Internal Decay of Onions Caused by *Enterobacter cloacae*

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ABSTRACT

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Enterobacter cloacae was consistently isolated from diseased onions (*Allium cepa*) collected from several fields of mature plants in California's San Joaquin Valley following a period of extreme heat (air temperatures 40–45 C). Several of the innermost leaf bases of affected bulbs were discolored (brown to black) and flaccid. All nine strains of *E. cloacae* tested, including those isolated from onions and other plant species and the type strain, reproduced these symptoms in inoculated onion bulbs incubated at 37 C; disease was less pronounced at 22 C. *Enterobacter cloacae* did not cause disease in young, growing onions.

More than half of the approximately 35,000 acres (total value of greater than $100 million) of onions (*Allium cepa* L.) produced annually in California are grown in the San Joaquin Valley (1). An internal decay of bulbs of unknown etiology affected approximately 1–5% of the crop in at least four fields of processing onions in the summer of 1988. The disease was observed in mature bulbs following a period of extreme heat during which air temperatures reached 40–45 C, not an uncommon occurrence in this region. Symptoms of the disease included brown to black discoloration and decay of the inner scales. No evidence of disease was present on the exterior of the bulbs. The objectives of this work were to identify the causal agent of this newly observed disease of onions and to investigate the effect of temperature on disease development.

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MATERIALS AND METHODS

Isolation from field specimens. Samples of mature onions were collected from three fields in Fresno County and one field in San Joaquin County, California, during August and September 1988. A few milligrams of tissue from flaccid, discolored inner scales were suspended in 1–3 ml of sterile, distilled water. A 0.1-ml aliquot from each of five 10-fold serial dilutions of the resulting suspension was spread on nutrient agar (NA) (Difco) and Miller-Schroth medium (MS) (10). Plates were incubated at room temperature (approximately 23 C). Colonies to be identified and tested for pathogenicity were picked from isolation plates after 2–4 days.

Identification of strains. Known strains of *Enterobacter cloacae* (Jordan) Hormaeche and Edwards used for reference in identification included the species type, ATCC 13047, isolated from human spinal fluid (American Type Culture Collection, Rockville, MD), EcH-1 and EcCT-501 isolated from cotton (*Gossypium* L. sp.) hypocotyls

(11), and NRRL B-14095 (ATCC 39978) and NRRL B-14096 isolated from seeds of cucumber (*Cucumis sativus* L.) (7). Additional bacteria used for comparison in pathogenicity tests included three recently isolated strains of *Erwinia chrysanthemi* Burkholder et al from Dickey's subdivision IV (6), CCPB 147, CCPB 188, and CCPB 189, isolated from decaying onion, soft rotted stems of potato (*Solanum tuberosum* L.), and rotting crowns of primrose (*Primula* L. sp.), respectively; and a strain of *Pseudomonas cepacia* (Burkholder) Palleroni and Holmes, CCPB 176, isolated from onions with sour skin disease (3). API20E strips (Analytab Products, Plainview, NY) were used according to the manufacturer's instructions, except that bacteria were suspended in sterile, distilled water rather than in saline and incubations were at 28 C. Pectolytic activity was determined by production of pits on crystal violet-pectate agar (5) and by reaction on modified pectate-yeast-extract agar (PecYA) (14) consisting of (per liter) 15 g of Bacto agar (Difco), 10 g of sodium polypectate (Sigma), and 1 g of Bacto yeast extract (Difco), adjusted to pH 7.8 before autoclaving. Plates of PecYA on which strains had grown for 2–3 days were flooded with 2 N HCl to precipitate pectate. Colonies of pectolytic strains were surrounded by a clear halo. Oxygen requirements and gas production from glucose were assessed in Hugh-Leifson's glucose O/F medium (8). All physiological tests were repeated at least two times.

Nutrient broth (Difco) shake cultures (24 hr) were held motionless for 40 min to allow regrowth of sheared flagella. A loopful of cultured broth was placed on carbon-coated Formvar film on a copper grid for 30 sec and then removed with absorbent paper. Patterns of flagellation were examined by transmission electron microscopy after negative staining of grids with 2% phosphotungstic acid.

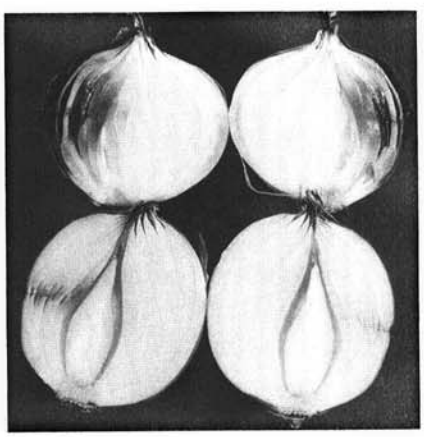

Fig. 1. Symptoms of internal decay of onion caused by *Enterobacter cloacae* two weeks after inoculation. Onion bulbs were injected with 0.2 ml of an aqueous suspension of approximately 10^8 cfu/ml of strains CCPB 162 (top) and CCPB 164 (bottom), and incubated at 37 C.

Pathogenicity tests. Inoculum was cultured for 1 day at 28 C on NA except for *P. cepacia*, which was grown on King's medium B (9). Cultures were suspended in sterile, distilled water and adjusted to a turbidity of 0.1 at A_{600} (approximately 10^8 colony forming units [cfu] per millileter). Growing onion plants (Southport White Globe), started from seed in one test and sets in another, were inoculated before bulb formation approximately 8 wk after sowing seed and 3 wk after planting sets. A syringe and hypodermic needle were used to inject the base of each plant with 0.1–0.2 ml of inoculum or sterile, distilled water. Three pots (three to seven plants per pot) inoculated with each strain were held in a greenhouse (approximately 21–27 C) or in a 37 C growth chamber. To inoculate mature, stored bulbs, 0.1–0.2 ml of inoculum or sterile, distilled water was injected into the center of each bulb. Three bulbs per strain were held in moist chambers at room temperature (approximately 22 C) or at 37 C. Two weeks later, inoculation bulbs were coded, bisected longitudinally in a plane including the inoculation wound, and rated for disease severity; growing plants were uprooted and treated in a similar fashion. Symptoms in bulbs were rated on a scale of 0–5 where 0 = no discolored or flaccid tissue, 1 = wound discolored, 2 = tissue up to 1 cm from wound discolored or flaccid, 3 = innermost leaf bases generally discolored or flaccid, 4 = half of bulb affected, and 5 = entire bulb affected. Fisher's protected LSD was used to compare treatments in a two-way analysis of variance.

RESULTS

Isolations from field specimens yielded yellow-orange, mucoid colonies on MS medium, and mucoid, cream-colored colonies on NA. All strains recovered (including two from each onion specimen) and all known strains of *E. cloacae* were positive in the following tests: motility by peritrichous flagellation; facultative anaerobiosis; beta-galactosidase; arginine dihydrolase; ornithine decarboxylase; utilization of citrate; production of acetoin; acid from glucose, mannitol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose; reduction of nitrate to nitrite; and gas production from glucose. Unknown bacteria recovered from onions and all reference strains of *E. cloacae* were negative in the following tests: pectolytic activity, lysine decarboxylase, H_2S production, urease, tryptophan deaminase, indole production, gelatinase, acid from inositol, denitrification, and oxidase. Based on the perfect correspondence between test results of the unknown strains isolated from onion and the reference strains, unknowns were identified as *E. cloacae*.

The pathogenicity of four strains from onion (one from each of the four field incidents, CCPB 162, CCPB 163, CCPB 164, and CCPB 170) was compared with that of the reference strains of *E. cloacae*, *E. chrysanthemi*, and *P. cepacia*. All strains of *E. cloacae* caused brown discoloration and loss of turgor of the innermost swollen leaf bases (Fig. 1). The bacteria were recovered in nearly pure culture from inoculated bulbs. Control bulbs inoculated with sterile, distilled water were free of symptoms. Inoculated bulbs incubated at 37 C were more severely affected ($P < 0.01$ and $P < 0.03$ in two independent trials, respectively) than those incubated at 22 C; variation among strains of *E. cloacae* was significant in one experiment, but not in another ($P < 0.02$ and $P > 0.20$, respectively; Table 1). None of the strains of *E. cloacae* were as destructive as *P.*

Table 1. Effect of temperature and strain on disease development in onion bulbs inoculated with *Enterobacter cloacae*[w]

Strain	Source	Trial 1		Trial 2	
		37 C	22 C	37 C	22 C
CCPB 170	Onion	1.0[x] a	0.7 a	3.0[y]	2.2
EcCT-501	Cotton	2.0 ab	1.0 ab	2.8	3.2
ATCC 13047	Man	2.0 ab	1.3 ab	2.5	3.0
CCPB 163	Onion	2.0 ab	2.0 ab	3.7	3.0
EcH-1	Cotton	2.3 ab	1.3 ab	2.2	2.0
CCPB 164	Onion	2.3 ab	2.3 b	3.2	2.7
NRRL B-14096	Cucumber	2.3 ab	2.3 b	4.5	2.7
CCPB 162	Onion	2.7 b	1.0 ab	3.2	2.3
NRRL B-14095	Cucumber	3.2 b	2.3 b	3.3	2.3
Temperature means[z]		2.2	1.6	3.2	2.6

[w] Disease development was rated 2 wk after onion bulbs were injected with approximately 0.2 ml of an aqueous bacterial suspension of approximately 10^8 cfu/ml or sterile, distilled water. Disease ratings: 0 = no discolored or flaccid tissue, 1 = wound discolored, 2 = tissue up to 1 cm from wound discolored or flaccid, 3 = innermost leaf bases discolored or flaccid, 4 = half of bulb affected, 5 = entire bulb affected.

[x] Each value is the mean of three replicates; sterile, distilled water controls were not affected (rating = 0). Means followed by the same letter in a given column are not significantly different based on Fisher's protected $LSD_{0.05} = 1.38$.

[y] Significant differences among strains were not detected in trial 2.

[z] Disease was more severe at 37 C than at 22 C in both trials, $P < 0.03$.

cepacia (CCPB 176; mean disease rating of 3.8 and 3.9 at 37 and 22 C, respectively) or the strain of *E. chrysanthemi* isolated from onion (CCPB 147; mean disease rating of 5.0 at both temperatures). Strains of *E. chrysanthemi* isolated from primrose and potato (CCPB 188 and CCPB 189) were weakly pathogenic at 37 C (no disease in one trial, mean disease rating of 1.2 in the second) and somewhat more aggressive at 22 C (mean disease ratings of 2.0 and 3.6 in two trials, respectively). None of the strains of *E. cloacae* tested nor the reference strain of *P. cepacia* were pathogenic in actively growing onions at either temperature. However, plants inoculated with strain CCPB 147 of *E. chrysanthemi* and incubated at 37 C were killed outright; 2 wk after inoculation the bases of these plants had disintegrated and the tops were completely necrotic. In contrast, plants inoculated with CCPB 147 and incubated at 22–27 C were wilted and chlorotic, with extensive soft rot of leaf bases.

DISCUSSION

This is the first report of *E. cloacae* causing a disease of plants in the field. Previous reports have implicated *E. cloacae* in a high-temperature breakdown of onions in storage, losses in mung bean (*Phaseolus aureus* Roxb.) sprout production, and internal yellowing of harvested papayas (*Carica papaya* L.) (4,12,16). *Enterobacter cloacae* was among several species of enteric bacteria isolated in the other report of its association with onions (4); in our study *E. cloacae* was the only bacterium consistently isolated. Variation in pathogenicity of *E. chrysanthemi* to onions based on source of strains has been reported before, though the subdivisions to which the strains tested belonged were not identified (15). Pathogenic variation has been observed in Dickey's subdivision IV of *E. chrysanthemi*, but specialization for onions has not been described previously (2).

There was no evidence of physiological or pathological specialization attributable to strains of *E. cloacae* isolated from onion as compared with those from animal or other plant hosts. Those isolated from other plant species caused disease in onion, as did the type strain originally isolated from human spinal fluid. These observations bear out Nishijima et al who found no variation in pathogenicity of *E. cloacae* strains isolated from papaya, water tanks, fruit fly (*Dacus dorsalis* Hendel), or the type strain (12). The difference in severity of disease between the two pathogenicity tests reported here may be attributable to differences in host plant material. This notion is supported by the observation that strains of *E. chrysanthemi* from primrose and potato, as well as strains of *E. cloacae*, caused more severe disease in the second test. Onions used in these experiments were purchased several months apart from different sources.

We consider *E. cloacae* to be an opportunistic pathogen of onions. Strains of *E. cloacae* are common components of the microflora in many environments, including plant surfaces, water, sewage, meat, soil, hospital environments, and skin and intestinal tracts of man and other animals (7,11–13). The species is widely distributed and lacks strain specialization. The disease we observed was associated with high temperature stress, and pathogenicity of strains from various sources was greater at higher temperatures. Investigation of the normal microflora of onions to determine whether or not *E. cloacae* is commonly present and analysis of the effects of heat stress on non-wounded onions with *E. cloacae* present in internal tissues would improve our understanding of this disease.

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