

In vitro および In planta における *Pseudomonas syringae* pv. *lachrymans* から非病原性 *Pseudomonas* 株へのストレプトマイシン耐性の伝達について

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***In Vitro* and *In Planta* Transfer of Dihydrostreptomycin Resistance from *Pseudomonas syringae* pv. *lachrymans* to Nonvirulent *Pseudomonas* strains**

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矢野 博*・藤井 溥**: *In vitro* および *In planta* における *Pseudomonas syringae* pv. *lachrymans* から非病原性 *Pseudomonas* 株への
ストレプトマイシン耐性の伝達について

Key Words: *Pseudomonas syringae* pv. *lachrymans*, drug resistance.

In the previous paper¹⁾, we reported the transmissibility of dihydrostreptomycin (DHSM) resistance of *Pseudomonas syringae* pv. *lachrymans* (hereafter referred to as *P. s.* pv. *lachrymans*). Transmission experiments were conducted by mixing culture method, and it was found that the resistance of 13 *P. s.* pv. *lachrymans* isolates out of 109 DHSM-resistant ones was distinctly transmitted to the recipient isolates of rifampicin (RFP) resistant *P. s.* pv. *lachrymans*. In this paper, it is described that DHSM resistance of *P. s.* pv. *lachrymans* can also be transmitted to nonvirulent pseudomonads under natural conditions.

Bacterial strains During the period from 1973 to 1977, 165 isolates of *P. s.* pv. *lachrymans* and nonvirulent pseudomonads were obtained from various districts of Japan and used in this experiment. Among them, 22 nonvirulent pseudomonads were generously provided by Dr. A. Ohuchi, National Institute of Agricultural Sciences²⁾. All of these nonvirulent pseudomonads were isolated from cucumber leaves or fruits, together with virulent *P. s.* pv. *lachrymans*, but different from *P. s.* pv. *lachrymans* or other recognized plant pathogens in bacteriological characteristics (Table 1). They were, monotrichous, gram-negative, aerobic rods, and utilize glucose, producing green fluorescent pigment. Therefore, they were simply described as "nonvirulent pseudomonads" in this report. So far as the strains used for the transmissibility test, *P. s.* pv. *lachrymans* 75101 (DHSM^r), and *P. s.* pv. *lachrymans* 7563 (RFP^r) were DHSM resistant and RFP resistant respectively¹⁾.

Drug resistance of nonvirulent pseudomonads Drug sensitivity was tested

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Table 1. Cultural and physiological characteristics of *Pseudomonas syringae* pv. *lachrymans* and nonvirulent *Pseudomonas* strains

Strains	Tests ^{a)}													
	Pathogenicity test	Gram stain	Green fluorescent pigment	Oxidation-fermentation (O-F) test	Production of levan	Cytochrome oxidase activity	Soft rot of potato slice	Arginine dihydrolase activity	Production of 2-keto gluconate	Utilization of tartrate	Utilization of malonate	Lecithinase activity	Reduction of nitrate	Agglutination reaction against antiserum <i>P. s. pv. lachrymans</i> 7568
<i>P. s. pv.</i>														
<i>lachrymans</i>														
75101 (SM ^r)	+	-	+	O	+	-	-	-	-	+	+	-	-	+
7563 (RFP ^r)	+	-	+	O	+	-	-	-	-	+	+	-	-	+
Nonvirulent														
<i>Pseudomonas</i>														
N-9	-	-	+	O	-	-	-	+	-	+	+	-	-	-
-10	-	-	+	O	-	-	-	+	-	+	+	-	-	-
-18	-	-	+	O	-	+	-	+	-	-	+	-	+	-
-27	-	-	+	O	+	+	-	+	-	-	+	+	-	-
-28	-	-	+	O	-	+	-	+	-	-	+	+	-	-
-29	-	-	+	O	-	+	-	+	-	-	+	-	-	-
-30	-	-	+	O	-	+	-	+	-	-	+	+	-	-
-31	-	-	+	O	+	+	-	+	-	+	+	+	-	-
-32	-	-	+	O	-	+	-	+	-	-	+	+	-	-
-47	-	-	+	O	-	+	-	-	-	+	+	-	-	-
-48	-	-	+	O	-	+	-	-	-	+	+	-	-	-

a) + : Positive.
 - : Negative.
 O : Oxidation.

by an agar dilution method as reported previously³⁾. Minimal inhibitory concentration (MIC) of 11 isolates out of 22 nonvirulent pseudomonads was shown in Table 2, some of which were extremely high ($>1600 \mu\text{g/ml}$). Cell free systems prepared from strain N-9, -10, -18 or -27 were able to inactivate DHSM, in the presence of disodium adenosine triphosphate as reported previously⁴⁾. These enzymically inactivated DHSM were regenerated by the treatment with alkaline phosphatase, and not by phosphodiesterase. The inactivation was, therefore, attributed to be phosphorylation reaction.

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Table 2. Drug resistance of nonvirulent *Pseudomonas* strains

Strains	Minimal inhibitory concentration ($\mu\text{g/ml}$) ^{a)}										Inactivation of SM ^{b)}
	SM	KM	CP	OTC	CBPC	NA	KSM	Hg	CL	RFP	
N-9	>1600	800	200	6.25	800	12.5	>100	>100	>100	6.25	Phosphorylation
-10	"	1.56	"	3.12	"	3.12	100	"	"	"	Phosphorylation
-18	"	3.12	"	1.56	400	12.5	50	3.12	"	12.5	Phosphorylation
-27	"	6.25	"	6.25	>1600	25	"	100	"	6.25	Phosphorylation
-28	6.25	50	"	"	"	"	"	6.25	"	12.5	—
-29	25	3.12	100	"	1600	12.5	>100	"	"	6.25	—
-30	6.25	"	200	"	>1600	25	100	"	"	12.5	—
-31	"	1.56	"	"	"	"	"	"	"	"	—
-32	"	"	"	"	"	12.5	"	"	"	"	—
-47	25	12.5	12.5	0.39	100	3.12	25	3.12	>100	1.56	—
-48	"	"	"	"	"	"	"	"	3.12	"	—

a) For the determination of MIC, agar streak method was applied on 2-fold serial dilution plates.

b) Inactivated SM were regenerated by alkaline phosphatase treatment, and not by phosphodiesterase. Abbreviation: SM, Streptomycin; KM, Kanamycin; CP, Chloramphenicol; OTC, Oxytetracycline; CBPC, Carbenicillin; NA, Nalidixic acid; KSM, Kasugamycin; Hg, HgCl₂; CL, Colistin; RFP, Rifampicin.

***In vitro* transfer of DHSM resistance** Mating was done by mixing culture method as reported previously¹⁾. After mixing cultures of DHSM resistant *P. s. pv. lachrymans* 75101 as a donor, and nonvirulent pseudomonad strains N-28, -29, -30, -31, -32, -47 or -48 as recipients, it was found that only N-28 strain could be transmitted DHSM resistance. The kinetics of transmission of isolate 75101 and N-28 was shown in Table 3. It was clarified that the transmission frequency rapidly increased with the lapse of time of mixing culture, which was 10^{-4} after 6 hr. Transconjugant N-28 (DHSM resistant) thus obtained could transmit its resistance further when *P. s. pv. lachrymans* 7563 (RFP^r) was used as a recipient, and the transmission frequency was 10^{-5} after 6 hr. Enzymic activities of these transconjugants were examined, and production of DHSM phosphotransferase was confirmed similar to their donor isolates. On the other hand, transmission of DHSM resistance was not observed from nonvirulent pseudomonads as donors to *P. s. pv. lachrymans* 7563 (RFP^r) as a recipient.

***In planta* transfer of DHSM resistance** Inoculum was prepared by mixing 1:1 suspensions of recipient and donor bacteria cultured 24 hr on peptone broth agar (PBA), and sprayed on leaflets of cucumber (TOKIWA, No 3, Type P) at 4th leaf stage. Inoculated plants were incubated at 25 C under 100 % humidity. After 6 days, inoculated leaf was cut in pieces about 3×3 mm in size, and pieces were plated on selective PBA media containing DHSM (100 $\mu\text{g/ml}$) and Colistin (100 $\mu\text{g/ml}$). Transconjugant nonvirulent *Pseudomonas* N-28 was selected by 3 times consecutive single colony isolation on selective media, and identified according to their colony type, growth, and also to the following tests; detection of DHSM phosphotransferase, *in vitro* transmission tests using *P. s. pv. lachrymans* 7563 (RFP^r) as a recipient, and serological reactions against *P. s. pv. lachrymans* 7568 antibody. Results of these experiments indicated that this transconjugant nonvirulent *Pseudomonas* N-28 received

Table 3. Transfer of SM resistance by conjugation between *Pseudomonas syringae* pv. *lachrymans* and nonvirulent *Pseudomonas* strains

Donor	Recipient	Selection ^{a)}	Transfer frequency ^{b)}
<i>P. s.</i> pv. <i>lachrymans</i> 75101 (SM)	Nonvirulent <i>Pseudomonas</i> N-28 (CL)	SM, CL	2.1×10^{-4}
Transconjugant nonvirulent <i>Pseudomonas</i> N-28 (SM, CL)	<i>P. s.</i> pv. <i>lachrymans</i> 7563 (RFP)	SM, RFP	1.3×10^{-5}
Nonvirulent <i>Pseudomonas</i> N-9 (SM)	<i>P. s.</i> pv. <i>lachrymans</i> 7563 (RFP)	SM, RFP	$<10^{-6}$

Transmissibility was tested by mixing culture method, 0.25 ml each of overnight cultures of donor and recipient was added to 2 ml of peptone broth and cultured for 6 hr, at 27 C.

a) Transconjugants were isolated on selective plate containing 100 µg/ml of each drug.

b) Transfer frequencies were represented by transconjugant/donor ratio.

its DHSM resistance from *P. s.* pv. *lachrymans* 75101 *in planta* (on leaf surface). On the other hand, *in planta* transmission of DHSM resistance was not observed from transconjugant nonvirulent *Pseudomonas* N-28 to *P. s.* pv. *lachrymans* 7563 (RFP^r).

Several isolates of nonvirulent pseudomonads showed resistance against DHSM. These specific isolates were commonly distributed on cucumber leaves, some of which inactivated DHSM enzymically. The inactivation of these isolates was attributed to be phosphorylation reaction. At the same time, DHSM resistance of *P. s.* pv. *lachrymans* 75101 was transmitted to the recipient isolate of DHSM sensitive *Pseudomonas* N-28 *in planta* as well as *in vitro*.

Several papers have been published that show phytopathogenic or phytosymbiotic species of bacteria were able to acquire plasmids *in vitro*^{5,6,7)}. If plasmids play a role in the epidemiology of DHSM resistant bacteria in plant diseases, they must be transmissible in plant tissue. *In vitro* transfer of plasmids has been reported in the intestinal tract of gnotobiotic or antibiotic treated mice, pig and humans. These results of studies suggest that DHSM resistance may be movable from bacterium to bacterium in various natural environments.

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