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Bacteria Responsible for Mucilage-Layer Decomposition in Kona Coffee Cherries[1]

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ABSTRACT

FRANK, HILMER A. (University of Hawaii, Honolulu), NORMA A. LUM, AND AMY S. DELA CRUZ. Bacteria responsible for mucilage-layer decomposition of Kona coffee cherries. Appl. Microbiol. **13**:201–207. 1965.—The predominant microbial flora present during decomposition of the mucilage layer of Kona coffee cherries were gram-negative bacteria which fermented lactose rapidly. Cultures isolated from coffee cherries undergoing fermentation included species of *Erwinia*, *Paracolobactrum*, and *Escherichia*. Unblemished cherry surfaces and coffee plantation soil also had a microflora containing a high proportion of bacteria belonging to these three genera. Of 168 isolates tested, the 44 strains capable of demucilaging depulped coffee cherries were all members of *Erwinia dissolvens*. Supernatant growth medium liquids, after removal of *E. dissolvens* cells, actively decomposed the mucilage layer of depulped cherries.

The mucilage layer in coffee cherries must be removed before the beans can be effectively dried and roasted (Goto and Fukunaga, 1956). The usual method for demucilaging coffee cherries is by a natural fermentation procedure resulting in decomposition of the mucilage layer (Goto and Fukunaga, 1956; Coste, 1959).

The two fermentation methods most commonly used have previously been described in detail (Stern, 1946; Pederson and Breed, 1946; Beaumont and Fukunaga, *unpublished data*; Goto and Fukunaga, 1956; Coste, 1959). The dry method consists of harvesting the cherries, allowing them to ferment naturally, and then to dry in the sun before mechanically removing the superfluous membranes surrounding the beans. In the Kona district of Hawaii, virtually all coffee is demucilaged by the wet method of fermentation. In the wet method, after harvest, the outer skins are removed (giving "depulped" beans). Fermentation proceeds naturally until the decomposed mucilage layer can be removed readily by washing with water. The demucilaged beans are then dried to the proper moisture level.

To date the pectinolytic, demucilaging organisms involved in coffee fermentation have not been identified with certainty. Loew (1907) attributed the fermentation process to yeasts that formed ethyl alcohol, carbon dioxide, and later acetic acid. From coffee cherry samples shipped by air from Mexico and Colombia to Geneva, N.Y., Pederson and Breed (1946) isolated *Leuconostoc mesenteroides*, two species of *Lactobacillus*, and *Streptococcus faecalis*. Although their isolates were probably responsible for the acid detectable in later stages of fermentation, Pederson and Breed doubted the possible involvement of these bacteria in mucilage-layer decomposition. Recently, Vaughn et al. (1958) reported that during the first 12 to 24 hr of fermentation the pectic material in Brazilian coffee cherries was degraded by coliform-like bacteria resembling *Aerobacter* and *Escherichia*. These organisms were abundantly present on the cherry surfaces. Other pectinolytic bacteria (*Bacillus*) as well as fungi were also isolated.

The purpose of this investigation was to determine which bacteria of the fermentation microflora were responsible for carrying out mucilage-layer decomposition of depulped coffee cherries grown in the Kona region on the island of Hawaii.

MATERIALS AND METHODS

Coffee cherries. Samples of Kona coffee cherries were obtained and handled as described previously (Frank and Dela Cruz, 1964).

Natural fermentation of depulped coffee cherries. Cherries were depulped by slitting one end and expressing the contents (beans plus adhering mucilage layer) into sterile Erlenmeyer flasks. Some decomposed cherries (10 to 20% of total) were always included to provide an inoculum of demucilaging organisms. Enough sterile tap water was then added to nearly cover the cherry mass. Periodically during incubation at 30 C, the flasks

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were swirled, and samples were removed from the supernatant liquid for *p*H measurements, for plating on several media, and for evaluating the progress of mucilage-layer decomposition. The results of a typical experiment are shown in Table 1.

Total microbial counts were estimated with nutrient agar; rapid lactose-positive colonies were determined with Eosin Methylene Blue Agar and Tergitol-7 Agar; and yeasts were counted with acidified Potato Dextrose Agar. *Leuconostoc* organisms were enumerated on selective medium (Hucker and Pederson, 1930; Mayeux and Colmer, 1961) containing 0.1% sodium azide.

Subcultures obtained from isolated colonies were identified (Table 2) as described below.

Mucilage-layer decomposition was evaluated empirically as described previously (Frank and Dela Cruz, 1964).

Fermentation in whole cherries. Subcultures were taken from brown spots on whole cherries by transferring a loopful of decomposing mucilage layer to Eosin Methylene Blue Agar surfaces. Rapid lactose-positive colonies were selected for identification (Table 2) and for demucilaging studies (Table 5).

Microflora of cherry surface and of coffee plantation soil. Ripe, unblemished cherries were shaken in sterile tap water blanks, diluted, and plated in several media for enumeration of total and rapid lactose-positive counts (Table 3). Serial dilutions of coffee plantation soil were also made for similar enumeration studies. Representative colonies selected from both samples were isolated, tested for demucilaging ability, and identified (Table 4).

Demucilaging studies with isolated strains. Inocula consisted of 25 ml of agitated (Dubnoff shaker) nutrient broth cultures obtained after incubation for 24 hr at 30 C. The inoculum was added directly to 250-ml Erlenmeyer flasks containing about 50 depulped, ripe, unblemished coffee cherries. Strains causing essentially complete decomposition of the mucilage layer within 5 hr at 30 C were regarded as demucilaging-positive. The results of demucilaging tests for 168 strains examined are given in Table 5.

Extracellular demucilaging activity of several demucilaging-positive strains was also tested in the cell-free liquid growth medium. Cells were removed by centrifugation and then reconstituted to original volume with sterile tap water. Cell-free growth medium and reconstituted aqueous cell suspension were then tested separately for demucilaging ability as described above.

Identification. The following properties were studied by conventional methods with incubation at 35 or 37 C: colony appearance on Levine's Eosin Methylene Blue Agar (Difco) and Tergitol-7 Agar (Difco); flagella with Leifson flagella stain (BBL); hydrogen sulfide production in Kliger Iron Agar (Difco) and in Triple Sugar Iron Agar (BBL); motility in hanging-drop preparations and in SIM Medium (BBL); indole production in Indole-Nitrite Medium (BBL) and in SIM Medium; methyl red and Voges-Proskauer reactions in MR-VP Medium (Difco); citrate utilization in Simmons Citrate Agar (Difco); reaction in litmus milk (Difco); urease test in urea medium (Difco); nitrate reduction in Nitrate Broth (Difco); carbohydrate fermentation reactions with Phenol Red Broth Base (Difco); growth in KCN medium and phenylalanine deamination in phenylalanine agar (Edwards and Ewing, 1962); starch hydrolysis with Phenol Red Broth Base (Rhodes, 1959), nutrient broth, and nutrient agar (Society of American Bacteriologists, 1957).

Liquefaction of Nutrient Gelatin (Difco) was tested in cultures incubated at 20 C.

Pectinolytic ability was studied at 30 C in Eosin Methylene Blue pectate medium (Vaughn et al., 1957), crystal violet polypectate gel (King and Vaughn, 1961), and in the pectate medium of Splittstoesser and Weltergreen (1964). Strains exhibiting a positive reaction on at least one of these three media were considered to be pectinolytic-positive.

Gram-negative bacterial isolates were identified from descriptions in *Bergey's Manual of Determinative Bacteriology* supplemented by information from several other sources (Elliott, 1951; Skerman, 1959; Edwards and Ewing, 1962; Martinec and Kocur, 1962).

Leuconostoc mesenteroides was tentatively identified from its cellular morphology, Gram reaction, gummy growth on orange serum agar, cottonlike appearance in sucrose-gelatin stab cultures (Berry, Folinazzo, and Murdock, 1954), and ability to ferment sucrose, arabinose, and xylose.

Yeasts were detected by their morphology in microscopic examinations of smears made from colonies appearing on acidified Potato Dextrose Agar.

RESULTS AND DISCUSSION

Natural fermentation. During preliminary experiments, we were unable to demucilage depulped cherries in less than 24 hr, the practical maximum for commercial processing. However, after including sufficient decomposed cherries (Fukunaga, *personal communication*), the demucilaging process was shortened to less than 6 hr.

Table 1 shows the results obtained in a typical natural fermentation of depulped cherries under suitable conditions. Complete demucilaging occurred in less than 5 hr, with little change in *p*H. Microscopic examination of the microflora present during decomposition revealed a mixed population containing predominantly gram-negative rods. The very low incidence of *Leuconostocs* and yeasts (Table 1) argues against their involvement in mucilage-layer decomposition. Subsequent experiments with pure cultures confirmed the inability of these latter organisms to decompose the cherry mucilage layer (Table 5). During extended incubation of depulped

cherries (i.e., up to several days), the number of lactic acid bacteria increased and was accompanied by a significant drop in *p*H.

Organisms that ferment lactose rapidly (i.e., coliform types) comprised about 25 to 40% of the total count in fermenting depulped cherries (Table 1). Subsequent investigation revealed that only *Erwinia dissolvens*, a rapid lactose-fermenter, was capable of demucilaging depulped cherries (Tables 2 and 5). Slow lactose-positive organisms, mainly species of *Paracolobactrum*, constituted the bulk of the remaining microflora in naturally fermenting depulped cherries (Table 2).

Because the inoculum for naturally fermented depulped cherries came from decomposing whole cherries, called *em coco* in Brazil (Vaughn et al., 1958), we subcultured material from brown spots of whole cherries. Demucilaging *E. dissolvens* strains were isolated easily from these decomposing mucilage layers of whole cherries (Table 2).

These results demonstrate that coffee demucilaging is associated with a high incidence of rapid lactose-fermenters, and that many of these are *E. dissolvens* strains capable of decomposing the mucilage layer when tested in pure culture. Quite probably these organisms are closely related to, if not identical with, the coliform-like bacteria described by Vaughn et al. (1958).

Habitat studies. The incidence of potential demucilaging bacteria on the surfaces of sound cherries and in coffee plantation soil was examined by determining the numbers of rapid lactose-fermenting bacteria present in samples from these two sources (Table 3). The proportions of rapid lactose-fermenting bacteria varied considerably, ranging from 20 to 60% of the total surface population in three samples tested and from 8 to 76% in the two soil samples.

Randomly selected colonies of rapid and non-rapid lactose-fermenters were subcultured, identified, and tested for demucilaging ability (Table 4). Of 30 rapid lactose-fermenters from cherry surfaces, 24 were demucilaging strains of *E. dissolvens*. On the other hand, only 1 of 22 rapid lactose-fermenters isolated from soil was a demucilaging *E. dissolvens*. A more comprehensive study should be conducted before excluding coffee plantation soil as a significant habitat for appreciable numbers of demucilaging bacteria.

TABLE 1. *Enumeration of the microbial population present during natural fermentation of depulped coffee cherries*

Time	Demucilaging	*p*H of liquid	No. of organisms per ml in liquid			
			Total	Rapid lactose-fermenters	Yeast	*Leuconostoc*
hr						
Initial	Nil	5.5	200×10^6	62×10^6	62×10^4	3×10^4
1.0	Very slight	5.4	230×10^6	53×10^6	51×10^4	2×10^4
2.5	Slight	5.4	254×10^6	63×10^6	66×10^4	$<1 \times 10^4$
3.75	Complete	5.3	185×10^6	78×10^6	60×10^4	$<1 \times 10^4$

TABLE 2. *Distribution of bacterial species isolated from coffee cherries undergoing natural fermentation*

Source	No. of isolates	Isolation medium	Organisms isolated					
			Erwinia dissolvens demucilagers	*E. dissolvens* non-demucilagers	*Escherichia intermedium*	*Paracolobactrum aerogenoides*	*P. intermedium*	Unidentified non-demucilagers
Depulped cherries	21	Mayeux-Colmer*	2	1	2	15	0	1
	23	Hucker-Pederson*	5	0	0	14	0	4
	20	Orange serum agar	4	0	2	13	0	1
	3	Tryptone glucose extract	1	0	0	2	0	0
	5	Nutrient agar	2	0	0	2	0	1
Whole cherries, brown spots	7	Eosin methylene blue	5	0	0	1	1	0

* Without sodium azide.

TABLE 3. *Enumeration of rapid lactose-fermenters present on surfaces of whole, unblemished cherries and in coffee plantation soil**

Sample no.	Source	Nutrient agar	Eosin methylene blue		Tergitol-7	
			Total	Rapid lactose-fermenters	Total	Rapid lactose-fermenters
1	Cherries	22	19	9.7	18	10.4
2	Cherries	90	26	10	28	13
3	Cherries	96	29	5	14	3
4	Soil	14	8.8	5.2	10.6	8.0
5	Soil	2.9	2.4	0.2	1.0	0.1

* Results in samples 1, 2, and 3 are expressed as organisms per coffee cherry $\times 10^4$; in samples 4 and 5, as organisms per gram $\times 10^6$.

TABLE 4. *Distribution of bacterial species isolated from unblemished whole cherries and from coffee plantation soil*

Experiment no.	Source	Isolation medium	Lactose fermentation		Organisms isolated						
			Rapid positive	Slow positive or negative	*Erwinia dissolvens* demucilagers	*E. dissolvens* nondemucilagers	*Escherichia intermedium*	*Paracolobactrum aerogenoides*	*P. coliforme*	*P. intermedium*	Unidentified nondemucilagers
1	Cherry	Eosin methylene blue	3		3	0	0	0	0	0	0
	Surface			2	0	0	0	2	0	0	0
2	Cherry	Eosin methylene blue	4		3	0	1	0	0	0	0
	Surface			8	0	0	0	4	1	1	2
3	Cherry	Eosin methylene blue	12		9	3	0	0	0	0	0
	Surface			4	0	0	0	4	0	0	0
4	Cherry	Tergitol-7	11		9	2	0	0	0	0	0
	Surface			5	0	0	0	3	0	0	2
5	Soil	Tergitol-7	11		1	8	0	0	0	0	2
				4	0	0	0	3	0	0	1
6	Soil	Tergitol-7	11		0	0	0		0	0	11
				4				4			0

Quite probably, demucilaging bacteria gain entrance into the mucilage layer through breaks in the outer skins caused by fruit flies. Visual evidence of insect damage can usually be detected in the browned areas of whole cherries.

Demucilaging studies. Of 168 isolates tested, 44 could completely decompose the mucilage layer in 5 hr or less (Table 5); these demucilaging-positive strains were all *E. dissolvens*. The 14 nondemucilaging *E. dissolvens* strains usually decomposed the mucilage layer slowly, generally taking 12 hr or more. Nevertheless, by the criterion used in this study, these latter strains were considered demucilaging-negative.

These observations establish that demucilaging of the coffee cherry is carried out by a specific organism, *E. dissolvens*, and that other members of the fermentation microflora, such as *Paracolobactrum*, lactic acid genera, and yeasts, are not involved in the early stages of fermentation. More likely, the lactic acid bacteria utilize pectic breakdown products and probably are responsible for imparting undesirable flavors and odors to the beans when fermentations are protracted.

Quite possibly, other genera are present that contribute to mucilage decomposition (Vaughn et al., 1957). The use of selective isolation media such as Eosin Methylene Blue and Tergitol-7 probably accounts for our inability to detect other demucilaging genera.

Because microbial pectinolytic enzymes are generally recovered from the growth medium liquors (Demain and Phaff, 1957), we compared demucilaging activities of growth medium supernatant liquids with that of the cells harvested. In five demucilaging *E. dissolvens* strains tested, an appreciable extracellular enzyme content was indicated by the observation of more rapid

TABLE 5. *Demucilaging ability of isolated species and of bacterial strains obtained from the American Type Culture Collection*

Organism	No. of strains	
	Tested	Demucilaging-positive
Our isolates		
Erwinia dissolvens	58	44
Escherichia intermedium	20	0
Leuconostoc mesenteroides	8	0
Paracolobactrum aerogenoides	53	0
P. coliforme	1	0
P. intermedium	2	0
Yeasts	2	0
Unidentified bacteria	24	0
American Type Culture Collection:		
Leuconostoc citrovorum 8082	1	0
Leuconostoc mesenteroides 8293	1	0
Lactobacillus brevis 4006	1	0
Lactobacillus plantarum 4008	1	0
Pediococcus cerevisiae 10791	1	0
Streptococcus faecalis 11700	1	0

demucilaging with supernatant liquids than with harvested cells.

Heating for 10 min at 80 C destroyed the enzyme activity of supernatant liquids and of cells.

Demucilaging activity was greater (i.e., faster) with agitated cultures than with still cultures.

Classification. These results conclusively show that the demucilaging bacteria belong to *E. dissolvens*, the only species in which mucilage-layer decomposition could be demonstrated in pure culture studies (Table 5). It appears certain that these organisms are closely related to the coliform-like bacteria found in Brazilian coffee undergoing decomposition *em coco* (Vaughn et al., 1958).

Waldee (1945), who studied a large number of gram-negative phytopathogens, suggested that *E. dissolvens* be placed in the genus *Aerobacter*. However, Waldee's *E. dissolvens* strains, while generally resembling the isolates reported in this study, were nonpectinolytic, motile, flagellated, and fermented lactose slowly.

We do not feel that the *E. dissolvens* strains isolated from coffee cherries undergoing fermentation should be classified as *Aerobacter*. Although *Aerobacter* and *Erwinia* are very similar and share many common characteristics, we were able to distinguish between these two genera on the basis of pectin degradation by the latter. Crystal violet polypectate gel (King and Vaughn, 1961) generally was the most satisfactory medium for detecting pectinolytic ability. For several weakly pectinolytic strains (all nondemucilagers), positive reactions were obtained with Eosin Methylene Blue pectate medium (Vaughn et al., 1957) or with the pectate medium of Splittstoesser and Weltergreen (1964). Furthermore, we do not agree with the suggestion of Martinec and Kocur (1962) that *Erwinia* should contain only two species, thereby placing *E. dissolvens* in the species *E. carotovora*. Sufficient differences exist (indole production, gelatin liquefaction, and starch hydrolysis) to warrant separation of these two species.

Except for differences in their ability to decompose coffee cherry mucilage layers, the demucilaging and nondemucilaging strains of *E. dissolvens* are indistinguishable from one another. The physiological properties observed in our study of these organisms are given in Table 6. In addition, the following properties also describe the *E. dissolvens* isolates: gram-negative, nonflagellated, asporogenic rods; mucoid, medium-size, cream-colored colonies on nutrient agar; mucoid, medium-size colonies having the typical

TABLE 6. *Physiological properties of 58 Erwinia dissolvens strains*

Property tested	Response
Motility	−
Pectin degraded	+
Indole produced	+
Voges-Proskauer reaction	+
Methyl red reaction	−
Citrate utilized	+
Gelatin liquefaction	−
Nitrate reduced to nitrite	+
Growth in presence of KCN	+
Phenylalanine deamination	−
Starch hydrolyzed slightly	+
Acid curd in litmus milk	+
Acid and gas from:	
Cellobiose	+
Dextrin	+ in 35 strains
Glucose	+
Glycerol	+
Inositol	+
Lactose	+
Maltose	+
Mannitol	+
Salicin	+
Sucrose	+
Urease present	+ in 55 strains
Acid only from dextrin	+ in 22 strains
Slight hydrogen sulfide production	+ in 25 strains

TABLE 7. *Spoilage delay in coffee cherries stored under refrigeration*

Sample no.	Incubation	Days of incubation								
		0	1	2	6	8	15	17	21	29
1	Ambient	0*	68	88	100	—	—	—	—	—
	Refrigerated	0	35	35	56					
2	Ambient	—	—	—	—	100	—	—	—	—
	Refrigerated	—	—	—	—	33	47	53	65	82

* Percentage of spoiled coffee cherries.

color of lactose-positive organisms on Eosin Methylene Blue (dark centers), Tergitol-7 (yellow with yellow zone), and MacConkey Agar (pink).

Spoilage of coffee cherries. Unsatisfactory demucilaging can result from insufficient as well as excessive fermentation. Under-fermentation interferes with the drying process, because the mucilage layer is not completely removed (Beaumont and Fukunaga, *unpublished data*). However, by periodically testing small samples from the fermentation tank, one can determine whether or not the beans are ready for washing prior to drying. If not, fermentation is continued until demucilaging is completed.

Overfermentation frequently results in adverse changes that affect the flavor and odor of the coffee produced (Beckley, 1930; Stern, 1946; Carbonell and Vilanova, 1952; Beaumont and Fukunaga, *unpublished data*; Goto and Fukunaga, 1956; Coste, 1959). The mucilage layer can support good microbial growth after the action of pectinolytic demucilagers (Coste, 1959; Frank and Dela Cruz, 1964). Most frequently, acidogenic fermentations occur (Loew, 1907; Stern, 1946; Pederson and Breed, 1946; Goto and Fukunaga, 1956), and these may possibly be the major cause for cherry spoilage problems arising during processing.

Overfermentation can best be avoided by testing small samples during processing to detect complete demucilaging at its earliest stage, so that washing can be instituted before undesirable changes occur (Goto and Fukunaga, 1956; Carbonell and Vilanova, 1956). Because of post-pectinolytic microbial buildup in machinery and equipment as the season progresses, thorough cleaning of the fermentation vats should be carried out frequently.

If possible, cherries should be processed soon after harvest to avoid overfermentation in the unpulped cherries (Goto and Fukunaga, 1956). However, farmers with limited labor forces often accumulate several days' cherry harvest before delivery to the processor. As a consequence, bags of harvested cherries lie in the open (often in the hot sun) before processing. Under these conditions, even putrefactive spoilage is common. Coffee made from such grossly spoiled cherries is poor in quality and frequently not potable.

We investigated the prevention of preprocessing spoilage by comparing spoilage in whole cherries stored at ambient temperatures with cherries kept under refrigeration (Table 7). Spoilage was indicated when the outer skins became completely brown and the cherry was flabby rather than firm when squeezed. Spoilage was often accompanied by an unpleasant odor as well.

Table 7 shows that spoilage in both samples of refrigerated cherries was appreciably slower than in unrefrigerated cherries. Sample 1 had a higher percentage of spoilage initially and, therefore, spoiled faster than sample 2 at both temperatures.

Though the economic feasibility of refrigeration as a method for slowing down spoilage of cherries in a commercial operation may be questionable, these results do demonstrate that low temperatures are effective in delaying growth of undesirable microorganisms.

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