Comparison of Ethylene Production by *Pseudomonas syringae* and *Ralstonia solanacearum*

H. Weingart, B. Völksch, and M. S. Ullrich

First and second authors: Friedrich-Schiller-University Jena, Institute of Microbiology, Philosophenweg 12, 07743 Jena, Germany; and third author: Max-Planck-Institute of Terrestrial Microbiology, Karl-von-Frisch-Strasse, 35043 Marburg, Germany.
Accepted for publication 3 February 1999.

ABSTRACT

Weingart, H., Völksch, B., and Ullrich, M. S. 1999. Comparison of ethylene production by *Pseudomonas syringae* and *Ralstonia solanacearum*. Phytopathology 89:360-365.

Strains of *Pseudomonas syringae* pv. *pisi* and *Ralstonia solanacearum* produced ethylene at rates 20- and 200-fold lower, respectively, than strains of *P. syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, and *sesami*. In the current study, we investigated which ethylene biosynthetic pathways were used by *P. syringae* pv. *pisi* and *R. solanacearum*. Neither the activity of an ethylene-forming enzyme nor a corresponding *efe* gene homolog could be detected in *R. solanacearum*, suggesting synthesis of ethylene via 2-keto-4-methyl-thiobutyric acid. In contrast, 2-oxoglutarate-dependent ethylene formation was observed with *P. syringae* pv. *pisi*, and Southern blot hybridization revealed the presence of an *efe* homolog in this pathovar. The *efe* genes from *P. syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, *pisi*, and *sesami* were sequenced. Nucleotide sequence comparisons indicated that the *efe* gene in pv. *pisi* was not as highly conserved as it was in other *P. syringae* pathovars. The pv. *pisi efe* homolog showed numerous nucleotide substitutions and a deletion of 13 amino acids at the C-terminus of the predicted gene product. These sequence alterations might account for the lower rate of ethylene production by this pathovar. All ethylene-producing *P. syringae* pathovars were virulent on bush bean plants. The overlapping host range of these pathovars suggests that horizontal transfer of the *efe* gene may have occurred among bacteria inhabiting the same host.

Among plant-pathogenic bacteria, *Ralstonia* (formerly *Pseudomonas* or *Burkholderia*) *solanacearum* has long been known to produce ethylene gas (3). However, the most efficient bacterial ethylene producers belong to the *P. syringae* group. Goto et al. (9) found that strains of *P. syringae* pv. *phaseolicola*, which are pathogenic to the Japanese weed *Pueraria lobata* (Willd.) Ohwi (common name: kudzu), form large amounts of ethylene. Strains of *P. syringae* pvs. *cannabina*, *glycinea*, and *sesami* also produce ethylene as efficiently as the pv. *phaseolicola* kudzu strains (21,22). In contrast, *P. syringae* pv. *pisi* strains showed a 20-fold lower ethylene production rate than the other pathovars (30). Recently, we demonstrated that strains of *P. syringae* pvs. *glycinea* and *phaseolicola* were also able to produce ethylene in planta, suggesting a role for ethylene in pathogenicity (30).

Microbial ethylene biosynthesis can occur via two different routes. Both pathways are distinct from that of higher plants, which use only one pathway involving 1-aminocyclopropane-1-carboxylic acid as the sole intermediate (12). Most microorganisms produce only trace amounts of ethylene via the 2-keto-4-methyl-thiobutyric acid (KMBA) pathway (13). KMBA is a transaminated derivative of methionine. In this pathway, a NADH:Fe(III)EDTA oxidoreductase catalyzes the formation of hydroxyl radicals from molecular oxygen (7). The hydroxyl radicals serve as oxidizing agents in the nonenzymatic oxidation of KMBA to ethylene, methanethiol, and carbon dioxide (19).

The precursor for the second pathway is 2-oxoglutarate. Four pathovars of *P. syringae* and the fungi *Penicillium digitatum*, *Penicillium cyclopium*, *Chaetomium globosum*, *Phycomyces nitens*, and *Fusarium oxysporum* have been reported to produce ethylene in a 2-oxoglutarate-dependent manner (5,22). A single protein, the ethylene-forming enzyme (EFE), was found to be responsible for the ethylene formation by these microorganisms. The EFE of *P. syringae* pv. *phaseolicola* has been characterized extensively (4,6, 14,15,17).

The EFE protein is a member of the superfamily of Fe^{2+}/ascorbate oxidases (4,5). It is encoded by the *efe* gene located on one of the indigenous plasmids of *P. syringae* (16,22,29). Two reactions are simultaneously catalyzed by the EFE. In the main reaction, 2-oxoglutarate is dioxygenated to produce one molecule of ethylene and three molecules of carbon dioxide. In the second reaction, both 2-oxoglutarate and L-arginine are monooxygenated. The end products of the subreaction are succinate, carbon dioxide, guanidine, and L-Δ^1-pyrroline-5-carboxylate (6). Ethylene and succinate are formed in a molar ratio of 2:1.

In this paper, we provide evidence that *P. syringae* pv. *pisi* strains belong to the group of 2-oxoglutarate-dependent ethylene producers. In contrast, EFE activity could not be detected in tested *R. solanacearum* strains, indicating that ethylene formation by these bacteria probably occurs via the KMBA pathway. The EFE encoding genes from five ethylene-forming *P. syringae* pathovars were sequenced. Sequence alignment revealed a high conservation of the *efe* gene among *P. syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, and *sesami*, whereas numerous differences were found in the nucleotide sequence of the *efe* gene from a low ethylene-producing strain of *P. syringae* pv. *pisi*. Using a polymerase chain reaction (PCR)-based approach, these differences were also found in four other strains of pv. *pisi* and may explain why this pathovar produces less ethylene.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. *Pseudomonas* strains were routinely cultured on bouillon glycerol agar (26) at 28°C. The references cited in Table 1 indicate the specific studies or laboratories in which the strains were isolated and characterized.

Corresponding author: M. S. Ullrich
E-mail address: ullrichm@mailer.uni-marburg.de

Publication no. P-1999-0304-03R

Determination of ethylene production and bacterial growth. Ethylene production and bacterial growth curves were determined in 5a medium (26) using 100-ml cultures in 500-ml flasks shaken at 140 rpm and incubated at 28°C. At the appropriate time points, 1 ml of the culture was transferred to a sterile 5-ml syringe sealed with a rubber cap and incubated on a rotary shaker at 90 rpm for 2 h at 28°C. After incubation, 1-ml gas samples were withdrawn using a gas-tight syringe, and ethylene was determined with a GC-14A gas chromatograph (Shimadzu Scientific Instruments, Inc., Duisburg, Germany) equipped with an active aluminum column and a flame ionization detector. Ethylene was determined every 3 h, and production rate was expressed in nanoliters per hour per cell. Bacterial growth was estimated by the optical density of the bacterial cultures at 578 nm using a spectrophotometer. Strains of eight previously unexamined *P. syringae* pathovars, *avellanae*, *dendropanices*, *garcae*, *hibisci*, *mellea*, *myricae*, *porri*, and *zizaniae*, were tested for their ability to produce ethylene.

Ethylene production by cell-free protein extracts. For the preparation of cell-free protein extracts, 100 ml of an overnight culture in 5a medium (26) was centrifuged at 8,000 × *g* for 15 min. Cells were washed twice with deionized water, resuspended in 5 ml of 10 mM phosphate buffer (pH 7.0), and disrupted by sonicating five times for 30 s with a cooling time of 1 min between bursts of sonication. Vials were immersed in an ice-water/acetone mixture during sonication. Cell debris was removed by centrifugation at 20,000 × *g* at 4°C for 30 min, and the supernatant was used as cell-free protein extract.

Ethylene production was determined using a reaction mixture (2 ml) that contained 0.4 ml of 200 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid/NaOH buffer (pH 8.0), 0.2 ml of 0.75 mM $FeSO_4$, 0.2 ml of 10 mM dithiothreitol, 0.2 ml of 10 mM L-arginine, 0.2 ml of 100 mM L-histidine, 0.2 ml of 10 mM 2-oxoglutarate, 0.4 ml of deionized water, and 0.2 ml of cell-free extract (8,13,14). Test tubes (12-ml capacity) containing 2 ml of the reaction mixture were sealed with rubber caps and incubated on a rotary shaker at 140 rpm for 1 h at 28°C. After incubation, a 1-ml gas sample was withdrawn using a gas-tight syringe, and ethylene was determined with a gas chromatograph as described above. The protein concentration of the cell-free extract was measured by the Bradford method (20). Ethylene production rate was expressed as nanoliters per hour per milligram of protein.

Southern blot analysis. Southern blots were performed as described by Sambrook et al. (20) using a digoxigenin (DIG) DNA labeling and luminescent detection kit (Boehringer-Mannheim, Mannheim, Germany). A DIG-labeled DNA probe was prepared from a PCR amplification product carrying the *efe* gene from *P. syringae* pv. *phaseolicola* PK2. Hybridizations were carried out with a hybridization temperature of 60°C and two 10-min washes with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 60°C.

PCR analysis. Two 18-mer oligonucleotides with the sequences 5′-GTCACCAGTAACGATAAG-3′ (primer EFE11) and 5′-TATGGATAAAGAAGAGAC-3′ (primer EFE12) were chosen for amplification of the genes encoding the EFE of different *P. syringae* pathovars. These primers were located 55 bp upstream and 22 bp downstream, respectively, of the *efe* open reading frame. For *P. syringae* pv. *pisi*, primer EFE11 was used together with primer ZR2 (5′-CTGCGATGAAAAAGT-3′) complementary to a nucleotide sequence from within a transposase gene that has been found on plasmid p4180A and other plasmids of *P. syringae* pv. *glycinea* PG4180 (25; M. Ullrich and C. L. Bender, *unpublished data*). Since the *efe* gene has been reported to reside on plasmids (16,22,29), use of this nucleotide primer was intended to obtain a PCR fragment containing the *efe* and transposase genes to facilitate nucleotide sequencing. Three additional primers were designed based on the *efe* sequence of *P. syringae* pv. *pisi* GSPB1206 to determine a possible conservation of nucleotide substitutions among other strains of this pathovar. The sequence of primer EFEP1 (5′-ACGCTGGGATGTTACTTG-3′) was complementary to nucleotides 1,012 to 1,029 of the sequence published in GenBank submission AF101061 (*efe* gene of *P. syringae* pv. *pisi* GSPB1206), whereas primer EFEP2 (5′-GCCTGTTCAAAACGTGTG-3′) was identical to nucleotides 727 to 744 of AF101061. The sequence of oligonucleotide primer EFEP3 (5′-GAGGTTATTGGCAGCGCC-3′) was identical to nucleotides 38 to 55 from AF101061. The standard reaction mixture (50 μl) contained 10× PCR reaction buffer (Qiagen, Hilden, Germany), 2 mM $MgCl_2$, 0.2 mM each of dNTPs, 1 unit *Taq* DNA polymerase (Qiagen), 25 pmol of each primer, and 50 ng of DNA. Amplification included initial denaturation (95°C for 3 min); followed by 30 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min); and a single final extension (72°C for 10 min). PCRs were carried out with a GenAmp 2400 thermocycler (Perkin-Elmer, Foster City, CA).

DNA sequencing. Nucleotide sequencing of PCR-generated DNA fragments for both strands was performed by the dideoxynucleotide method (20) with the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham-Buchler, Braunschweig, Germany). Automated DNA sequencing was accomplished with an ALF Express sequencing apparatus (Pharmacia, Freiburg, Germany). Sequence data were aligned and processed with the Lasergene version 4.1 software package (DNASTAR Inc., Madison, WI).

Pathogenicity tests. Plants were grown in individual pots in a greenhouse at 20 to 30°C with supplemental light for a 14-h photoperiod. Bush bean plants (*Phaseolus vulgaris* L. cv. Red Kidney) were inoculated by spraying the abaxial surfaces of leaves using a glass atomizer until water-soaking appeared. Bacterial suspensions (about 10^6 CFU per ml) were applied to the first fully expanded trifoliate leaves (about 20 days old). Plants were observed daily for development of symptoms. Bacterial populations in leaves were monitored by punching 15 discs (7 mm in diameter) from the inoculated leaf area 7 days after inoculation. The discs were homogenized in 15 ml of isotonic NaCl and serially diluted for plating onto King's medium B (10).

Nucleotide sequence accession numbers. The reported nucleotide sequences were deposited with GenBank and EMBL under accession numbers AF101057 (*P. syringae* pv. *glycinea* 7a/90), AF101058 (*P. syringae* pv. *phaseolicola* GSPB669), AF101059 (*P. syringae* pv. *cannabina* GSPB2553), AF101060 (*P. syringae* pv. *sesami* 962), and AF101061 (*P. syringae* pv. *pisi* GSPB1206).

TABLE 1. Bacterial strains used in this study

Species	Strain designation	Source or reference
Pseudomonas syringae pv.		
avellanae	CFBP10963	L. Gardan[a]
cannabina	GSPB2553	K. Rudolph[b]
dendropanices	CFBP3226	L. Gardan
garcae	CFBP1634	L. Gardan
glycinea	7a/90	B. Völksch
hibisci	CFBP11294	L. Gardan
mellea	CFBP2344	L. Gardan
myricae	CFBP11005	L. Gardan
phaseolicola	6/0	(1)
	PK2	(24)
	GSPB669	K. Rudolph
pisi	GSPB104	K. Rudolph
	GSPB105	K. Rudolph
	GSPB1206	K. Rudolph
	GSPB1477	K. Rudolph
	GSPB1787	K. Rudolph
	PP01	K. Naumann
porri	CFBP1908	L. Gardan
sesami	962	S. Prathuangwong
zizaniae	CFBP11040	L. Gardan
Ralstonia solanacearum	K60	C. Allen
	GSPB1960	K. Rudolph

[a] Collection Francaise de Bacteries Phytopathogenes, Anger, France.
[b] Göttinger Sammlung Phytopathogener Bakterien, Göttingen, Germany.

RESULTS

Ethylene production by cell-free protein extracts. Nagahama et al. (13) reported that cell-free extracts are useful for the determination of 2-oxoglutarate-dependent ethylene production in bacteria. Cell-free protein extracts of the *P. syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, and *sesami* showed strong ethylene production using this system (Table 2). In addition, cell-free protein extracts of the ethylene-producing *P. syringae* pv. *pisi* GSPB1206 showed 2-oxoglutarate-dependent ethylene formation, but had about 20-fold lower activity than those of the other *P. syringae* pathovars. *P. syringae* pv. *pisi* GSPB104 and *R. solanacearum* K60 failed to produce ethylene from 2-oxoglutarate, suggesting that *R. solanacearum* might belong to the group of KMBA-dependent ethylene producers.

Ethylene production and growth kinetics. The kinetics of ethylene production and bacterial growth were determined for *P. syringae* pv. *sesami* 962, a typical 2-oxoglutarate-dependent ethylene producer, *P. syringae* pv. *pisi* GSPB1206, and *R. solanacearum* K60 as shown in Figure 1. Ethylene production by *P. syringae* pvs. *sesami* and *pisi* was strictly growth associated. The highest rates of ethylene formation were detected in the late exponential phase. In contrast, ethylene synthesis by *R. solanacearum* had its maximum in the early exponential phase.

P. syringae pv. *sesami* had a production rate of about 4×10^{-7} nl of ethylene per h per cell, which was 20-fold higher than that determined for *P. syringae* pv. *pisi* (about 2×10^{-8} nl per h per cell) and 200-fold higher than that determined for *R. solanacearum* (about 2×10^{-9} nl per h per cell). A similar high production rate of about 5×10^{-8} nl per h per cell was previously reported for strains of *P. syringae* pvs. *cannabina*, *glycinea*, and *phaseolicola* (28).

Southern blot analysis. The *efe* gene of *P. syringae* pv. *phaseolicola* PK2 was amplified by DIG-labeled PCR and used as probe for Southern blot hybridization studies under conditions of moderate stringency. Bacterial genomic DNA was digested with *Eco*RI prior to blotting. The *efe* probe hybridized to all ethylene-producing *P. syringae* strains tested (Fig. 2). This result suggested that *P. syringae* pv. *pisi* GSPB1206 also harbored a gene encoding the EFE. No hybridization signal was observed with DNA from the ethylene-negative *P. syringae* pv. *pisi* GSPB104. The absence of a hybridization signal from genomic DNA of *R. solanacearum* strains K60 and GSPB1960 was an additional indication that ethylene synthesis by this bacterium apparently occurs via the KMBA pathway.

Nucleotide sequence analysis and comparison. Although *P. syringae* pv. *pisi* GSPB1206 showed 2-oxoglutarate-dependent ethylene production and hybridized to the *efe* gene probe, this strain produced 20-fold less ethylene than the other EFE$^+$ *P. syringae* pathovars. To determine whether the decreased ethylene production was a result of mutations within the *efe* gene, the nucleotide sequences of the *efe* genes from strains of all five ethylene-producing *P. syringae* pathovars were determined. The *efe* genes were amplified by PCR using the primer set EFE11/EFE12 and directly sequenced (GenBank submissions AF101057 to AF101061). The sequences of the open reading frame from *P. syringae* pv. *cannabina* GSPB2553 and *P. syringae* pv. *sesami* 962 coincided completely with the sequence from *P. syringae* pv. *glycinea* 7a/90, which in turn differed in only two single-base pair substitutions from the previously published *efe* nucleotide sequence of *P. syringae* pv. *phaseolicola* PK2 (4), leading to only one change in the amino acid sequence. The nucleotide sequence of the *efe* gene from *P. syringae* pv. *phaseolicola* GSPB669 was completely identical to that derived from *P. syringae* pv. *phaseolicola* PK2. In contrast to the high degree of sequence conservation among the mentioned pathovars, a total of 79 base pair substitutions were found when the *efe* gene from *P. syringae* pv. *pisi* GSPB1206 was compared with that of *P. syringae* pv. *glycinea* 7a/90. These substitutions led to 29 changes in the deduced amino acid sequence (Fig. 3). Moreover, the *efe* gene from *P. syringae* pv. *pisi* GSPB1206 was found to lack 39 base pairs in the C-terminal region, generating a new

TABLE 2. Rate of ethylene production by cell-free extracts from strains of *Pseudomonas syringae* pathovars and *Ralstonia solanacearum*

Strains	Ethylene production[a] (nl/h/mg of protein)	
	With 2-oxoglutarate	Without 2-oxoglutarate
P. syringae pv.		
cannabina GSPB2553	369.2 ± 32.3	0.4 ± 0.1
glycinea 7a/90	472.0 ± 60.1	0.7 ± 0.2
phaseolicola GSPB669	1,165.7 ± 95.9	1.3 ± 0.4
sesami 962	497.3 ± 40.7	1.1 ± 0.7
pisi GSPB1206	26.2 ± 3.1	0.2 ± 0.1
pisi GSPB104	1.3 ± 0.4	0.7 ± 0.3
R. solanacearum K60	1.1 ± 0.7	0.4 ± 0.1

[a] The data are the means and standard errors of five independent determinations.

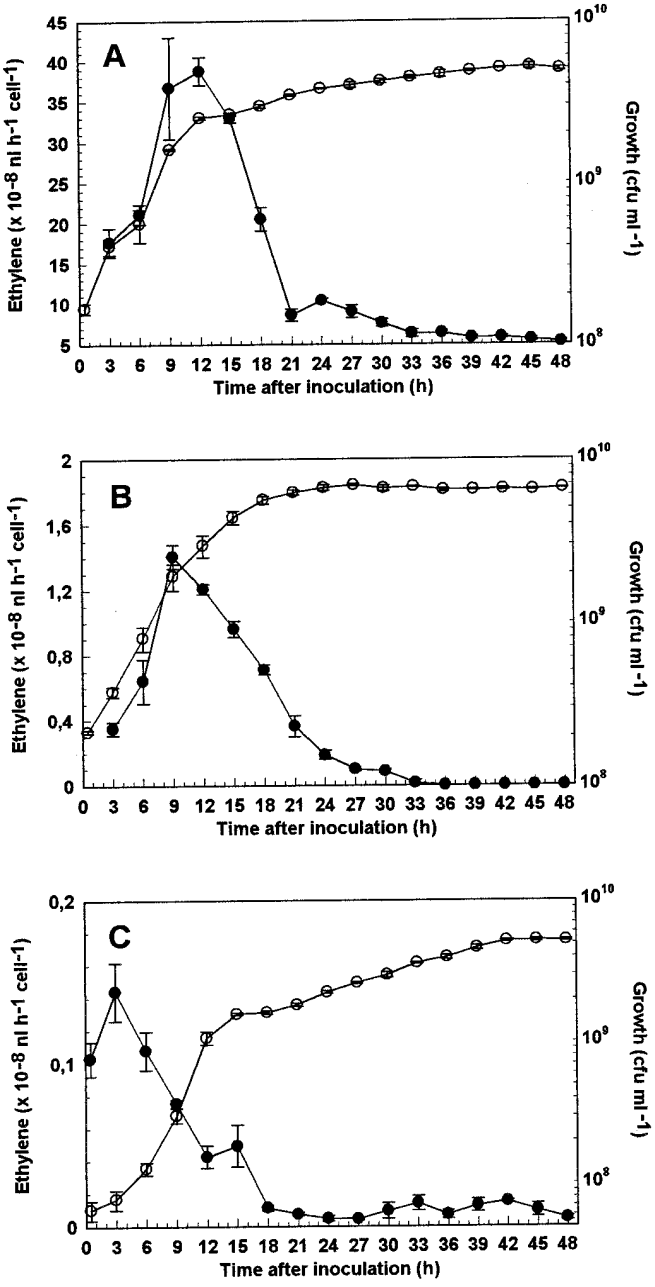

Fig. 1. Growth kinetics (○) and ethylene production (●) by **A,** *Pseudomonas syringae* pv. *sesami* 962; **B,** *P. syringae* pv. *pisi* GSPB1206; and **C,** *Ralstonia solanacearum* K60 in shaken cultures. The data are the means and standard errors of three independent experiments.

stop codon at positions 1,012 to 1,014 (data not shown) and resulting in the loss of 13 amino acids at the C-terminus of the predicted protein (Fig. 3). The nucleotide sequence downstream of the new stop codon differed greatly from nucleotides 1,015 to 1,058 of the *efe* gene sequence from *P. syringae* pv. *glycinea* 7a/90.

Screening for sequence alterations in the *efe* genes of other *P. syringae* pv. *pisi* strains. To determine whether the observed nucleotide substitutions in the *efe* gene of GSPB1206 were characteristic for this pathovar, *P. syringae* pv. *pisi* strains GSPB105, GSPB1477, GSPB1787, and PP01 were screened for these sequence alterations by PCR. The primer sets EFEP1/EFEP2 and EFEP1/EFEP3 were used to amplify 0.3- and 1-kb DNA fragments, respectively. The sequences of these primers corresponded to DNA regions of the GSPB1206 *efe* gene, which were significantly divergent from *efe* sequences derived from other pathovars. Both PCR products were amplified from genomic DNA of all tested *P. syringae* pv. *pisi* strains, but not from that of representatives of pv. *glycinea* or pv. *phaseolicola* (data not shown), indicating that the observed sequence alterations were typical for pv. *pisi* strains.

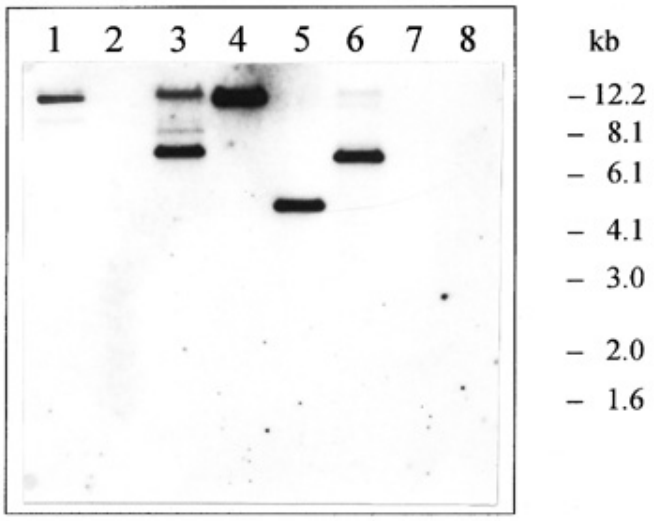

Fig. 2. Southern blot hybridization of *Eco*RI-digested genomic DNA from *Pseudomonas syringae* pathovars and *Ralstonia solanacearum* probed with the *efe* gene from *P. syringae* pv. *phaseolicola* PK2. Lane 1, *P. syringae* pv. *pisi* GSPB1206; lane 2, *P. syringae* pv. *pisi* GSPB104; lane 3, *P. syringae* pv. *glycinea* 7a/90; lane 4, *P. syringae* pv. *phaseolicola* GSPB669; lane 5, *P. syringae* pv. *sesami* 962; lane 6, *P. syringae* pv. *cannabina* GSPB2553; lane 7, *R. solanacearum* K60; and lane 8, *R. solanacearum* GSPB1960.

Pathogenicity on bean plants. Sato et al. (22) proposed that ethylene production is advantageous for colonization of *Leguminosae* plants, because the ethylene-producing *P. syringae* pvs. *cannabina*, *glycinea*, and *phaseolicola* can infect plants of this family. In this study, *R. solanacearum* and all ethylene-producing pathovars of *P. syringae* were tested for pathogenicity on bush bean plants (*Phaseolus vulgaris*) (Table 3). We used *P. syringae* pv. *phaseolicola* 6/0 isolated from bean as a positive control (27). Strains of the *P. syringae* pvs. *cannabina*, *glycinea*, and *phaseolicola* grew very well in the bean plants (Table 3). They reached population densities of about 5×10^7 to 2×10^8 CFU per cm^2 of leaf area. Symptoms caused by these pathovars were water-soaked

TABLE 3. Pathogenicity of *Ralstonia solanacearum* and of the ethylene-forming *Pseudomonas syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, *pisi*, and *sesami* on bean

Strains	Bacterial growth[a,b] (CFU per cm^2 of leaf area)	Symptoms[a]
P. syringae pv.		
phaseolicola 6/0 (bean strain, control)	2×10^8	Chlorosis, necrosis
cannabina GSPB2553	5×10^7	Chlorosis, weak necrosis
glycinea 7a/90	1×10^8	Chlorosis, weak necrosis
phaseolicola GSPB669 (kudzu strain)	2×10^8	Chlorosis, strong necrosis
sesami 962	5×10^5	Chlorosis
pisi GSPB1206	1×10^6	Chlorosis, weak necrosis
R. solanacearum K60	3×10^3	None

[a] Determined 7 days after inoculation.
[b] The data are the means of three independent determinations.

```
7a/90 M T N L Q T F E L P T E V T G C A A D I S L G R A L I Q A W Q K D G I F Q I K T D S E Q D  45
1206  M T N L Q T F E L P T E V I G S A A D I S L G R A L I Q A W Q K D G I L Q I K T D S E Q N  45

7a/90 R K T Q E A M A A S K Q F C K E P L T F K S S C V S D L T Y S G Y V A S G E E V T A G K P  90
1206  R K T Q E A M A A S K Q F C K E P L T F K S S C V S D L T Y S G Y V A S G E E V T A G K P  90

7a/90 D F P E I F T V C K D L S V G D Q R V K A G W P C H G P V P W P N N T Y Q K S M K T F M E  135
1206  D F P E I F T V C K D L P V S D Q R V K A G W P C H G P V P W P N N T Y Q K S M K A F M G  135

7a/90 E L G L A G E R L L K L T A L G F E L P I N T F T D L T R D G W H H M R V L R F P P Q T S  180
1206  E L G L A G E R L L K L T A L G F E L P I N T F T D L T R N G W H H M R V L R F P P Q T S  180

7a/90 T L S R G I G A H T D Y G L L V I A A Q D D V G G L Y I R P P V E G E K R N R N W L P G E  225
1206  T M S S G I G A H T D Y G L L V I A A Q D D V G G L Y I R P P V E G E K R N R N W L P G E  225

7a/90 S S A G M F E H D E P W T F V T P T P G V W T V F P G D I L Q F M T G G Q L L S T P H K V  270
1206  S S A G M F E H D D P W T Y V T P V Q N V W T V F P G D I L Q F M T C G Q L L S T P H K V  270

7a/90 K L N T R E R F A C A Y F H E P N F E A S A Y P L F E P S A N E R I H Y G E H F T N M F M  315
1206  R L N T R E R F A C A Y F H E P N F E A C A Y Q V F E P S G N E R I H Y G E H F T S M F M  315

7a/90 R C Y P D R I T T Q S I N K E N R L A H L E D L K K Y S D T R A T G S                    350
1206  R C Y P D R I T T K R I H K D N R L A H F K                                          337
```

Fig. 3. Alignments of the predicted amino acid sequences for the *efe* gene from *Pseudomonas syringae* pv. *glycinea* 7a/90 and *P. syringae* pv. *pisi* GSPB1206. Amino acid residues are numbered on the right. Boxes mark amino acid substitutions in the sequence from the *P. syringae* pv. *pisi* strain.

lesions that turned chlorotic and then necrotic after 7 days. *P. syringae* pv. *phaseolicola* strains from kudzu caused very intensive chlorosis that turned into necrotic lesions surrounded by yellow halos. Following inoculation of the bean plants with *P. syringae* pvs. *pisi* and *sesami*, symptoms appeared that were similar to those induced by the other *P. syringae* pathovars. *P. syringae* pv. *pisi* GSPB1206 caused chlorosis that turned to brown and necrotic lesions, whereas the *P. syringae* pv. *sesami* strain produced only chlorotic spots. The latter two strains reached population densities of only 1×10^6 and 5×10^5 CFU per cm^2 of leaf area, respectively. *R. solanacearum* was not pathogenic on bean plants. After inoculation, the *R. solanacearum* strain did not cause any symptoms and the bacterial population density reached only 3×10^3 CFU per cm^2 of leaf area. These results demonstrated that all five ethylene-producing *P. syringae* pathovars, but not *R. solanacearum*, were virulent on bean plants.

Screening of additional *P. syringae* pathovars for ethylene production. To reveal the distribution of ethylene production among various pathovars of *P. syringae*, strains of the pvs. *avellanae*, *dendropanices*, *garcae*, *hibisci*, *mellea*, *myricae*, *porri*, and *zizaniae* were tested for their ability to produce ethylene. No strains of these pathovars had ever been examined for ethylene production. All tested strains failed to produce detectable amounts of ethylene.

DISCUSSION

Ethylene production is a widespread characteristic of microorganisms and most synthesize ethylene at low rates via the KMBA pathway (13). In contrast, the most potent ethylene producers utilize the 2-oxoglutarate-dependent pathway; for example, *Penicillium digitatum* and certain pathovars of *P. syringae* (5,13). In this study, we characterized ethylene production in *P. syringae* pv. *pisi* GSPB1206, which produced 20-fold less ethylene than other *P. syringae* pathovars, and in *R. solanacearum* K60, which produced only trace amounts of ethylene. We could not detect any EFE activity or an *efe* gene in *R. solanacearum*. Moreover, ethylene production in *R. solanacearum* was maximal in the early exponential phase in contrast to the *P. syringae* pathovars, in which ethylene production peaked in the late exponential phase. It is, therefore, likely that *R. solanacearum* may utilize the KMBA pathway.

Cell-free protein extracts from *P. syringae* pv. *pisi* GSPB1206 produced ethylene from 2-oxoglutarate in an assay for the EFE (14). Additionally, Southern blot analysis demonstrated the presence of an *efe* gene in this strain. We, therefore, concluded that ethylene formation by *P. syringae* pv. *pisi* was mediated by the 2-oxoglutarate-dependent pathway. This subsequently raised the question of why this *P. syringae* pv. *pisi* strain produced ethylene at such a low rate. To show whether sequence alterations in the *efe* gene were responsible for the lower production rate, the nucleotide sequences of the *efe* gene from strains of all five ethylene-forming *P. syringae* pathovars were determined. Sequence comparisons indicated that the predicted *efe* gene products of the pvs. *cannabina*, *glycinea*, *phaseolicola*, and *sesami* were identical, with only a single amino acid difference in the enzyme of *P. syringae* pv. *phaseolicola*. Obviously, this change did not interfere with the EFE function, since strains of this pathovar produced ethylene very efficiently. In contrast, numerous nucleotide substitutions were found in the *efe* gene of *P. syringae* pv. *pisi*. These changes resulted in 29 amino acid substitutions. Additionally, we identified a deletion of 39 nucleotides at the end of the gene, which led to a C-terminal truncation of 13 amino acids for its gene product. It appears very likely that these sequence alterations in the *efe* gene of *P. syringae* pv. *pisi* were responsible for the lower efficiency. The observed differences in the *efe* gene sequence were a typical characteristic of this pathovar as demonstrated by PCR with genomic DNA of four additional pv. *pisi* strains.

Nagahama et al. (17) individually substituted each of the 10 histidine codons in the *efe* gene of *P. syringae* pv. *phaseolicola* PK2 with codons for glutamine, thereby identifying two histidine residues that were essential for iron-binding and catalytic activity. The other site-directed mutations led to lower catalytic activities, demonstrating that single nucleotide substitutions could cause a decrease in the enzymatic activity.

Highly conserved secondary metabolite synthesis-associated gene sequences in different pathovars of *P. syringae* were also found by Sawada et al. (23) when comparing the *argK* genes from phaseolotoxin-producing *P. syringae* pvs. *actinidiae* and *phaseolicola*. In contrast, Bereswill et al. (2) sequenced a PCR product of the *cfl* gene, involved in coronatine synthesis, from different toxin-producing *P. syringae* pathovars and found numerous differences in the nucleotide sequences.

The high conservation of the *efe* gene sequences among the *P. syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, and *sesami* suggests that horizontal gene transfer between these pathovars may have occurred. Recently, Watanabe et al. (29) demonstrated transfer of a plasmid carrying the *efe* gene from *P. syringae* pv. *glycinea* to another pathovar of *P. syringae* in vitro. Normander et al. (18) reported that the phylloplane is conducive to conjugative gene transfer. In our study, the ethylene-producing pathovars of *P. syringae* were tested for pathogenicity on a common host. Bush bean plants were chosen for this experiment because *P. syringae* pvs. *cannabina*, *glycinea*, and *phaseolicola* were known to be pathogenic on this plant species (22). Our results demonstrated that pvs. *pisi* and *sesami* were also pathogenic on bean plants. However, in contrast to the other ethylene-producing *P. syringae* pathovars, they reached population densities that were two orders of magnitude lower. The *efe* gene could have been horizontally transferred between these pathovars by conjugation in the phyllosphere when they simultaneously colonized the same host.

One additional aim of this work was to screen previously untested pathovars of *P. syringae* for ethylene production. For the first time, pvs. *avellanae*, *dendropanices*, *garcae*, *hibisci*, *mellea*, *myricae*, *porri*, and *zizaniae* were tested for ethylene production, but none of them were able to produce ethylene. Although our current study was limited to only one representative strain from each of these pathovars, previous experience indicated that ethylene production is a stable characteristic in those pathovars that produce it (22,30). The only exception was the low ethylene-producing pv. *pisi*, in which strain GSPB104 turned out to be ethylene negative (30). In a previous report, ethylene has been found to be produced by all 50 tested strains of *P. syringae* pv. *glycinea* isolated from plants of various regions (30). *P. syringae* is currently subdivided into 55 pathovars (31). A total of 51 pathovars has now been tested for the production of ethylene, and to date, only *P. syringae* pvs. *cannabina*, *glycinea*, *phaseolicola*, *pisi*, and *sesami* are known to produce ethylene (9,22,28).

In infected leaf tissue, significant amounts of ethylene were produced by strains of *P. syringae* pvs. *glycinea* and *phaseolicola*, suggesting a role for ethylene synthesis in the pathogenicity of these bacteria (30) that preferentially infect leaves. In contrast, strains of *P. syringae* pv. *pisi*, causing bacterial blight of pea, primarily colonize the stem (11). Perhaps, the lower ethylene production by these strains may not have been disadvantageous for disease development in stem tissue. Over time, the randomly mutated *efe* gene of *P. syringae* pv. *pisi* might not have been affected by selection pressure. In contrast, foliar pathogens of *P. syringae* may be under selection pressure to maintain high ethylene production rates. To evaluate the role of ethylene in plant-bacteria interactions, further investigations are in progress in our laboratories.

ACKNOWLEDGMENTS

We thank C. Allen, S. Prathuangwong, L. Gardan, K. Naumann, K. Nishiyama, and K. Rudolph for providing bacterial strains.

LITERATURE CITED

1. Arndt, H., Henning, C., Völksch, B., and Fritsche, W. 1989. Beziehung zwischen Virulenz und Phaseolotoxinbildungsvermögen bei verschiedenen *Pseudomonas syringae* pv. *phaseolicola*-Stämmen. Arch. Phytopathol. Pflanzenschutz 25:347-357.
2. Bereswill, S., Bugert, P., Völksch, B., Ullrich, M., Bender, C. L., and Geider, K. 1994. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. Appl. Environ. Microbiol. 60:2924-2930.
3. Freebairn, H. T., and Buddenhagen, I. W. 1964. Ethylene production by *Pseudomonas solanacearum*. Nature 202:313-314.
4. Fukuda, H., Ogawa, T., Ishihara, K., Fujii, T., Nagahama, K., Omata, T., Inoue, Y., Tanase, S., and Morino, Y. 1992. 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.
5. Fukuda, H., Ogawa, T., and Tanase, S. 1993. Ethylene production by micro-organisms. Pages 275-306 in: Advances in Microbiology and Physiology. Vol. 35. A. H. Rose, ed. Academic Press, New York.
6. Fukuda, H., Ogawa, T., Tazaki, M., Nagahama, K., Fujii, T., Tanase, S., and Morino, Y. 1992. 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.
7. Fukuda, H., Takahashi, M., Fujii, T., Tazaki, M., and Ogawa, T. 1989. An NADH:Fe(III)EDTA oxidoreductase from *Cryptococcus albidus*: An enzyme involved in ethylene production in vivo? FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 60:107-112.
8. Goto, M., and Hyodo, H. 1987. Ethylene production by cell-free extract of the kudzu strain of *Pseudomonas syringae* pv. *phaseolicola*. Plant Cell Physiol. 28:405-414.
9. Goto, M., Ishida, Y., Takikawa, Y., and Hyodo, H. 1985. Ethylene production by the kudzu strains of *Pseudomonas syringae* pv. *phaseolicola* causing halo blight in *Pueraria lobata* (Willd.) Ohwi. Plant Cell Physiol. 26:141-150.
10. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44: 301-307.
11. Kleinhempel, H., Naumann, K., and Spaar, D. 1989. Bakterielle Erkrankungen der Kulturpflanzen. Gustav Fischer Verlag, Jena, Germany.
12. Mattoo, A. K., and Suttle, J. C. 1991. The Plant Hormone Ethylene. CRC Press, Boca Raton, FL.
13. Nagahama, K., Ogawa, T., Fujii, T., and Fukuda, H. 1992. Classification of ethylene-producing bacteria in terms of biosynthetic pathways to ethylene. J. Ferment. Bioeng. 73:1-5.
14. Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Goto, M., and Fukuda, H. 1991. L-Arginine is essential for the formation *in vitro* of ethylene by an extract of *Pseudomonas syringae*. J. Gen. Microbiol. 137:1641-1646.
15. Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Tanase, S., Morino, Y., and Fukuda, H. 1991. Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. J. Gen. Microbiol. 137:2281-2286.
16. Nagahama, K., Yoshino, K., Matsuoka, M., Sato, M., Tanase, S., Ogawa, T., and Fukuda, H. 1994. 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. Microbiology 140:2309-2313.
17. Nagahama, K., Yoshino, K., Matsuoka, M., Tanase, S., Ogawa, T., and Fukuda, H. 1998. Site-directed mutagenesis of histidine residues in the ethylene-forming enzyme from *Pseudomonas syringae*. J. Ferment. Bioeng. 85:255-258.
18. Normander, B., Christensen, B., Molin, S., and Kroer, N. 1998. Effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of the bush bean (*Phaseolus vulgaris*). Appl. Environ. Microbiol. 64:1902-1909.
19. Ogawa, T., Takahashi, M., Fujii, T., Tazaki, M., and Fukuda, H. 1990. The role of NADH:Fe(III)EDTA oxidoreductase in ethylene formation from 2-keto-4-methylthiobutyrate. J. Ferment. Bioeng. 69:287-291.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Sato, M., Urushizaki, S., Nishiyama, K., Sakai, F., and Ota, Y. 1987. Efficient production of ethylene by *Pseudomonas syringae* pv. *glycinea* which causes halo blight in soybeans. Agric. Biol. Chem. 51:1177-1178.
22. Sato, M., Watanabe, K., Yazawa, M., Takikawa, Y., and Nishiyama, K. 1997. Detection of new ethylene-producing bacteria, *Pseudomonas syringae* pvs. *cannabina* and *sesami*, by PCR amplification of genes for the ethylene-forming enzyme. Phytopathology 87:1192-1196.
23. Sawada, H., Takeuchi, T., and Matsuda, I. 1997. Comparative analysis of *Pseudomonas syringae* pv. *actinidiae* and pv. *phaseolicola* based on phaseolotoxin-resistant ornithine carbamoyltransferase gene (*argK*) and 16S-23S rRNA intergenic spacer sequences. Appl. Environ. Microbiol. 63:282-288.
24. Takikawa, Y., Ando, Y., Hamaya, E., Tsuyumu, S., and Goto, M. 1988. Identification of the pathogens responsible for bacteriosis of tea plant occurred in 1983. Ann. Phytopathol. Soc. Jpn. 54:224-228.
25. Ullrich, M., and Bender, C. L. 1994. The biosynthetic gene cluster for coronamic acid, an ethylcyclopropyl amino acid, contains genes homologous to amino acid-activating enzymes and thioesterases. J. Bacteriol. 176:7574-7586.
26. Völksch, B., Laplace, F., and Fritsche, W. 1984. Untersuchungen zur Variabilität der Phaseolotoxinbildung bei *Pseudomonas syringae* pv. *phaseolicola*. Zentralbl. Mikrobiol. 139:109-118.
27. Völksch, B., and Weingart, H. 1997. Comparison of ethylene-producing *Pseudomonas syringae* strains isolated from kudzu (*Pueraria lobata*) with *Pseudomonas syringae* pv. *phaseolicola* and *Pseudomonas syringae* pv. *glycinea*. Eur. J. Plant Pathol. 103:795-802.
28. Völksch, B., and Weingart, H. 1998. Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. J. Basic Microbiol. 38:135-145.
29. Watanabe, K., Nagahama, K., and Sato, M. 1998. A conjugative plasmid carrying the *efe* gene for the ethylene-forming enzyme isolated from *Pseudomonas syringae* pv. *glycinea*. Phytopathology 88:1205-1209.
30. Weingart, H., and Völksch, B. 1997. Ethylene production by *Pseudomonas syringae* pathovars in vitro and in planta. Appl. Environ. Microbiol. 63:156-161.
31. Young, J. M., Saddler, G. S., Takikawa, Y., De Boer, S. H., Vauterin, L., Gardan, L., Gvozdyak, R. I., and Stead, D. E. 1996. Names of plant pathogenic bacteria 1864-1995. Rev. Plant Pathol. 75:721-762.