIleTyrGlnGlnSerAlaGlyGlnAsnGlyGlyTrpHisGlyIleArgProPheArgIleValAlaLy
F I Q R E S D I Y Q Q S A G Q N G G W H G I R P F R I V A K

721 ACAGCCCCAGAGCAGCCTGATCACCAGCTTACGCTGGAACCGGTGGATGGCGCCGATCGCCGCATTCCGTCCCGGCCAGTATCTGGC
sGlnProGlnSerSerLeuIleThrSerPheThrLeuGluProValAspGlyGlyProIleAlaAlaPheArgProGlyGlnTyrLeuAl
Q P Q S S L I T S F T L E P V D G G P I A A F R P G Q Y L A

811 GGTCTACATCCCGATAAGCGCTTTGAATATCAGGAGATTGCTCAGTACTACTAAACGAACGAACCCAAACGGGCGCTATTACCGTATCGC
aValTyrIleArgAspLysArgPheGluTyrGlnGluIleArgGlnTyrSerLeuThrAsnGluProAsnGlyArgTyrTyrArgIleAl
V Y I R D K R F E Y Q E I R Q Y S L T N E P N G R Y Y R I A

901 GGTCAAACGGGAAACGATGGGCAGCGTATCGGGCTATTTGCACGATGTGCGCCGGAAGGCGATGTGATTGAACTGGCCGCCCCGACGG
aValLysArgGluThrMetGlySerValSerGlyTyrLeuHisAspValAlaArgGluGlyAspValIleGluLeuAlaAlaProHisGl
V K R E T M G S V S G Y L H D V A R E G D V I E L A A P H G

991 CGATTTCTACCTTGAGGTACGCCAGAGACGCCGTAGCGCTGATTTCCGCCGGTGTGGTCAAACACCGATGCTCAGCATGCTGCACAG
yAspPheTyrLeuGluValThrProGluThrProValAlaLeuIleSerAlaGlyValGlyGlnThrProMetLeuSerMetLeuHisSe
D F Y L E V T P E T P V A L I S A G V G Q T P M L S M L H S

1081 CCTGAAAAATCAGCAGCATCAGGCGGATATCTTCTGGTTACACGCGGCTGAAAAACACAGAAGTACATGCCTTCGCGACGAAATCGCCGA
rLeuLysAsnGlnGlnHisGlnAlaAspIlePheTrpLeuHisAlaAlaGluAsnThrGluValHisAlaPheAlaAspGluIleAlaAs
L K N Q Q H Q A D I F W L H A A E N T E V H A F A D E I A D

1171 TGTGCGCGCTACGCTGCCGAGTTGCAATCCTATGTCTGGTATCGGGAAGCCTCATCGGAAGCGGCACGTTCCGGCGCATGCTTTTCACGG
pValAlaAlaThrLeuProGlnLeuGlnSerTyrValTrpTyrArgGluAlaSerSerGluAlaAlaArgSerAlaHisAlaPheHisGl
V A A T L P Q L Q S Y V W Y R E A S S E A A R S A H A F H G

1261 ATTGATGGCGTTGAAGACCTCCGACGCGCTGCCGATGACAAACCTGCACTGTTATTTATGCGGCCCGGTGCGCTTTATGCAATTCCG
yLeuMetAlaLeuLysAspLeuProThrProLeuProMetThrAsnLeuHisCysTyrLeuCysGlyProValAlaPheMetGlnPheAl
L M A L K D L P T P L P M T N L H C Y L C G P V A F M Q F A

1351 CGCCCCACAATTGTTGGAGTTGGGGATTACGAATCGCAGATTTCATTACGAATGCTTTGGTCCGCACAAGGTTATATAACCGATAAAAGG
aAlaArgGlnLeuLeuGluLeuGlyIleThrGluSerGlnIleHisTyrGluCysPheGlyProHisLysValIle
A R Q L L E L G I T E S Q I H Y E C F G P H K V I

1441 TTATATTTATTCACACCAGCAGGCCGGGAATCCGGCCTGTTTTATTTTCCATTTATTTCCAAAAATAAACACCAGTACAGAAATAACCT
-----
1531 GACGAGGTAACCGTTACTGTTTCGTAATAGTTTCATTATACCACCGCGTCATGCTGACCAGTAAAAACAACGCTCCCTCCCGCTTCTCATT
-----

1621 TTTTATTTCCGAATTGAAAAATAGTTAAAGTAAACCTTCATAAATAAGAAATAAAAAACACAATGTTACATTTAAA

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Fig. 2. For legend see facing page.

Fig. 3. Autoradiograms of ^{35}S -labelled proteins expressed in AR 1062 minicells containing plasmid p065 (lane 1) or vector alone (lane 2). Samples (10 μg protein in lanes 1 and 2) were run on a 12% SDS-polyacrylamide gel. The HmpX protein is indicated by an arrow. Molecular mass standards (M) were stained with Coomassie blue.

typical signal peptide sequence was identified, suggesting that the protein is cytoplasmic.

Identification of HmpX protein

To identify the HmpX protein, plasmid p0165 was introduced into the minicell-producing strain *E. coli* AR 1062. Analysis of the ^{35}S -labelled products expressed from p0165 is shown in Fig. 3, lane 1. In addition to the pUC-encoded polypeptides, mainly the β -lactamase, a polypeptide of apparent molecular mass 42 kDa was produced.

HmpX protein belongs to a family of flavohaemoglobins also present in *E. coli* and yeasts

Screening of protein sequence databases revealed that the sequence of HmpX is highly similar to flavohaemoprotein (HMP) sequences. Over its whole sequence, HmpX showed 64% identity with *E. coli* Hmp (Vasudevan *et al.*, 1991) and 36% identity with HMP from the yeasts *Saccharomyces cerevisiae* and *Candida norvegiensis* (Zhu & Riggs, 1992; Iwaasa *et al.*, 1992) (Fig. 4). HMPs from *Alcaligenes eutrophus* and *Vibrio parahaemolyticus* were

recently found (respective accession numbers GenPept: AEFHPG and GenPept: VPU09005); these showed respectively 47% and 65% sequence identity with HmpX.

HMPs possess an N-terminal domain similar to haemoglobins and a C-terminal region similar to those of a family of reductases (Zhu & Riggs, 1992; Andrews *et al.*, 1992).

Amino acids 1 to 143 of HmpX show 45% identity with the complete amino acid sequence of the haemoglobin from the filamentous bacterium *Vitreoscilla* (Wakabayashi *et al.*, 1986; Fig. 4). The similarity between the N-terminal part of HmpX and plant leghaemoglobin or animal haemoglobin sequences was low but significant, ranging from 15 to 21% (over 150 amino acids). Comparison of protein sequences at the secondary structure level by HCA showed that HmpX possesses a globin fold and a conserved haem pocket (the HCA score of HmpX and human haemoglobin was as high as 76%). HmpX possesses hydrophobic residues in 33 specific positions, which in globins form the conserved hydrophobic core described by Bashford *et al.* (1987). From the alignment of HmpX and globin sequences we also deduced that the distal and proximal haem ligands in HmpX could be the glutamine Gln⁵³ in helix E and the histidine His⁸⁴ in helix F, respectively. These residues are strictly conserved in the other HMPs and in *Vitreoscilla* haemoglobin (Perutz, 1986; Fig. 4). From these comparisons, we conclude that HmpX possesses a haemoglobin domain in its N-terminal part. Furthermore, colonies of *E. coli* harbouring plasmid pB413 or p065 have a red coloration that could result from a large amount of haem in these cells.

In addition to its haemoglobin domain, HmpX possesses at its C-terminus (amino acids 150 to 395) a region having striking similarity with sequences of the ferredoxin-NADP-reductase-like proteins (FenR-like proteins). These homologous proteins belong to a new flavoenzyme family (Karplus *et al.*, 1991). Comparisons of HmpX with various members of this family showed that the sequence identity ranged from 15% – with ferredoxin reductase (FenR) – to 24% with the nitrate reductases (NitR) and the NAD-cytochrome *b₅* reductases (Cb5R, also called methaemoglobin reductase). The sequence identity with other oxido-reductase families was lower than 10%. The atomic structure of the spinach FenR (Karplus *et al.*, 1991) allowed the identification of the active-site residues, corresponding to the FAD- and NADP-binding sites. They are highly conserved in the FenR-like proteins (Karplus *et al.*, 1991) and also in HmpX (Fig. 4). By similarity, we assume that Arg²⁰⁴, Tyr²⁰⁶, Gly²²⁷, Ser²³⁰ and Phe³⁸⁹ are involved in the FAD-binding of HmpX. The GxGxxP motif specific to the NAD(P)-binding site in FenR-like proteins is conserved in all the HMPs (²⁸⁷GVGQTP²⁷² in HmpX; see Fig. 4). Specific NADP-

Fig. 2. Nucleotide sequence of the *hmpX* gene of *E. chrysanthemi* 3937, with the deduced amino acid sequence of HmpX. The putative –35 and –10 regions and the Shine–Dalgarno sequence (SD) are indicated by dashed lines. The potential rho-independent termination sites are indicated by facing arrows underlining the inverted repeat of G+C-rich dyad symmetry preceding the poly(T) sequence.

Fig. 4. Sequence alignment of *Erwinia chrysanthemi* HmpX and homologous proteins. The alignment of the sequences of *Vitreoscilla* haemoglobin (V), *Escherichia coli* Hmp (E), *Erwinia chrysanthemi* HmpX (R), *Saccharomyces cerevisiae* Hmp (Y), *Candida norvegiensis* Hmp (C) and spinach ferredoxin reductase (S) is shown as deduced from HCA comparison. The one-letter amino acid code is used. Strictly conserved residues are indicated in black and conservatively changed residues in grey. Secondary structure elements are indicated under the alignments: α stands for α -helix and β for β -strand. The globin-like domain spans residues 1 to 143 and the FenR-like domain spans residues 150 to 395.

binding by bacterial HMP could be suggested from the conservation of Arg³²⁵. Despite the low sequence identity, the sequence alignment by the HCA method indicated that HmpX shares the specific structural features of the proteins from this new family of reductases. The HCA score of the alignment of HmpX and FenR was as high as 76.5%. We conclude that these proteins possess a similar fold.

The Hmp protein of *E. coli* (Vasudevan *et al.*, 1991) has been shown to be associated with a dihydropteridine reductase activity (DHPR). However, no increase in DHPR activity could be demonstrated in *E. coli* harbouring pB413, and no difference in DHPR activity could be found between *E. chrysanthemi* 3937 and an HmpX⁻ mutant (PMV4125).

Role of HmpX in PL production

Since oxygen may play a role in soft rot disease (De Boer & Kelman, 1978) and HmpX possesses the structural features of a haemoglobin, we studied the effect of an *hmpX* mutation on PL production in aerobic or in microaerobic conditions. Bacteria were grown in minimal medium with glycerol. When we measured PL activity in the culture medium under aerobic conditions, no dif-

Table 2. PL specific activity in 3937 (wild-type) and *hmpX* mutant strains

Strains were grown in M9 medium supplemented with 0.5% glycerol. Specific activity is expressed as μmol unsaturated products liberated min^{-1} (mg bacterial dry weight)⁻¹. The experiments were carried out at least three times. The values obtained for a typical experiment are given. ND, Not determined.

Strain	PL specific activity		
	Aerobic	Microaerobic	In planta*
3937	0.1	5	30
PMV4125	0.1	0.4	ND

* Bacterial numbers were determined by dilution plating (3937: 10^9 c.f.u. ml^{-1} ; PMV4125: $< 10^7$ c.f.u. ml^{-1}).

ference was observed between *E. chrysanthemi* strains 3937 (wild-type) and PMV4125 (HmpX⁻). In microaerobic conditions, however, PL activity in strain 3937 was 50-fold higher than in aerobic conditions, whereas in the HmpX⁻ strain it was only four- to fivefold higher (Table 2). The same results were observed when cell lysates were

assayed for PL activity, suggesting that PL production and not secretion was affected (data not shown). In addition, the HmpX⁻ mutant grew similarly to the wild-type strain in aerobic and in microaerobic conditions.

We next studied the effect of *hmpX* mutation on PL synthesis *in planta*. Isolated Saintpaulia leaves were infiltrated with strain 3937 or PMV4125; the intercellular fluid was recovered after 24 h and PL activity measured. The wild-type strain exhibited a 300-fold increase in PL production (10^9 c.f.u. ml⁻¹) (Table 2). PMV4125 did not grow *in planta* (5×10^6 c.f.u. ml⁻¹). To confirm the role of the *hmpX* mutation, plasmid p0165 harbouring the *hmpX* gene was introduced into PMV4125, and PL synthesis was measured in bacteria infiltrated into Saintpaulia leaf tissue: wild-type levels of PL synthesis and maceration were observed. These results, showing the interrelation between PL synthesis and HmpX expression, shed light on the reduced pathogenicity of HmpX mutants observed on Saintpaulia plants.

HmpX expression in coculture with tobacco cells

As HmpX mutants were unable to grow *in planta*, we used co-culture with tobacco cells to study the effect of a plant environment on the transcription of *hmpX*. When grown in M9 minimal medium, strains X2 and X8 showed no difference in Gus activity (6 a.u.). However, when these strains were grown in co-culture with tobacco cells we found a 60-fold increase in *gus* expression after 20 h for strain X8 (370 a.u.) but no increase (7 a.u.) for X2, which possesses an insertion in the reverse orientation. These results might suggest that *hmpX* is induced by factor(s) released by the infected plant.

Conclusions

In this paper we have presented the sequence of the *hmpX* gene of *E. chrysanthemi* and analysed that of its corresponding protein. HmpX belongs to a family of proteins which possess two distinct regions, a haemoglobin domain in their N-terminal part and a flavin-containing reductase region in their C-terminal part.

We have shown that HmpX protein is essential for *E. chrysanthemi* pathogenicity on Saintpaulia plants and that mutation in this gene affects the survival of the bacteria *in planta*. In addition, when bacteria harbouring a *hmpX::gus* fusion were co-cultivated with tobacco cells, a strong increase in Gus activity was observed, suggesting that *hmpX* is induced *in planta*. However, no *hmpX* induction was observed in microaerobic conditions, suggesting that its activation is induced by both plant factors and environmental conditions. Interestingly, sequencing of the upstream region of the *pelA* gene of *E. chrysanthemi* strain EC16 (Tamaki *et al.*, 1988) showed that in this strain, the *hmpX* gene is partially deleted. The remaining two short segments of the N- and C-terminal domains of HmpX lack the active-site regions (Andrews *et al.*, 1992). It is notable that the pathogenicity of strain EC16 on several hosts is severely reduced (M. Boccarda, unpub-

lished results). Although this strain possesses several deletions in its genome, it is tempting to correlate the deletion in *hmpX* and the loss of pathogenicity.

In microaerobic conditions, in the absence of HmpX, the level of PL activity is very low. Using *pel::gus* fusions we found that the *hmpX* mutation affected PL synthesis at the transcriptional level (M. Boccarda, data not shown). Reduced PL activity in an HmpX⁻ mutant would result in a reduced pathogenicity. As we have shown that *hmpX* mutants are non-pathogenic, these results might suggest that in the plant intercellular fluid, oxygen is not available for *E. chrysanthemi*.

What function can be attributed to HmpX? A similar protein, Hmp, has been isolated in *Escherichia coli* based on its DHPR activity. Recently, a ferrisiderophore reductase activity has also been found associated with *E. coli* Hmp (Andrews *et al.*, 1992). A ferrisiderophore reductase activity for HmpX is an attractive hypothesis as it has been shown that an iron assimilation system is necessary for *E. chrysanthemi* pathogenicity (Enard *et al.*, 1988). However, the reductase activities associated with *E. coli* Hmp have different substrate specificities, which could suggest that the reductase activity of Hmp assayed in aerobic conditions is not highly specific. Furthermore, the major DHPR activity in *E. coli* is associated with a protein which does not contain a haemoglobin domain (Vasudevan *et al.*, 1988) and no haem domain is required for the ferrisiderophore reductase activity (Fontecave *et al.*, 1987; Andrews *et al.*, 1992). We found no DHPR activity associated with HmpX in crude bacterial extracts. As multiple ferrisiderophore reductase activities are supposed to be present in *E. chrysanthemi* as in *E. coli*, the purification of HmpX should help to determine its function and confirm the presence of the predicted domains of this protein. Recently, we showed that the purified haemoglobin domain binds haem and that the holo-haemoglobin domain possesses a visible absorption spectra similar to human haemoglobin spectra (G. Labesse, unpublished results).

An alternative function can be hypothesized from the sequence comparisons. *Vitreoscilla*, a bacterium living in low-oxygen environments, produces a haemoglobin, VtHb (Wakabayashi *et al.*, 1986), and an associated reductase has been recently purified (Jakob *et al.*, 1992). VtHb is similar to the N-terminal region of HmpX, and the NAD-methaemoglobin reductase possesses properties very similar to the family of reductases homologous to the C-terminal domain of HmpX. VtHb is auto-oxidizable, and the methaemoglobin reductase keeps VtHb physiologically functional. Similarly, mammal Cb5R (also named methaemoglobin reductase) reduces the oxidized haem and, among the FenR-like proteins, is the most similar to HmpX. It has also been suggested that the *Vitreoscilla* haemoglobin might function as a terminal oxidase in oxygen-limiting conditions (Dikshit *et al.*, 1992). By analogy, HmpX could be a haemoglobin plus a NADP-methaemoglobin reductase on a single polypeptide chain. Its function could be to provide, shortly after infection, a new electron pathway to the bacterium in the unfavour-

able plant environment. As plants are known to generate oxygen radicals after microbial infections (for a review see Sutherland, 1991), it could be suggested that the reductase activity of HmpX recycles the auto-oxidizable haemoglobin of HmpX. To test this hypothesis, the purification of HmpX is under way.

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