A Conjugative Plasmid Carrying the *efe* Gene for the Ethylene-Forming Enzyme Isolated from *Pseudomonas syringae* pv. *glycinea*

K. Watanabe, K. Nagahama, and M. Sato

First and third authors: National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305-8634, Japan; and second author: Kumamoto Institute of Technology, Ikeda, Kumamoto 860-0082, Japan.
Accepted for publication 9 August 1998.

ABSTRACT

Watanabe, K., Nagahama, K., and Sato, M. 1998. A conjugative plasmid carrying the *efe* gene for the ethylene-forming enzyme of *Pseudomonas syringae* pv. *glycinea* Phytopathology 88:1205-1209.

Previous work suggested that the *efe* gene encoding the ethylene-forming enzyme was present in the plasmids of three pathovars of *Pseudomonas syringae* including *glycinea*, *phaseolicola* (kudzu strains), and *cannabina*. However, no direct evidence to support this assumption had been presented. In the current study, we isolated the conjugative plasmid harboring the *efe* gene (ethylene plasmid) designated pETH2 from *P. syringae* pv. *glycinea* MAFF301683. pETH2 was detected by Southern blot hybridization using the *efe* probe, marked with the transposon mini-

Tn5-Km1, and transferred into *P. syringae* Ni27ⁿ, which does not produce ethylene. The transconjugant Ni27ⁿ (pETH2) produced ethylene at a level similar to pv. *glycinea* MAFF301683. In addition, the plasmid designated pCOR2, which encodes coronatine biosynthesis genes, was detected in the same strain. Although the molecular size of the plasmid pCOR2 was not easily distinguishable from pETH2, pCOR2 transferred independently into Ni27ⁿ and the transconjugants produced coronatine. These findings suggested that the *efe* gene has been horizontally dispersed among pathovars of *P. syringae* by plasmid-mediated conjugation in nature.

Additional keywords: gene transfer, plant hormone, polymerase chain reaction.

Ethylene is a plant hormone involved in the regulation of numerous physiological processes that may play some role in pathogenicity. Ethylene is also produced by microorganisms including a limited number of plant-pathogenic bacteria (4,7,20,21,24). Four pathovars of *Pseudomonas syringae* including *phaseolicola* (kudzu strain), *glycinea*, *cannabina*, and *sesami* produce ethylene very efficiently through a biochemical pathway using 2-oxoglutarate, which is controlled by the gene *efe* (7,20,21,24). These findings suggested that *efe* had been horizontally dispersed among pathovars of *P. syringae* through conjugation, natural transformation, or transduction.

Conjugation may play a major role in the dispersion of *efe*. Nagahama et al. (11) reported that *efe* may be encoded in indigenous plasmids in the pathovars *phaseolicola* and *glycinea* by Southern blot analysis using an *efe* gene probe. Sato et al. (21) showed that plasmids in all the strains of the pathovars *glycinea* and *phaseolicola* hybridized with the *efe* probe. Furthermore, Sato et al. (21) also obtained data using Southern blot analysis and polymerase chain reaction (PCR) detection of *efe* in the plasmid indicating that pv. *cannabina* MAFF302256 may harbor an ethylene plasmid. However, no direct evidence for the plasmid location of *efe* of *P. syringae* has been obtained, since the assumption was based only on analyses by Southern blot hybridization and PCR.

On the other hand, it remained to be determined whether the ethylene-producing phenotype of these pathovars is advantageous for survival on their host *Leguminosae* plants (pathovars *phaseolicola*, *glycinea*, and *cannabina*) and *Sesamum indicum* (pv. *sesami*). A previous study suggested that ethylene production in planta may play an important role in the pathogenicity of *P. syringae* pv. *glycinea* (24). The purpose of this study was to produce ethylene-

producing strains by transferring the ethylene plasmid into nonproducing strains; these strains could be used for analyses of the role of *efe* in the pathogenicity or survival of *P. syringae*.

In the current study, we obtained direct evidence that a plasmid of *P. syringae* pv. *glycinea* MAFF301683 carries the *efe* gene and is self-transmissible.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. The plasmid pKEFE (5.6 kb), harboring *efe* of pv. *phaseolicola* strain PK2, was used as a DNA probe for Southern blot hybridization. To estimate the molecular size of the indigenous plasmids, the following plasmid molecular size standards were used: three indigenous plasmids of *P. syringae* pv. *mori* S6801 (91, 76, and 38 kb) (18) and two plasmids from *P. syringae* pv. *tabaci* BR2 (RSF1010) (19), which harbors the indigenous plasmid pBPW1 (45 kb) and the *Escherichia coli* R-plasmid RSF1010 (8.9 kb).

The molecular size was measured by Kodak Digital Science 1D-2.02 software (Eastman Kodak Co., Rochester, NY).

Growth of bacteria. For the PCR assay and plasmid isolation, bacteria were grown on modified Luria-Bertani (LB) agar medium (10 g of peptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar per 1 liter of distilled water; tryptone in the original LB was replaced by peptone) or in modified LB broth overnight at 25°C, respectively. To detect ethylene production, the bacteria were grown in modified LB medium with shaking.

Plasmid DNA isolation. Plasmid DNA was extracted according to the method of Birnboim and Doly (3) and separated by electrophoresis in Tris-borate buffer (0.89 M Tris-borate and 0.089 M boric acid, pH 8) at 8 V/cm in 0.6% agarose for 3 h.

Transposon mutagenesis. To obtain transposon-induced mutants, matings were performed on membranes (Nihon Millipore Ltd., Tokyo) overnight at 25°C. *E. coli* S17 (λ pir) harboring

Corresponding author: K. Watanabe; E-mail address: nabek@nises.affrc.go.jp

pUT::mini-Tn5-Km1 (8) was used as the donor strain, and strain 301683-r, which was obtained spontaneously as a rifampicin-resistant mutant of MAFF301683 in this study, was used as the recipient strain. An equal ratio of donor/recipient cells was used. To select transconjugants, modified LB medium containing 20 µg of rifampicin per ml and 20 µg of kanamycin per ml was used.

PCR analysis. To amplify the entire *efe* gene (1.05 kb), a pair of primers (21), ETH-1 (5'-ATGACCAACCTACAGACT-3') and ETH-2 (5'-TCATGAGCCTGTCGCGCG-3'), were designed from sequence data reported by Fukuda et al. (5). To amplify the *cfl* region (one of the coronatine biosynthesis genes), a pair of primers (26), Cor1 (5'-GGCGCTCCCTCGCACTT-3') and Cor2 (5'-GGTATTGGCGGGGTGC-3'), were constructed from sequence

TABLE 1. Bacterial strains and plasmids

Designation	Relevant properties ^a	Source or reference
Strains		
<i>Pseudomonas syringae</i>		
<i>pv. glycinea</i>		
MAFF301683	WT	MAFF ^b
MAFF302676	WT	MAFF
MAFF302683	WT	MAFF
MAFF302694	WT	MAFF
MAFF302754	WT	MAFF
301683-r	RIF ^r	This study
301683-r::mini-Tn5-P1	RIF ^r , KM ^r	This study
301683-r::mini-Tn5-P2	RIF ^r , KM ^r	This study
301683-r::mini-Tn5-P3	RIF ^r , KM ^r	This study
301683-r::mini-Tn5-P5	RIF ^r , KM ^r	This study
<i>pv. atropurpurea</i>		
NIAES1309	WT	K. Nishiyama
<i>pv. mori</i>		
S6801	Plasmid size marker	M. Sato
<i>pv. tabaci</i>		
BR2 (RSF1010)	Plasmid size marker	Sato et al. (19)
unknown <i>pv.</i>		
Ni27 ⁿ	RIF ^r , NAL ^r	This study
<i>Escherichia coli</i> S17 (λ pir)		
(pUT::mini-Tn5-Km1)	Transposon vector, KM ^r	Lorenzo et al. (7)
Plasmids		
pTKB	Mini-Tn5-Km1 probe	Watanabe et al. (23)
pKEFE	<i>efe</i> Probe	K. Nagahama

^a RIF^r, KM^r, and NAL^r: resistance to rifampicin (20 µg/ml), kanamycin (20 µg/ml), and nalidixic acid (20 µg/ml), respectively. WT = wild type.
^b MAFF: strains from the Ministry of Agriculture, Forestry and Fisheries, Japan.

Fig. 1. Plasmid profiles of **A**, *Pseudomonas syringae* *pv. glycinea* strains and Southern blots of the plasmids probed with **B**, the *efe* gene or **C**, the *cfl* gene. Lane a, *pv. glycinea* MAFF301683; b, MAFF302676; c, MAFF302683; d, MAFF302694; e, MAFF302754; f, *pv. mori* S6801; and g, *pv. tabaci* BR2 (RSF1010). Lanes f and g, molecular size markers (91-, 76-, and 38-kb plasmids of S6801, and 45- and 8.9-kb plasmids of BR2). Chr (arrow) shows the linear chromosomal DNA band.

data reported by Bereswill et al. (2). Template DNAs were prepared by boiling a bacterial suspension (10⁸ CFU/ml) for 5 min. The standard reaction mixture (50 µl) contained 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 U of *Taq* DNA polymerase (Promega Corp., Madison, WI), 25 pmol of each primer, 5 µl of 10× reaction buffer, and 2.5 µl of boiled bacterial suspension. Amplification included 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 2 min), and extension (72°C for 3 min).

Southern blot analysis. Several probes were used in this study. To detect transposon insertions, a Tn5-Km1 ribo-probe (1.7-kb *Bam*HI fragment of mini-Tn5-Km1) was prepared from pTKB as described previously (23). To detect *cfl*, a *cfl* probe was constructed from the PCR product of *P. syringae* *pv. atropurpurea* NIAES1309 obtained when the primer set Cor1-Cor2 (26) was used. To detect the *efe* region, an *efe* probe was constructed from the PCR product of pKEFE obtained when the primer set ETH1-ETH2 (21) was used. These probes were labeled with a digoxigenin DNA-labeling kit (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's protocol and used for Southern blot hybridization. Southern blot analysis was performed according to standard procedures (14) with some modifications (23).

Ethylene production. Ethylene production was measured by the method described by Nagahama et al. (11). To measure the rate of ethylene production by bacterial strains, the bacteria were inoculated into modified LB liquid medium (200 ml) in a 500-ml Erlenmeyer flask and incubated with slow rotary shaking (180 rpm) at 25°C overnight. A portion of each culture (1 ml) was then transferred to a 34-ml test tube for the ethylene assay. The remainder of each culture was used for the measurement of the dry weight of bacteria after they were pelleted by centrifugation at 8,000 × g. The test tube for the ethylene assay was sealed with a rubber serum cap and incubated with slow rotary shaking (180 rpm) at 25°C for 1 h. A gas sample (1 ml) was withdrawn through the rubber seal with a gas-tight syringe and analyzed with a Yanaco G3810 gas chromatograph (GC) (Yanaco Co., Kyoto, Japan) equipped with an activated alumina column and a hydrogen flame ionization detector. The rate of ethylene production was expressed as nl of ethylene (mg of cell dry weight) h⁻¹.

Coronatine assay. To detect the production of coronatine by bacterial strains, the potato assay previously described by Nishiyama (12) was used.

Fig. 2. Southern blot hybridization to the plasmids of MAFF301683::mini-Tn5-Km1 with the Tn5-Km1 ribo-probe. Lane a, MAFF301683 wild type; and b, c, d, and e, transconjugants with mini-Tn5-Km1 designated as P1, P2, P3, and P5, respectively. An arrow shows the linear chromosomal DNA band.

RESULTS

Detection of plasmids. Five strains of *pv. glycinea* (MAFF301683, MAFF302676, MAFF302683, MAFF302694, and MAFF302754) were examined for their plasmid content. All of the strains harbored several plasmids as follows: MAFF301683 (93, 68, 59, and 47 kb), MAFF302676 (105, 69, 62, 48, and 9 kb), MAFF302683 (103, 68, 61, 48, 12, and 9 kb), MAFF302694 (102, 72, 60, 46, 9, and 6 kb), and MAFF302754 (102, 89, 75, 60, 51, 15, and 12 kb) (Fig. 1A). Plasmid DNA was then transferred to nylon membranes and used for Southern hybridization experiments to detect plasmids that carry *efe*. The *efe* probe hybridized to a 93-kb plasmid band in *pv. glycinea* MAFF301683 and to a 102- to 105-kb plasmid band in other strains. It also hybridized weakly to a 59-kb plasmid band in *pv. glycinea*; but this is thought to be a nonspecific reaction. On the other hand, the *cfl* probe hybridized to both the 93-kb and 102- to 105-kb plasmid bands, suggesting that 93-kb and 102- to 105-kb plasmids in these strains may carry both *efe* and *cfl*. In contrast, both probes failed to hybridize with other plasmids ranging from 6 to 89 kb in *pv. glycinea* (Fig. 1B and C) and with plasmids in *pv. mori* S6801 and *pv. tabaci* BR2 (RSF1010) (data not shown).

Transposon mutagenesis and PCR. Strain 301683-r was mated with *E. coli* S17 (λ pir) harboring pUT::mini-Tn5-Km1. Many transposon-insertion mutants were obtained in the mating, and 38 mutants were screened by Southern blot hybridization for transposon insertions into indigenous plasmids. Among them, four mutants (designated P1, P2, P3, and P5) harbored a plasmid shown to contain mini-Tn5-Km1 (Fig. 2). Southern blot analysis using the Tn5-Km1 ribo-probe revealed that mini-Tn5-Km1 was inserted into the largest plasmid (93 kb) in P2 and P3, but into smaller plasmids in P1 and P5 (Fig. 2).

To separate the 93-kb plasmid from the other indigenous plasmids of MAFF301683, P2 and P3 (93-kb plasmid::mini-Tn5-Km1) were mated separately with *P. syringae* Ni27ⁿ on membranes. To select the transconjugants, modified LB medium containing 20 μ g of nalidixic acid per ml and 20 μ g of kanamycin per ml was used. P2 and P3 transconjugants were obtained at a frequency of 1.3×10^{-8} and 5.0×10^{-8} per recipient, respectively, in these matings. These transconjugants were designated C2-1, C2-2, and C2-3 (obtained from mating with P2) and C3-1, C3-2, and C3-3 (obtained from mating with P3). A 93-kb plasmid was detected in all the C2 (Fig. 3A, lanes b, c, and d) and C3 (Fig. 3A, lanes f, g, and h) transconjugants. Next, plasmids from the C2 and C3 derivatives were hybridized with *efe* and *cfl* probes in Southern blots. The *efe* probe hybridized to the plasmid in the C2 derivatives, but not to the one in the C3 derivatives (Fig. 3B). In contrast, the *cfl* probe hybridized to the plasmid in the C3 derivatives, but not to the one in the

C2 derivatives (Fig. 3C). These results revealed that the 93-kb plasmid band of MAFF301683 was composed of two different similar size plasmids.

Likewise, a PCR assay using primers ETH1-ETH2 to amplify *efe* produced a product only with the C2 derivatives, whereas PCR using primers Cor1-Cor2 to amplify *cfl* was positive only for the C3 derivatives (Fig. 4). The *Bam*HI restriction patterns of the plasmids in C2 and C3 transconjugants were different (data not shown). Therefore, we designated the plasmid from the C2 derivatives as pETH2 (ethylene plasmid) and the plasmid from C3 derivatives as pCOR2 (coronatine plasmid).

Ethylene and coronatine production. Eight strains including P2, P3, C2 derivatives, C3 derivatives, MAFF301683, and Ni27ⁿ were examined for their ethylene-producing ability by GC analysis. As shown in Table 2, Ni27ⁿ (pETH2) (the C2 derivatives), and wild-type strain MAFF301683 and its derivatives (P2, P3) produced ethylene efficiently, whereas Ni27ⁿ, with and without pCOR2, did not. The amount of ethylene produced by MAFF301683 was 2,055 nl per mg of cells per h. Likewise, a similar high level of ethylene production was detected in Ni27ⁿ transconjugants harboring pETH2, but not pCOR2, indicating that *efe* was adequately expressed in a different pathovar of *P. syringae*. On the other hand, coronatine production was only observed in the transconjugants that had acquired pCOR2, but not pETH2. Thus, the production of ethylene or coronatine in a nonproducing strain of *P. syringae* (unknown pathovar) was verified for transconjugants that had acquired pETH2 or pCOR2, respectively.

DISCUSSION

In microorganisms, two pathways for ethylene production have been reported. One pathway synthesizes ethylene via 2-oxoglutarate using an ethylene-forming enzyme (*efe*) (9,10) and the other pathway makes it via 2-keto-4-methyl-thiobutyric acid using an NADH: Fe(III)EDTA oxidoreductase (13). Only four pathovars of *P. syrin-*

Fig. 4. Polymerase chain reaction (PCR) amplification of *efe* or *cfl* from *Pseudomonas syringae* *pv. glycinea* MAFF301683 and *P. syringae* Ni27ⁿ (pETH2) or Ni27ⁿ (pCOR2) transconjugants. Lane M, molecular size markers (λ HindIII/EcoRI); a, 301683-r::mini-Tn5-P2; b, c, and d, Ni27ⁿ (pETH2) transconjugants designated as C2-1, C2-2, and C2-3, respectively; e, 301683-r::mini-Tn5-P3; f, g, and h, Ni27ⁿ (pCOR2) transconjugants designated as C3-1, C3-2, and C3-3, respectively; and j, Ni27ⁿ. Arrows show PCR products amplified by primers ETH1-ETH2 (ETH) or primers Cor1-Cor2 (COR).

TABLE 2. Production of ethylene and coronatine by *Pseudomonas syringae* Ni27ⁿ transconjugants that acquired pETH2 or pCOR2 plasmids by mating with MAFF301683-r::mini-Tn5

Strains (plasmid)	Ethylene production ^a (nl/mg cells/h)	Coronatine production ^b
301683-r::mini-Tn5-P2	1,715	+
Ni27 ⁿ (pETH2) C2-1	1,507	—
Ni27 ⁿ (pETH2) C2-2	1,002	—
301683-r::mini-Tn5-P3	1,502	+
Ni27 ⁿ (pCOR2) C3-1	0	+
Ni27 ⁿ (pCOR2) C3-2	0	+
MAFF301683 wild type	2,055	+
Ni27 ⁿ	0	—

^a The values represent means obtained from three independent experiments.

^b The potato assay of Nishiyama (12) was used.

Fig. 3. Plasmid profiles of **A**, *Pseudomonas syringae* Ni27ⁿ strains that acquired pETH2 or pCOR2 and Southern blot hybridization to the plasmids probed with **B**, the *efe* gene or **C**, the *cfl* gene. Lane a, 301683-r::mini-Tn5-P2; b, c, and d, Ni27ⁿ (pETH2) transconjugants designated as C2-1, C2-2, and C2-3, respectively; e, 301683-r::mini-Tn5-P3; f, g, and h, Ni27ⁿ (pCOR2) transconjugants designated as C3-1, C3-2, and C3-3, respectively; and j, Ni27ⁿ. An arrow shows the linear chromosomal DNA band.

gae including pv. *glycinea* have been found to possess *efe* (21) and use the former pathway. In the current study, the plasmid location of *efe* in pv. *glycinea* MAFF301683 was verified. This is the first proof of plasmid-borne ethylene production in microorganisms, although this possibility was previously suggested by Nagahama et al. (11) and Sato et al. (21). Our findings further suggest that the 102- to 105-kb plasmids in four other strains of pv. *glycinea* used in this study are also ethylene plasmids. Likewise, several plasmids from pv. *cannabina* and kudzu strains of pv. *phaseolicola*, which hybridized to the *efe* probe, are likely to contain *efe* (21).

The pETH2 plasmid was transmissible from *P. syringae* pv. *glycinea* to *P. syringae*, and the transconjugants were able to efficiently produce ethylene. Strain Ni27ⁿ, which was isolated from mulberry leaves, is only very weakly pathogenic (or nonpathogenic) to mulberry and can be easily distinguished from strains of pv. *mori* and the "pv. *glycinea* and pv. *phaseolicola*" group by PCR restriction fragment length polymorphism (RFLP) analysis of *hrp* genes (K. Watanabe, K. Nishiyama, and M. Sato, unpublished data). Therefore, Ni27ⁿ is currently considered to be an unidentified pathovar, and it is different from pv. *glycinea* based on the above-mentioned PCR-RFLP and its inability to produce coronatine and ethylene. The finding of in vitro transfer of an ethylene plasmid to another pathovar of *P. syringae* suggests that similar conjugal transfer of *efe* can occur among pathovars of *P. syringae* in nature.

The transfer frequency of pETH2 to Ni27 was very low, only 1.3×10^{-8} per recipient. In a previous report, the transfer frequency of pBPW1 (an indigenous plasmid of *P. syringae* pv. *tabaci* BR2) to the same recipient strain (Ni27-Rif) was 1.0×10^0 (16). Unlike pBPW1, which is naturally derepressed for transfer, pETH2 appears to have repressed fertility functions. It is also possible that pETH2 could be mobilized by other indigenous plasmids of MAFF301683 (Fig. 1). The low frequency of transfer of pETH2 suggests that horizontal dispersion among bacteria by conjugation does not occur frequently in nature, although some factors that enhance plasmid transfer may exist.

Interestingly, the molecular size of pETH2 was similar to that of pCOR2. However, these plasmids could be distinguished from each other by endonuclease cleavage patterns (data not shown). In other strains of pv. *glycinea*, probes of both *efe* and *cfl* genes hybridized to the largest plasmid(s), which were similar in size (102 to 105 kb). To determine whether or not these genes reside on the same plasmid in each strain, more studies will be required. Several sizes of plasmids encoding coronatine biosynthesis genes (Cor-genes) have been detected in the *P. syringae* pathovars *atropurpurea* (17), *tomato* (1), *glycinea* (25), and *maculicola* (26). Among them, at least two plasmids, pCOR1 of pv. *atropurpurea* and pPT23A of pv. *tomato*, were conjugative (1,15). In this study, pCOR2 was also shown to be conjugative. Comparative studies between these coronatine plasmids would be valuable in understanding the evolution of Cor-genes in *P. syringae*.

Weingart and Völksch (24) have recently reported that pv. *glycinea* produces ethylene in planta, suggesting a potential role for ethylene in pathogenicity. Coronatine has been found to be associated with the development of some symptoms of pv. *glycinea* infection (6). All strains of pv. *glycinea* produce ethylene (20,22) and most strains produce coronatine (22, this study). These findings suggest that both phenotypes are advantageous for the survival of pv. *glycinea* in soybeans. Ni27ⁿ strains harboring both plasmids obtained in this study could be used for studying the role of *efe* and Cor-genes in pathogenicity or survival. In this study, it was found that most strains of pv. *glycinea* harbor both the ethylene plasmid and the coronatine plasmid. No other plant-pathogenic bacteria harboring two plasmids encoding *efe* and Cor-genes are known. These findings are important from the viewpoint of the evolution of pv. *glycinea*. For example, we can speculate that an ethylene plasmid first may have been intro-

duced into pro-pv. *glycinea*, followed by a coronatine plasmid, to create pv. *glycinea*.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries, Japan. We thank K. Nishiyama and the Ministry of Agriculture, Forestry and Fisheries, Japan (MAFF) for supplying bacterial strains.

LITERATURE CITED

- Bender, C. L., Malvick, D. K., and Mitchell, R. E. 1989. Plasmid-mediated production of the phytotoxin coronatine in *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 171:807-812.
- 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.
- Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.
- Freebairn, H. T., and Buddendenhagen, I. W. 1964. Ethylene production by *Pseudomonas solanacearum*. Nature 202:313-314.
- 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.
- Gnanamanickam, S. S., Starratt, A. N., and Ward, E. M. B. 1982. Coronatine production *in vitro* and *in vivo* and its relation to symptom development in bacterial blight of soybean. Can. J. Bot. 60:645-650.
- Goto, M., Ishida, Y., Takikawa, Y., and Hyodo, H. 1985. Ethylene production by kudzu strains of *Pseudomonas syringae* pv. *phaseolicola* causing halo blight in *Pueraria lobata* (Willd.) Ohwi. Plant Cell Physiol. 26:141-150.
- Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. J. Bacteriol. 172:6568-6572.
- 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.
- 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*. J. Gen. Microbiol. 137: 2281-2286.
- 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.
- Nishiyama, K. 1981. Studies on the pathogenicity-related substances in *Pseudomonas coronafaciens* var. *atropurpurea*. Bull. Natl. Inst. Agric. Sci. C 35:1-15.
- 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-methylthiobutrate. J. Ferment. Bioeng. 69:287-291.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1982. Plasmid vectors. Pages 1.1-1.110 in: Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sato, M. 1988. *In planta* transfer of the genes for virulence between isolates of *Pseudomonas syringae* pv. *atropurpurea*. Ann. Phytopathol. Soc. Jpn. 54:20-24.
- Sato, M., Nishiyama, K., and Panopoulos, N. J. 1985. Conjugative transmission and replicative properties of plasmid pBPW1, and antigen alteration induced by the plasmid in pathovars of *Pseudomonas syringae*. Ann. Phytopathol. Soc. Jpn. 51:426-434.
- Sato, M., Nishiyama, K., and Shirata, A. 1983. Involvement of plasmid DNA in the production of coronatine by *Pseudomonas syringae* pv. *atropurpurea*. Ann. Phytopathol. Soc. Jpn. 49:522-528.
- Sato, M., Staskawicz, B. J., and Panopoulos, N. J. 1982. Indigenous plasmids of *Pseudomonas syringae* pv. *mori*, the causal agent of bacterial blight of mulberry. Ann. Phytopathol. Soc. Jpn. 48:27-33.
- Sato, M., Staskawicz, B. J., Panopoulos, N. J., Peters, S., and Homma, M. 1981. A host-dependent hybrid plasmid suitable as a suicidal carrier for transposable elements. Plasmid 6:325-331.

20. Sato, M., Urushizaki, K., 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.
21. 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.
22. 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.
23. Watanabe, K., Katsuhara, M., Nakao, H., and Sato, M. 1997. Detection and molecular analysis of plant- and insect-associated bacteria harboring aconitate isomerase involved in biosynthesis of trans-aconitic acid as antifeedant in brown planthoppers. *Curr. Microbiol.* 35:97-102.
24. Weingart, H., and Völksch, B. 1997. Ethylene production by *Pseudomonas syringae* pathovars *in vitro* and *in planta*. *Appl. Environ. Microbiol.* 63:156-161.
25. Young, S. A., Park, S. K., Rodgers, C., Mitchell, R. E., and Bender, C. L. 1992. Physical and functional characterization of the gene cluster encoding the polypeptide phytotoxin coronatine in *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* 174:1837-1843.
26. Zhu, Y., Tamura, K., Watanabe, M., Matsuda, I., and Sato, M. 1995. Plasmid-mediated coronatine production in *Pseudomonas syringae* pv. *maculicola*. *Ann. Phytopathol. Soc. Jpn.* 61:569-574.