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Ethylene Production by *Pseudomonas syringae* Pathovars In Vitro and In Planta

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Significant amounts of ethylene were produced by *Pseudomonas syringae* pv. glycinea, pv. phaseolicola (which had been isolated from viny weed *Pueraria lobata* [Willd.] Ohwi [common name, kudzu]), and pv. pisi in synthetic medium. On the other hand, the bean strains of *P. syringae* pv. phaseolicola and strains of 17 other pathovars did not produce ethylene. *P. syringae* pv. glycinea and *P. syringae* pv. phaseolicola produced nearly identical levels of ethylene (about 5×10^{-7} nl h^{-1} $cell^{-1}$), which were about 10 times higher than the ethylene level of *P. syringae* pv. pisi. Two 22-bp oligonucleotide primers derived from the ethylene-forming enzyme (*efe*) gene of *P. syringae* pv. phaseolicola PK2 were investigated for their ability to detect ethylene-producing *P. syringae* strains by PCR analysis. PCR amplification with this primer set resulted in a specific 0.99-kb fragment in all ethylene-producing strains with the exception of the *P. syringae* pv. pisi strains. Therefore, *P. syringae* pv. pisi may use a different biosynthetic pathway for ethylene production or the sequence of the *efe* gene is less conserved in this bacterium. *P. syringae* pv. phaseolicola isolated from kudzu and *P. syringae* pv. glycinea also produced ethylene in planta. It could be shown that the enhanced ethylene production in diseased tissue was due to the production of ethylene by the inoculated bacteria. Ethylene production in vitro and in planta was strictly growth associated.

Ethylene is a well-studied gaseous plant hormone. It is involved in the regulation of numerous physiological processes, such as seed germination, seedling growth, fruit ripening, flower development, leaf abscission, and plant senescence. A variety of environmental factors, including wounding, drought, chilling temperature, and exposure to chemicals, induce ethylene production (20). Enhanced formation of ethylene is also an early response of plants to recognition of a pathogen attack (5). Therefore, it has been suggested that ethylene has a possible function as a signal inducing defense reaction. However, the results are contradictory. Treatment with exogenous ethylene induces pathogenesis-related proteins in a number of plant tissues (5). On the other hand, ethylene is not causally required for the systemic acquired resistance or the hypersensitive response (2, 7, 17). Ethylene may play a role in symptom development, because it can cause chlorosis, abscission, and senescence and thus may be important in the predisposition of plant tissue to disease (20, 28).

In addition to plants, a variety of microorganisms, including phytopathogenic fungi and bacteria, are capable of synthesizing ethylene (10). In 1985 Goto et al. (13) found that strains of *Pseudomonas syringae* pv. phaseolicola pathogenic to the Japanese weed *Pueraria lobata* (Willd.) Ohwi (common name, kudzu) form large amounts of ethylene. Two years later Sato et al. (27) reported that the causal agent of halo blight of soybean (*Glycine max* L.), *P. syringae* pv. glycinea, produces ethylene as efficiently as the kudzu strains. Microorganisms and higher plants synthesize ethylene by different biochemical pathways (10). In plants, ethylene is produced from methionine via 1-aminocyclopropane-1-carboxylic acid. In microorganisms, ethylene is produced either via 2-keto-4-methylthiobutyric acid (KMBA), a transaminated derivative of methionine, by an NADH:Fe(III)EDTA oxidoreductase-mediated reaction, as in *Escherichia coli* and *Cryptococcus albidus*, or via 2-oxoglutarate by an ethylene-forming enzyme (EFE) as in *P. syringae* pv. phaseolicola, *P. syringae* pv. glycinea, and *Penicillium digitatum* (12, 21). The genes coding for the EFE in *P. syringae* pv. phaseolicola and *P. syringae* pv. glycinea were previously shown to reside on an indigenous plasmid(s) and to have homologous DNA sequences (9, 21). However, the N-terminal amino acid sequence of the EFE from *Penicillium digitatum* was different from those of the two bacteria (21).

For *Pseudomonas solanacearum* (18, 24) and *Xanthomonas citri* (14), an attempt to assess the bacterial contribution to increased ethylene formation during symptom development has been made, with the conclusion that the ethylene was mostly of host origin.

Little information is available about ethylene production by other *P. syringae* pathovars. No information exists about ethylene production in planta and the possible role of ethylene in the pathogenicity of these bacteria.

The aims of this study were to reveal the distribution of ethylene production among the pathovars of *P. syringae* and to determine if *P. syringae* pv. glycinea and phaseolicola are able to produce ethylene in planta.

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 (31) at 28°C. The references cited in Table 1 indicate the specific studies or laboratories in which the strains were isolated and characterized.

Plants and inoculation. The plants grew in individual pots in a greenhouse. About 20-day-old soybean plants (cv. Maple Arrow) and bean plants (cv. Red Kidney) were inoculated by tissue infiltration with a pressure sprayer. Bacterial suspensions (about 10^6 CFU ml^{-1}) and coronatine (5×10^{-5} M) were applied to the first fully expanded trifoliate leaves. 2-Aminoethoxyvinyl-glycine (AVG; 100 μM) an inhibitor of plant ethylene biosynthesis, was used to investigate the origin of ethylene. Plants were infiltrated with AVG with a pressure sprayer 24 h after inoculation with coronatine and 4 days after inoculation with bacteria. Six hours after inoculation with AVG, levels of ethylene were determined. For comparison, leaves of untreated plants were infiltrated with AVG and levels of ethylene were also determined after 6 h. After treatments, the plants remained in the greenhouse.

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TABLE 1. *P. syringae* strains used in this study

Pathovar	Strain designation(s)	Host	Source or reference
aptata	1092, 1080, 1067, and 1084	Sugar beet	K. Rudolph[a]
atrofaciens	1392 and 1440	Wheat	K. Rudolph
	1742	Barley	K. Rudolph
atropurpurea	MAFF301017, MAFF301304, MAFF301309 and MAFF301313	Italian ryegrass	K. Nishiyama
cannabina	64	Hemp	K. Rudolph
coronafaciens	1357	Unknown	K. Naumann[b]
	50261 and 50262	Unknown	DSM[c]
glycinea	R2, R5, and R6	Soybean	K. Naumann
	G4180	Soybean	C. Bender
	8/83, 16/83, 6/83, 5/83, 15/83, 7/84, 25/84, 19/84, 26/84, 26/O/84, 21/84, 40B/85, 20/G/gr/85, 20/O/85, 28/B/85, 27/B/85, 21/O/kl/85, 25/G/kl/85, 25/O/B/kl/85, 1/88, 21/88, 3/88, 23/88, 24a/89, 9al/89, 6a/89, 23al/89, 7a/90, 27a/90, 43a/90, 33a/90, 16a/90, 6a/94, 11a/94, 14a/94, 15a/94, 15a8/94, 30a1/94, 30a2/94, 30a8/94, 30a15/94, 30a21/94, 33a/94, 34a/94, 39a/94, and 42a/94	Soybean	B. Völksch
lachrymans	61/15	Cucumber	K. Naumann
	77 and 1497	Cucumber	K. Rudolph
maculicola	2146	Cauliflower	K. Rudolph
	921 and 438	Crucifer	D. Cuppels
mori	1840	Mulberry	K. Rudolph
morsprunorum	D5	Plum	K. Naumann
	886	Cherry	K. Rudolph
	896	Pear	K. Rudolph
	1013	Unknown	K. Rudolph
phaseolicola	Hb-1b and 1321	Bush bean	K. Naumann
	6/0, M2/1, 26/0, 268/16, F2, and 106/1	Bush bean	1
	KZ2w and PK2	Kudzu vine	29
	664 and 667	Kudzu vine	K. Rudolph
persicae	1025	Peach	K. Rudolph
pisi	104, 105, 1206, 1477, and 1787	Pea	K. Rudolph
	pp01	Pea	K. Naumann
primulae	108	Primrose	K. Rudolph
savastanoi	2259	Oleander	K. Rudolph
	2260	Ash	K. Rudolph
	2264	Olive	K. Rudolph
striafaciens	1850	Oat	K. Rudolph
syringae	W50 and C72	Pear	K. Naumann
	J59	Apple	K. Naumann
	860 and 880	Cherry	K. Rudolph
tabaci	pp112	Tobacco	K. Naumann
	111, 113, and 117	Tobacco	K. Rudolph
tagetis	99	Marigold	K. Naumann
tomato	483, 479, 487, and 119	Tomato	K. Rudolph
	33 and 55	Tomato	K. Naumann
	DC3000	Tomato	D. Cuppels

[a] Strain collection of the Institut für Pflanzenpathologie und Pflanzenschutz, Göttingen, Germany.
[b] Strain collection of the Bundesanstalt für Züchtungsforschung an Kulturpflanzen, Aschersleben, Germany.
[c] Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

Determination of ethylene and bacterial growth in vitro. For the routine determination of ethylene production by *P. syringae* pathovars, 1 ml of an overnight culture in synthetic 5a medium (31) was transferred in a sterile 5-ml syringe sealed with a rubber cap and incubated on a rotary shaker at 90 rpm and room temperature (22 to 24°C) for 2 h. After incubation, gas samples (1 ml) were withdrawn with a gas-tight syringe (Hamilton) and ethylene was determined with a gas chromatograph (model GC-14A; Shimadzu) equipped with an active alumina column and a flame ionization detector. Ethylene production in vitro and bacterial growth curves were obtained from 60-ml shaking cultures of 5a medium in 250-ml flasks (140 rpm, 28°C). Ethylene was determined every 3 h as described above, and production was expressed in nanoliters per hour per cell. Bacterial growth was estimated with a spectrophotometer (578 nm).

Determination of ethylene and bacterial growth in planta. Fifteen discs (7 mm in diameter) were excised with a cork borer from infected leaves at designated intervals, transferred into 3-ml syringes (five discs per syringe) sealed with rubber caps, and incubated at room temperature (22 to 24°C) for 6 h. A 1-ml gas sample was taken from each syringe, and ethylene production was determined as described above. After we determined the ethylene concentrations, the bacterial populations in the leaf discs were monitored. The five discs of a syringe were macerated together in 5-ml isotonic NaCl. Appropriate serial 10-fold dilutions were plated onto King's medium B (16). Plates were incubated at 28°C, and colonies from three replicate plates were counted after 3 to 4 days. The amount of ethylene produced was expressed in nanoliters per hour per square centimeter.

PCR techniques. The ethylene-producing *P. syringae* pathovars were detected with a specific primer pair derived from the *efe* gene of *P. syringae* pv. phaseolicola PK2 (9). Two 22-mer oligonucleotides with the sequences 5′-GCATGACC AACCTACAGACTTT-3′ (primer 1) and 5′-CTCCTTGTTGATCCTCTGGGT G-3′ (primer 2) were chosen for amplification; these were located 516 and 1,486 bp from the 5′ end of the *efe* gene, respectively. The primers were synthesized with a DNA-RNA synthesizer (model 394; Applied Biosystems) at the Institute of Virology, Friedrich-Schiller-University, Jena, Germany. The standard reaction mixture (50 μl) contained 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 160 μg of bovine serum albumin, 5% dimethylsulfoxide, 5% Tween 20, 200 μM (each) deoxynucleoside triphosphates, 0.8 U of *Taq* DNA polymerase (Pharmacia), and 25 pmol of each primer (3). About 10^6 bacterial cells were directly applied to the reaction mixture. Amplification included initial denaturation (95°C, 3 min) followed by 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min) with a single final extension (72°C, 10 min). PCRs were carried out with a Gene ATAQ Controller from Pharmacia. After the reactions, 10-μl samples of the PCR

TABLE 2. *P. syringae* pathovars tested for their ability to produce ethylene

Pathovar	No. of tested strains	Ethylene production (10^{-8} nl h^{-1} $cell^{-1}$)
aptata	4	0
atrofaciens	3	0
atropurpurea	4	0
cannabina	1	0
coronafaciens	3	0
glycinea	50	5–100
lachrymans	3	0
maculicola	3	0
mori	1	0
morsprunorum	4	0
phaseolicola (from kudzu)	4	30–70
phaseolicola (from bean)	8	0
persicae	1	0
pisi	5	1–3
pisi	1	0
primulae	1	0
savastanoi	3	0
striafaciens	1	0
syringae	5	0
tabaci	4	0
tagetis	1	0
tomato	7	0

products were separated on 1% agarose gels, stained with ethidium bromide, and photographed with Polaroid type 667 film.

Southern analysis. Southern blots were performed as described by Sambrook et al. (26) with digoxigenin DNA labelling and detection kits (Böhringer Mannheim). A digoxigenin-labelled DNA probe was made from the 0.99-kb PCR fragment of the *efe* gene.

RESULTS AND DISCUSSION

Ethylene production in vitro. Strains of 20 *P. syringae* pathovars were tested for their ability to produce ethylene in shaking cultures. The strains grew well in the synthetic medium, resulting in a high cell density. Only three pathovars produced ethylene. Of six *P. syringae* pv. pisi strains, five produced ethylene. All strains of *P. syringae* pv. glycinea ($n = 50$) isolated from soybean plants of various regions and all strains of *P. syringae* pv. phaseolicola ($n = 4$) isolated from kudzu produced ethylene (Table 2). However, strains of *P. syringae* pv. phaseolicola ($n = 8$) isolated from beans and all other tested pathovars failed to produce detectable amounts of ethylene (Table 2).

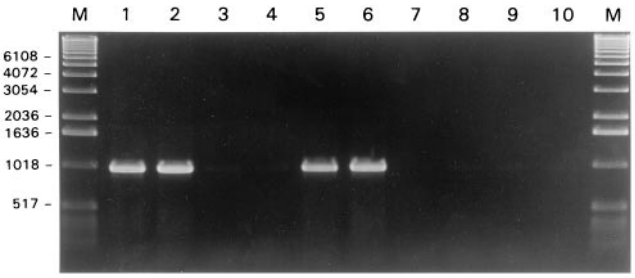

FIG. 1. PCR products amplified from various *P. syringae* pathovars. Lanes 1 and 2, *P. syringae* pv. glycinea 8/83 and 7a/90; lanes 3 and 4, *P. syringae* pv. phaseolicola (from bean) 6/0 and 1321; lanes 5 and 6, *P. syringae* pv. phaseolicola (from kudzu) PK2 and KZ2w; lanes 7 and 8, *P. syringae* pv. pisi 1787 and pp01; lane 9, *P. syringae* pv. syringae C72; lane 10, *P. syringae* pv. tomato 483; lanes M, molecular weight markers. The amplification was carried out with a specific primer pair derived from the *efe* gene of *P. syringae* pv. phaseolicola PK2. Molecular weights are noted at the left.

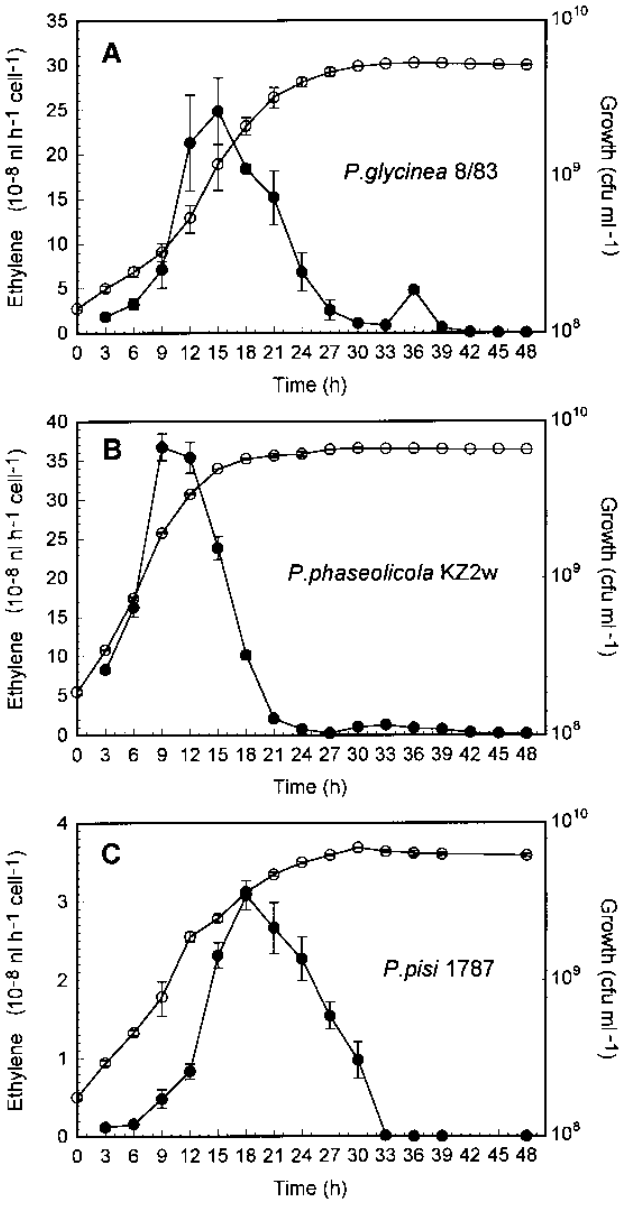

FIG. 2. Growth kinetics (○) and ethylene production (●) by *P. syringae* pv. glycinea 8/83 (A), *P. syringae* pv. phaseolicola KZ2w (B), and *P. syringae* pv. pisi 1787 (C) in vitro. The data are the means and standard errors of three independent experiments.

PCR amplification with *efe* primers 1 and 2 resulted in the detection of a specific 0.99-kb fragment in all ethylene-producing *P. syringae* pv. glycinea and phaseolicola strains (Fig. 1, lanes 1, 2, 5, and 6). The 0.99-kb fragment was absent when PCR was carried out with the ethylene-producing *P. syringae* pv. pisi strains (Fig. 1, lanes 7 and 8) and with pathovars which did not produce ethylene (Fig. 1, lanes 9 and 10). Primers 1 and 2 specifically amplified a portion of the *efe* gene which encodes the enzyme responsible for the formation of ethylene and succinate from 2-oxoglutarate. Southern blot hybridization of genomic DNA of *P. syringae* pv. pisi strains with the *efe* gene probe failed to yield hybridization signals. *P. syringae* pv. glycinea and ethylene-producing *P. syringae* pv. phaseolicola strains gave a strong hybridization signal (data not shown). It is

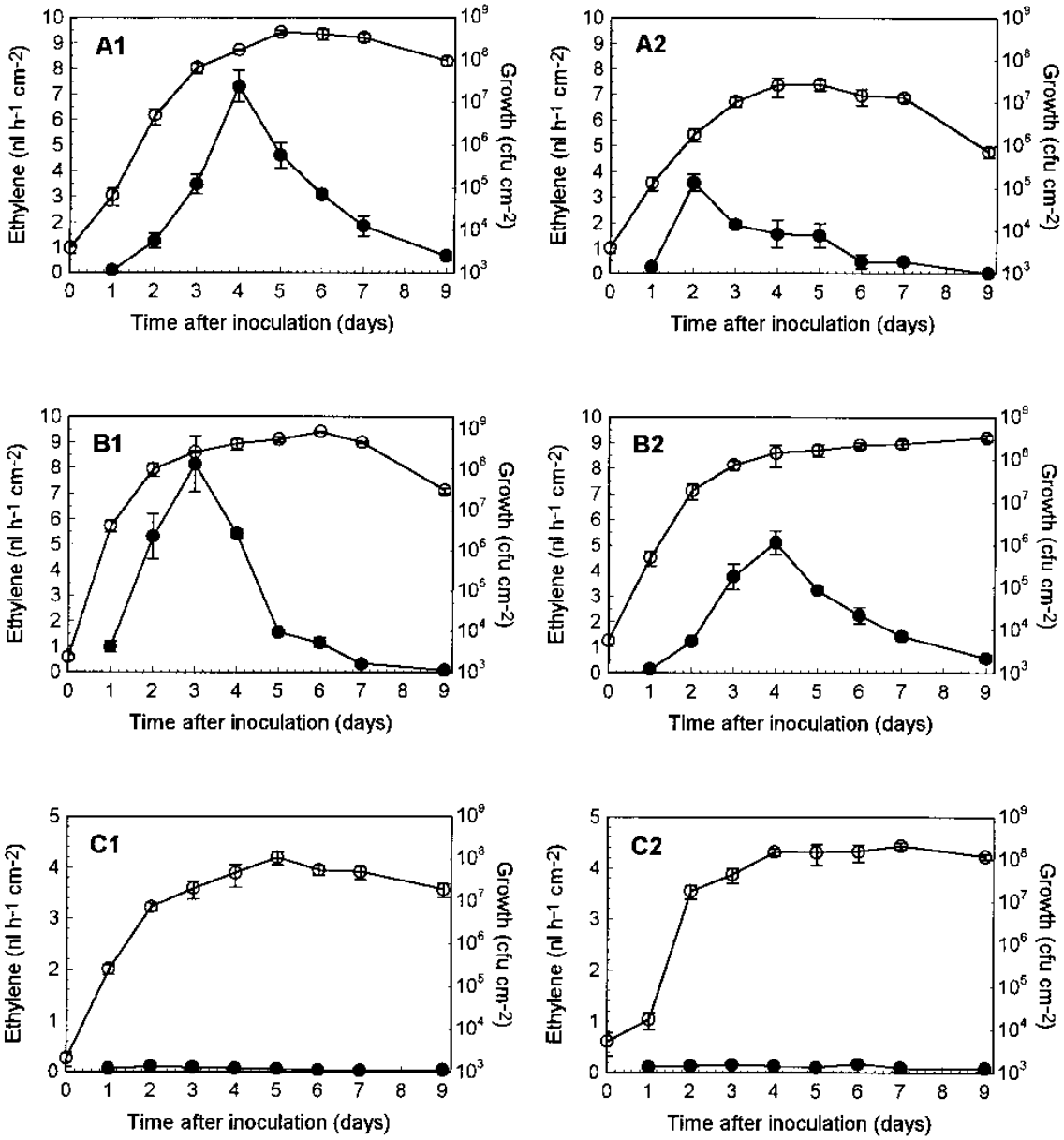

FIG. 3. Growth kinetics (○) and ethylene production (●) by *P. syringae* pv. glycinea 8/83 (A), *P. syringae* pv. phaseolicola KZ2w from kudzu (B), and *P. syringae* pv. phaseolicola 6/0 from bean (C) in soybean (1) and bean (2) leaves. The data are the means and standard errors of three independent experiments.

TABLE 3. Inhibitory effects of 100 μM AVG on ethylene production of bean leaves inoculated with coronatine, ethylene-forming *P. syringae* pv. *glycinea* 8/83, and *P. syringae* pv. *phaseolicola* KZ2w

Treatment	Ethylene production (nl h^{-1} cm^{-2})[a] of:	
	Untreated plants	Plants treated with AVG
Control (bean)	0.044 ± 0.005	0.011 ± 0.002
Inoculation with coronatine	0.221 ± 0.042	0.012 ± 0.002
Inoculation with 8/83	1.820 ± 0.330	1.260 ± 0.080
Inoculation with KZ2w	9.230 ± 0.840	11.440 ± 2.520

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

known that there are two biosynthetic pathways for the production of ethylene in microorganisms. The absence of the 0.99-kb PCR product when PCR experiments utilized *P. syringae* pv. pisi strains and the absence of a hybridization signal suggest that these strains might use the KMBA pathway for ethylene formation. This is consistent with the results of Billington et al. (4), who showed that KMBA is a common metabolic product of *P. syringae* pv. pisi. However, it also is possible that the sequence of the *efe* gene of *P. syringae* pisi is different from that of *P. syringae* pv. phaseolicola PK2.

In contrast to the *P. syringae* pv. pisi strains with an ethylene production rate of about 3×10^{-8} nl h^{-1} $cell^{-1}$, the ethylene-producing *P. syringae* pv. glycinea and phaseolicola strains showed a 10-fold higher production rate of about 5×10^{-7} nl h^{-1} $cell^{-1}$. This rate was several times higher than those re-

ported for *P. solanacearum* and for *Penicillium digitatum*, which are potent ethylene producers (6, 18). It is difficult to compare the data from the literature directly with ours, because cell densities are often not recorded and different incubation methods for ethylene detection were used. Nevertheless, strains like *P. syringae* pv. glycinea and *P. syringae* pv. phaseolicola, which transform 2-oxoglutarate into ethylene, may be more efficient ethylene producers than microorganisms which form ethylene via KMBA.

For the *P. syringae* pv. phaseolicola and glycinea strains the rate of ethylene production was tested at 18°C, which is favorable for toxin production, and at 28°C, which is the optimum growth temperature. In contrast to the formation of the phytotoxins phaseolotoxin (22) and coronatine (30), which are produced maximally at 18°C, the rate of ethylene production at 28°C (e.g., *P. syringae* pv. phaseolicola KZ2w's rate was 4×10^{-7} nl h^{-1} $cell^{-1}$) was about two times higher than that at 18°C (e.g., *P. syringae* pv. phaseolicola KZ2w's rate was 2×10^{-7} nl h^{-1} $cell^{-1}$).

The kinetics of ethylene production and bacterial growth of typical cultures of *P. syringae* pv. glycinea (8/83), *P. syringae* pv. phaseolicola (KZ2w), and *P. syringae* pv. pisi (1787) are shown in Fig. 2. The ethylene production of all kinds of pathovars is strictly growth associated, with the highest rate of ethylene production being detected in the late exponential phase. Lu et al. (18) obtained similar results with *P. solanacearum*, which causes bacterial wilt of tomato plants. In contrast to these observations, ethylene synthesis by *E. coli* does not occur during the exponential phase of growth (25).

Ethylene production in planta. The results of ethylene production and growth of *P. syringae* pv. glycinea, *P. syringae* pv. phaseolicola (from kudzu), and *P. syringae* pv. phaseolicola (from bean) in inoculated soybean and bean leaves are presented in Fig. 3. Untreated leaf discs of soybean and bean produced almost no ethylene (soybean, about 20 pl h^{-1} cm^{-2}; bean, about 80 pl h^{-1} cm^{-2}). Soybean and bean leaves inoculated with the *P. syringae* pv. phaseolicola 6/0 produced ethylene at a very low rate (about 100 pl h^{-1} cm^{-2}) throughout the experimental period (Fig. 3, graphs C1 and C2). However, in soybean and bean leaves inoculated with *P. syringae* pv. glycinea (8/83) and *P. syringae* pv. phaseolicola (KZ2w) isolated from kudzu, respectively, ethylene production began after inoculation at the beginning of the multiplication of the bacterial strains and continued to increase until the bacteria entered stationary phase (Fig. 3A and B). The kinetics of ethylene production in planta conform with the kinetics in vitro. The strains also showed in planta growth-associated production of ethylene. Ethylene production did not increase in leaves infected with *P. syringae* pv. phaseolicola 6/0, an ethylene nonproducer, even though the bacterial populations in both plants increased from the time of inoculation from about 5×10^3 CFU cm^{-2} to about 10^8 CFU cm^{-2} 4 to 5 days after inoculation (Fig. 3, graphs C1 and C2), and typical disease symptoms developed in bean leaves after 3 to 4 days. The phytotoxin coronatine also enhanced ethylene production in plant tissues (Table 3) (8, 15). Coronatine-induced ethylene production could be inhibited by AVG, an effective inhibitor of ethylene biosynthesis in higher plants. This fact indicates that the ethylene was of plant origin. AVG strongly inhibits pyridoxal enzyme 1-aminocyclopropane-1-carboxylic acid synthase activity in vitro as well as in vivo (20). In contrast, the ethylene produced in inoculated soybean and bean plants was not inhibited by AVG (Table 3; data of soybean plants are not shown). It is thus apparent that the ethylene in soybean and bean leaves infected with ethylene-producing *P. syringae* pv. glycinea or phaseolicola was mostly of bacterial origin. The plant contribution to ethylene production was negligible in contrast to the ethylene production of citrus leaves infected with *X. citri* (14) and of tomato plants inoculated with *P. solanacearum* (18, 24).

Many ethylene-forming microorganisms exist in nature, and most of them biosynthesize ethylene from methionine via KMBA at a low rate (19). The last step of this reaction occurs nonenzymatically (23). In contrast to the 2-oxoglutarate-dependent system in *P. syringae* pv. glycinea and phaseolicola, ethylene is not directly produced by an ethylene-forming enzyme (11, 23). Ethylene is a common metabolic product of the *P. syringae* pv. glycinea and the kudzu strains. These strains produce ethylene at a rate of about 5×10^{-7} nl h^{-1} $cell^{-1}$. In contrast to the known phytotoxins coronatine (30) and phaseolotoxin (1, 31), which are produced by some but not all *P. syringae* pv. glycinea and phaseolicola strains, respectively, ethylene was produced by all tested *P. syringae* pv. glycinea and phaseolicola strains from kudzu. Our results may indicate that ethylene production is important in the pathogenicity process. The role that it plays in the interaction between plants and bacteria is still being investigated.

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