JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1977, p. 379-386
Vol. 6, No. 4
Copyright © 1977 American Society for Microbiology
Printed in U.S.A.

Enzymatic Degradation of Polygalacturonic Acid by *Yersinia* and *Klebsiella* Species in Relation to Clinical Laboratory Procedures

MORTIMER P. STARR,* ARUN K. CHATTERJEE, PHOEBE B. STARR, AND GORDON E. BUCHANAN

Department of Bacteriology, University of California, Davis, California 95616

Received for publication 16 May 1977

As scored by several specified plating procedures, clinical and environmental strains of *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Klebsiella pneumoniae* "Oxytocum" showed detectable, albeit generally weak, ability to digest polygalacturonic (pectic) acid. None of these bacterial strains had the vigorous and rapid pectolytic activity on these polygalacturonic acid-containing media that is typical of soft-rot *Erwinia* species, although some of the Oxytocum strains came fairly close. Analyses of the pectolytic enzyme contents of the cells and culture supernatants of the *Yersinia* and *Klebsiella* species revealed that readily detectable quantities of cell-bound polygalacturonic acid *trans*-eliminase and hydrolytic polygalacturonase were formed by the *Yersinia* and *Klebsiella* species; however, the total units of enzyme activity produced by these bacteria were, in general, lower than were produced by soft-rot *Erwinia* species. Furthermore, unlike the situation in soft-rot *Erwinia* cultures, these pectolytic enzymes of *Yersinia* and *Klebsiella* species were not excreted rapidly and massively into the growth medium. Cultures of other enterobacteria (*Citrobacter* species, *Enterobacter* species, *Erwinia amylovora, Erwinia herbicola, Escherichia coli, Proteus* species, *Salmonella typhimurium*, and *Serratia marcescens*) showed no pectolytic ability whatsoever by any of the plating procedures used and (to the extent they were so examined) produced no pectolytic enzymes detectable either in their cells or culture supernatants. This slow or weak release of pectolytic enzymes by *Yersinia* and *Klebsiella* species has a bearing on clinical laboratory procedures suitable for detecting their pectolytic activity; methods adequate for this purpose are detailed.

The assortment of polysaccharides based on linear chains of α-1,4-linked galacturonic acid, collectively called "pectic substances," occurs in nature only in higher plants. Pectic substances, particularly the partially methoxylated material (called pectin), constitute much of the plant middle lamella and primary cell wall, intergraded regions outside the cellulosic secondary cell wall (2, 3, 29). Demethoxylated pectin is called polygalacturonic acid or pectic acid. Until quite recently, the only enterobacteria known to have the capacity to degrade pectic substances—as scored by various procedures (19, 30)—were members of the soft-rot section of the genus *Erwinia* (12, 13, 16, 21, 32). This degradative ability is an important component of the phytopathogenicity of these soft-rot *Erwinia* species because it results in the maceration (rotting) of plant tissue (3, 4). Breakdown of pectic substances is mediated by one or another of a great array of pectolytic enzymes formed by soft-rot phytopathogenic *Erwinia* species (14, 15, 18, 20, 24, 25, 28, 33). The two pectolytic enzymes treated here are hydrolytic polygalacturonase (PG) and polygalacturonic acid *trans*-eliminase (PATE). PG cleaves polygalacturonic acid hydrolytically to yield the monomer, galacturonic acid, and various saturated oligomers (i.e., polysaccharides consisting of galacturonic acid chains shorter than the starting material). PATE cleaves polygalacturonic acid eliminatively to yield unsaturated galacturonic acid (4-deoxy-L-threo-5-hexoseulose uronic acid), unsaturated oligomers (i.e., shorter polysaccharide chains in which the terminal residues are the unsaturated galacturonic acid), and saturated oligomers.

Given this ecological truism, it was indeed surprising to learn from two recent reports by von Riesen (34, 35) that animal-pathogenic members of two other enterobacterial genera, *Yersinia enterocolitica* and *Y. pseudotubercu-*

losis (but not *Y. pestis*) and "Oxytocum" strains of *Klebsiella pneumoniae*, none of which has any known phytopathogenic relationship to plants, are able to digest the kind of calcium-stabilized polygalacturonate gels (31) used in earlier studies of soft-rot *Erwinia* species. The purposes of the present report are: (i) to confirm and extend these findings of von Riesen; (ii) to demonstrate that the pectolytic action stems from production by these bacteria of certain pectolytic enzymes that (unlike the situation in soft-rot *Erwinia* strains) are often cell bound and usually not excreted by these organisms into the growth medium in any appreciable quantity; (iii) to present explicit directions for preparing and using plating media suitable for demonstrating pectolytic action in the clinical laboratory and for other uses; and (iv) to raise some issues concerning the possible ecological, evolutionary, and taxonomic significance of the occurrence of pectolytic enzymes in such animal-pathogenic bacteria.

MATERIALS AND METHODS

Bacteria studied. The bacterial cultures studied are listed in Tables 1 and 2. These cultures include some of the *Yersinia* and *Klebsiella* strains used by von Riesen (34, 35) as well as strains of these bacteria obtained from other investigators (1, 5, 7). Authentic reference strains of soft-rot *Erwinia* species (known from previous studies to be pectolytic) and of other enterobacterial sorts (generally believed not be pectolytic) were included. The cultures were maintained on Luria agar (9) or on yeast extract-glucose-$CaCO_3$ agar (21). Unless otherwise stated, all incubations were at 30°C.

PEC medium. The calcium-stabilized pectate (polygalacturonate) gel (PEC) medium was prepared along the lines previously outlined (31), except that the sodium ammonium pectate (no. 24 of Sunkist Growers, Ontario, Calif.), a product that no longer is commercially available, was replaced by sodium polygalacturonate (Sigma Chemical Co., St. Louis, Mo., no. P-1879, or ICN, Cleveland, Ohio, no. 102921). The exact procedure we presently use in preparing PEC medium follows. Distilled water (100 ml) and a magnetic stirring bar were placed in a 1-liter Erlenmeyer flask (situated on a heater fitted with a magnetic stirrer). The following ingredients were then added while stirring: 0.6 ml of 10.0% aqueous $CaCl_2 \cdot 2H_2O$; 1.0 ml of 0.1% aqueous bromothymol blue (BTB) in 6.4×10^{-4} N NaOH; 0.5 g of Difco yeast extract; and (very slowly, so that each particle is wetted) 3.0 g of sodium polygalacturonate (one of the brands mentioned above). After the polygalacturonate was uniformly wetted and suspended, the heater was turned on, and the temperature was brought almost to boiling with continuous stirring. The material had a strong tendency to foam (the reason that a rather large flask is specified); this foaming can be avoided by removing the flask from the heater before the solution actually boils. While the solution was hot and stirred, its pH was adjusted (by monitoring the color of the BTB indicator) to 7.3 with 1 N NaOH (taking care not to overshoot, because polygalacturonate is quite labile at elevated pH). The medium was autoclaved (121°C, 15 min) and allowed to cool to about 95°C before opening the autoclave (again, to avoid foaming). It was then poured directly into sterilized petri dishes, which should be held at room temperature until the gel sets. PEC medium thus prepared will, after sterilization and gelling, usually be at pH 6.8 to 7.0 (light green in color). It is important to emphasize that the PEC medium cannot be remelted and, consequently, that the plates must be poured while the medium is still fluid, soon after it comes from the autoclave, since gelation may occur even at elevated temperatures. Surplus moisture on the covers of the petri dishes or the surfaces of the medium can be controlled by pouring the plates after the PEC medium has cooled for 5 to 15 min in a 50°C water bath (the brand of polygalacturonic acid will dictate how long the medium can be held and cooled before irreversible gelation begins), by replacing the petri dish covers with dry ones, and/or by drying the plates for a day or two at 25°C. Plates of PEC medium can be stored for several weeks at room or refrigerator temperatures, sealed within the plastic sleeves in which plastic petri dishes are commonly packed. Because PEC medium cannot be remelted, if tubes of medium (slants or stabs) are required, the unsterilized PEC medium is dispensed into tubes, which are then autoclaved and allowed to gel in a slanting or upright position.

PEC-SSA medium. When working with strongly pectolytic bacteria (such as soft-rot *Erwinia* strains), particularly when it is not possible to observe the plates frequently, it is often desirable to use a semisolid agar version (PEC-SSA) of PEC medium. By this device, the vigorous and rapid digestion of the polygalacturonate, as well as the consequent liquefaction of PEC medium, remained localized around individual pectolytic colonies rather than resulting in complete fluidity of the contents of the plate. To make PEC-SSA medium, 0.3% agar (Difco) was added to the PEC medium after the sodium polygalacturonate (either Sigma no. P-1879 or ICN no. 102921) was completely wetted; the agar and polygalacturonate were completely dissolved by further heating and stirring; the pH was adjusted to 7.3; and the PEC-SSA medium was autoclaved and dispensed—all as recommended for PEC medium.

Several cultures were spotted on each plate of the PEC and PEC-SSA media, and the ability to degrade polygalacturonic acid was scored by periodically observing the plates for liquefaction of the medium and/or sinking of the colonies.

PEC-YA medium. Another procedure was used that is based on the precipitability of undecomposed polygalacturonic acid with 2 N HCl. Pectolytic colonies in this procedure are surrounded by clear halos in a turbid background (10). The brand of polygalacturonic acid suitable for this procedure is very critical; neither Sigma no. P-1879 nor ICN no. 102921 was suitable; Sunkist Growers no. 24 and 3491 worked quite well, but neither product is now commercially available; the polygalacturonic acid no. P 21750 of

Pfaltz-Bauer (Stamford, Conn.) is both suitable and available. The pectate-yeast extract-agar (PEC-YA) medium was prepared (following the same precautions given above for PEC medium) as follows: to 100 ml of cold distilled water (held in a 1-liter Erlenmeyer flask, fitted with a magnetic stirring bar, that is situated on a magnetic stirrer-heater) were added slowly with continuous stirring 1.0 g of polygalacturonic acid (Sunkist Growers no. 3491 or Pfaltz-Bauer P 21750), 1.0 g of yeast extract (Difco), 1.0 ml of 0.1% aqueous BTB in 6.4×10^{-4} N NaOH, and 1.5 g of agar (Difco). After the components were completely wetted, the heater was turned on, and the medium was brought almost to boiling with continuous stirring. When all of the components were dissolved, the pH was adjusted while stirring to approximately 7.3 with 1 N NaOH (taking care not to overshoot) by monitoring the color change of the BTB indicator dye and periodically checking with pH paper (pHydrion paper; Micro Essential Laboratory, Brooklyn, N.Y.). The medium was autoclaved at 121°C for 15 min. The PEC-YA medium was poured into plates after the medium had cooled to about 50°C. The pH of the medium after autoclaving was about 6.8 to 7.0 (light green in color). Because polygalacturonic acid is alkali labile, especially at elevated temperatures, any desired adjustment of the PEC-YA medium to a higher pH should be effected, after autoclaving and cooling somewhat, with sterile 1 N NaOH solution while stirring continuously; in this case, it might be necessary to substitute a more appropriate ph indicator for BTB. To use this PEC-YA medium, cultures were streaked or spotted on the surface and allowed to grow. The plates were then flooded with 2 N HCl, held at room temperature for a few minutes, and scored for the appearance of clear halos around and beneath the colonies in the otherwise turbid medium. Since the HCl-scoring method kills the organisms, it was necessary to prepare replicate plates if the colonies were to be recovered or if the time course of pectolytic action was to be followed.

Survey of pectolytic action of enterobacteria on plating media. The enterobacterial strains (16-h cultures in Luria broth; 9) listed in Table 1 were spotted on plates of PEC, PEC-SSA, and PEC-YA media. The inoculated plates were incubated at 30°C and observed daily for 6 days (in the case of the PEC and PEC-SSA media) or, by means of replicate preparations, at 2, 4, and 6 days (in the case of the PEC-YA medium).

Culture medium for enzyme assays. Bacterial cultures for enzyme assays were grown in a complex medium that consisted of (in grams per liter): polygalacturonic acid (Sigma no. P-1879), 2.0; Casamino Acids (Difco), 10.0; yeast extract (Difco), 1.0; potassium phosphate buffer (final concentration, 0.055 M at pH 7.0); $(NH_2)_2SO_4$, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.1. This medium was sterilized by autoclaving. The inorganic salts were autoclaved separately and added aseptically to the autoclaved solution containing the rest of the components.

Preparation of cultures for enzyme assays. After growth for 24 h at 30°C (for *Erwinia* species) or 37°C (for *Klebsiella* and *Yersinia* species) on a gyratory shaker in the specified medium, the cells and culture supernatants were collected by a 10-min centrifugation (17,000 × *g*) at 4°C. The culture supernatants were dialyzed at 4°C overnight against 200 volumes of tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.005 M, pH 7.0) and assayed for enzyme activity with no further treatment. The cells were washed twice with tris(hydroxymethyl)-aminomethane-hydrochloride buffer (0.01 M, pH 7.0). The washed cells were resuspended in the same buffer and disrupted either by sonic treatment, using the Biosonik IV (Bronwill Scientific Co., San Francisco, Calif.) with four 30-s bursts at maximum output each followed by a cooling period of 1 min, or by two cycles through the Aminco (Silver Spring, Md.) French Pressure Cell Press operating at 18,000 lb/in^2.

Enzyme assays. The following enzyme assays were carried out: PG (EC 2.3.1.15; 28); PATE (EC 4.2.99.3; 24, 26, 33); and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) with glucose 6-phosphate as substrate (22). Since some modifications were made in the published procedures for PATE and PG assays, the actual protocols follow. For assay of PATE activity, the reaction mixture contained: 1.2 ml of a stock solution containing 5.75 mg of polygalacturonic acid (Sigma P-1879) per ml; tris(hydroxymethyl)aminomethane-hydrochloride buffer, 0.6 ml of 0.5 M at pH 8.5; $CaCl_2$, 0.02 ml of 0.05 M; enzyme preparation; and distilled water to a total volume of 3.0 ml. The rate of reaction was measured at 25°C (room temperature) at 235 nm, using a Cary recording spectrophotometer. One unit of PATE activity is defined as the amount of this enzyme that produces a change in absorbancy at 25°C of 1.0 at 235 nm. The specific activities of PATE, reported in Table 2, are expressed in terms of enzyme units per milligram of protein. The percentage of total PATE activity in supernatant, reported in Table 2, is calculated in terms of the units of enzyme activity present in equivalent volumes of dialyzed culture supernatants and cell extracts.

For assay of PG activity, the reaction mixture contained: 0.5% (wt/vol) polygalacturonic acid (Sigma P-1879); 0.2 M NaCl in 0.05 M sodium acetate buffer at pH 5.2; enzyme preparation; and distilled water to a total volume of 10.0 ml. At the desired time interval, a 3.0-ml portion of the reaction mixture was removed. To this 3.0-ml sample, 2.0 ml of distilled water and 0.35 ml of 1 M Na_2CO_3 were added, followed by 2.0 ml of 0.05 N iodine in KI. The mixture was incubated for 20 min at 25°C in the dark and then was acidified by adding 0.8 ml of 2 N H_2SO_4. The free iodine was titrated, with continuous stirring, against 0.01 N $Na_2S_2O_3$, using soluble starch (0.5%; Difco) as the indicator. One unit of PG activity is defined as the amount of this enzyme that liberates from polygalacturonic acid 1.0 μmol of aldehyde-equivalent per h at pH 5.2 and 25°C; specific activity is expressed as units per milligram of protein.

Protein was determined by the method of Lowry et al. (23); crystallized bovine serum albumin was used as the standard.

Maceration of plant tissues. Ability to macerate celery petioles, potato tubers, and carrot roots was determined on pieces of such plant parts previously surface sterilized with ethanol, cut into slices aseptically, and placed on sterilized paper toweling sup-

ported by a sterilized wire rack in a sterilized plastic box fitted with a tight cover. A small amount of sterile water was kept at the bottom of the box to maintain humid conditions. The plant samples were inoculated by spotting 0.02 ml of bacterial cell suspension (about 10^9 cells per ml) in a sterile 0.05 M potassium phosphate buffer (pH 7.0) and were incubated for up to 1 week at 30°C. Tissue maceration (rotting) was scored by touching the tissue periodically with a flamed needle and determining in this way whether it had softened in the vicinity of the bacterial growth (if there was any growth).

RESULTS

As summarized in Table 1, all (14 out of 14) tested cultures of soft-rot *Erwinia* species showed vigorous and rapid pectolytic action when scored on plates of PEC, PEC-SSA, or PEC-YA media. This pectolytic action was readily evident by liquefaction of the pectate gels, and/or sinking of the colonies on PEC-SSA media, or by the halos around colonies visualized after flooding plate cultures on PEC-YA medium with 2 N HCl. The PEC medium was noticeably liquefied around colonies of the soft-rot *Erwinia* strains almost as soon as the colonies were first visible, and, within a day or so, much of the PEC plate was totally liquefied—which is the reason that we recommend use of PEC-SSA or PEC-YA media for such strongly pectolytic cultures.

None of the *Yersinia* or *Klebsiella* strains are

TABLE 1. *Pectolytic action of enterobacterial strains as scored on various plating media*[a]

Species[b]	Source[c]	Strains tested (no.)	Cumulative no. of strains showing pectolytic action on: PEC[d] after (h):			PEC-SSA[e] after (h):			PEC-YA[f] after (h):	
			24	48	96	24	48	96	48	96
Erwinia carotovora	ICPB	11	11			11			11	
Erwinia chrysanthemi	ICPB	2	2			2			2	
	CDC	1	1			1			1	
Klebsiella pneumoniae[g]	CDC	7	0	1	1	1	1	1	1	1
	RJS	37	0	3	4	1	3	3	0	3
Klebsiella pneumoniae (Oxytocum)[g]	CDC	8	7	8		7	8		3	8
	RJS	24	8	18	19	13	19	19	10	19
	LvR	3	2	3		3			1	3
Yersinia enterocolitica	CDC	14	2	13	13	7	11	13	3	13
	LvR	5	2	5		2	5		2	5
Yersinia pseudotuberculosis	CDC	6	0	0	4[h]	0	4[h]	4[h]	0	1
	LvR	5	0	2[h]	5[h]	0	4[h]	5[h]	0	1

[a] For details of the experimental procedures, including compositions of the various media, refer to the Materials and Methods section.

[b] The species designations generally are those used by the donors of the cultures; the main exception is in *Erwinia carotovora* (which here includes its "varieties"). No attempt has been made to characterize further the strains used (see, also, footnote *g*). Representative cultures (number of strains tested in parentheses) of the following enterobacterial species, tested at the same time, showed no pectolytic action on any of the three media within 4 to 6 days: *Citrobacter* species (three strains), *Enterobacter aerogenes* (one strain), *Enterobacter cloacae* (two strains), *Enterobacter hafnia* (one strain), *Erwinia amylovora* (four strains), *Erwinia herbicola* (two strains), *Escherichia coli* (two strains), *Proteus* species (two strains), *Salmonella typhimurium* (one strain), *Serratia marcescens* (two strains).

[c] ICPB, International Collection of Phytopathogenic Bacteria, Davis, Calif.; CDC, Center for Disease Control, Atlanta, Ga.; RJS, R. J. Seidler, Corvallis, Ore.; LvR, L. von Riesen, Omaha, Neb.

[d] PEC, Calcium-stabilized pectate (polygalacturonate) gel medium, as described in the section on Materials and Methods, using either the Sigma or ICN brand of polygalacturonate. Pectolytic action was scored in terms of liquefaction of the gel and/or sinking of the colonies.

[e] PEC-SSA, Semisolid agar version of the pectate (polygalacturonate) gel medium. Other remarks as in footnote *d*.

[f] PEC-YA, Polygalacturonate-yeast extract agar medium made with the Pfaltz-Bauer product. Pectolytic action was scored in terms of clear areas (halos) around and below colonies after the plates were flooded with 2 N HCl. Results with the weakly pectolytic *Yersinia* and *Klebsiella* strains were not clear-cut at 24 h; 48 to 96 h is the earliest that reliable results can be obtained with such strains by this procedure.

[g] The distinction between *K. pneumoniae* and *K. pneumoniae* (Oxytocum) strains was made by the donors of the cultures, presumably on the basis that Oxytocum strains produce indol and may liquefy gelatin (35). We do not known how sharply circumscribed these two groups are, but there seems to be some overlap in terms of pectolytic action or lack of it.

[h] In *Y. pseudotuberculosis*, weakly and questionably positive reactions are included. None of these strains were as strongly pectolytic as the *Y. enterocolitica* strains. Some strains degraded both Sigma and ICN brands of polygalacturonate, and others degraded one brand or the other but not both.

as actively pectolytic (in rate or degree of digestion) as the soft-rot *Erwinia* strains, although some *K. pneumoniae* Oxytocum strains come fairly close (Table 1). Most (30 out of 35) strains of *K. pneumoniae* Oxytocum are actively pectolytic and could be scored as such on any of the three media within one day. Few (5 out of 44) strains of *K. pneumoniae* (other than the Oxytocum sort) are pectolytic, and those that are pectolytic generally are somewhat less vigorously so than the Oxytocum strains (Table 1). Essentially all (18 out of 19) strains of *Y. enterocolitica* (including representatives of all four hybridization groups; 5) were detectably pectolytic, although incubation for at least 2 days was usually required to score these cultures as pectolytic (Table 1). The *Y. pseudotuberculosis* strains (9 out of 11) are at best weakly pectolytic, requiring incubation for as long as 4 days before assignment was possible of a weakly or questionably positive reaction (Table 1). Moreover, there were variations in reactions of *Y. pseudotuberculosis* strains that depended on the brands of polygalacturonate used (Table 1, footnote *h*). In most cases, PEC and PEC-SSA media seemed to be more sensitive indicators of pectolytic action of the *Yersinia* species than was the PEC-YA(HCl) procedure. In general, PEC-SSA medium is to be recommended for testing pectolytic action of the *Yersinia* species, with the reaction scored in terms of sinking of the colonies rather than liquefaction of the medium.

The pectolytic activities reported in Table 1 were those determined at 30°C. A representative assortment of the pectolytic strains of *Klebsiella* and *Yersinia* species was scored also at 37°C, with results comparable to those obtained at 30°C. This finding, together with the fact that PATE and PG activities were detectable in cells grown at 37°C (Table 2), suggests that pectolytic activity can be detected on these media at either temperature (assuming that the organism grows at that temperature).

None of the enterobacterial strains tested (Table 1, footnote *b*), other than the aforementioned, were demonstrably pectolytic as scored

TABLE 2. *PATE and PG activities (after 24 h) in culture supernatants and cells of Erwinia, Yersinia, Klebsiella, and other enterobacterial strains*[a]

Species	Strain designation[b]		Specific activity[c] of PATE in:		Total PATE activity in supernatant[c] (%)
	ICPB	Other	Supernatant	Cells	
Erwinia aroideae	EA144		4.7	0.2	94.0
Erwinia carotovora	EC153[d]	ATCC 15359	3.6	2.3	13.0
Erwinia chrysanthemi	EC16[d]	ATCC 11662	58.4	0.03	98.0
Klebsiella pneumoniae (Oxytocum)	3739[d]	LvR 75009	0.02	0.3	0.7
	3740	LvR 75012	0	0.11	
	3745	RJS UOMS 10879	0	0.07	
	3747	RJS V112	0	0.20	
	3749	RJS MH21	0	0.03	
	3750	RJS ATCC 13182	0	0.03	
	3752[d]	RJS UG5	0	0.25	
	3755	RJS ATCC 13183	0.06	0.10	5.0
Yersinia enterocolitica	3734[d]	LvR 74427	0.08	2.5	0.1
	3735	LvR 74428	0	0.04	
	3737	LvR 74338	0	0.08	
	3741	LvR 76035	0	0.06	
Yersinia pseudotuberculosis	3821[e]	LvR 74334	0.01	0.42	0.6
	3824[e]	LvR 74425	0	<0.01	

[a] There was no detectable PATE activity either in cell extracts or culture supernatants of *Erwinia amylovora* (ICPB EA213), *Erwinia herbicola* (ICPB EH103 and EH133), *Escherichia coli* K-12 and B (ICPB 2195 and 2262, respectively), *Salmonella typhimurium* LT2 (ICPB 3335), and *Serratia marcescens* (ICPB 2031). However, the cell extracts of these PATE-negative strains contained detectable glucose 6-phosphate dehydrogenase activity, indicating that the handling of the cell extracts did liberate another, typical, cell-bound enzyme. See footnotes *d* and *e*, below, for remarks about PG activity; these were the only strains in which PG activity was determined.

[b] ICPB, International Collection of Phytopathogenic Bacteria, Davis, Calif.; ATCC, American Type Culture Collection, Rockville, Md.; RJS, R. J. Seidler, Corvallis, Ore.; LvR, L. von Riesen, Omaha, Neb.

[c] See Materials and Methods section for definitions of these units.

[d] Produces substantial amounts (specific activity greater than 5.0) of PG activity and excretes much of this enzyme activity into the culture medium in 24 h.

[e] Produces slight amounts (specific activity less than 5.0) of PG activity; essentially none of this enzyme activity is excreted into the culture medium in 24 h, but remains bound in the cells.

by any of the three plating procedures.

The pectolytic abilities of the *Yersinia* and *Klebsiella* cultures were not evident on stab cultures in PEC medium (detailed data not presented here), even when such degradative abilities were readily detected on plate cultures in PEC medium.

The distribution (in culture supernatants and cell-free extracts) of PATE is given in Table 2. In the various soft-rot *Erwinia* strains, this pectolytic enzyme usually occurred both in the culture medium and in the cells; the excretion of PATE varies (18 to 98% of the total activity is excreted) in these strains (Table 2). In the majority of the *Yersinia* and *Klebsiella* strains thus examined, PATE activity was found only in the cells. Small amounts of PATE (not over 5% of the total activity) were excreted into the culture fluids within 24 h only by *K. pneumoniae* Oxytocum strains ICPB 3739 and 3755, *Y. enterocolitica* strain ICPB 3734, and *Y. pseudotuberculosis* strain ICPB 3821 (Table 2). The other tested enterobacteria (*Erwinia amylovora, Erwinia herbicola, Escherichia coli, Salmonella typhimurium,* and *Serratia marcescens*) produced no PATE detectable in either the cells or the culture supernatants (Table 2, footnote *a*), as might be expected from their inability to degrade polygalacturonate in any of the plating media (Table 1, footnote *b*). The cell extracts of these PATE-negative enterobacterial strains did show detectable glucose 6-phosphate dehydrogenase activity, indicating that our handling of these preparations was adequate for demonstrating another typical cell-bound enzyme activity.

Our examination of the distribution of the PG was limited to only a few representative strains (footnotes *d* and *e*, Table 2). The excretion patterns of PG were, in general, similar to those of PATE.

A definite ability to macerate (rot) potato, carrot, or celery tissues could not be detected in any of the 15 *Yersinia* or *Klebsiella* strains thus tested (detailed data not presented here). In contrast, the soft-rot *Erwinia* strains, which served as positive controls, caused the typical (rapid and massive) maceration of these plant tissues within 24 h. Few of the *Yersinia* or *Klebsiella* strains grew at all or well on the plant material under the conditions used; of these 15 strains, 5 strains showed barely detectable growth on one or more of the plant tissues, 5 showed scanty growth, and 5 did not seem to grow at all.

DISCUSSION

The several plating procedures that are described here for demonstrating pectolytic ability of clinical strains of *Yersinia* and *Klebsiella* species each has advantages and disadvantages. If this ability is scored on PEC medium, in terms of liquefaction of the pectate gel and sinking of the colonies, it is necessary to look at the plates very carefully over a prolonged incubation period because the slow liquefaction might be balanced by evaporation, so that the net visible fluidification would be nil. However, the pectolytic colonies will sink below the surface of the surrounding undigested gel, and careful examination will reveal this sinking even if a definite liquefaction cannot be observed. As already remarked, although PEC medium provides a sensitive indicator of pectolytic ability, it has a disadvantage (complete liquefaction of the plateful of medium within a day or two) when it is used with soft-rot *Erwinia* species, and colonies must be recovered before this total liquefaction occurs. This latter disadvantage can be circumvented by adding enough agar to make a semisolid medium. This PEC-SSA medium seems to be as sensitive an indicator of pectolytic action as is PEC medium, and, hence, PEC-SSA medium is recommended also for scoring the weakly pectolytic bacteria such as the *Yersinia* and *Klebsiella* species.

The foregoing remarks are based on the use of PEC and PEC-SSA media in plates. A limited comparison was made between plate and stab cultures in PEC medium. Stab cultures in PEC medium were not adequate for demonstrating the pectolytic action of most *Yersinia* and *Klebsiella* strains. Hence, the use of PEC medium stab cultures cannot be recommended for this application in the clinical laboratory, although it is decidedly useful for the strongly pectolytic bacteria (such as the soft-rot *Erwinia* species) for which the procedure was originally developed (31).

The HCl-precipitation technique (with PEC-YA medium) seems to be a somewhat less sensitive method for detecting the weakly pectolytic *Yersinia* and *Klebsiella* strains than is PEC-SSA medium. It is necessary to use pectic acid of sufficiently high molecular weight—such as Sunkist Growers no. 24 or 3491 (presently not commercially available, to our knowledge) or Pfaltz-Bauer no. P 21750—so that the undecomposed polygalacturonic acid forms an easily visible precipitate with the HCl. Moreover, the method is destructive; this difficulty can be circumvented by making replicate plates, only one of which would be flooded with the HCl at each time period, with colonies to be recovered from another, untreated plate. However, the PEC-YA(HCl) procedure is indeed useful for scoring the strongly pectolytic action of some bacteria

(such as soft-rot *Erwinia* species and some *Klebsiella* strains) and for mutant isolation, uses which are perhaps more relevant to other activities of our group than to some of the present aims.

It is evident that we have been able to confirm the observations of von Riesen (34, 35) to the effect that *Yersinia* and Oxytocum *Klebsiella* strains are indeed demonstrably pectolytic. Considering the extensive series of such strains (Table 1) that we have shown to be pectolytic by one or another of the procedures detailed herein, it now seems appropriate to recommend trial of the aforementioned media and procedures in the primary isolation and/or identification of such bacteria in the clinical laboratory.

Our first notion on verifying and extending the observations of von Riesen (34, 35) was that this relatively weak ability of *Yersinia* and *Klebsiella* strains to digest commercial grades of polygalacturonic acid in plate cultures on PEC, PEC-SSA, and PEC-YA media (Table 1) was an artifact of one sort or another stemming from the fact that such polygalacturonic acid preparations are by no means homogeneous (2, 3). Several possibilities in this direction occurred to us, but they are for the present irrelevant because it is clear, as shown in Table 2, that pectolytic enzymes that, superficially at least, are the same sorts as some produced by phytopathogenic soft-rot *Erwinia* species (14, 24, 25, 28) can actually be demonstrated in preparations from strains belonging to the genera *Klebsiella* and *Yersinia*. In most of the examined pectolytic *Yersinia* and *Klebsiella* cultures, the pectolytic enzymes are entirely localized within the cells with no traces of them in the culture supernatants following a 24-h growth period, quite unlike the situation in cultures of soft-rot *Erwinia* species where such enzymes occur both intracellularly and extracellularly (Table 2; 24, 26).

In preliminary trials, we have found an increased excretion of PATE activity into the culture supernatants of *Klebsiella* and *Yersinia* strains after longer growth periods (48 to 72 h, rather than the 24 h on which the data in Table 2 are based). A detailed comparative description of the PATE excretion process in various enterobacteria (*Erwinia*, *Klebsiella*, and *Yersinia* species) will be reported by us elsewhere. However, in general, the results of the PATE and PG assays of cells and culture supernatants (Table 2) correlated quite well with the observations of pectolytic activity on plates of the several polygalacturonate media (Table 1).

It is indeed an ecological and taxonomic puzzle that these animal enterobacteria—*Yersinia* species and Oxytocum *Klebsiella* strains—can digest pectic substances. As far as is presently known, the *Yersinia* species do not occur in plant habitats. Some *K. penumoniae* strains, but possibly not the Oxytocum sorts, are common on plants (7), yet it is predominantly the Oxytocum strains that do produce pectic enzymes capable of attacking substances that are uniquely of plant origin.

What might be some possible explanations of these facts? The entirely separate development of plant and animal bacteriology, which we have elsewhere referred to as "disciplinal insularity" (32), might have resulted in the application by plant and animal bacteriologists of separate generic names to bacteria that actually are closely related. This putative explanation is much more pertinent to *Klebsiella* than it is to *Yersinia*; the soft-rot *Erwinia* species (apart from their plant pathogenicity) seem relatively closely related in some respects (6, 11, 27, 32) to some of the organisms in the *Klebsiella-Enterobacter-Serratia* complex, whereas the *Yersinia* species (5) seem much less closely related to this enterobacterial assemblage. The *Yersinia* and *Klebsiella* strains that we have examined, albeit capable of producing active cell-bound pectolytic enzymes, usually do not excrete more than a trace of these enzymes into the environment (which is why they usually scored as only "weakly pectolytic" on the various plating media). Moreover, none of the *Yersinia* or *Klebsiella* strains thus tested could macerate plant tissues, and it is rather unlikely that they could cause frank plant diseases. Some *Klebsiella* species (but, perhaps, not the Oxytocum sort) are known to occur on plants (e.g., 1, 7), and some of the *Yersinia* and *Klebsiella* species have been reported from waters (5, 8, 17) and could have entered these aquatic habitats from plants. Exploration of the habitats of these sorts of *Yersinia* and *Klebsiella* species—other than their occurrence in animal disease—might provide the kind of ecological information that is now lacking. Such undertakings might be facilitated by making use of the procedures for scoring pectolytic ability that are detailed herein.

It is also possible that such *Yersinia* and *Klebsiella* strains might have descended, in evolutionary time, from organisms that once lived on plants and that the present-day members of these bacterial groups have retained the capacity for producing pectolytic enzymes that seem no longer to be ecologically relevant. Alternatively, it may be the case that conjugational events occurred (e.g., in the intestinal tracts of herbivores or on plant surfaces or in soil) with the result that *Erwinia* genes specifying pectolytic enzyme production were incorporated into

Yersinia and *Klebsiella*. The likelihood that such genetic recombination had in the past occurred in nature would be strengthened if recombinational events could be shown to occur between present *Erwinia* strains and present *Yersinia* or *Klebsiella* strains. Our recent construction of donor strains in the soft-rot *Erwinia* group (A. K. Chatterjee and M. P. Starr, J. Bacteriol., in press) now enables such an examination to be made, and we intend to devote our attention to the matter in the near future.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-08426 from the National Institute of Allergy and Infectious Diseases.

We appreciate very much the assistance of V. L. von Riesen, R. J. Seidler, and D. J. Brenner in providing us with *Yersinia* and *Klebsiella* cultures and much helpful advice.

LITERATURE CITED

1. **Aho, P. E., R. J. Seidler, H. J. Evans, and P. N. Raju.** 1974. Distribution, enumeration and identification of nitrogen-fixing bacteria associated with decay in living white fir trees. Phytopathology **64**:1413–1420.
2. **Aspinall, G. O.** 1970. Pectins, plant gums, and other plant polysaccharides, p. 515–536. *In* W. Pigman and D. Horton (ed.), The carbohydrates, chemistry and biochemistry, 2nd ed., vol. IIB. Academic Press Inc., New York and London.
3. **Bateman, D. F., and H. G. Basham.** 1976. Degradation of plant cell walls and membranes, p. 316–355. *In* R. Heitefuss and P. H. Williams (ed.), Physiological plant pathology. Springer-Verlag, Berlin, Heidelberg, and New York.
4. **Bateman, D. F., and R. L. Millar.** 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. **4**:119–146.
5. **Brenner, D. J., A. G. Steigerwalt, D. P. Falcão, R. E. Weaver, and G. R. Fanning.** 1976. Characterization of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by deoxyribonucleic acid hybridization and by biochemical reactions. Int. J. Syst. Bacteriol. **26**:180–194.
6. **Brenner, D. J., A. G. Steigerwalt, G. V. Miklos, and G. R. Fanning.** 1973. Deoxyribonucleic acid relatedness among Erwiniae and other *Enterobacteriaceae*: the soft-rot organisms (genus *Pectobacterium* Waldee). Int. J. Syst. Bacteriol. **23**:205–216.
7. **Brown, C., and R. J. Seidler.** 1973. Potential pathogens in the environment: *Klebsiella pneumoniae*, a taxonomic and ecological enigma. Appl. Microbiol. **25**:900–904.
8. **Campbell, L. M., G. Michaels, R. D. Klein, and I. L. Roth.** 1976. Isolation of *Klebsiella pneumoniae* from lake water. Can. J. Microbiol. **22**:1762–1767.
9. **Chatterjee, A. K., R. F. Buss, and M. P. Starr.** 1977. Unusual susceptibility of *Erwinia amylovora* to antibacterial agents in relation to the barrier function of its cell envelope. Antimicrob. Agents Chemother. **11**:897–905.
10. **Chatterjee, A. K., and M. P. Starr.** 1972. Genetic transfer of episomic elements among *Erwinia* species and other enterobacteria: F′*lac*⁺. J. Bacteriol. **111**: 169–176.
11. **Cowan, S. T.** 1974. *Enterobacteriaceae*, p. 290–293. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Co., Baltimore.
12. **Davis, B. R., and W. H. Ewing.** 1964. Lipolytic, pectolytic, and alginolytic activities of Enterobacteriaceae. J. Bacteriol. **88**:16–19.
13. **Dye, D. W.** 1969. A taxonomic study of the genus *Erwinia*. II. The "carotovora" group. N. Z. J. Sci. **12**:18–97.
14. **Garibaldi, A., and D. F. Bateman.** 1971. Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. Physiol. Plant Pathol. **1**:25–40.
15. **Goto, M., and N. Okabe.** 1962. Studies on pectin-methylesterase secreted by *Erwinia carotovora* (Jones) Holland, with special reference to the cultural conditions relating to the enzyme production and the difference of enzyme activity due to the kind of strains. Ann. Phytopathol. Soc. Jpn. **27**:1–9.
16. **Graham, D. C.** 1964. Taxonomy of the soft-rot coliform bacteria. Annu. Rev. Phytopathol. **2**:13–42.
17. **Harvey, S., J. R. Greenwood, M. J. Pickett, and R. A. Mah.** 1976. Recovery of *Yersinia enterocolitica* from streams and lakes of California. Appl. Environ. Microbiol. **32**:352–354.
18. **Hatanaka, C., and J. Ozawa.** 1973. Effect of metal ions on activity of exopectic acid transeliminase of *Erwinia* sp. Agric. Biol. Chem. **37**:593–597.
19. **Hildebrand, D. C.** 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens. Phytopathology **61**:1430–1436.
20. **Kilgore, W. W., and M. P. Starr.** 1959. Catabolism of galacturonic and glucuronic acids by *Erwinia carotovora*. J. Biol. Chem. **234**:2227–2235.
21. **Lakso, J. U., and M. P. Starr.** 1970. Comparative injuriousness to plants of *Erwinia* spp. and other enterobacteria from plants and animals. J. Appl. Bacteriol. **33**:692–707.
22. **Langdon, R. G.** 1966. Glucose-6-phosphate dehydrogenase from erythrocytes. Methods Enzymol. **9**:126–131.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
24. **Moran, F., S. Nasuno, and M. P. Starr.** 1968. Extracellular and intracellular polygalacturonic acid *trans*-eliminases of *Erwinia carotovora*. Arch. Biochem. Biophys. **123**:298–306.
25. **Moran, F., S. Nasuno, and M. P. Starr.** 1968. Oligogalacturonide *trans*-eliminase of *Erwinia carotovora*. Arch. Biochem. Biophys. **125**:734–741.
26. **Moran, F., and M. P. Starr.** 1969. Metabolic regulation of polygalacturonic acid *trans*-eliminase in *Erwinia*. Eur. J. Biochem. **11**:291–295.
27. **Murata, N., and M. P. Starr.** 1974. Intrageneric clustering and divergence of *Erwinia* strains from plants and man in the light of deoxyribonucleic acid segmental homology. Can. J. Microbiol. **20**:1545–1565.
28. **Nasuno, S., and M. P. Starr.** 1966. Polygalacturonase of *Erwinia carotovora*. J. Biol. Chem. **241**:5298–5306.
29. **Northcote, D. H.** 1972. Chemistry of the plant cell wall. Annu. Rev. Plant Physiol. **23**:113–132.
30. **Skerman, V. B. D. (ed.).** 1969. Abstracts of microbiological methods. Wiley-Interscience, New York.
31. **Starr, M. P.** 1947. The causal agent of bacterial root and stem disease of guayule. Phytopathology **37**:291–300.
32. **Starr, M. P., and A. K. Chatterjee.** 1972. The genus *Erwinia*: enterobacteria pathogenic to plants and animals. Annu. Rev. Microbiol. **26**:389–426.
33. **Starr, M. P., and F. Moran.** 1962. Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. Science **135**:920–921.
34. **von Riesen, V. L.** 1975. Polypectate digestion by *Yersinia*. J. Clin. Microbiol. **2**:552–553.
35. **von Riesen, V. L.** 1976. Pectinolytic, indole-positive strains of *Klebsiella pneumoniae*. Int. J. Syst. Bacteriol. **26**:143–145.