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Production of exopolysaccharide and levels of protease and pectinase activity in pathogenic and non-pathogenic strains of *Xanthomonas campestris* pv. *campestris*

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Abstract. *Xanthomonas campestris* pv. *campestris* is the phytopathogenic bacterium causing black rot in crucifers. A wild-type strain of *X. campestris*, XC11, was found to have lost its pathogenicity spontaneously after frequent subculturing for years. This non-pathogenic derivative, designated XC11A, was compared with its wild-type strain XC11 and a newly isolated pathogenic XC17 from an infected cabbage leaf for some phenotypic characteristics which have previously been implicated as factors contributing to pathogenicity. Colony morphology, growth characteristics and phage susceptibility were all the same for XC17, XC11 and XC11A. The exopolysaccharide (EPS)-synthesis capability of the non-pathogenic XC11A was about 3- to 4-fold lower than those of the pathogenic strains, whereas the levels of pectinase and protease(s) were the same in all three strains. Therefore, our data confirm the previous report showing that correlation exists between EPS production and pathogenesis, but do not favor the recent ones that implicating the extracellular protease and pectinase as the factors contributing to pathogenicity.

Key words: α-Amylase; Exopolysaccharide; Pathogenicity; Pectinase; Protease.

Introduction

Xanthomonas campestris pv. *campestris* causes black rot in crucifers worldwild resulting in tremendous loss in agriculture (Williams, 1980).

Some products including exopolysaccharide (EPS) (Corey and Starr, 1957; Jeans *et al.*, 1961; Sutton and Williams, 1970b), pectolytic, proteolytic and cellulolytic enzymes (Knosel and Garber, 1967; Reddy *et al.*, 1971; Starr and Nasuno, 1967.) and toxic metabolites (Noda *et al.*, 1980; Perreaux *et al.*, 1982) produced by *Xanthomonas* have been attributed to be factors contributing to pathogenicity. Daniel *et al.* (1984a; b) and Turner *et al.* (1985) have recently demonstrated that, in *X. campestris*, simultaneous loss of exocellular protease and pectinase was accompanied with the loss of pathogenicity in mutant strains; and, restoration of these enzyme activities by complementation with cloned genes resulted in concomitant regainment of pathogenicity.

X. campestris strain 11 (called XC11 for short), initially as a wild-type pathogenic strain, has been used in our past studies (Pan, 1985; Tsai, 1987; Tseng *et al.*, 1984; Tseng and Lu, 1986; Tseng and Peng, 1985; Yang, 1985; S.T. Hsu, personal communication). No changes had been noticed in those years in terms of colony morpho-

logy, pigmentation, EPS formation, starch hydrolysis and sensitivity to phages. However, in a recent study of EPS metabolism, we surprisingly found that XC11 had lost pathogenicity when we were trying to see the effects of the mutation leading to non-mucoidy by comparing the non-mucoid mutants with their parental strain XC11. This observation prompted us to examine some phenotypic alterations accompanied with the loss of pathogenicity in this non-pathogenic derivative. Our results on production of exopolysaccharide and some exocellular enzymes by this non-pathogenic strain, compared with those produced by its wild-type XC11 and other pathogenic strain, seem not to coincide with the data reported by Daniels *et al.* (1984b), but rather confirm the previously reproted data showing the correlation of exopolysaccharide production with pathogenicity (Sutton and Williams, 1970b).

Materials and Methods

Bacterial Strains, Phage and Conditions of Cultivation.

X. campestris pv. *campestris* 11 (XC11) was a wild-type strain received from Dr. S. T. Hsu, and has been routinely used in our laboratory for several years. Stock culture was kept on L agar slant (Miller, 1972) at 4°C. A strain designated XC11A was a non-pathogenic mutant derived spontaneously from XC11. Strain XC17 was newly isolated in our laboratory from infected cabbage leaf by using SX agar (Schaad and White, 1974), and verified by some tests including colony morphology, electron microscopy, starch-, pectin- and casein- hydrolyzing activities, phage sensitivity and pathogenicty.

Virulent phage ØL7, which infects *X. campestris* specifically, was isolated from a local garden soil and has been described previously (Tseng *et al.*, 1984).

Bacterial cultures were incubated at 28°C. Vigorous shaking was needed for liquid cultures. L broth and L agar were routinely used for cultivation of the bacterium. For some measurements, the chemically defined medium, XOL (Tseng *et al.*, 1983), was used to grow the cells. Unless otherwise described, carbon sources were added to a final concentration of 20 mM of six carbon equivalent. For example, when glycerol or sucrose was needed, 40 mM and 10 mM, respectively, were added. Four-carbon compound was calculated as a three-carbon compound. 0.125% tryptone was supplemented as an organic nitrogen source to the XOL medium when needed.

Electron Microscopy

For electron microscopic observation, the bacterial cells grown overnight were stained with uranyl acetate (2%) and examined under Jeol JEM-200 CX.

Pathogenicity Test

Overnight cultures grown in L broth were used as inocula for pathogenicity test. A pair of sterilized scissors were dipped into the culture (ca. 5×10^8 cells/ml) and then used to make a cut of about 1 cm from the leaf edge toward midrib of 2-week old potted cabbage seedlings.

Assay of Exocellular Enzymes

Crude exocellular enzymes were prepared by removing the cells after centrifugation of the cultures at 10,000 ×g for 10 min at 4°C. Activity of α-amylase was assayed as previously described (Tseng and Peng, 1985) by following the release of reducing sugars, using maltose as a standard. Protease activity was measured by determination of acid-soluble material hydrolyzed from casein. The assay mixture of 2.0 ml contained 25 mM Tris-HCl buffer (pH 8.3), 0.25% casein and crude culture fluid of an overnight culture (16 h) as the enzyme source. The mixture was incubated at 37°C and terminated after 10 min by adding cold trichloroacetic acid to 5% final concentration and keeping in ice for 30 min. The acid-soluble fraction was recovered by centrifugation at 10,000×g, for

15 min at 4°C. Then 3.0 ml of 0.5 N NaOH was added to obtain an alkaline pH. The product was then measured by Lowry method (Lowry *et al.*, 1951) using tyrosine as a standard. One unit of enzyme was defined as the amount that released one μmole tyrosine per min.

Pectinase activity was measured according to the method described by Keen *et al.* (1984). The reaction mixture of 3 ml contained 1 ml of 0.75% sodium polypectate, 1 ml of 3 mM $CaCl_2$, 0.5 ml of 0.2 M Tris-HCl, pH 8.0 and 0.5 ml of culture fluid. The reaction was carried out at 30°C for 5 min. The unsaturated uronide released was monitored by following the increase of OD at 235 nm. A conversion factor of 1.73 OD at 235 nm unit per μmole was employed to calculate the amount of uronide. One unit of enzyme was defined as the amount that released one μmole uronide per min.

Other Measurements

Cell growth was followed by reading OD at 550 nm. Amont of exopolysaccharide was prepared and measured as described elsewhere (Lin and Tseng, 1979). Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Phage susceptibility was tested by dropping 5 μl (2×10^{12} pfu/ml) of phage ØL7 (Tseng *et al.*, 1984) on a double layered LA plate wite bacterial cells in the top agar. Cleared zones appeared if the host cells were sensitive to the phage.

Results

Characterization of ***X. campestris*** pv. ***campestris***

A strain produced yellow-pigmented, mucoid colonies with strong starch-hydrolyzing activity on the selective medium SX agar (Schaad and White, 1974) were freshly isolated from infected cabbage leaf. The strain was designated *X. campestris* 17, or XC17 for short, and characterized in this study. Firstly, characterization of this new strain was carried out using wild-type strain XC11 as a control. The colony morphology of both XC7 and XC11 were the same—yellow, glossy and mucoid on an agar plate. When the cells were examined under a transmission electron microscope, typical short rod cells with polar monotrichous flagellum were observed (Fig. 1).

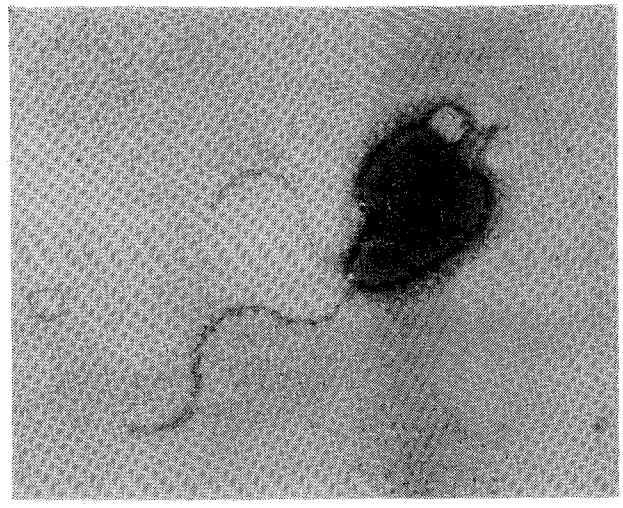

Fig. 1. Electron micrograph of *X. campestris* pv. *campestris* XC17 (magnified 14,400×).

The cells of XC17 grew well in either L broth or XOL medium (Tseng *et al.*, 1983) supplemented with an appropriate sugar. A generation time of 2.5 h, which was very close to that of XC11, was calculated when the cells were grown in XOL containing 20 mM glucose and 0.125% trypton. Using the XOL agar containing a sugar to test the bacterial cells' ability to grow, it was found that the patterns were the same for both XC17 and XC11. In summary, the growth was the the best on sucrose, glucose and galactose, good on starch, maltose, xylose and fructose, and poor on lactose and glycerol, while no growth was found on succinic acid.

Finally, phage susceptibility test showed that XC17 was sensitive to the infection of ØL7, a specific phage for *X. campestris*, which was previously isolated using XC11 as the indicator host (Tseng *et al.*, 1984).

Pathogenicity

When XC11, XC11A and XC17 were inoculated

onto the young leaves of cabbage, obvious difference in the symptoms caused by the pathogenic and non-pathogenic strains was observed within two weeks. XC11 and XC17 caused a typical chlorosis, resulting in a yellowing and then browning of the area around the cut, while XC11A caused no symptom at all. Neither did the various mutant derivatives of XC11A, including three non-mucoid mutants, one rough mutant, and one pigmentation mutant (white). The symptoms shown on the lower righ-hand side of Fig. 2 are the typical ones caused by pathogenic strains.

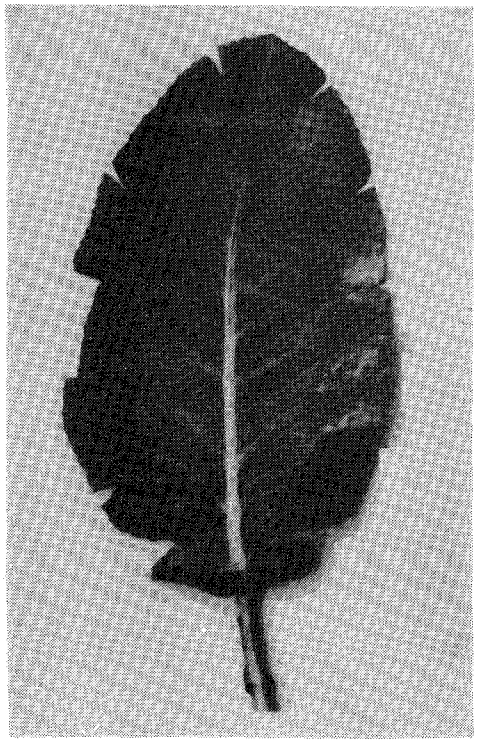

Fig. 2. Typical symptoms caused by strain XC11 or XC17 of *X. campestris* pv. *campestris* in a cabbage leaf.

Exopolysaccharide Formation

Formation of exopolysaccharide (EPS) by *X. campestris* is prominent. On an agar plate, it turns the colony watery, and in a liquid medium renders the culture fluid viscous. XC11, XC11A and XC17 appeared similar in having the watery colony morphology and the extremely viscous culture fluid. Accumulation of the EPS in a liquid culture was found to reach its maximum after about 24 h—a few hours after onset of the stationary phase. Essentially no difference was observed among XC11, XC11A and XC17 upon the time course of EPS accumulation. However, significant differences were found in the amounts of EPS produced by the three strains. As shown in Table 1, XC17 and XC11 produced about the same amount of EPS which was almost three times that produced by XC11A. When the EPS-producing capability was expressed as μg EPS per μg protein, the values were 13.8, 9.5 and 3.4 for XC17, XC11 and XC11A, respectively; those for the pathogenic XC11 and XC17 were 2.8- and 4.1-fold, respectively, higher than that for the non-pathogenic XC11A.

Table 1. *Exopolysaccharide formation by X. campestris* pv. *campestris*

Exopolysaccharide was prepared from cultures at 24 h.

Strain	μg EPS/ml	μg Protein/ml	μg EPS/μg Protein
XC11A	3274.3	978.5	3.4
XC17	9308.6	676.9	13.8
XC11	9300.6	980.5	9.5

Levels of Exocellular Enzymes

It has been documented that *X. campestris* possesses starch-, casein- and pectin-hydrolyzing activities (Bradburg, 1984; Starr and Nasuno, 1966). We have previously purified and characterized the α-amylase of XC11 as an exocellular enzyme (Tseng and Peng, 1985), while characterization of the protease and pectinase from this strain has not been reported. In this study, we were able to assay the exocellular activities of protease and pectinase in the culture fluid of *X. campestris*, in addition to the previously described α-amylase. The results are summarized in Table 2. The activity of protease and pectinase in the three strains were at the same level, while the α-amylase

Table 2. *Levels of α-amylase, protease and pectinase in X.* ***campestris*** pv. ***campestris***

Cells were grown in XOL medium plus 0.125% peptone supplemented with 0.1% starch, 0.1% casein or 0.5% sodium polypectate, respectively, for induction of α-amylase, protease and pectinase. For measurements of pectinase and protease activities, the cultures were harvested at 16 hr to obtain the crude enzyme (exocellular fraction), wherease the crude enzyme for α-amylase assay was from 48 hr cultures. Enzyme assays were carried out as described in Materials and Methods. Enzyme levels for α-amylase, protease and pectinase are expressed as μmoles maltose, tyrosine, and uronide, respectively, per mg cellular protein per min.

Strain	α-amylase	Protease	Pectinase
XC11A	4.4	0.12	1.31
XC17	16.9	0.14	1.70
XC11	6.4	0.11	1.89

levels in XC11 and XC17 were approximately 1.5- and 4-fold, respectively, higher than that in XC11A.

Discussion

The newly isolated strain designated XC17 was identified as a strain of *X. campestris* based on colony morphology, pigmentation, flagellation, starch hydrolysis, phage sensitivity and pathogenicity. The reason for us to obtain a new strain directly from the natural environment was that in a recent experiment, we surprisingly found that the XC11 we used for the past studies had lost pathogenicity when we tried to compare the virulence of this strain with its derivative mutants of cell surface alteration. So, a pathogenic strain was thus needed in hand for our research on this bacterium as a phytopathogen.

Daniels *et al.* (1984a) have recently obtained non-pathogenic mutants of *X. campestris* by using transposon (Tn5) mutagenesis. The mutants were found to possess much recuced levels of protease and pectinase activities. By cloning the wild-type genes and complementation tests they have identified at least two genes in a cluster that specify pathogenicity; and introduction of the cloned genes to the mutants caused a concomitant restoration of the pathogenicity (Daniels, 1984b; Turner, 1985). These findings interested us to see if the loss of pathogenicity in XC11 was resulted from similar alterations in exocellular enzyme levels. The data of enzyme assay showed that the protease and pectinase activities in the non-pathogenic and pathogenic strains are quite close (Table 2). These results, therefore, do not agree with the above mentioned observations by Daniels *et al.* (1984b) showing the concomitant loss of the pathogenicity with the level reduction of these two enzymes.

Information concerning the role of amylase activity as one of the possible factors causing pathogenicity is not available. In this study, we measured the α-amylase activity merely because of the ease to assay, and found that XC11A has an enzyme activity which was 1.5- and 4.0-fold lower than XC11 and XC17, respectively. Whether a 1.5- to 4-fold reduction in this enzyme contributes an important part to the loss of pathogenicity needs further investigation. We have cloned the α-amylase gene from XC11A in *Escherichia coli*, which can be transferred conjugally back to *X. campestris*. When mobilized into XC11A, approximately 6-fold increase in enzyme activity was detected (Tsai, 1987). A clone which carries gene specifying protease activity has also been obtained, and elevation of enzyme activity in XC11A was also detected (unpublished data). All these overproduction clones will be used for pathogenicity test. More light may be shed upon the roles these exocellular enzymes play in pathogenesis in the near future.

The production of EPS has been associated with virulence in several plant pathogenic bacteria (Ayers *et al.*, 1979; Corey and Starr, 1975; Eden-Geen and Knee, 1974; Gorin and Spencer, 1983; Husain and Kelman, 1985; Misaki *et al.*, 1962; Strobel, 1977). These polysaccharides are thought

to be involved in xylem plugging which decreases vascular flow and thus implicated as an early and important event in lesion development (Sutton and Williams, 1970a). Also, it has been suggested that the production of EPS may interefere with plant defenses which involve recognition of other bacterial surface component such as lipopolysaccharide (Sequeria and Graham, 1977). The *in vitro* EPS-producing capability of pathogenic XC17 and XC11 are 4.1 and 2.8-fold higher than that of non-pathogenic XC11A. These data confirm the correlation of virulence with EPS formation in *X. campestris* reported previously (Sutton and Willams, 1970b). We have recently cloned EPS synthesis genes from XC11. The expression of these genes are being evaluated. Preliminary data showed that in XC11A carrying the cloned genes, the EPS formation is much elevated (Yang *et al.*, 1987). Whether the elevation of EPS formation is accompanied by restoration of pathogenicity will be tested. If the results are positive, then the role *X. campestris* EPS plays in pathogenesis will be further confirmed.

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Literature Cited

Ayers, A. R., S. B. Ayers, and R. N. Goodman 1979. Extracellular polysaccharide of *Erwinia amylovora*: a correlation with virulence. App. Eviron. Microbiol. **38**: 659-666.

Bradburg, J. F. 1984. Genus II. Xanthomonas *Dowson. In* N. R. Krieg (ed.), Bergey's Manual of Systematic Bacterilogy, Vol. I. Williams and Wilkins, Baltimore, MD.

Corey, R. R., and M. P. Starr. 1957. Colony types of *Xanthomonas phaseoli*. J. Bacteriol. **74**: 137-140.

Daniels, M. J., C. E. Barber, P. C. Turner, W. G. Clearry, and M. K. Sawczyc. 1984a. Isolation of mutant of mutant of *Xanthomonas campestris* pv. *campestris* showing altered pathogenicity. J. Gen. Microbiol. **130**: 2447-2455.

Daniels, M. J., C. E. Barber, P. C. Turner, M. K. Sawczyc, R. J. W. Byrde, and A. H. Fielding. 1984b. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. **3**: 3323-3328.

Eden-Green, S. J. and M. Knee 1974. Bacterial polysaccharide and sorbitol in fireblight exudate. J. Gen. Microbiol. **81**: 509-512.

Gorin, P. A. J. and J. F. T. Spencer. 1963. Structural relationships of extracellular polysaccharides from phytopathogenic *Xanthomonas* spp. Can. J. Chem. **41**: 2357-2361.

Husain, K. and A. Kelman. 1958. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Phytopathology **48**: 155-165.

Jeans, A., J. E. Pittsley, and F. R. Senti. 1961. Polysac charide-B-1459; a new hydrocolloid polyelectrolyte produced from glucose by bacterial fermentation. J. Appl. Polymer Sci. **5**: 519-526.

Keen, N. T., D. Dahlbeck, B. Staskawicz, and W. Belser. 1984. Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. J. Bacteriol. **159**: 825-832.

Knosel, D. and E. D. Graber. 1967. Pektolytische und cellulytische enzyme bei *Xanthomonas campestris* (Pammel) Dowson. Phytopathol. Z. **59**: 194-202.

Lin, H. M. and Y. H. Tseng. 1979. Exopolysaccharide synthesis in *Xanthomonas oryzae*. Proc. Natl. Sci. Council, R. O. C. **3**: 279-284.

Lowry, O. H., N. J. Rosebrough, A. L. Farrand, and R. J. Randall. 1951. Protein measumement with the folin phenol reagent. J. Biol. Chem., **193**: 265-271.

Misaki, A., S. kirkwood, J. V. Scaletti, and F. Smith. 1962. Structure of the extracellular polysaccharide produced by *Xanthomonas oryzae*. Can. J. Chem. **40**: 2204-2213.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Noda, T., Z. Sato, H. Kobayahi, S. Iwasaki, and S. Okuda. 1980. Isolation and structure elucidation of phytotoxic substance produced by *Xanthomonas campestris* pv. *oryzae*. Ann. Phytopath. Soc. Japan, **46**: 663-666.

Pan, C-C. 1985. Transposon mutagenesis of *Xamthomonas campestris* pv, *campestris*. Master Thesis of National Chung Hsing University.

Perreaux, D. H., H. Maraite, and J. A. Meyer. 1982. Identification of 3 (methylthio) propionic acid as a blight inducing toxin produced by *Xanthomonas campestris* pv. *manihotis in vitro*. Physiol. Plant Pathol. **20**: 313-319.

Reddy, M. N., D. L. Stuteville, and E. L. Sorensen. 1971. Protease production during pathogenesis of bacterial leaf spot of alfalfa and by *Xanthomonas alfalfae in vitro*. Phytopathology **61**: 361-365.

Schaad, N. W. and W. C. White. 1974. A selective medium for soil isolation and enumeration of *Xanthomonas campestris*. Phytopathology. **64**: 876-880.

Sequeira, L. and T. L. Graham. 1977. Agglutination of avirulent strains of *Psoudomonas solanacearum* by potato lectin. Physiol. Plant Pathol. **11**: 43-54.

Starr, M. P. and S. Nasuno. 1966. Pectolytic activity of

phytopathogenic *Xanthomonas*. J. Gen. Microbiol. **46**: 425-433.

Strobel, G.A. 1977. Bacterial phytotoxins. Ann. Rev. Microbiol. **31**: 205-244.

Sutton, J.C. and P.H. Williams. 1970a. Relation of xylem plugging to black rot lesion development in cabbage. Can. J. Bot. **48**: 391-401.

Sutton, J.C. and P.H. Willams. 1970b. Comparison of extracellular polysaccharide of *Xanthomonas campestris* from culture and from infected cabbage leaves. Can. J. Bot. **48**: 645-651.

Tsai, H-F. 1987. Cloning, expression and DNA sequence of amylase gene from *Xanthomonas campestris* pv. *campestris*. Master Thesis of National Chung Hsing University.

Tseng, Y-H., and M-C. Lu. 1986. A novel filamentous phage of *Xanthomonas campestris* pv. *campestris*. Abstr. Amer. Soc. Microbiol. Ann. Meet. p. 236.

Tseng, Y-H. and K-C. Peng. 1985. Characterization of α-amylase from phytopathogeic *Xanthomonas campestris* pv. *campestris*. Proc. Natl. Sci. Counc. B. ROC 9: 103-109.

Tseng, Y-H., M-C. Su, and N-T. Hu 1984. Characterization and trasfection of *Xanthomonas campestris* pv. *campestris* phage L7. Abstr. Sixth International Congress of Virology, Sendai, Japan, p. 196.

Tseng, Y.H., S.F. Weng, H.M. Lin, and Y.F. Lai. 1983. Pleotropic effects in non-mucoid mutants of *Xanthomonas campestris* pv. *oryzae*. Proc. Natl. Sci. Counc. B. ROC **7**: 44-50.

Turner, P., C. Barber, and M. Daniels. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. **199**: 338-343.

Williams, P.H. 1980. Black rot: A continuing threat to world crucifers. Plant Dis. **64**: 736-742.

Yang, B-Y. 1985. Mutants of *Xanthomonas campestris* pv. *campestris* related to exopolysaccharide synthesis. Master Thesis of National Chung Hsing University.

Yang, B-Y., H-F. Tsai, and Y-H. Tseng. 1987. Molecular cloning of exopolysaccharide synthesis genes of *Xanthomonas campestris* pv. *campestris*. Proceedings, ROC-USA Agricultural Biotechnology Workshop. (In press)

十字花科蔬菜黑腐病菌 *Xanthomonas campestris* pv. *campestris* 有、無病原性菌株的胞外多醣產量及蛋白酶與果膠酶之活性

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Xanthomonas campestris pv. *campestris* 爲十字花科蔬菜黑腐病之病原細菌。本實驗室所收藏之野生株 XC11，經過數年一再移植之後被發現已經喪失致病性。此一無病原性之菌株因而改名爲 XC11A，而原始之保存菌種（−70°C）仍稱爲 XC11。爲了要了解喪失病原性之同時，細菌有何性狀上的改變，我們特地由罹病之甘籃葉片上分離一具致病力的菌株，並命名爲 XC17。然後，將 XC11A、XC11 與 XC17 一起進行一些分析比較，以了解何者爲致病因子。結果顯示，三者之菌落形態，生長能力和性狀及噬菌體敏感性均相同。XC11A 產生胞外多醣之能力低於 XC11 及 XC17 三到四倍，而蛋白酶與果膠酶之活性則三者間並無差別。因此，本試驗之結果與前人指出 EPS 與致病性有相關之報告相符；但與最近指蛋白酶與果膠酶爲致病因子之說不同。