Relationship of Pectolytic Clostridia and *Erwinia carotovora* Strains to Decay of Potato Tubers in Storage

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ABSTRACT

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Prevalence of pectolytic clostridia and *Erwinia* spp. was assessed in samples of 100 partially decayed potatoes collected from each of seven commercial storage bins maintained at 5 C. Pectolytic clostridia were present in 22%, *Erwinia carotovora* subsp. *carotovora* (Ecc) in 13%, and *Erwinia carotovora* subsp. *atroseptica* (Eca) in 45% of the samples. In 58% of the samples from which clostridia were isolated, Ecc and/or Eca were also present. When healthy tubers from storage bins were injured uniformly and incubated at 20 C for 96 hr in a mist chamber, the proportion of clostridia to *Erwinia* isolates was significantly higher than in isolations directly from storage bin samples. In vitro, clostridia and Ecc, but not Eca, grew more rapidly at temperatures from 18 to 36 C.

Additional key words: blackleg, *Solanum tuberosum* L.

An association between spore-forming anaerobic bacteria and rotting in potatoes was observed by van Tieghem as early as 1884 (24). Although other investigators made similar observations (15,25), the direct evidence that clostridia may be involved in potato soft rot in commercial storage in England was first presented by Rudd-Jones and Dowson (21). They found that under experimental conditions *Clostridium* caused decay only when *Erwinia carotovora* (Jones) Bergey, Harrison, Breed, Hammer, and Huntoon was also introduced at the same inoculation points; lesions produced by inoculations with both organisms were larger than those produced by *Erwinia* alone. Conclusive data that certain pectolytic clostridia can cause decay in tubers in the absence of *Erwinia* were presented by Lund and Nicholls (14). Other recent reports provide additional evidence that clostridia can act as primary pathogens in laboratory inoculations (10,11,18). However, the extent to which clostridia are responsible for decay of potatoes under commercial storage conditions in the United States has not been reported.

The objective of this study was to determine the prevalence of pectolytic clostridia in relation to *Erwinia* strains in the decay of potatoes in commercial storage bins in Wisconsin.

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MATERIALS AND METHODS

Potato samples. Partially decayed Russet Burbank potatoes were removed from seven bins (100 tubers per bin) in a commercial storage facility in Bancroft, WI. The storage capacity per bin was 900 metric tons. The samples represented seven different farmers' fields in the Central Sands region of Wisconsin and five different seed sources. Tubers were obtained at weekly or biweekly intervals coincident with removal of tubers from the bins for shipment. The first sample was taken 126 days and the last 190 days after harvest. In each case, bin conditions after curing were maintained at 90–95% relative humidity and 5 C. The potato samples were collected at random from various levels on the vertical face of the pile in each bin as the tubers were removed for shipment. Tubers selected for isolation had at least 25% sound tissue and were wrapped separately in small plastic bags for transport to the laboratory. Samples were held at 5 C for 20 hr before processing.

In addition to the decayed tubers, 10 healthy tubers were also selected from each bin at every sampling period to evaluate the soft rot potential of injured tubers. Ten lenticels per tuber were punctured with sterile toothpicks and then incubated in a mist chamber at 20 C for 4 days (13).

Isolation of pectolytic bacteria. Gram-negative pectolytic bacteria were isolated from decayed potatoes as follows: 0.1–0.2 g of tissue adjacent to the decayed area was removed and suspended in 10 ml of sterile distilled water. Plates of crystal violet pectate medium (CVP) were streaked with the water suspensions and incubated for 48 hr at 20–25 C (4). Pectolytic *Erwinia* spp. were tentatively identified on the basis of their distinctive colony morphology and the type of pit produced in the CVP medium (4). Pure cultures were obtained by transferring single colonies forming typical *Erwinia*-like pits on CVP to a casamino acid-peptone-glucose medium (CPG) (9) and incubating them for 48 hr at 20–25 C.

E. carotovora subsp. *atroseptica* (Van Hall) Dye (Eca) was differentiated from *E. carotovora* subsp. *carotovora* (Jones) Bergey, Harrison, Breed, Hammer, and Huntoon (Ecc) by the absence of growth at 36 C, production of reducing sugars from sucrose and acid from α-methyl glucoside (7), and reaction with an immunofluorescent antibody stain specific for Eca (1).

Pectolytic clostridia were isolated from the water suspensions prepared for the isolation of Gram-negative bacteria after the suspensions had been incubated for 1 wk at 28 C. Optimum recovery of pectolytic clostridia from decaying tissue was possible with this technique. Loopfuls of the suspensions were streaked on nutrient-dextrose agar (NDA), then exposed to chloroform for 10 min to eliminate nonspore-forming bacteria (2). The NDA plates were incubated in Gas Pak Anaerobe jars (Baltimore Biological Labs, Baltimore, MD) in an atmosphere of 10% (v/v) H_2, 10% (v/v) CO_2, and 80% (v/v) N_2 for 4 days at 28 C. Pectolytic clostridia were tentatively identified on the basis of characteristic colony morphology on NDA, cell morphology in Gram stain, catalase test after exposing the colonies to O_2 for 1 hr, and development of a slimy decay in potato tubers (Fig. 1) inoculated with pure cultures.

After inoculation with strains of *Clostridium*, tubers were incubated in a mist chamber (13) or a dew chamber

(Percival Co., Boone, IA) or were wrapped in moist paper towels and Saran wrap (5) and incubated at 20 C for 4 days.

Fungal isolations. Isolations for fungi were made from each of 101 partially decayed tubers selected at random in the course of isolations for soft rot bacteria. Small pieces of potato tissue were excised from the margins of decayed areas and placed on acidified potato-dextrose agar plates (23) and incubated for 8 days at 20–25 C. Isolated fungi were identified by R. W. Caldwell (Department of Plant Pathology, University of Wisconsin, Madison).

Growth curves. The effect of temperature on growth of two stock strains of Ecc (SR 206) and Eca (SR 8) was evaluated by comparing turbidity measurements of 100-ml CPG broth cultures in 250-ml side arm flasks using a Klett Summerson Photoelectric Colorimeter (Klett Manufacturing Co., Rochester, NY). Cultures were shaken only at the O.D. reading times.

RESULTS

Isolation of pectolytic bacteria. Several different types of pectolytic bacteria were isolated from 563 of the 688 decayed tubers sampled (Table 1). Data from each of the seven bins were consolidated since the percentages of clostridia and *Erwinia* obtained in each of the bins remained constant.

Clostridia alone or in combination with other pectolytic bacteria were isolated from 22% of the tubers sampled directly from the storage bins. In only 5% of the isolations were clostridia the only pectolytic organism obtained. The pectolytic bacterium isolated most frequently was Eca.

In the isolations from tubers that decayed after lenticel injury and mist chamber incubation, the proportion of Eca:Ecc:clostridia changed markedly from that which characterized isolations from tubers that had decayed in storage. Alone or in combination with other pectolytic bacteria, clostridia were obtained from 83% of the tubers that decayed in the mist chamber; in contrast, Eca was obtained from 13% and Ecc from 39%.

Clostridia were readily isolated from decayed tuber tissue when water suspensions prepared from the tissue were incubated for 1 wk at 28 C and then plated on NDA plates that were immediately chloroformed and incubated anaerobically at 28 C. Furthermore, pectolytic clostridia produced distinctive pulvinate, raised, slimy colonies that could be easily distinguished from colonies of nonpectolytic clostridia (Fig. 2). Cultures of Gram-positive strains exhibiting this distinctive colony type were tested for pathogenicity by inoculation into potato tubers (6). The development of a characteristic slimy, gaseous decay after 2–6 days of incubation indicated that a given strain was probably a pectolytic *Clostridium*.

Clostridia obtained by this procedure varied in colony color on the NDA medium. White colonies were isolated three times more frequently than pink or yellow colonies. All three colony types formed a very viscous slime that was extremely difficult to disperse in water. Other clostridia differing in colony morphology were frequently present in the primary plates but consistently failed to produce decay in inoculated potato tubers.

Slime production was observed in 180 of the 688 water suspensions incubated for 1 wk at 28 C. Pectolytic clostridia were isolated from 137 of these 180 tubes. To determine whether the formation of slime in the water suspensions was due to *Clostridium* spp., tubes containing small cubes (1 cm^3) of raw potato tissue in 10 ml of sterile distilled water were inoculated with a representative sample of pure cultures of pectolytic clostridia. After 1–3 days of incubation at 28 C, each cube of potato tissue was elevated by gas bubbles to the surface of the water in the tube; by the end of the first week, the cubes had become totally macerated and only slimy masses of degraded tissue remained (Fig. 3). No bacterial growth was observed in noninoculated control tubes. Production of slime was not observed in similar tests when tubes were inoculated with Ecc or Eca.

A representative group of 30 strains of pectolytic clostridia obtained in this study was characterized by methods described previously (8). Testing included the L_3 and LP_1 types previously isolated in Wisconsin (18) as well as representative cultures of other pectolytic species of *Clostridium*. Initial data indicated that the clostridia isolated from potato are closely related to the "butyric acid group" of *Clostridium* (3,15) but differ in a number of characteristics from other species of *Clostridium*.

Potatoes inoculated with clostridia alone and incubated in the mist chamber at 20 C decayed more rapidly than those inoculated with Eca alone. When potatoes were first inoculated with clostridia (10^5 cells per tuber), then injected at the same site with Eca (10^5 cells per tuber) at intervals of 0, 12, 24, and 36 hr, and incubated in a mist chamber at 20 C, no significant increase in the amount of decayed tissue was observed over that in samples inoculated with clostridia alone. Potato tubers inoculated with Eca and incubated in a dew chamber for 2 wk at temperatures between 7 and 15 C developed typical

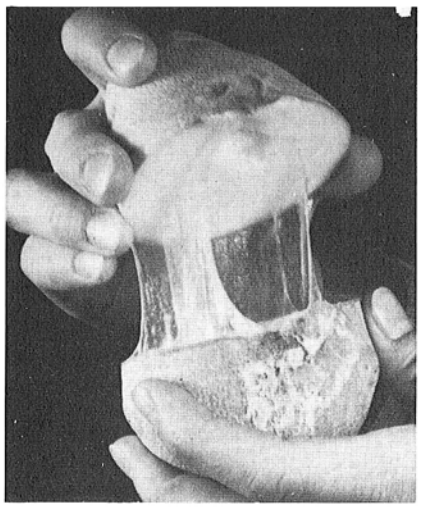

Fig. 1. Symptoms of decay and slime production in a potato tuber inoculated with a pure culture of a pectolytic *Clostridium*.

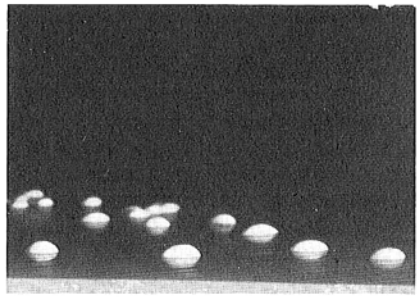

Fig. 2. Colony morphology of a pectolytic strain of *Clostridium* on nutrient-dextrose agar after anaerobic incubation for 4 days at 28 C.

Table 1. Frequency of isolation of pectolytic clostridia, *Erwinia*, and other Gram-negative bacteria from decaying potato tubers

Bacteria isolated	Tubers	
	No.[a]	Percent
Clostridia present		
Clostridia alone	36	5
Clostridia with:		
Erwinia carotovora subsp. *atroseptica*	67	10
Erwinia carotovora subsp. *carotovora*	20	3
Other Gram-negative pectolytic bacteria	27	4
Total	150	22
Clostridia absent		
Erwinia carotovora subsp. *atroseptica*	241	35
Erwinia carotovora subsp. *carotovora*	66	10
Other Gram-negative pectolytic bacteria	106	15
No Gram-negative pectolytic bacteria	125	18
Total	538	78

[a] No. of tubers of 688 sampled from which designated bacteria were obtained.

Erwinia lesions only when the potatoes were covered by a film of water and oxygen levels were decreased (6). At temperatures between 7 and 15 C, clostridia alone failed to cause decay, even when tuber surfaces were wet.

Fungal isolations. Seven different fungi were isolated from 70 of 101 decayed tubers: 26, *Stysanus*; 18, unknown yellow nonsporulating fungi; 7, *Trichocladium*; 6, *Fusarium oxysporum* Schlecht. emend Snyd. & Hans.; 4, *F. roseum* (Lk.) Snyd. & Hans.; 4, *F. solani* (Mart) App. & Wr. emend Snyd. & Hans.; 3, *F. acuminatum* Ellis & Everh.; and 2, *F. equiseti* (Corda) Sacc. Pathogenicity of representative isolates was determined by inoculation of whole tubers with agar plugs of fungal mycelium followed by incubation in a moist chamber for 7–14 days at 22 C. Although strains of *Stysanus* and the nonsporulating yellow fungi were isolated more frequently than others, their pathogenicity could not be demonstrated by the tuber inoculation technique used. Representative isolates of the various *Fusarium* species tested were pathogenic, causing typical dry rot symptoms.

Growth curves. The temperatures of incubation had a marked effect on both the growth rate and the growth yield of Eca and Ecc (Fig. 4) in vitro. At temperatures from 18 to 36 C and above, Ecc grew faster and yielded higher populations than Eca; at temperatures below 8 C, the opposite was true. Growth yields of both bacteria were highest at temperatures between 10 and 18 C; however, the growth rates were lower than those at higher temperatures.

Attempts to quantitate the effects of temperature on the growth of pectolytic clostridia were unsuccessful since the large amounts of slime produced made it difficult to obtain accurate turbidity measurements. However, bacterial colonies on solid media grew most rapidly between 26 and 29 C. Optimal temperatures for most species of those clostridia reported to be able to degrade plant tissue are relatively high compared with those for the potato strains isolated in this study (3).

DISCUSSION

Although clostridia alone or in combination with other pectolytic bacteria were isolated from 22% of the tubers sampled directly from commercial storage bins, in only 5% of the isolations were clostridia the only pectolytic organism obtained. In the latter cases *Erwinia* strains may have been present but not detected. The data indicate that, in commercial storages held at low temperatures, clostridia may not have a major role in tuber decay although they are commonly present in association with other pectolytic organisms. However, in

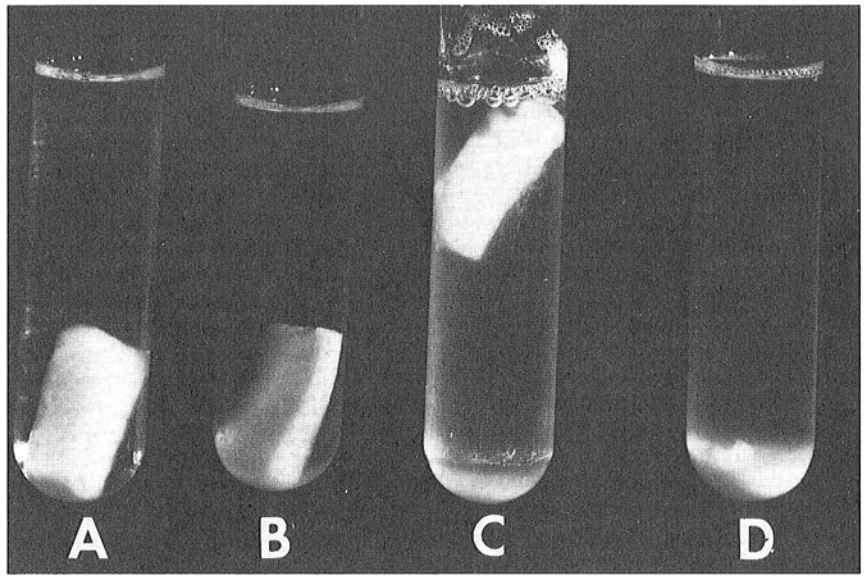

Fig. 3. Stages in the maceration of cubes of potato tissue by a pectolytic *Clostridium*: **(A)** Noninoculated control, and **(B)** 8 hr, **(C)** 3 days, and **(D)** 1 wk after inoculation.

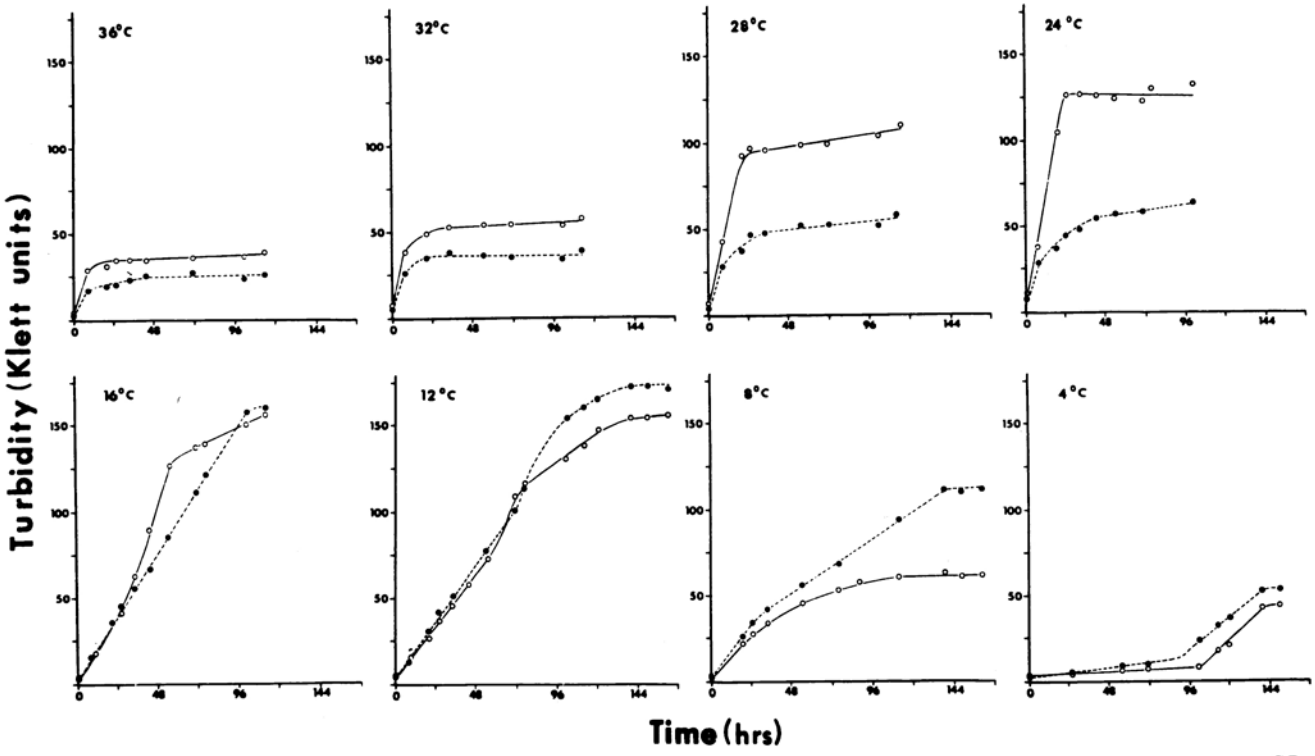

Fig. 4. Growth of *Erwinia carotovora* subsp. *carotovora* SR 206 (—o—) and *Erwinia carotovora* var. *atroseptica* SR 8 (-- • --) in a casamino acids-peptone-glucose broth medium at various temperatures.

the experiment in which healthy tubers from the storage bins were wounded and incubated at 20 C in the mist chamber for 4 days, the proportion of clostridia isolated in relation to *Erwinia* changed significantly, and clostridia became the bacteria most frequently isolated from decaying tubers. Pérombelon et al (19) obtained similar results when they injected mixtures of clostridia and *Erwinia* and noted that the clostridia became the dominant components of the populations when tubers were incubated at temperatures above 20 C. In isolations from freshly harvested potatoes that had been incubated in a mist chamber, Lund and Kelman (13) also noted that clostridia could be obtained at a high frequency from decaying tubers.

Ecc grew more rapidly than Eca in vitro as the temperature increased above 18 C; below 8 C, the converse was true (Fig. 4). Since the frequency of isolation of both Ecc and pectolytic clostridia increased in tubers that decayed after mist chamber incubation at 20 C, the effect of temperature on growth of pectolytic clostridia may be similar to that on growth of Ecc.

These results are consistent with those presented in other studies. After inoculations with mixtures of Ecc and Eca and anaerobic incubation at various temperatures, Eca was the primary isolate recovered at 16 C; at 20–22 C, Ecc predominated (19). Frequency of isolation of Ecc also increases during the summer months (Maher, Kelman, and De Boer, *unpublished data*; 20). In contrast, Eca was recovered mainly during early spring and late fall (16,17,19,22). The data also indicate the influence of the conditions under which potatoes are held before isolation on the relative proportions of the strains of *E. carotovora* that are obtained (17). Observations of decay symptoms in many samples of apparently healthy tubers after injury and incubation under anaerobic conditions indicate that pectolytic clostridia are probably widespread and commonly present on stored potatoes.

Slime production in potato tissue suspensions was highly correlated with the presence of pectolytic clostridia. Demonstration of abundant slime production may provide a simple preliminary screening procedure for the presence of clostridia.

Tubers with external symptoms of decay by *Fusarium* spp. were not included in the samples of decayed potatoes obtained from the bins. Among the 688 decayed tubers collected, however, 15% had internal symptoms typical of fungal infections. Although most of the fungal isolates from decayed tissue were avirulent, isolates of *Fusarium* caused typical dry rot symptoms in inoculation experiments.

Classification of the nonpigmented pectolytic clostridia isolated from potatoes in this study has not been completed. Preliminary tests indicate that the potato strains belong to the "butyric acid group" described by McCoy et al (15). This is consistent with the recent description of *Clostridium puniceum* Lund, Brocklehurst and Wyatt (a pink colony-type) (12) as well as other reports (10,18).

No attempts were made in this study to characterize to species the other pectolytic bacteria that were isolated in addition to strains of *Erwinia* and *Clostridium*. Samples recorded as pectolytic Gram-negative bacteria other than Ecc and Eca included pseudomonads such as *Pseudomonas marginalis* (Brown) Stevens and *Flavobacterium* species. Pectolytic strains of certain other bacteria such as species of *Bacillus* were not detected by the procedures used, although they may have been present. These strains may have been responsible for the decay in 18% of the potatoes from which Gram-negative pectolytic bacteria were not isolated.

Inasmuch as prior surveys to identify bacterial decay organisms in potato tubers in commercial storage facilities or in transit did not employ anaerobic culture procedures, it appears that the significance of pectolytic clostridia as agents of decay of potato tubers has been overlooked in the United States. Our data and results of other recent studies (11,12) indicate that clostridial infections may be the major factor in decay losses in potatoes when temperature control is lost in storage or when tubers are in transit under anaerobic conditions without adequate cooling.

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