Pectolytic Activity of Phytopathogenic Xanthomonads

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SUMMARY

Cultures of 10 of 19 nomenspecies of the genus *Xanthomonas* liquefied a nutrient pectate gel. None of the 19 nomenspecies produced either hydrolytic polygalacturonase or pectin *trans*-eliminase. Seventeen of the nomenspecies showed a detectable, at times weak, pectinesterase activity. Seven of the 10 pectate-liquefying nomenspecies excreted polygalacturonic acid *trans*-eliminase in culture fluids containing pectin as growth substrate. The enzymological basis, if any, is not at present known for the liquefaction of the nutrient pectate gel in the remaining three xanthomonads, which all lacked both the hydrolytic and the eliminative enzymes. Pectinesterase and polygalacturonic acid *trans*-eliminase were produced inducibly on pectin by most *X. campestris* cultures which were examined; some strains of this phytopathogen formed polygalacturonic acid *trans*-eliminase constitutively on glucose, but pectinesterase was never constitutive. The polygalacturonic acid *trans*-eliminase excreted by the tested xanthomonads degraded polygalacturonic acid in a random manner. The major end-products were unsaturated di- and tri-galacturonic acids, accompanied by lesser amounts of saturated mono-, di-, and tri-galacturonic acids.

INTRODUCTION

The symptoms caused in plants by phytopathogenic bacteria of the genus *Xanthomonas* are sufficiently unlike those induced by the pectolytic soft-rot *Erwinia*, *Bacillus* and *Pseudomonas* species to foster the impression that xanthomonads lack ability to degrade pectic substances. Nevertheless, several reports attest to the pectolytic activity of xanthomonads. For example, one of the present writers had observed two decades ago (Burkholder & Starr, 1948) that 38 of 77 xanthomonad cultures (14 of 25 *Xanthomonas* spp.) could liquefy a pectate gel, although more slowly and less vigorously than did soft-rot *erwinias*. Similar observations and crude enzymological surveys were subsequently presented by other workers (Sabet & Dowson, 1951; Smith, 1958*a, b*; Dye, 1960). By present standards, a certain confusion about the nomenclature of pectic substances and enzymes is evident in some of these writings. An assortment of methodological and interpretative procedures, ingenious or naive, were used; these make almost impossible any intelligent comparisons among the results obtained by individual workers. Moreover, following the discovery by Albersheim, Neukom & Deuel (1960) of fungal pectin *trans*-eliminase (PTE), there have been elucidated eliminative splits of pectic substances in soft-rot phytopathogenic bacteria such as *Erwinia carotovora* (Starr & Moran, 1962) and *Bacillus polymyxa* (Nagel & Vaughn, 1961*b*), in addition to the classical hydrolytic action (Kraght & Starr, 1953) which has recently been re-examined with purified enzyme in *Erwinia carotovora* (Nasuno &

Starr, 1966*a*). Except for our preliminary abstract (Starr & Nasuno, 1963), there has been no published work on the eliminative split of pectic substances by xanthomonads; indeed, not much is known about the enzymology of pectic substances by *Xanthomonas*, by comparison with the knowledge now available for other groups of microbes. A comprehensive study was undertaken with the hope of unravelling the kinds of pectolytic activity in various xanthomonads, and their distribution among the various so-called species (actually, nomenspecies; see Stolp, Starr & Baigent, 1965). The present report surveys the pectolytic capacities of a representative assortment of xanthomonads; the enzymological details with respect to a typical strain will be published separately (Nasuno & Starr, 1967).

METHODS

Chemicals. Pectin N.F. (68 % esterified) and polygalacturonic acid (no. 491) were obtained from Sunkist Growers, Inc. (Corona, California); they were used without additional treatment. 0-(4-Deoxy- β -L-threo-hexopyranos-4-enyluronic acid)-(1 $\rightarrow$ 4)-D-galacturonic acid (unsaturated digalacturonic acid) was kindly supplied by Dr C. W. Nagel (Department of Horticulture, Washington State University, Pullman, Washington). Di-, tri-, and tetra-galacturonic acids were the generous gifts of Dr H. J. Phaff (Department of Food Science and Technology, University of California, Davis, California). Pure D-galacturonic acid was prepared from the commercial product by recrystallization from acetone.

Cultural methods. Stock cultures of 27 species or strains of the genus *Xanthomonas* were obtained from the International Collection of Phytopathogenic Bacteria (ICPB), maintained in this Department; they were grown on slopes of yeast extract, glucose and CaCO_3 (YDC) agar during the course of this programme.

A nutrient sodium pectate gel (Starr, 1947) was used for liquefaction tests. This medium consisted of Exchange Brand sodium ammonium pectate (Sunkist Growers, Inc., Corona, California), 3 g.; 10 % (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.6 ml., N-NaOH, 0.9 ml.; yeast extract (Difco), 1 g.; distilled water, 100 ml.; pH 6.4 after autoclaving.

The basal medium consisted of two parts: (i) pectin N.F., 5 g./200 ml.; (ii) KH_2PO_4 , 1.6 g.; Na_2HPO_4 , 1.6 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g.; yeast extract (Difco Laboratories, Inc., Detroit, Michigan), 5 g.; total volume, 800 ml. Both solutions were adjusted to pH 6.8 autoclaved separately, and mixed before use. For the calcium-deficient medium, the CaCl_2 was omitted.

Bacterial growth in liquid culture was determined as the dry weight of bacteria/ml. culture.

Enzyme preparations. The bacteria were grown at 28° in a basal medium, or in a calcium-deficient medium, containing pectin, on a rotary shaker (3 cm. amplitude and 100 rotations/min.) for 48 hr. When pectin was replaced with glucose, the bacteria were grown for 24 hr, at which time maximum growth had been attained. The culture fluid was centrifuged at 6000 g for 10 min., filtered through a 0.45 μ Millipore filter (Millipore Filter Corp., Bedford, Massachusetts), dialysed against distilled water for 24 hr at 4°, and tested for the presence of pectic enzymes.

Assays of pectic enzymes. Heated enzyme was used as a control in all enzyme assays. Pectinesterase (PE) activity was determined by a titration method. The reaction mixture contained 1 % (w/v) pectin N.F., 0.1 M-NaCl., and 10 % (v/v) enzyme solution in a total volume of 10 ml. The reaction mixtures were adjusted to pH 7.0 after

addition of enzyme, incubated at 30° for 1 hr, and titrated to pH 7.0 with 0.01 N-NaOH at the end of the incubation period. PE activity was expressed in terms of μ moles ester hydrolysed/hr./ml. reaction mixture, under the above conditions.

Polygalacturonic acid *trans*-eliminase (PATE) activity was determined, by spectrophotometry. Reaction mixtures contained 10% (v/v) enzyme solution, in 0.05 M-glycine-NaOH buffer (pH 9.5), in a total volume of 6 ml. Samples (0.5 ml.) were removed immediately after enzyme was added and again after 1 hr incubation at 30°, placed in 4.5 ml. or 9.5 ml. of 0.01 N-HCl to stop enzyme action, and the extinction measured in the Beckman model DU spectrophotometer. The relative activity of PATE is expressed as the increase in extinction at 235 m μ /hr, under the above conditions.

The same principle used in the PATE assay was used for the attempted detection of pectin *trans*-eliminase (PTE) activity. Reaction mixtures containing 10% (v/v) enzyme solution, 0.5% (w/v) pectin N.F., and 0.4 M-NaCl in 0.05 M-sodium acetate buffer (pH 5.2) were incubated at 30° for 1–24 hr, and the extinction measured at 235 m μ (Edstrom & Phaff, 1964 *a*).

The iodometric method of Yemm (1935) was used to detect polygalacturonase (PG) activity by following the release of reducing groups during hydrolysis of the substrate. Replicate flasks were set up containing 10% (v/v) enzyme solution, 0.5% (w/v) polygalacturonic acid, and 0.1 M-NaCl in 0.05 M-sodium acetate buffer (pH 5.2) in a total volume of 5 ml. After suitable periods of reaction (1 hr and 24 hr), the reaction was stopped by adding 0.35 ml. of M-Na₂CO₃ and then 2 ml. of the iodine solution were added; the initial values were obtained by adding the Na₂CO₃ before the enzyme. After 20 min., the reaction mixture was acidified with 0.8 ml. of 2N-H₂SO₄ and the liberated iodine was titrated with 0.01 N-Na₂S₂O₃ with starch as indicator.

Paper chromatography. After 24 hr of incubation, samples (1 ml.) were taken from each reaction mixture for paper chromatography. The tubes containing these samples were immersed in boiling water for 5 min., and the samples dried at 70° over CaCl₂ under vacuum after removing cations with Dowex 50 cation exchange resin in the hydrogen form. The residues were dissolved in 0.1 ml. distilled water, and 10 μ l. of each sample was chromatographed on Whatman no. 4 paper. The solvent was a mixture of pyridine+ethyl acetate+acetic acid+water (5+5+1+3 by vol.) applied for 18 hr at room temperature. The marker spots were mono-, di-, tri-, and tetra-galacturonic acids, and unsaturated di-galacturonic acid. The silver nitrate reagent (Block, Durrum & Zweig, 1955) was used to detect saturated and unsaturated oligo-galacturonides and galacturonic acid. Under these conditions, 0.02 μ mole galacturonic acid gives a definite black spot at room temperature. Unsaturated compounds were also located by spraying with a solution containing 0.01% (w/v) quinine sulphate and 0.04 N-sulphuric acid in 95% (v/v) ethanol in water (Edstrom & Phaff, 1964 *b*). After drying at room temperature, the presence of the unsaturated compounds was detected under ultraviolet radiation as a dark spot on a light violet to white fluorescent field. Amounts of less than 0.2 μ mole of unsaturated digalacturonic acid were readily located by this method.

RESULTS AND DISCUSSION

Pectic enzymes produced by Xanthomonas species

Tables 1 and 2 summarize the results of the pectate gel liquefaction tests and the assays for the various pectic enzymes in xanthomonad culture supernatant fluids. Even though every culture examined showed a good growth on the pectate gel medium (probably at the expense of the yeast extract in the medium), cultures of only 10 of 19 species were able to bring about prompt liquefaction of the gel; viz. *Xanthomonas badrii*, *X. campestris*, *X. carotae*, *X. geranii*, *X. lespedezae*, *X. manihotis*, *X. nakatae-olitorii*, *X. papavericola*, *X. pelargonii*, *X. ricinicola*. These results are generally in accord with those presented earlier by Burkholder & Starr (1948) and Dye (1960).

Table 1. *Distribution of pectolytic enzymes in Xanthomonas species*

The strains are designated by their accession numbers in the International Collection of Phytopathogenic Bacteria (ICPB); many of the cultures are deposited also in the National Collection of Plant Pathogenic Bacteria, Harpenden. Liquefaction (a) of nutrient pectate gel (Starr, 1947) was scored after 24 hr at 28°. Growth (b) is expressed as mg. dried cells per ml. culture fluid. Pectinesterase (PE) activity (c) is reported in terms of the increase in μ moles of carboxyl groups per ml. reaction mixture per hour. Polygalacturonic acid *trans*-eliminase (PATE) activity (d) is recorded as change per hour in absorbancy at 235 m μ ; reaction mixtures were diluted 20-fold with 0.01 N-HCl before measuring absorbancy.

Species	Strain ICPB	Liquefaction (a)	Growth (b)	Enzymic activity*	
				PE (c)	PATE (d)
<i>X. badrii</i>	XB 103	+	0.76	1.10	1.63
<i>X. beticola</i>	XB 109	—	0.39	0.05	0
<i>X. campestris</i>	XC 135	+	0.98	0.09	0.69
<i>X. carotae</i>	XC 139	+	1.17	0.57	0.14
<i>X. corylina</i>	XC 12	—	0.46	0.05	0
<i>X. desmodii-gangetici</i>	XD 106	—	0.63	0	0
<i>X. geranii</i>	XG 10	+	1.05	0.02	0.06
<i>X. holcicola</i>	XH 3	—	0.83	0.06	0
<i>X. hyacinthi</i>	XH 110	—	0.83	0	0
<i>X. juglandis</i>	XJ 103	—	0.51	0.10	0
<i>X. lespedezae</i>	XL 2	+	0.48	0.10	0
<i>X. manihotis</i>	XM 12	+	1.06	0.02	0.55
<i>X. nakatae-olitorii</i>	XN 101	+	0.95	0.01	0
<i>X. papavericola</i>	XP 5	+	1.24	0.61	0.02
<i>X. pelargonii</i>	XP 8	+	0.84	0.04	0
<i>X. phaseoli</i>	XP 104	—	0.83	0.01	0
<i>X. ricinicola</i>	XR 4	+	0.33	0.67	0.83
<i>X. taraxaci</i>	XT 11	—	0.48	0.04	0.12
<i>X. vesicatoria</i>	XV 3	—	0.10	0.09	0

* Not even a trace of hydrolytic polygalacturonase or pectin *trans*-eliminase activity was detectable in any of the culture filtrates.

Some pectinesterase (PE) activity was found in all pectate-liquefying species and even in some cultures which did not liquefy the gel. The level of PE activity in *Xanthomonas badrii*, *X. campestris* (strains ICPB-XC 147 and ICPB-XC 149), *X. carotae*, *X. papavericola* and *X. ricinicola* is comparable to that reported for *Pseudomonas solanacearum* (Winstead & Walker, 1954), *Erwinia carotovora* (Goto & Okabe, 1962a) and *X. malvacearum* (Abo-El-Dahab, 1964), based on the same enzyme concentration and

the same reaction time. The lesser amounts of PE produced by other species or strains also are comparable on the same bases to that excreted by *Pseudomonas marginalis* (Ceponis & Friedman, 1959; Nasuno & Starr, 1966*b*).

Table 2. *Production of pectolytic enzymes by various strains of Xanthomonas campestris grown in the medium containing glucose or pectin as substrate**

Strain ICPB	Plant sources	Lique- faction (a)	Glucose-grown organisms			Pectin-grown organisms		
			Growth (b)	Enzymic activity		Growth (b)	Enzymic activity	
				PE (c)	PATE (d)		PE (c)	PATE (d)
xc 4	Horseradish	—	1·24	0	0	0·47	0·04	0
xc 7	Cabbage	+	0·99	0	0·04	0·52	0·12	0·12
xc 10	Turnip	+	1·42	0	0·12	0·42	0·06	0·95
xc 15	Cabbage	+	1·10	0	0·01	0·44	0·02	0·30
xc 117	Cauliflower	+	1·12	0	0·05	0·41	0·02	0·04
xc 132	Candytuft	+	1·26	0	0·10	0·71	0·02	0·45
xc 147	Cabbage	+	1·00	0	0·43	0·37	0·78	0·36
xc 149	Brussels sprout	—	1·62	0	0·04	0·51	0·47	0·07

* See the sublegend of Table 1 for notes; the only exception in the present instance is that the samples for determination of PATE activity (d) were diluted ten-fold with 0·01 N-HCl before measuring absorbancy.

The excretion of polygalacturonic acid *trans*-eliminase (PATE) was observed in all pectate-liquefying species except *Xanthomonas lespedezae*, *X. nakatae-olitorii* and *X. pelargonii*. Since these three cultures do not produce any polygalacturonase (PG), although they do form pectinesterase, the basis for liquefaction of the pectate gel deserves further study. Dye (1960), who observed a similar pattern, suggested that weak liquefaction of a pectate gel might well have been caused by other than enzymic factors. To further complicate the picture, it must be noted that PATE is shown in the present study to be produced by *X. taraxaci* which does not, however, detectably liquefy the pectate gel.

Under the assay-conditions used in the present study, neither polygalacturonase nor pectin *trans*-eliminase activity could be detected, even after 24 hr of reaction, in the culture supernatant fluids of any tested xanthomonads. According to Goto & Okabe (1962*b*), *Erwinia carotovora* can produce a pectic glycosidase active at pH 4·0 only when grown in a medium at very low calcium ion levels. However, polygalacturonase was not excreted by any xanthomonad cultures, even in a calcium-deficient medium. It might be noted that polygalacturonic acid *trans*-eliminase production in this calcium-deficient medium was either remarkably reduced (*Xanthomonas badrii*, *X. campestris*, *X. carotae*, *X. ricinicola*) or completely halted (*X. geranii*, *X. manihotis*, *X. papavericola*, *X. taraxaci*) in the case of xanthomonads which normally form PATE in the presence of calcium. Static culture conditions were ineffective in stimulating polygalacturonase excretion by xanthomonads, although this enzyme is produced by yeasts in static culture (Luh & Phaff, 1954 *a, b*).

The production of a 'pectin polygalacturonase' was reported by Dye (1960) to occur in many xanthomonads. It is more likely that the enzyme so designated by Dye is actually a combination of polygalacturonic acid *trans*-eliminase (PATE) and pectinesterase rather than the hydrolytic enzyme. He determined this enzyme activity

in growing cultures on a pectic agar media adjusted to pH 6.2–7.0 at which pH value the PATE of xanthomonads is still somewhat active (Nasuno & Starr, 1967). Moreover, a shift in pH value of the growth medium to the alkaline range favourable for PATE normally occurs when xanthomonads are grown on the medium used by Dye. The distribution of PATE+pectinesterase among the various xanthomonad species coincides well with Dye's (1960) report of what he terms 'protopectinase' ('PP') activity in xanthomonads (actually measured by Dye as maceration of potato tuber tissue). The good agreement between distribution of 'PP' and PATE+pectinesterase suggests that the so-called 'PP' of xanthomonads in most cases is probably a combination of polygalacturonic acid *trans*-eliminase (PATE) and pectinesterase.

Effects of substrate on pectic enzyme production

Various studies have shown that some pectic enzymes are inducible, whereas others seem to be constitutive; most pectic enzymes produced by phytopathogenic bacteria are inducible. Kraght & Starr (1953), Ozawa & Okamoto (1956) and Goto & Okabe (1962*b*) reported that pectin was superior to glucose as the carbon source for production of pectic glycosidase by *Erwinia* species. Similar results were found for *Bacillus polymyxa* by Nagel & Vaughn (1961*a*), and for *Xanthomonas malvacearum* by Abo-El-Dahab (1964). When eight strains of *X. campestris* were grown in a medium containing glucose but no pectic substances, there was no pectinesterase activity in the culture fluids of any strain; however, polygalacturonic acid *trans*-eliminase (PATE) was produced to some extent under these conditions by some strains (Table 2). On the other hand, in all these strains, pectinesterase was induced by the presence in the growth medium of pectin, which remarkably stimulated PATE production by some strains. Constitutive production of PATE is, thus, clear in several *X. campestris* strains. Somewhat similar results were recently found in *Pseudomonas marginalis* (Nasuno & Starr, 1966*b*), in which one of two strains excreted PATE regardless of the presence of a pectic substrate in the culture medium.

End-products from pectic substances formed by Xanthomonas species

Table 3 shows the results of paper chromatographic analysis of the breakdown products from polygalacturonic acid by polygalacturonic acid *trans*-eliminase (PATE) excreted by various *Xanthomonas* species or strains. The experimental conditions were 10% (v/v) enzyme solution, 0.5% (w/v) substrate, and 0.001 M-CaCl₂ in 0.05 M-glycine-NaOH buffer (pH 9.5), and a reaction time of 24 hr at 30°. With enzyme preparations showing high activity, the reaction products were unsaturated di- and tri-galacturonic acids accompanied by saturated mono-, di-, tri- and tetra-galacturonic acids. With weakly active enzyme preparations, the mono- and di-galacturonic acid and sometimes even the unsaturated di-galacturonic acid were not detectable in the case of some xanthomonads.

In earlier work it was believed that some 'polygalacturonase' preparations degrade the substrate completely to galacturonic acid, whereas others seemed to produce only oligalacturonides but not galacturonic acid. For example, in the reaction mixture with the pectic glycosidase from *Pseudomonas marginalis* and a pectic substrate, Ceponis & Friedman (1959) could not detect galacturonic acid which recently was found to be formed with concentrated enzyme under similar conditions (Nasuno &

Starr, 1966*b*). With tomato polygalacturonase, the reaction product was first thought to be pentagalacturonic acid (McColloch & Kertesz, 1949), then tri-, di-, and galacturonic acids (Roelofsen, 1953); eventually, mono- and di-galacturonic acids were confirmed as the real end-products (Luh, Leonard & Phaff, 1956).

Table 3. *Paper chromatographic analysis of the reaction products from polygalacturonic acid by polygalacturonic acid trans-eliminase produced by various Xanthomonas species*

		Galacturonides formed*						
Species	Strain ICPB	Saturated				Unsaturated		$\Delta E_{253}\ddagger$
		Mono-	Di-	Tri-	Tetra-	Di-	Tri-†	
<i>X. badrii</i>	XB 103	(+)	++	+	(+)	+++	++	0.500
<i>X. campestris</i>	XC 7	.	+	+	+	++	++	0.305
<i>X. campestris</i>	XC 10	(+)	+	+	+	++	++	0.480
<i>X. campestris</i>	XC 15	.	.	.	+	(+)	.	0.046
<i>X. campestris</i>	XC 117	.	.	.	+	(+)	.	0.035
<i>X. campestris</i>	XC 132	(+)	+	+	+	++	++	0.320
<i>X. campestris</i>	XC 135	(+)	+	+	(+)	++	++	0.470
<i>X. campestris</i>	XC 147	(+)	+	+	+	++	++	0.445
<i>X. campestris</i>	XC 149	.	+	+	+	+	+	0.110
<i>X. carotae</i>	XC 139	.	+	+	++	++	++	0.300
<i>X. geranii</i>	XG 10	.	.	+	+	+	.	0.135
<i>X. lespedezae</i>	XL 2	.	(+)	+	++	(+)	++	0.085
<i>X. manihotis</i>	XM 12	+	++	+	(+)	+++	++	0.545
<i>X. papavericola</i>	XP 5	.	.	.	+	(+)	.	0.035
<i>X. ricinicola</i>	XR 4	+	++	+	(+)	+++	++	0.504
<i>X. taraxaci</i>	XT 11	.	.	+	+	+	+	0.164

* (+) represents very weak spots; +++ intense spots.

† From their relative positions on the paper, these spots were only tentatively assumed to represent unsaturated trigalacturonic acid.

‡ Reaction mixtures were diluted 100-fold with 0.01 N-HCl for extinction measurement. Reaction period, 24 hr.

Although various patterns of breakdown products were transiently found in the reaction mixtures when culture supernatant fluids of xanthomonads were used as the enzyme preparations, the major reaction products from polygalacturonic acid acted upon by concentrated crude polygalacturonic acid *trans*-eliminase from all *Xanthomonas* cultures were unsaturated di- and tri-galacturonic acids with lesser amounts of saturated mono- and di-galacturonic acids. It can now be concluded that reports in the earlier literature to the effect that the monomer was not formed by some xanthomonads might stem from: (a) the use of samples too small to permit detection of the galacturonic acid, or (b) enzyme activity insufficient to produce monomer in a quantity detectable by the methods used.

The sequential appearance of the several products of pectic digestion by polygalacturonic acid *trans*-eliminase-forming *Xanthomonas* species was followed in culture fluids by paper chromatography of samples taken at 24 hr intervals of incubation. In the medium containing pectin, saturated and unsaturated oligogalacturonides first appeared after 24 hr and then monomer was detected after 48 hr. Saturated mono-, di-, and tri-galacturonic acids and unsaturated di- and tri-galacturonic acids were found as major end-products after 7 days of incubation. The sequence was the same,

but this process was shortened, when polygalacturonic acid replaced the pectin in the culture medium; this suggested that pectinesterase activity might be limiting. After incubation for 4 days in the polygalacturonic acid medium, the sole end-product was galacturonic acid which cannot be utilized further by any *Xanthomonas* culture examined. Therefore, it is plausible that this uronic acid is one of the real end-products of pectin digestion by *Xanthomonas* species. These results contrast markedly with the non-accumulation of galacturonic acid in the culture fluid of *Erwinia carotovora* (Kraght & Starr, 1953); but this organism can rapidly metabolize galacturonic acid through the pathway elucidated by Kilgore & Starr (1959).

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