Isolation and Characterization of Strains of *Erwinia ananas* from Honeydew Melons

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ABSTRACT

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Strains of a yellow-pigmented bacterium causing firm, brown lesions on honeydew melons were isolated from shipments from South and Central America and from California. The bacteria were gram-negative, non-acid fast, motile, facultative anaerobic rods (0.5–1.0 × 1.0–2.5 μm) that produced a yellow pigment on nutrient agar with major visible absorption peaks at 420, 446, and 467 nm, and R_f of 0.75–0.83 on silica gel plates with methanol as the solvent. Strains were positive for catalase, beta-galactosidase, gelatinase, beta-lactamase, phosphatase, H_2S, and indole production and formed acid from cellobiose, glycerol, and melibiose. *Erwinia ananas* (ATCC 11530 and 23822), a pathogen of pineapple fruits, was identical to the melon isolates. All were pathogenic to pineapple and melon, had identical fatty acid profiles and mole percent guanine and cytosine in the DNA, and cross-reacted serologically. Strains isolated from California melons were highly pathogenic and differed in the intensity of indole and beta-lactamase reactions and in serological properties.

In December 1981, a boat shipment of 8,000 boxes (72,000 kg) of honeydew melons (*Cucumis melo* L.) arrived in New York from Ecuador. Twenty percent of the melons were affected by conspicuous yellow-brown, smooth, firm lesions up to 40 mm in diameter, which severely reduced market value. Isolations of infected rind tissues on nutrient agar yielded yellow colonies of rod-shaped bacteria. The bacterium was also isolated in 1982 from similar lesions on melons in the New York markets received from other shipping points in South and Central America and from melons originating in California. In wound inoculation tests, isolates caused firm, yellow-brown lesions on melons after incubation for 10 days at 21 C. The disease was called "brown spot," and based on preliminary tests, the causal bacterium was identified as a strain of *Erwinia herbicola* (Lohnis) Dye (2,21).

The Herbicola-Agglomerans group of *Erwinia* includes bacteria that are saprophytic and parasitic to plants (5,8,12). In the first edition of Bergey's Manual of Systematic Bacteriology Vol. 1, the species designation *Erwinia ananas* Serrano was constructed to accommodate the only member of the *E. herbicola* group that was phytopathogenic (12). The bacterium was previously known as *E. herbicola* var. *ananas* (Serrano) Dye, causing soft rot of pineapple fruitlets (5). The existence of only one pathogenic form in the *E. herbicola* group has led to the uncertainty of its status as a separate species (7). The extension of the host range of *E. ananas* to honeydew melons, as reported herein, would help confirm its distinct taxonomic position.

This report further documents the pathogenicity of the yellow-pigmented bacterium to fruit of honeydew melon and presents physiological, biochemical, and serological evidence that the bacterium is the same as *E. ananas*.

MATERIALS AND METHODS

Isolation and maintenance of bacterial strains. Honeydew melons showing symptoms of brown spot were obtained from wholesale importers in New York and from the New York City Terminal Market at Hunt's Point. Melon surfaces were sterilized with 95% ethanol for 1 min, and 2-mm sections of surface rind at lesion margins were aseptically cut and transferred to trypticase-soy agar (TSA) or King's Medium B (KB). Plates were incubated at 21 C for 7 days, by which time yellow-pigmented bacteria developed around the rind sections. Bacteria were successively cloned three times from single colonies. The following strains were used in this study: X4 (ATCC 35396) and X10 from melons imported from Guatemala; X5 (ATCC 35397) and X12 from melons from Venezuela; X6 (ATCC 35398), X7 (ATCC 35399), and X14 from melons from Ecuador; X9 (ATCC 35400) and X9R from melons shipped from California; and *E. h.* var. *ananas*, ATCC 11530 and ATCC 23822 obtained from the American Type Culture Collection (ATCC), Rockville, MD. Cultures were maintained on glucose-yeast extract-calcium carbonate agar (18) at 1 C and subcultured every 6 wk. Freeze-dried pure cultures were maintained in the laboratory and in deposit at the ATCC. Cells, as required for tests, were grown on trypticase-soy broth (Difco), harvested after 24 hr (late log phase) by centrifugation, and washed three times with physiological saline.

Pathogenicity tests. Pathogenicity of strains was tested on sound honeydew melons and on pineapple fruit obtained from the wholesale market. Melons were surface-sterilized and wounded with a series of 2–3-mm scalpel cuts across the surface. Wounds were inoculated with 10 μl of a bacterial suspension prepared by suspending bacteria from a 24-hr TSA culture in 10 ml of 0.01 M phosphate buffer (pH 7) to a density of approximately 10^7 colony-forming units (cfu)/ml. Pineapples were similarly inoculated on fruitlet surfaces. Typically, there were 12–15 inoculation sites per fruit with three to four sites per strain tested. Three wound sites on each fruit were inoculated with buffer only. Inoculated fruit were held at 21 C and about 95% relative humidity for 2 wk. A brown to yellow-brown margin of firm discolored tissue extending at least 2 mm beyond the inoculation site was considered evidence of infection. Bacteria were reisolated on TSA from tissues from infected areas to confirm the identity of the pathogen.

Morphological examinations and general culture methods. Colonies were examined at 25× with a dissecting microscope, and unstained cells examined at 450× with a phase contrast microscope. Gram staining was performed by the method of Huckner and acid-fast stains by the method of Ziehl–Neelsen (4). Oxygen requirements at 27 C were determined by growth in stab-inoculated tubes containing semi-solid nutrient agar layered with mineral oil and by culturing in nutrient broth flasks attached to a nitrogen gas atmosphere for 72 hr. A known aerobe, *Xanthomonas campestris* pv. *cucurbitae* (Bryan) Dye was used as a control. Other tests useful in characterizing *Erwinia* and used in this work were growth on Miller–Schroth (MS) medium (19), mucoidal growth on

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nutrient agar plus 5% glucose, and observations for motility in stab-inoculated semi-solid medium.

Analysis of pigments. Methanol-soluble pigments were extracted and concentrated from 24-hr TSA cultures (11). Extracts were chromatographed on thin-layer chromatography (TLC) sheets precoated with silica gel 60 (E. Merck, Gibbstown, NJ) using spectrophotometric-grade methanol as the developing solvent. Visible absorption spectra of pigments in methanol were determined with a Beckman Model 26 spectrophotometer (Beckman Instruments, Fullerton, CA). Xanthomonadin pigments extracted by the same method from cultures of *X. c.* pvs. *cucurbitae*, *vesicatoria*, and *phaseolicola* (from E. Civerolo, USDA, Beltsville, MD) were used for comparison.

Physiological tests. Acid production from adonitol, arabinose, cellobiose, dulcitol, galactose, glucose, glycerol, maltose, and melibiose was tested with phenol red broth base, pH 7.4, to which was added the filter-sterilized carbohydrate solutions (15). Four milliliters of phenol red broth containing carbohydrate were inoculated with 0.1 ml of a bacterial suspension (approximately 4×10^6 cfu/ml) and incubated at 27 C for 72 hr. Asparagine utilization was tested by the method of Dye (6) with acid-washed glassware and inorganic salt-asparagine medium inoculated and incubated with agitation at 27 C for 10 days.

Catalase production was tested by mixing bacteria from 24-hr TSA cultures on a slide with 3% hydrogen peroxide. Oxidase was tested by applying bacteria to filter paper saturated with 1% tetramethyl-*p*-phenylenediamine dichloride. (No color change within the first minute indicated a negative reaction.) Coagulase was tested with commercial plasma (Bacto-coagulase plasma, Difco) by the tube method; phosphatase activity was determined on nutrient broth supplemented with 1% sodium phenolphthalein diphosphate and urease activity on Rustigian and Stuart's urea broth (15). Beta-lactamase was detected by the paper strip method described by Oberhofer and Towle (18). Gelatinase production was determined by liquefaction of commercial gelatin medium (Difco) in plates. Beta-galactosidase was detected by the ortho-nitrophenol-beta-galactopyranoside broth method 1 (15). Lipase production was tested on nutrient agar amended with 1% Tween 80 plus 0.01% $CaCl_2 \cdot H_2O$ and also on egg yolk agar (20).

Hydrogen sulfide production was tested by suspending acetate-impregnated strips (Difco) over nutrient broth cultures. Indole production was determined by the microtechnique method of Arnold and Weaver using broth 2 (1). Test for nitrate reduction was conducted as described in MacFaddin (15). Pectate degradation was determined on the crystal-violet pectate agar medium of Cuppels and Kelman (3).

Serology. Serological tests were by indirect enzyme-linked immunosorbent assay (ELISA) using the avidin-biotin-peroxidase complex technique (9) and an EIA Gilford PR-50 automatic analyzer system (Gilford Instrument Labs, Oberlin, OH). Membrane proteins of different strains of *E. ananas* were used as antigens. Cells broken in a French press were washed twice in saline (0.15 M NaCl), and 10 g (dry wt.) was then extracted by shaking with glass beads for 2 hr in 200 ml of 0.2 M LiCl. The cell homogenate was clarified by centrifugation at 27,000 *g* for 25 min at 4 C. The protein in the supernatant was washed twice in saline, pelleted by centrifugation at 100,000 *g* for 2 hr, and diluted to 20 μg of protein per milliliter in 0.05 M carbonate buffer (pH 9.6) for ELISA tests (23). Antibodies were prepared from serum from BALB/c mice immunized with membrane protein of *E. ananas* strain X5 and used at 10× and 100× concentration in ELISA tests. Antibodies were also prepared from hybridoma cells produced by fusion between NS-1 myeloma cells and spleen cells. Hybridoma fusion protocol and hybridoma selection procedures using hypoxanthine-aminopterin-thymidine medium are described elsewhere (10,13). Hybridomas were screened for antibody production by ELISA using undiluted culture fluids.

Fatty acid analysis. Fatty acids were determined by gas-liquid chromatography (GLC) using a Varian Model 3700 Gas Chromatograph (Varian Instruments, Palo Alto, CA) equipped with a stream splitter. Cells were grown on KB agar for 1, 3, and 6 days at 27 C. Saponification and methylation were by a modification of the method of Sasser (14). The hexane-diethyl ether extract was concentrated under a stream of prepurified N gas, and 1 μl of concentrate was injected into the GLC. Chromatography conditions were: a 15 M, 0.25-μm fused-silica capillary column, helium carrier gas flowing at 30 ml/min; air at 300 ml/min; and hydrogen at 30 ml/min. Temperature was programmed at 100–225 C at 8 C per minute. Data were handled with a Model 4270 Integrator (Varian Associates) and expressed as percentage of total peak area. Individual fatty acids were identified by co-chromatography using known standards (Supelco, Supelco Park, Bellefonte, PA). Confirmation of unsaturated, hydroxy-substituted, and cyclopropane acids was by methods previously described (22). Data were averaged from extractions of 1-, 3-, and 6-day-old cultures per strain and expressed as percentage of total fatty acids. Major fatty acids were defined as those composing at least 1% of the total.

DNA analysis. DNA was isolated by the method of Marmur (16) from cells of *E. ananas* strains X5, X9, and type strain (ATCC 11530). The purity of extracted DNA was checked spectrophotometrically at 260 and 280 nm. Mole percent guanine and cytosine content (G + C) was determined by melting point (T_m) analysis with a Coleman 575 thermo-spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). T_m data were converted to mole percent G + C by the equation $T_m = 69.3 + 0.41$ (G + C) (17). DNA from the type strain ATCC 11530 was used as a reference.

TABLE 1. Pathogenicity of strains of *Erwinia ananas* to honeydew melon and to pineapple fruits

E. ananas		Melon		Pineapple	
Strain	Source	Inoculation sites[a]	Infection (%)	Inoculation sites[b]	Infection (%)
X4	Guatemala	38	66	18	33
X5	Venezuela	40	72	21	43
X7	Ecuador	40	60	18	50
X9	California	40	92	18	45
T1	ATCC 11530	22	55	12	50
Control[c]		20	0	9	0

[a] Three to four inoculation sites per melon, replicated three times.
[b] Three inoculation sites per fruit.
[c] Fruit wounded by scalpel only.

RESULTS

Morphology and cultural characteristics. Cells of all strains were motile, single, straight rods averaging 0.5–1.0 by 1.0–3.5 μm. Colonies on KB agar were typically yellow, domed, shining, and mucoid. Growth on KB, nutrient agar, and TSA at 27 C was abundant with colony diameters reaching 5–9 mm. All strains grew at 36 C, grew on MS agar, and produced mucoid growth on nutrient agar supplemented with 5% glucose. Mucoid growth and yellow pigmentation were more abundant with the California strains X9 and X9R than in the other strains. All strains grew under anaerobic conditions in deep, stab-inoculated tubes or in nutrient broth under nitrogen atmosphere—conditions that inhibited the growth of *X. c.* pv. *cucurbitae*, a known aerobe.

Pigmentation. The major absorption peak of the methanol-soluble pigments of all strains of *E. ananas* was at 446 nm with shoulders at 420 and 467. A minor peak occurred at 334 nm. The R_f (methanol) values of the *E. ananas* pigments were 0.75–0.83. In contrast, the absorption maxima of the xanthomonadin from *X. campestris* was at 420, 445 (major), and 460 nm; and the R_f value of the single band was 0.45.

Pathogenicity. All strains tested caused lesions on inoculation sites on honeydew melons and pineapples (Table 1). Lesions on melons began with necrosis around wound margins and developed into firm brown areas measuring up to 25 mm across the surface but were confined to tissue within 10 mm of the surface. In repeated tests, isolates X9 and X9R were noticeably more pathogenic to melon, causing an average of 92% infection of wound sites compared with 55–72% with the other strains. Tissue penetration

was also deeper, sometimes extending to the cavity if inoculation was more than 4–6 mm deep. Statistical analysis of the infection data on melon confirmed the differences as significant.

All strains were pathogenic on pineapple fruits (Table 1). Lesions were firm and were shaded brown because of discoloration of tissues underneath the epidermal layer. Rate of lesion development was approximately the same on pineapple as on melons, but tissue penetration was deeper in pineapple. After 2 wk of incubation at 21 C, discoloration spread an average of 22 mm across the surface, but an average of 57 mm into the center.

Physiological characteristics. Reactions of the 11 strains to selected physiological tests were characteristic of those of *E. ananas*. Tests for which all strains were positive were: acid production from arabinose, cellobiose, galactose, glucose, glycerol, maltose, and melibiose; and production of catalase, phosphatase, beta-lactamase, gelatinase, beta-galactosidase, H_2S, and indole. Tests for which all strains were negative were: acid production from adonitol and from dulcitol; production of oxidase, nitrogen reductase, coagulase, urease, and lipase; and pectin degradation. Except for the Ecuadorean strains (X6, X7, and X14), all were able to use asparagine. Significant differences among strains in the degree of positive reactions were also obtained with the beta-lactamase and indole tests. The California strains, X9 and X9R, had a weaker beta-lactamase reaction and a stronger indole reaction than the other strains. Tests considered critical in distinguishing *E. herbicola* from *E. ananas* were consistently typical for *E. ananas*. Among them were acid utilization from cellobiose (+), glycerol (+), melibiose (+), and the indole (+) and nitrogen reductase (−) tests.

Serology. Serum antibodies from blood of mouse immunized with strain X5 reacted with all strains of *E. ananas* isolated from melon and with the two ATCC pineapple strains (Table 2). Reactions to strain X9, isolated from California melons, were consistently weaker than those with other strains. A stable hybridoma was selected that secreted antibodies that reacted with strains X5 and X7 but not with X9, ATCC 11530, or ATCC 23822.

Fatty acid analyses. Relative distribution and quantities of cellular fatty acids were similar for all strains tested (Table 3). Major constituents of the saturated, straight-chain acids in 1-, 3-, and 6-day-old cells (and their average concentrations) were: 12:0 (4.1%), 14:0 (3.9%), and 16:0 (34.6%). Major unsaturated acids were 16:1 (14.5%) and 18:1 (20.0%). Other identified components were a hydroxy-substituted acid, 14:0-30H (6.5%), and one cyclopropane acid, cyclo-17:0 (6.3%). As cells aged from 1 to 6 days, the concentration of total saturated, even-carbon straight chains (± standard error) decreased from 43.5 ± 0.3 to 41.9 ± 0.5%. Saturated odd-carbon chains increased from 0.4 ± 0.3 to 2.3 ± 0.8%, and unsaturated acids decreased from 38.1 ± 1.4 to 32.0 ± 1.2%. There was also a tendency for increases in the percentage of hydroxy-substituted acids (7.6 ± 0.7 to 8.8 ± 0.6%) and of cyclopropane acids (5.5 ± 0.6 to 7.4 ± 0.8%) between 1 and 6 days. The ratio of saturated to unsaturated acids ranged from 1.16 to 1.47.

DNA analyses. The mole percent G + C of melon strains X5 and X9 and *E. ananas* ATCC 11530 was in the range of 53.71 and 54.71—comparable to the previously published mole percent G + C values for the species (12). The thermal denaturation curves of all three strains were similar, and T_m values were 92.0, 92.0, and 91.75 for X5, X9, and ATCC 11530, respectively.

DISCUSSION

The yellow bacteria isolated from firm, brown lesions on honeydew melon were identified as strains of *E. ananas*, a species newly established as a distinct taxonomic group. Because *E. ananas* was previously known as a pathogen only of pineapple fruits, the characterization of strains pathogenic to another host strengthens the concept of this bacterium being a species distinct from *E. herbicola*. It is likely that in the future other hosts will be discovered and that *E. ananas* will prove to be a more diversified pathogen.

The strains of *E. ananas* studied for this report were all similar in pigmentation, physiological properties, biochemical composition, and pathogenicity. A limited range of variation was nevertheless evident. The California strains X9 and X9R were more aggressive in their pathogenicity (in terms of rapidity and severity of lesion development) than the South and Central American strains or the ATCC strains from pineapple. They were also distinctive in their weak beta-lactamase and strong indole reactions, not typical of the other strains.

The serological and some biochemical data of the melon strains from California suggested they were more closely related to the ATCC pineapple strains than to the melon strains from South and Central America. The most direct evidence was serological. Hybridoma antibody, reacting with the South American strains

TABLE 2. Avidin-biotin-peroxidase complex ABC reactions, by enzyme-linked immunosorbent assay, to membrane protein of different strains of *Erwinia ananas* with immunized mouse blood antibody and with antibody produced by hybridoma cells

E. ananas strain	Antibody[a]	
	Mouse blood serum[b]	Hybridoma[c]
X5	0.297 ± 0.006[d]	3.215 ± 0.128
X7	0.223 ± 0.006	3.283 ± 0.020
X9	0.157 ± 0.022	0
ATCC 11530	0.232 ± 0.015	0
ATCC 23822	0.225 ± 0.012	0
Buffer control	0	0

[a] Antibody controls, serum from normal mouse and hybridoma culture medium, were negative.
[b] Mouse immunized against membrane protein of *E. ananas* strain X5.
[c] Hybridomas from fusion between NS-1 myeloma cells and spleen cells from mouse immunized with *E. ananas* strain X5.
[d] Average absorbance at 460 nm ± standard error, based on three replicated tests.

TABLE 3. Class analysis of cellular fatty acids from *Erwinia ananas* isolated from honeydew melons and from isolates obtained from the American Type Culture Collection

Fatty acid class	Percent total cellular fatty acids[a]						
	Melon isolates					ATCC[b]	
	X4	X5	X6	X7	X9	11530	23822
Saturated straight chain							
Even-carbons	42.3 ± 0.9	42.9 ± 0.2	42.3 ± 0.7	45.3 ± 0.5	45.4 ± 0.8	42.0 ± 1.0	43.5 ± 0.7
Odd-carbons	0.8 ± 0.3	1.5 ± 0.7	1.2 ± 0.6	0.9 ± 0.7	0.9 ± 0.5	2.1 ± 0.8	1.2 ± 0.7
Unsaturated acids	37.1 ± 1.4	37.1 ± 1.9	37.9 ± 1.6	35.0 ± 1.7	32.2 ± 1.5	30.0 ± 1.8	33.8 ± 1.2
Hydroxy-substituted acids	7.9 ± 0.7	7.9 ± 0.6	7.5 ± 0.7	6.2 ± 0.6	6.9 ± 0.7	9.8 ± 0.8	8.1 ± 0.4
Branched-chain acids	1.5 ± 0.5	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.4 ± 0.4	0.9 ± 0.2
Cyclopropane acids	6.4 ± 0.8	5.0 ± 0.1	5.2 ± 0.3	7.4 ± 0.8	8.6 ± 0.8	8.2 ± 1.6	7.4 ± 1.1
Unidentified components	3.6 ± 0.5	4.6 ± 0.6	4.6 ± 0.7	3.1 ± 0.5	4.2 ± 0.5	6.2 ± 1.1	4.6 ± 0.5
Ratio saturated/unsaturated	1.16	1.20	1.15	1.36	1.45	1.46	1.47

[a] Average percentage of total fatty acids from 1-, 3-, and 6-day-old cultures per strain.
[b] Originally isolated from pineapple fruitlets (5).

X5 and X7, apparently interacted with antigenic determinants not present or masked in strain X9 or in the ATCC strain. Strain X9 and the ATCC strains also tended to have lower percentages of unsaturated fatty acids (30.0–33.8%) than the other strains (35.0–37.9%). These percentages affected the physiologically important saturated/unsaturated acid ratios. The ratio of the California strain X9 (1.45) was almost identical to those of the ATCC strains (1.46 and 1.47) and consistently higher than those of the other melon strains (1.15–1.36).

No conclusions can be drawn regarding any natural strain groupings within the species *E. ananas*. Although there seem to be observable differences in a number of characteristics, a greater number of strains need to be studied. The diversity among the *E. ananas* strains can be expected to be as broad as that among the nonpathogenic *E. herbicola* group. Further studies are also needed on the differences between *E. herbicola* and *E. ananas*, particularly on any analytical techniques that may provide rapid identification of the pathogenic forms. An antiserum or biochemical test that is unique to the pathogens would also provide clues to the existence of any gene products associated with pathogenicity.

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