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, Kuniaki Yoshino, Masayoshi Matsuoka, Mamoru Sato, Sumio Tanase, Takahira Ogawa and Hideo Fukuda

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 microorganisms, including plant pathogens (Freebairn & Buddlenagen, 1964; Goto et al., 1985; Sato et al., 1987). There are two biosynthetic pathways for the production of ethylene by microorganisms (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 pPS1, and the gene was cloned and expressed in *Escherichia coli* J1019 (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 *P. syringae* pv. *phaseolicola* PK2. 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 (1 g): 5 g Polypepton, 3 g yeast extract, 3 g meat extract, 2 g NaCl, 5 g
Table 1. Strains of P. syringae

<table>
<thead>
<tr>
<th>Strain</th>
<th>Place of isolation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pv. phaseolicola&lt;br/&gt;PK2</td>
<td>Shizuoka</td>
<td>Goto et al. (1985)</td>
</tr>
<tr>
<td>KUZ1</td>
<td>Ibaragi</td>
<td>Goto et al. (1985)</td>
</tr>
<tr>
<td>KUZ5</td>
<td>Ibaragi</td>
<td>Goto et al. (1985)</td>
</tr>
<tr>
<td>KUZ7</td>
<td>Yamagata</td>
<td>Goto et al. (1985)</td>
</tr>
<tr>
<td>pv. glycinea&lt;br/&gt;KN35</td>
<td>Iwate</td>
<td>Nishiyama et al. (1986)</td>
</tr>
<tr>
<td>KN41</td>
<td>Akita</td>
<td>Nishiyama et al. (1986)</td>
</tr>
<tr>
<td>KN44</td>
<td>Ibaragi</td>
<td>Nishiyama et al. (1986)</td>
</tr>
<tr>
<td>KN130</td>
<td>Ibaragi</td>
<td>Nishiyama et al. (1986)</td>
</tr>
<tr>
<td>pv. mori&lt;br/&gt;M5</td>
<td>Miyazaki</td>
<td>Sato (1983)</td>
</tr>
</tbody>
</table>

* 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 (Bransonic model B, 30 s) in an ice bath. The 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 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 8.3), 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 Maekawa & 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 HindIII-EcoRI fragment that contained the full-length gene for EFE from pPS1, 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-Sepharose 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
Ethylene production by P. syringae

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

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

*The values represent the means of three independent experiments with a reproducibility of ±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 ±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.
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 *Penicillium* 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.

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**REFERENCES**


Ethylene production by \textit{P. syringae}


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