Ethylene production by strains of the plant-pathogenic bacterium *Pseudomonas syringae* depends upon the presence of indigenous plasmids carrying homologous genes for the ethylene-forming enzyme

Kazuhiro Nagahama,¹ Kuniaki Yoshino,¹ Masayoshi Matsuoka,¹ Mamoru Sato,² Sumio Tanase,³ Takahira Ogawa¹ and Hideo Fukuda¹

Author for correspondence: Kazuhiro Nagahama. Tel: +81 96 326 3111 ext. 5135. Fax: +81 96 326 3000.

¹ Department of Applied Microbial Technology, Kumamoto Institute of Technology, Ikeda 4-22-1, Kumamoto 860, Japan

² National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305, Japan

³ Department of Biochemistry, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860, Japan

The molecular characteristics of the ethylene-forming enzymes of strains of *Pseudomonas syringae* were tested. The ethylene-producing activities of the nine strains as measured *in vivo* and *in vitro* were similar, except for that of *P. syringae* pv. *mori* M5. A polyclonal antibody and a DNA probe for the ethylene-forming enzyme from *P. syringae* pv. *phaseolicola* PK2 were prepared to investigate homologies among the proteins and genes for the ethylene-forming enzymes. With the exception of *P. syringae* pv. *mori* M5, eight strains tested expressed the same antigen as the ethylene-forming enzyme from *P. syringae* pv. *phaseolicola* PK2 and were homologous to DNA sequences on indigenous plasmids. Molecular masses of antigenic proteins from all ethylene-producing strains were 40 kDa. The N-terminal amino acid sequence of the purified ethylene-forming enzyme from *P. syringae* pv. *glycinea* KN130 was identical to that of the enzyme from *P. syringae* pv. *phaseolicola* PK2. These results show that the ethylene-forming enzymes encoded by the indigenous plasmid(s) in the pathogenic bacteria examined were similar.

Keywords: ethylene production, *Pseudomonas syringae* pv. *glycinea*, *Pseudomonas syringae* pv. *phaseolicola*

INTRODUCTION

Ethylene is a plant hormone that is produced by plants (Adams & Yang, 1979) and a variety of micro-organisms, including plant pathogens (Freebairn & Buddenhagen, 1964; Goto *et al.*, 1985; Sato *et al.*, 1987). There are two biosynthetic pathways for the production of ethylene by micro-organisms (Fukuda & Ogawa, 1992; Fukuda *et al.*, 1993). In one pathway, ethylene is produced via 2-keto-4-methyl-thiobutyric acid by an NADH: Fe(III)EDTA oxidoreductase (Fukuda *et al.*, 1989b; Ogawa *et al.*, 1990). In the other pathway, ethylene is produced via 2-oxoglutarate as, for example, in *Penicillium digitatum* (Fukuda *et al.*, 1986, 1989a) and in *Pseudomonas syringae* pv. *phaseolicola* PK2 (Fukuda *et al.*, 1992b; Nagahama *et al.*, 1991a, b).

Recently, the gene coding for the ethylene-forming enzyme (EFE) of *P. syringae* pv. *phaseolicola* PK2 was

found to be located in an indigenous plasmid, designated pPSP1, and the gene was cloned and expressed in *Escherichia coli* JM109 (Fukuda *et al.*, 1992a). Nucleotide sequence analysis of the clone revealed an open reading frame that could specify 350 amino acids. The ethylenogenic capability of several pathovars of *P. syringae* was studied (Sato *et al.*, 1987) and it was found that strains of *P. syringae* pv. *glycinea* isolated from various regions of Japan, which cause halo blight in soybean plants, produced ethylene as efficiently as the Kudzu strains of *P. syringae* pv. *phaseolicola*. In this study, we describe homologies among the EFEs from several strains of *P. syringae* using polyclonal antibodies and DNA probes derived from the EFE gene of *P. syringae* pv. *phaseolicola* PK2.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. All strains were maintained on modified nutrient broth agar that contained (l⁻¹): 5 g Polypepton, 3 g yeast extract, 3 g meat extract, 2 g NaCl, 5 g

Abbreviation: EFE, ethylene-forming enzyme.

Table 1. Strains of *P. syringae*

Strain	Place of isolation	Reference
<i>pv. phaseolicola</i>		
PK2	Shizuoka	Goto <i>et al.</i> (1985)
KUZ1	Ibaragi	Goto <i>et al.</i> (1985)
KUZ5	Ibaragi	Goto <i>et al.</i> (1985)
KUZ7	Yamagata	Goto <i>et al.</i> (1985)
<i>pv. glycinea</i>		
KN35	Iwate	Nishiyama <i>et al.</i> (1986)
KN41	Akita	Nishiyama <i>et al.</i> (1986)
KN44	Ibaragi	Nishiyama <i>et al.</i> (1986)
KN130	Ibaragi	Nishiyama <i>et al.</i> (1986)
<i>pv. mori</i>		
M5	Miyazaki	Sato (1983)

* *P. syringae* *pv. phaseolicola* PK2 was obtained from Professor M. Goto (Shizuoka University, Shizuoka, Japan). Other strains were obtained from the collection of the National Institute of Sericultural and Entomological Science, Tsukuba, Japan (Nishiyama *et al.*, 1986; Sato, 1983).

glucose and 15 g agar (pH 7.0). All chemicals were from Wako Pure Chemical Industries unless otherwise stated. A portion (2 ml) of the seed culture of each strain of *P. syringae* was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of modified nutrient broth medium and was incubated at 30 °C for 12 h on a rotary shaker at 180 r.p.m. (70 mm amplitude).

Preparation of cell-free extracts and immunoblot analysis.

Bacterial cells in 1 litre of culture broth were pelleted by centrifugation, suspended in 10 ml 100 mM potassium phosphate buffer (pH 7.0) and disrupted by sonication (Branson Sonifier, model 350) for 10 × 30 s (cooled on ice between each 30 s period of sonication). Cell debris was removed by centrifugation (15000 g, 40 min, 4 °C) and the supernatants were used for the *in vitro* assay of EFE activity. Proteins in extracts (300 µg protein per lane) were fractionated by SDS-PAGE on a 10% (w/v) gel as described by Laemmli (1970) and were transferred electrophoretically onto nitrocellulose membranes (Bio-Rad) in 25 mM Tris/HCl (pH 8.3), 192 mM glycine and 20% (v/v) methanol. The membranes were blocked with PBS to which 8% (w/v) skim milk was added, and incubated with polyclonal antibodies, raised against the purified EFE (Nagahama *et al.*, 1991b). Unbound antibodies were washed out twice with PBS containing 0.05% Tween 20. Antibodies bound to the membranes were detected using peroxidase-conjugated antibodies against rabbit IgG raised in donkeys (Amersham) in 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, containing 6 mg 3,3'-diaminobenzidine tetrahydrochloride ml⁻¹.

Molecular genetic techniques. Plasmid DNA was isolated by the alkaline lysis method (Birnboim & Doly, 1979). Southern blotting was performed essentially as described by Meinkoth & Wahl (1984) using DIG (digoxigenin) DNA Labelling and Luminescence Detection Kits (Boehringer Mannheim). A DIG-labelled DNA probe was made from the 1.5 kb *Hind*III-*Eco*RI fragment that contained the full-length gene for EFE from pPSP1, an indigenous plasmid of *P. syringae* *pv. phaseolicola* PK2 (Fukuda *et al.*, 1992a). The concentration of DNA probe used in hybridization solution was 4 ng ml⁻¹.

Measurement of ethylene-forming activity *in vivo*. One millilitre of culture broth (each strain was cultured for 11 h at

30 °C) was transferred to a sterile test tube, which was then sealed with a sterile rubber stopper and incubated on a reciprocal shaker at 30 °C for 1 h. After incubation, 1 ml air space was withdrawn through the stopper by a syringe and analysed by gas chromatography (gas chromatograph G3800; Yanako), under the following conditions: column size, 3 mm i.d. × 2 m; solid phase, active alumina; temperature, 100 °C; carrier gas, nitrogen at a flow rate of 40 ml min⁻¹; flame ionization detector. The rate of production of ethylene was calculated as previously described (Nagahama *et al.*, 1991b) and expressed as µl ethylene (mg cell dry wt)⁻¹ h⁻¹.

Measurement of ethylene-forming activity *in vitro*. The standard reaction mixture (1 ml) was composed of 2.5 mM 2-oxoglutarate, 2 mM L-arginine, 2 mM ferrous sulfate, 10 mM L-histidine, and cell-free extract in 40 mM HEPES buffer, pH 8.0. A test tube containing the standard reaction mixture was sealed with a rubber stopper and then incubated with gentle shaking at 25 °C for 10 min. After incubation, the amount of ethylene formed was determined by gas chromatography, as described above. The activity was expressed as µl ethylene (mg protein)⁻¹ h⁻¹. Protein concentrations were determined from the absorbance at 280 nm or by the Lowry method.

Purification of EFE and N-terminal amino acid analysis.

Purification of EFE was performed essentially as described by Nagahama *et al.* (1991b), using chromatography columns of Butyl-Toyopearl 650M, DEAE-Sephacrose CL-6B, Bio-Gel HT and Sephadex G-100. The N-terminal amino acid residues of the purified enzyme of *P. syringae* *pv. glycinea* KN130 (20 mg, equivalent to approximately 480 pmol) were determined by automated Edman sequencing with an amino acid sequencer (model 477A; Applied Biosystems).

RESULTS AND DISCUSSION

Comparison of ethylene-forming activities *in vivo* and *in vitro* among different strains of *P. syringae*

With the exception of *P. syringae* *pv. mori* M5, all strains of *P. syringae* tested produced ethylene from 2-oxoglutarate (Table 2). Since activities of EFE *in vivo* and *in vitro* were

Table 2. Rates of ethylene formation by strains of *P. syringae*

Strain	Specific activity*	
	<i>in vivo</i> †	<i>in vitro</i> ‡
<i>pv. phaseolicola</i>		
PK2	1.2	0.9
KUZ1	1.4	0.8
KUZ5	1.7	2.5
KUZ7	1.0	3.8
<i>pv. glycinea</i>		
KN35	0.5	0.3
KN41	0.7	0.4
KN44	1.1	0.8
KN130	1.9	1.2
<i>pv. mori</i>		
M5	0.0	0.0

* The values represent the means of three independent experiments with a reproducibility of $\pm 5\%$ or better.

† μl ethylene (mg cell dry wt) $^{-1}$ h $^{-1}$.

‡ μl ethylene (mg protein) $^{-1}$ h $^{-1}$.

affected by the culture conditions and the duration of cultivation, these results do not correspond to the maximum activities of ethylene production for each strain. Nevertheless, within the range of standard conditions of *in vivo* and *in vitro* assays (error $\pm 5\%$) *P. syringae* pv. *mori* M5 did not produce ethylene at all.

Plasmids carrying homologous EFE genes

A DNA fragment carrying the entire coding region of EFE from the indigenous plasmid, pPSP1, of *P. syringae* pv. *phaseolicola* PK2 (Fukuda *et al.*, 1992a) was used to probe the related genes in *P. syringae* strains. Size separation analysis of indigenous plasmids from different bacterial strains gave different electrophoretic patterns

(Fig. 1a). Southern blot analysis showed that eight of the ethylene-producing strains harboured indigenous plasmids carrying sequences homologous to the EFE gene (Fig. 1b). *P. syringae* pv. *glycinea* KN130 contained two plasmids, and *P. syringae* pv. *glycinea* KN35 contained several plasmids carrying the homologous EFE gene sequence (Fig. 1b, lanes 8 and 5). In other strains, only one plasmid hybridized. In contrast, genomic and plasmid DNAs from *P. syringae* pv. *mori* M5 did not hybridize. These results suggest that in strains of *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *glycinea* indigenous plasmids may be involved in ethylene production. So far, plasmids have been detected in a number of plant-pathogenic bacteria but the plasmids differed in their sizes even within the same species (Coplin *et al.*, 1981; Curiale & Mills, 1983).

Ethylene-forming enzyme in various strains

Immunoreactive protein bands with the same mobility (about 40 kDa) as the purified EFE from *P. syringae* pv. *phaseolicola* PK2 were detected in the extracts of the different ethylene-producing strains (Fig. 2). Minor staining bands, especially in the extracts from *P. syringae* pv. *mori* M5, were due to the nonspecific binding of peroxidase-conjugated anti-rabbit IgG antibody. This nonspecific binding was confirmed in the control experiment in which polyclonal antibodies were removed from the assay system (Fig. 2b, lane 10).

The analysis of the N-terminal amino acid sequence (Fig. 3) of EFE from *P. syringae* pv. *glycinea* KN130 revealed that the first 30 residues were identical to those from *P. syringae* pv. *phaseolicola* PK2 with the possible exception of an unidentified residue at position 16.

These data suggested that all eight ethylene-producing strains expressed proteins having the same antigenic properties as the EFE from *P. syringae* pv. *phaseolicola* PK2, and that the EFes from *P. syringae* pv. *glycinea* KN130 and *P. syringae* pv. *phaseolicola* PK2 were identical proteins.

Fig. 1. Electrophoretic and Southern analysis of plasmid DNA from *Pseudomonas syringae* strains. (a) Ethidium bromide-stained gel showing several plasmid patterns. (b) Southern blot analysis with the EFE probe (1.5 kb *Hind*III-*Eco*RI fragment of the full-length gene for EFE from plasmid pPSP1). Lanes: 1, *P. syringae* pv. *phaseolicola* PK2; 2, *P. syringae* pv. *phaseolicola* KUZ1; 3, *P. syringae* pv. *phaseolicola* KUZ5; 4, *P. syringae* pv. *phaseolicola* KUZ7; 5, *P. syringae* pv. *glycinea* KN35; 6, *P. syringae* pv. *glycinea* KN41; 7, *P. syringae* pv. *glycinea* KN44; 8, *P. syringae* pv. *glycinea* KN130; 9, *P. syringae* pv. *mori* M5. The arrow indicates the position of chromosomal DNA.

Fig. 2. SDS-PAGE and immunoblot analysis of cell-free extracts from strains of *Pseudomonas syringae*. (a) SDS-PAGE of the cell-free extract. (b) Western blot with the EFE-specific polyclonal antibody raised against EFE from *P. syringae* pv. *phaseolicola* PK2. Lanes: 1, *P. syringae* pv. *phaseolicola* PK2; 2, *P. syringae* pv. *phaseolicola* KUZ1; 3, *P. syringae* pv. *phaseolicola* KUZ5; 4, *P. syringae* pv. *phaseolicola* KUZ7; 5, *P. syringae* pv. *glycinea* KN35; 6, *P. syringae* pv. *glycinea* KN41; 7, *P. syringae* pv. *glycinea* KN44; 8, *P. syringae* pv. *glycinea* KN130; 9, *P. syringae* pv. *mori* M5; 10, negative control for *P. syringae* pv. *mori* M5 without the EFE antibody in the reaction mixture for Western blotting. Arrows indicate the molecular markers and the purified EFE of *P. syringae* pv. *phaseolicola* PK2. The arrowhead indicates a band for nonspecific binding of peroxidase-conjugated anti-rabbit IgG antibody.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Met-Thr-Asn-Leu-Gln-Thr-Phe-Glu-Leu-Pro-Thr-Glu-Val-Thr-Gly-
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
X-Ala-Ala-Asp-Ile-Ser-Leu-Gly-Arg-Ala-Leu-Ile-Gln-Ala-Lys-

Fig. 3. N-terminal amino acid sequence of the EFE from *P. syringae* pv. *glycinea* KN130. X, Unidentified residue.

CONCLUSIONS

Among the nine strains studied here, *P. syringae* pv. *glycinea* strains KN35 and 130 seemed to possess several copies of the EFE gene on different plasmids (Fig. 1b). The EFE protein from *P. syringae* pv. *glycinea* KN130 was

purified from a cell-free extract and was found to have an N-terminal amino acid sequence the same as that of the previously characterized enzyme from *P. syringae* pv. *phaseolicola* PK2 (Fig. 3). Moreover, immunoblot analysis showed that the EFE proteins from different strains had the same molecular mass (Fig. 2b). These results are consistent with the hypothesis that the EFE expressed in *P. syringae* pv. *glycinea* KN130 is identical to or closely related to that in other EFE-producing strains of *P. syringae* and all genes for EFEs are present on indigenous plasmids.

The purified EFE from *Penicillium digitatum* IFO 9372 produces ethylene from 2-oxoglutarate (Fukuda *et al.*, 1986, 1989a), but did not react with the polyclonal antibody raised against the EFE from *P. syringae* pv. *phaseolicola* PK2 (data not shown). The N-terminal amino acid sequence of this fungal EFE was different from that of the bacterial enzyme (Nagahama *et al.*, 1991b). Since at least two strains of *Pseudomonas* possess EFE proteins with the same N-terminal sequence, important information about ethylene production by 2-oxoglutarate-dependent micro-organisms may be obtained by comparing the bacterial EFEs with fungal EFE such as that from *Penicillium digitatum* IFO 9372.

ACKNOWLEDGEMENT

We are grateful to Dr K. Nishiyama, National Institute of Agro-Environmental Science, for supplying bacterial strains.

REFERENCES

- Adams, D. O. & Yang, S. F. (1979). Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc Natl Acad Sci USA* **76**, 170–174.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Coplin, D. L., Rowan, R. G., Chisholm, D. A. & Whitmoyer, R. E. (1981). Characterization of plasmids in *Erwinia stewartii*. *Appl Environ Microbiol* **42**, 599–604.
- Curiale, M. S. & Mills, D. (1983). Molecular relatedness among cryptic plasmids in *Pseudomonas syringae* pv. *glycinea*. *Phytopathology* **73**, 1440–1444.
- Freebairn, H. T. & Buddenhagen, I. W. (1964). Ethylene production by *Pseudomonas solanacearum*. *Nature* **202**, 313–314.
- Fukuda, H. & Ogawa, T. (1992). Microbial production. In *The Plant Hormone Ethylene*, pp. 279–292. Edited by A. K. Matto & J. C. Suttle. London: CRC Press.
- Fukuda, H., Fujii, T. & Ogawa, T. (1986). Preparation of a cell-free ethylene-forming system from *Penicillium digitatum*. *Agric Biol Chem* **50**, 977–981.
- Fukuda, H., Kitajima, H., Fujii, T., Tazaki, M. & Ogawa, T. (1989a). Purification and some properties of a novel ethylene-forming enzyme produced by *Penicillium digitatum*. *FEMS Microbiol Lett* **59**, 1–6.
- Fukuda, H., Takahashi, M., Fuji, T., Tazaki, M. & Ogawa, T. (1989b). An NADH:Fe(III)EDTA oxidoreductase from *Crypto-*

coccus albidus: an enzyme involved in ethylene production *in vivo*? *FEMS Microbiol Lett* **60**, 107–112.

Fukuda, H., Ogawa, T., Ishihara, K., Fujii, T., Nagahama, K., Omata, T., Inoue, Y., Tanase, S. & Morino, Y. (1992a). Molecular cloning in *Escherichia coli*, expression and nucleotide sequence of the gene for the ethylene-forming enzyme of *Pseudomonas syringae* pv. *phaseolicola* PK2. *Biochem Biophys Res Commun* **188**, 826–832.

Fukuda, H., Ogawa, T., Tazaki, M., Nagahama, K., Fujii, T., Tanase, S. & Morino, Y. (1992b). Two reactions are simultaneously catalyzed by a single enzyme: the arginine-dependent simultaneous formation of two products, ethylene and succinate, from 2-oxoglutarate by an enzyme from *Pseudomonas syringae*. *Biochem Biophys Res Commun* **188**, 483–489.

Fukuda, H., Ogawa, T. & Tanase, S. (1993). Ethylene production by microorganisms. In *Advances in Microbial Physiology*, pp. 275–306. Edited by A. H. Rose. London: Academic Press.

Goto, M., Ishida, Y., Takikawa, Y. & Hyodo, H. (1985). Ethylene production by the kudze strains of *Pseudomonas syringae* pv. *phaseolicola* causing halo blight in *Pueraria lobata* (Willd) Ohwi. *Plant Cell Physiol* **26**, 141–150.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Meinkoth, J. & Wahl, G. (1984). Hybridization of nucleic acids immobilized on solid supports. *Anal Biochem* **138**, 267–284.

Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Goto, M. &

Fukuda, H. (1991a). L-Arginine is essential for the formation *in vitro* of ethylene by an extract of *Pseudomonas syringae*. *J Gen Microbiol* **137**, 1641–1646.

Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Tanase, S., Morino, Y. & Fukuda, H. (1991b). Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae*. *J Gen Microbiol* **137**, 2281–2286.

Nishiyama, K., Azegami, K., Osada, S., Nakasone, W., Ezuka, A. & Watanabe, Y. (1986). Bacterial diseases of soybean and their pathogens in Japan. *Bull Natl Inst Agro-Environ Sci* **1**, 83–94.

Ogawa, T., Takahashi, M., Fujii, T., Tazaki, M. & Fukuda, H. (1990). The role of NADH:Fe(III)EDTA oxidoreductase in ethylene formation from 2-keto-4-methylthiobutyrate. *J Ferment Bioeng* **93**, 177–181.

Sato, M. (1983). Phage-induction from lysogenic strains of *Pseudomonas syringae* pv. *mori* by an extract from mulberry leaves. *Ann Phytopathol Soc Japan* **49**, 259–261.

Sato, M., Urushizaki, S., Nishiyama, K., Sakai, F. & Ota, Y. (1987). Efficient production of ethylene by *Pseudomonas syringae* pv. *glycinea* which causes halo blight in soybeans. *Agric Biol Chem* **51**, 1117–1178.

Received 17 January 1994; revised 17 March 1994; accepted 20 April 1994.