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Studies on the Relationship between Virulence and Bacteriological Properties in *Erwinia carotovora* subsp. *carotovora*

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Nineteen wild-type strains of *Erwinia carotovora* subsp. *carotovora* isolated in Japan were classified into 3 groups on the basis of their virulence to slices of potato tuber. On the other hand, these strains were divided into 6 biovars on the basis of the differences in both methyl red reaction and acid production from maltose and sorbitol. No relationship was found between virulence and biovars. The activities of pectolytic, cellulolytic and proteolytic enzymes excreted from these strains were also examined by means of agar plate method. Indistinct correlation between virulence and enzyme activities in wild-type strains was observed. The mutant strains attenuated in virulence which were originated from the virulent strains by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment, also attenuated in enzyme activities, though several exceptions were observed. Among virulent mutant strains, those negative in enzyme activity were also found.

INTRODUCTION

A single species or pathovars of plant pathogenic bacteria usually involves many strains which are different in the pathogenicity, virulence and bacteriological properties. From the pathological point of view, it is important to find out some bacteriological properties related to pathogenicity or virulence. Because, these finding may provide useful informations for understanding the factors responsible for pathogenicity or virulence.

The studies on the relationship between virulence and physiological properties in *Erwinia carotovora* subsp. *carotovora* have been carried out by Garber *et al.* (1956) and Friedman (1962) with biochemical mutants. They reported that positive correlation existed between some physiological properties and virulence. The correlation between productivity of several extracellular enzymes and virulence in this bacterium has also been studied by some workers. Beraha *et al.* (1971, 1974) reported that the activities of tissue-macerating enzymes (pectin esterase, polygalacturonase, pectate lyase, cellulase and phosphatidase) were remarkably low in the avirulent strains in contrast with the high activities of these enzymes in the virulent parent and revertant strains. Besides these experiments, a close correlation was also confirmed between enzyme activity and virulence with some other plant pathogenic bacteria such as *Pseu-*

domonas solanacearum (Friedman and Ceponis, 1959; Kelman and Cowling, 1965) and *Xanthomonas campestris* pv. *oryzae* (Fujii and Uematsu, 1975).

In the present study, variability in virulence and bacteriological properties of *E. carotovora* subsp. *carotovora* collected from different regions in Japan were made clear. Besides, the relationships between virulence and activities of tissue-macerating enzymes such as pectolytic, cellulolytic and proteolytic enzymes were evaluated with wild-type and mutant strains induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment.

MATERIALS AND METHODS

Bacterial strains used

Nineteen wild-type strains of *E. carotovora* subsp. *carotovora* used in this experiment were selected from stock cultures in the Laboratory of Plant Pathology, Kyushu University. Fifteen of these strains had been supplied from National Institute of Agricultural Sciences, Ibaraki, Japan.

Besides wild-type strains, 11 mutant strains derived from a parent strain N7129 ST were also used. The parent strain N7129 ST was a spontaneous mutant derived from a wild-type strain, N7129, by plating a suspension of its exponential phase cells (ca. 10^9 CFU/ml) directly onto potato semisynthetic agar (PSA) medium (Wakimoto, 1955) containing streptomycin (100 μg/ml). All mutants used in this experiment were those induced by treating cells of the parent strain N 7129 ST with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). For inducing mutants with altered virulence, the following procedures were carried out. Cells of the parent strains were cultured under shaking condition in nutrient broth (NB) medium (10 g peptone, 5 g yeast extract, 10 g NaCl in 1 liter of distilled water, pH 7.0) at 30°C for 24 hr. The cells were washed twice in sterile Tris-HCl buffer (pH 7.0) by centrifugation. The final suspension containing approximately 1×10^7 viable cells/ml was treated with 50 μg NTG/ml in 0.1 M Tris-HCl buffer, pH 7.0. After incubation at 30°C in a water-bath for 30 min, cells were washed twice in the same buffer to remove NTG. A portion of the suspension was fransferred to fresh broth and incubated overnight for multiplication of mutants. Another portion of the suspension was serially diluted, spread on the plates of nutrient agar (NA) medium, subsequently incubated at 30°C to check the rate of survival. Approximately 50 % of the treated cells survived. The colonies were then transferred by replica methodon NA medium containing streptomycin at a concentration of 100 μg/ml and on the minimal medium described by Chatterjee and Starr (1972) to check up streptomycin resistance and prototrophy. Colonies grown on both media were inoculated onto potato slices to test their virulence.

List of the strains used in this experiment is shown in Table 1. All cultures were maintained by means of lyophilization. Unless otherwise stated, PSA medium was used for multiplication of bacterial strains.

Test of virulence

Virulence of bacterial strains was tested on fresh slices of potato tuber

(var. May Queen, 1 cm in thickness). The potato slice was placed on the disc of filter paper impregnated previously with 10 ml of sterile distilled water in a 9 cm Petri dish. One loopful amount of bacteria cultured on PSA slant at 30°C for 48 hr was placed on the slices. Virulence of the bacteria was estimated on the basis of the soft-rotted area developed on the surface of slices. The virulence was also tested by inoculating bacteria against the slices of radish, carrot and detached leaves of Chinese cabbage.

Bacteriological properties

Unless specifically cited, the methods described by Tominaga (1971) were followed. All wild-type strains were precultured at 30°C for 24 hr on PSA slants, and were subjected to bacteriological tests. The bacteria inoculated on each test medium were incubated at 25°C.

Production of pigment: King's A and B media were adopted. Pigmentation was observed up to 5 days after inoculation.

Gelatin liquefaction: The bacteria were inoculated on Beef extract (Difcoj bouillon stabs containing 20 % gelatin. The results were estimated after 20 days of inoculation.

Litmus milk reaction: The inoculated litmus milk medium was observed for acid production and coagulation or digestion every week for one month.

Reduction of nitrate: Peptone broth supplemented by 0.1% KNO_3 was tested with naphthylamine and sulfanilic acid reagents for the presence of nitrite 3 days after inoculation. Unreduced nitrate was detected with zinc pieces.

Nitrate respiration: The method of Komagata *et al.* (1965) was applied. The nutrient broth containing 1 % KNO, was inoculated with bacteria and immediately sealed with sterile liquid paraffin. After 2 days incubation, bacterial growth and bubbling of gas were observed.

Production of indole, ammonia and H_2S: The bacterial culture in peptone broth was tested for indole by Kovac's reagent. Ammonium production in bouillon broth was tested with Nessler's reagent after 7 days incubation. H_2S production in bouillon broth (pH 6.5) was detected by lead acetate paper-strips up to 3 weeks after inoculation.

Mode of glucose utilization (O-F test) : The test was conducted with Hugh and Leifson's medium. The bacteria were inoculated to two tubes of the medium, one of which was overlayed with liquid paraffin. After 5 days of incubation, fermentative bacteria changed color of both tubes to yellow. Motility of the bacterium was also tested at the same time.

Acid production from carbohydrates: Peptone broth was used as basal medium. Each sugar or related compound was added at a concentration of 0.5 % together with suitable concentration of bromothymol blue as indicator. Acid production was indicated by yellowing of the medium.

Hydrolysis of aesculin and arbutin : Peptone broth containing 0.1 % aesculin and 0.05 % ferrous citrate was used for aesculin test. For testing arbutin hydrolysis, the culture medium consisting of 5 g arbutin, 10 g peptone, 3 g yeast extract, 1 g glucose, 0.5 g ferrous citrate and 1 liter of distilled water was inoculated. The color change of the medium to brown or dark brown indicated

positive hydrolysis of the substrates.

Catalase activity: One loopful amount of bacteria was transferred to a drop of 3 % H_2O_2 and bubbling was observed.

Oxidase activity: A filter paper was dropped with 1 % tetramethylparaphenylenediamine dihydrochloride and was smeared with bacterial mass. The oxidase positive bacteria changed its color to dark purple.

Citrate utilization: A large amount of bacteria was transferred to Simmons' medium containing 0.2 % sodium citrate. When the medium is changed to blue (alkalized) within 14 days after inoculation, the reaction indicates utilization of citrate as a sole carbon source, and is scored as positive.

Enzyme assays

The agar plate method (Jayasankar and Graham, 1969; Hankin et al., 1971; Hankin and Anagnostakis, 1977) was adopted for assaying polysaccharide-degrading and proteolytic enzyme activities. The basal medium described by Lee et *al.* (1975) was modified to contain the following ingredients; 2g $(NH_4)_2SO_4$, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.001 g $CaCl_2 \cdot 2\ H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, 1 g yeast extract, 20 g glucose and 15 g agar, in 1 liter of distilled water.

Pectin (from apple), pectic acid or carboxymethyl cellulose (CMC) (Wako Pure Chemical Industries, Ltd.) was added as a substrate instead of glucose to the basal medium at a concentration of 0.5 % (w/v). The pH value of the media containing pectin, pectic acid and CMC were adjusted to 7.6, 7.8 and 6.9, respectively, after autoclaving.

The casein medium for testing proteolytic enzyme activity was prepared by supplementing basal medium by 0.5 % of casein (Katayama Chemical Co. Ltd.) and 0.4 % of glucose, and pH of the medium was adjusted to 6.8 after autoclaving.

Fifteen ml of each medium was poured to a Petri dish and solidified. Bacterial strains to be tested were cultured under shaking condition in PS or NB broth for 24 hr at 30°C. For testing enzyme activity of each bacterium, a filter paper (6 mm diameter) impregnated with 0.01 ml of each bacterial suspension (conc. ca. 10^9 cells/ml) was placed on each medium. The plates thus

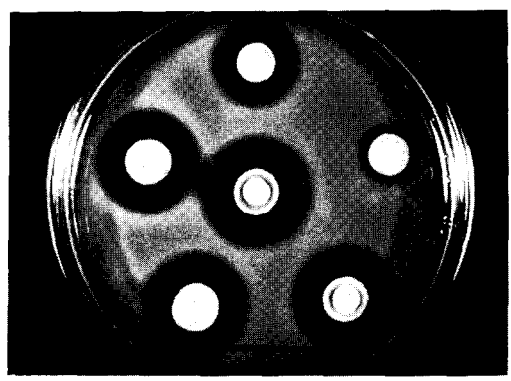

Fig. 1. Clear zones formed around the colonies as a result of digestion of substrate (pectic acid) containing in the medium.

prepared were incubated at 30°C for 24 hr. After incubation, the colony size was measured and the plate was carefully flooded with 1% (w/v) aqueous solution of cetyltrimethylammonium bromide and allowed to stand for a few minutes. Since this reagent precipitates only undegraded long-chain polysaccharides, the degraded area remained as a transparent zone. The production of pectolytic or cellulolytic enzyme (Cx) was evaluated as H/C, that is, the ratio of zone size to colony diameter. In the case of casein medium, the degraded zone was visible without using any reagent (Fig. 1).

RESULTS

Virulence of wild-type strains

All wild-type strains caused soft rot on the slices of potato tuber, carrot, radish and detached tissue of Chinese cabbage. Variations in their virulence

Table 1. List of ***Erwinia carotovora*** subsp. carotovora used.

Bacterial strain	Host	Collected in	Virulence[1]
Wild-type strain			
N7101	Chinese cabbage	Fukui, Japan	++++
N7107	Cabbage	Aichi, Japan	
N7109	Chinese cabbage	Ishikawa, Japan	++++
N7114	Cabbage	Kyoto, Japan	+++
N7116	Cabbage	Gunma, Japan	++++
N7118	Arum	Fukushima, Japan	+
N7122	Tobacco	Iwate, Japan	++++
N7126	Cabbage	Nagano, Japan	+++
N 7127	Carrot	〃 〃	+++
N7128	Celery	〃 〃	++++
N7129	Radish	〃 〃	++++
N7131	Tomato	〃 〃	++++
N7135	Tomato	Tochigi, Japan	+++
N7157	Chinese cabbage	Miyagi, Japan	++++
N11	Chinese cabbage	Kanagawa, Japan	++++
Ku 7514	Watermelon	Fukuoka, Japan	++++
Ku 7515	Chinese cabbage	〃 〃	+++
Ku 7516	Chinese cabbage	〃 〃	+
Ku 7611	Lettuce	〃 〃	++++
Parent strain			
N7129ST			++++
Mutant strain			
ST-1			+++
ST-4			+++
ST-8			+++
ST-14			+++
ST-25			+++
ST-26			+++
ST-5			+
ST-10			+
ST-27			+
ST-38			+
ST-40			+

[1] Rating was made by measuring rotted area developed on the potato tuber slices (var. May Queen) 24 hr after inoculation.

were noticed among strains, but host specificity was indistinct. As shown in Table 1, virulence of strains was graded on the basis of their ability to cause soft rot in potato slices. With highly virulent strains (##), symptom appeared about 6 hr after incubation and the slice was completely degraded at inoculation site by 24 hr with considerable watery exudate. In the case of moderately virulent (#) or weakly virulent (+) strains, a little or no enlargement of lesions localizing at inoculation site with or without watery exudate was observed. No relationship was found between virulence to patato slice and those original host plants from which the bacteria were isolated.

NTG-induced mutant strains with attenuated virulence were also graded according to their virulence on potato slices (Table 1). Avirulent strains which have completely lost their virulence were not obtained in this experiment.

Bacteriological properties of wild-type strains

All of the wild-type strains of *E. carotovora* subsp. *carotovora* showed motility in Hugh-Leifson's medium, grew in Fermi's and Uschinsky's solutions, but did not grow in Cohn's solution. The strains presented marked similarities in most of the physiological properties except for methyl red test (Table 2). Acid productivity of the strains from 16 carbohydrates was also similar in most reactions with only exception in acid productivity from maltose and sorbitol (Table 3).

Table 2. Physiological properties of wild-type strains of *E. carotovora* subsp. *carotavora.*

Test	*Erwinia* carotovora subsp. *carotovora* (Bergey's Manual 8 th)	N7101 N7116 N7122 N7127 N7107 N7109 *N7128 N7129 N7131 N7135* N7114 *N7118* N7157 N11 Ku7514 Ku7515 N7126 Ku7516 Ku7611
Gram reaction		−
Motility	+	+
Production of pigments	−	
Gelatin liquefaction	+	+
Litmus milk reaction		+
Reduction of nitrate	+	+
Nitrate respiration		+
Indole production		
H_2S production	+	+
Ammonia production	+	+
O-F test (glucose)	F	F
Hydrolysis of aesculin		+
Hydrolysis of arbutin		+
Voges-Proskauer test		+
Methyl red test		+
Catalase activity		+
Oxidase activity		−
Citrate utilization	+	+

On the basis of the differences in the properties, the bacterial strains were divided into 6 biovars (Table 4). Most of the strains with high virulence tended to belong to biovars A, B and C, while the remaining strains with moderate or weak virulence were miscellaneous (Tables 1, 4).

Table 3. Acid production from carbohydrates by wild-type strains of *E.* carotovora subsp. *carotovora*.

Compound	*Erwinia carotovora* subsp. *carotovora* (Bergey's Manual 8 th)	N7101 N7107 N7116 N7126 N7128 N7131 Ku 7515	N7109 N7114 N7122 N7127 N7135 N7157 N11 Ku7516	N7118 N7129 Ku 7514 Ku 7611
Glucose	+	+[1]	+	+
Galactose		+	+	+
Fructose		+	+	+
Mannose	+	+	+	+
Xylose	+	+	+	+
Arabinose	+	+	+	+
Sucrose		+	+	+
Maltose	d	+	−	−
Lactose	+	+	+	+
Raffinose	+	+	+	+
Starch		−	−	−
Inulin		−	−	−
Glycogen		−	−	−
Mannitol	+	+	+	+
Sorbitol	+	+	+	−
Inositol	d	+	+	+

[1] + : Acid produced; −: no acid produced.

Table 4. Biovars of *E. carotovora* subsp. *carotovora* different in bacteriological properties.

Methyl red reaction	Acid production from Maltose	Acid production from Sorbitol	Strains	Biovar
+	+	+	N7101 N7116 N7128 N7131 Ku7515	A
+	−	+	N7122 N7127 N7135 N7157 N11 Ku7516	B
+	−	−	N7129 Ku 7514 Ku 7611	C
−	+	+	N7107 N7126	D
−	−	+	N7109 N7114	E
−	−	−	N7118	F

Enzyme assays

The activities of pectolytic, cellulolytic (Cx) and proteolytic enzymes of the wild-types and mutant strains were evaluated and analyzed statistically for 6 replications in each experiment.

As shown in Table 5, some differences were found in the degree of pectolytic, cellulolytic and proteolytic activities among wild-type strains. The ranges of variation were greater in the groups of moderately or weakly virulent strains than those of virulent strains. The activities of these enzymes of induced mutant strains were shown in Table 6. The mutant strains showed lower values of H/C ratio than the parental strain, and the ratios of mutant strains varied with a range wider than those of wild-type strains. Although inverse relationship was observed between virulence and enzyme activity in some mutants, the values were not significantly different. Two mutant strains, ST-10 and ST-26, were characterized by production of no lytic enzymes on any media tested, even though they retained virulence.

Table 5. Comparison of pectolytic, cellulolytic and proteolytic enzyme activities of wild-type strains of ***E. carotovora*** subsp. ***carotovora.***

Strain	Virulence	Enzyme activity			
		Pectin (pH 7.6)	Pectic acid (pH 7.8)	Carboxymethyl cellulose (pH 6.9)	Casein (pH 6.8)
N7101	+++	1.621' cd	1.85 ab[2)]	1.73 a	1.20 bcde
N7109	+++	1.77 abcd	2.03 a	1.97 a	1.38 abc
N7116	+++	1.70 bcd	1.90 a	1.88 a	1.48 a
N7122	+++	1.68 bcd	1.97 a	1.80 a	1.35 abc
N7128	+++	1.70 bcd	1.91 a	1.96 a	1.43 ab
N7129	+++	1.73 abcd	1.97 a	1.97 a	1.52 a
N7131	+++	1.72 abcd	2.03 a	1.84 a	1.27 bcd
N7157	+++	1.66 bcd	1.93 a	1.78 a	1.35 abc
N11	+++	1.81 abc	2.13 a	1.90 a	1.24 bcd
Ku 7514	+++	1.74 abcd	1.97 a	1.71 a	1.26 bcd
Ku 7611	+++	1.72 abcd	1.95 a	1.76 a	1.35 abc
N7114	++	1.78 abcd	2.10 a	1.96 a	1.36 abc
N7126	++	1.58 d	1.58 b	1.27 b	1.24 bcd
N7127	++	1.67 bcd	1.86 ab	1.82 a	1.10 de
N7135	++	1.86 ab	2.03 a	2.00 a	1.31 abc
Ku 7515	++	1.75 abcd	1.98 a	1.96 a	1.04 e
N7107	+	1.22 e	1.13 c	1.06 b	1.03 e
N7118	+	1.91 a	2.17 a	2.00 a	1.49 a
Ku 7516	+	1.83 ab	2.01 a	1.77 a	1.18 cde

[1)] Diameter of hydrolysis zone/colony diameter (H/C ratio) at 24 hr after incubation.
[2)] In each row, means followed by a common letter are not significantly different at the 5% level by the Duncan's multiple range test.

Table 6. Comparison of pectolytic, cellulolytic and proteolytic enzyme activities of induced mutant strains of ***E. carotovora*** subsp. ***carotovora.***

Strain	Virulence	Enzyme activity			
		Pectin (pH 7.6)	Pectic acid (pH 7.8)	Carboxymethyl cellulose (pH 6.9)	Casein (pH 7.0)
Virulent strain					
N7129ST	+++	1.61[1)] a	1.87 a	1.77 a	1.37 a
Attenuated mutant strain					
ST-1	++	1.42 ab	1.50 abc	1.44 abc	1.08 bc
ST-4	++	1.43 ab	1.65 ab	1.78 a	1.06 bc
ST-8	++	1.43 ab	1.56 ab	1.40 bc	1.14 b
ST-14	++	1.30 bc	1.16 cde	1.00 d	1.09 bc
ST-25	++	1.42 ab	1.41 bcd	1.50 ab	1.00 c
ST-26	++	1.00 d	1.00 e	1.00 d	1.00 c
ST-5	+	1.43 ab	1.66 ab	1.36 bcd	1.08 bc
ST-10	+	1.00 d	1.00 e	1.00 d	1.00 c
ST-27	+	1.43 ab	1.45 bc	1.25 bcd	1.00 c
ST-38	+	1.11 cd	1.06 de	1.46 abc	1.00 c
ST-40	+	1.20 bcd	1.28 bcde	1.10 cd	1.00 c

[1)] See legend in Table 5.

DISCUSSION

In the present study, the virulence of bacterial strains was assessed semi-

quantitatively by the potato slice method which was similar to the carrot slice method used for the detection of *E. carotmora* subsp. *carotovora* in the soil (Tsuyama, 1962). By this method, some variations in virulence were observed among 19 wild-type strains of *E. carotovora* subsp. *carotmora* (Table 1) as Garber *et al. (1956)* and Kobayashi (1962) have already reported. The mutant strains induced by NTG treatment also showed much variation in virulence on potato tuber slices (Table 1). No host specificity was observed among strains, that is, the strains which is highly virulent to potato also showed high virulence to carrot, radish and Chinese cabbage.

Graham (1964) reported in taxonomic studies of soft rot coliform bacteria that 37 and 26 strains out of 57 strains tested showed positive reactions in methyl red test and acid production from maltose, respectively. Although the strains were similar in most of the bacteriological properties tested (Tables 2, 3), variations were observed in methyl red reaction and acid production from maltose and sorbitol. On the basis of these differences, bacterial strains were grouped into 6 biovars, A, B, C, D, E and F (Table 4).

With respect to the relationship between virulence and biovars, the highly virulent wild-type strains generally tended to belong to biovar A, B and C, while the strains attenuated in virulence were distributed irregularly (Tables 1, 4).

The physiological bases for the virulence of the erwinias which cause soft rot diseases of plants have been explored to some extent. *E. carotovora* subsp. *carotovora* is known to produce several enzymes such as cellulase (Cx), proteinase, pectin esterase (PE), polygalacturonase (PG), polygalacturonic acid *trans*-eliminase (PATE), pectin *trans*-eliminase (PTE) and phosphatidase (Jansen and McDonell, 1945; Friedman, 1962; Starr and Moran, 1962; Moran *et al.*, 1968; Tseng and Bateman, 1968; Beraha and Garber, 1971; Beraha *et al.*, 1974; El-Goorani et *al.*, 1976; Cabezas de Herrera and Sanchez Maeso, 1980).

In connection with the relationship between activities of tissue macerating enzymes and virulence in this bacterium, the activities of pectin esterase, polygalacturonase, pectate lyase, cellulase and phosphatidase in the virulent, avirulent and virulent revertant strains have been compared. Loss of virulence was associated with reduction of all pectic and cellulolytic enzyme activities tested and phosphatidase activity (Beraha and Garber, 1971; Beraha *et al.*, 1974). Such positive correlation between enzyme activities and virulence in this bacterium was also reported by other workers (Garber *et al.*, 1956; Friedman, 1962).

In this experiment, all wild-type strains produced more or less amount of extracellular enzymes into solid agar medium containing respective substrates. In the case of induced mutant strains, almost all of the strains attenuated in virulence showed enzyme activities lower than those of parent strain. These activities varied with strains and a few strains such as ST-10 and ST-26 were lacking in all enzyme activities in spite of the fact that these strains retained virulence (Table 6). Although the correlation between virulence and activities of pectolytic enzymes, cellulase or protease was indistinct in the wild-type strains of *E. carotovora* subsp. *carotmora,* NTG-induced mutants attenuated

in virulence were associated with reduction in all enzyme activities tested.

Explanations for the absence of all enzyme activities in mutant strains such as ST-10 and ST-26 which still retained virulence to potato were not offered, but some possible suggestion may be employed. These mutant strains might have other enzymes or isozymes necessary for pathogenesis, which could not be detected in this experiment. The isozymes of endopectatelyase were purified already from both *E. carotovora* subsp. *carotovora* (Garibaldi and Bateman, 1971) and *E. chrysanthemi* (Basham and Bateman, 1975), which were heterogeneous electrophoretically. Beraha *et al.* (1974) offered two explanations to account for the coordinate alterations in the activity of the tissue-macerating enzymes and phosphatidase of the mutant strains of *E. carotovora* subsp. *carotovora*. One explanation assumed a mutation in one or more genes controlling regulatory mechanisms for those extracellular enzymes. The other explanation suggested that mutations altered the composition or structure of the bacterial cell membrane, thereby interfering with the up-take of possible inducers from the medium or host-tissue.

It would be worthwhile to select greater number of mutants of this bacterium with no detectable or greatly reduced activities for extracellular enzymes. A better understanding of the physiological bases of virulence might come from comparative physiological and biochemical studies on these mutants and their virulent parent.

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