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Pediococci Residing on Plants

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The pediococci residing on plants resemble the lactobacilli, but they differ from the streptococci in their limited distribution and low population level on plants. They are a subgroup within the genus *Pediococcus* which grow freely in neutral media and require neither NaCl nor CO_2. They are most readily recognized by the ability to initiate growth in liquid media, acidified to *p*H 5.0, which contain 1.5% sodium acetate. In stained preparations the cells occur singly and in pairs, short chains, and clusters. The occurrence of two-dimensional tetrads may be rare; this varies with the individual culture and with the culture medium. The terminal *p*H in 2% glucose broth varies from 3.6 to 4.3. Ability to initiate growth at 45 C, production of ammonia from arginine, dissimilation of malate, and fermentation of arabinose are confirmatory characteristics. The subgroup contains only two quite similar, but differentiable, species. *P. acidilactici* initiates growth at 50 C and produces catalase on heated blood medium but does not produce acid-sensitive catalase; a majority of the strains fail to initiate growth at 10 C and many fail to ferment maltose and lactose. *P. pentosaceus* initiates growth at 10 C but not at 50 C and produces acid-sensitive catalase; catalase production on heated blood medium is transient; a majority of the cultures ferment maltose, salicin, and trehalose. No carbohydrate serves reliably to differentiate between the species. The guanine plus cytosine ratio of *P. pentosaceus* deoxyribonucleic acid (DNA) was determined to be 35.1 ± 1.2 and that of *P. acidilactici* DNA is 38.5 ± 0.8.

Three physiologically diverse groups are included within the genus *Pediococcus* (15, 19). Each group is known in context with a specific type of environment or habitat. Within the plant group, two species have been recognized, *P. acidilactici* and *P. pentosaceus*. They are associated with fermentations of a variety of vegetables (8), and they have been isolated, although in small numbers, from plants (18). The group has erroneously been given the name *P. cerevisiae* (16). Guenther and White (7) suggested that this name be assigned to the plant group, although *P. cerevisiae* Balcke is an organism of unusual properties and is well known to the brewing and the yeast industries (17). In this paper, the terminology as reaffirmed by Nakagawa and Kitahara (15) and by Whittenbury (19) is employed.

The plant pediococci studied were obtained almost exclusively from fermenting vegetables. It is not known whether the number of species within the subgroup is limited to those now described, or whether these are the species which become dominant during fermentations, comparable to the dominance to which a few species of lactobacilli ascend, although it is now known that many species of *Lactobacillus* exist on plants (13). Little is known about the frequency of occurrence or the population levels of pediococci in nature.

Pediococci were isolated during surveys for the occurrence of streptococci (12) and lactobacilli (13) on plants. During the latter survey they composed approximately 15% of the isolated cultures. Subsequently, a survey directed toward the pediococci was initiated for the quantitative enumeration of both the plant and the brewing pediococci. As the cultures were studied, discrepancies were noted between our results and the published descriptions of the plant species; cultures from all sources were then subjected to differential studies.

MATERIALS AND METHODS

It was determined during preliminary studies that the quantitative detection and recovery of the plant pediococci was optimal in an acetate-containing liquid medium adjusted to *p*H 5.4, and Rogosa's SL medium was selected for the purpose. An added advantage of the liquid medium is the lack of limitation on the size of the primary sample for the detection of pediococci occurring in populations of less than 10/g.

Incubation of enrichment media was at both 32 and 45 C, because it was not known initially whether all pediococci were capable of growth at the higher temperature. At 45 C, only lactobacilli and pediococci will grow in SL broth. Since pediococci initiate growth

and reproduce more rapidly than do the lactobacilli, recovery was simplified by streaking from tubes containing cocci, as revealed by staining, promptly upon development of noticeable turbidity. At 32 C, both yeasts and *Leuconostoc mesenteroides* grow. The former are suppressed completely through the addition of 0.01% cycloheximide. No modification of the broth, including reduction in *p*H to 5.0, the addition of 7% ethyl alcohol (13), or the use of hop extract, inhibited the *Leuconostoc* without repression of the pediococci.

Triplicate serial dilutions employing 1-g samples and 10% aqueous homogenates of plant material were prepared for incubation at each temperature. Fluids containing cocci were streaked on MRS (2) agar from which colonies were transferred to SL broth.

The medium of Nakagawa (14) was prepared and sterilized in 10-fold concentrations, and then diluted with nine volumes of bottled beer for use in attempted recovery of *P. cerevisiae*. Tubes of the medium were closed with cotton stoppers and were incubated in an atmosphere of CO_2 at room temperature for 5 days. A known culture was carried in parallel with the plant inocula.

Cultural studies. The criteria of Nakagawa and Kitahara (15) and of Whittenbury (19) were employed in characterization of the cultures. Basal media were MRS and tryptone-glucose-0.5% yeast extract broths, which can be used interchangeably. Incubation at 32 C was done in an air incubator; at all other temperatures, water baths were used. Except in the determination of terminal *p*H, performed in broth with 2% concentration of glucose, carbohydrates were used at 0.5 and 0.75% concentrations, with no differences noted in the results. Other procedures have been recorded elsewhere (12, 13).

Deoxyribonucleic acid (DNA) was extracted according to the procedure of Marmur (10), and the guanine plus cytosine (GC) content was calculated from values obtained by thermal melting point analysis (11) with a Beckman DB-G spectrophotometer equipped with a T_m analyzer and a temperature bridge. In contrast to the streptococci, the pediococci proved insensitive to the action of the lysozyme until subjected to 15,000 psi in a French pressure cell.

Samples. Nearly all small-grain heads and 1-g samples of soil were added directly to tubes of SL broth. Other plant materials were prepared as homogenates. The samplings of the small grains and the forage grasses represent all stages of development from pre-inflorescence to maturity of the seed head. Samples of corn included inflorescences from the time of emergence through fertilization and portions of sheath and stem areas taken at and above the nodes. Moistened cotton swabs were rubbed over areas of equipment delineated at 4 inches^2 (10 cm^2) within a template. Of 300 samples, 226 were taken in the field. The remainder consisted of products undergoing processing or during fermentations, or of swabs of harvesting equipment.

RESULTS AND DISCUSSION

Distribution. *P. cerevisiae* was not recovered from any sample of plant or of soil. The relatively small range in plant species sampled does not justify a firm conclusion regarding the absence of *P. cerevisiae* in the Southeastern United States, although its preference for a lower temperature does suggest its inability to tolerate the relatively high environmental temperature.

Plant pediococci were obtained from 8% of 226 field samples of plants (Table 1) implanted into SL broth. Incubation of the media at either 32 or 45 C had little effect on the number of recoveries or their distribution among the species. The distribution on plants compared with a frequency of 37.5% for the lactobacilli (13) and 85.3% for the streptococci (12). The maximum number per

TABLE 1. *Distribution and levels of population of the plant pediococci*

Source of samples	No. of samples cultured	Isolations	Per cent	No./g of sample	
				Min	Max
Small grains	73	3	4	ND[a]	ND[a]
Corn	32	2	6	23	23
Forage grasses	39	4	10		10
Garden vegetables	35	1	2		10
Vegetables received for processing	16	7	43	4	10
Vegetables undergoing processing	16	11	68	9	10^3
Processing equipment and waste	14	9	64	10	10^5
Vegetables after post-blanch handling	9	0	0		
Fermenting vegetables	12	6	50	9	2×10^4
Fodder at silo	3	1	33		3
Fodder-harvesting machinery	11	7	63	3	2×10^3
Silage	3	3	100	2×10^3	2×10^4
Soils	30	0	0		

[a] Not determined; recovery from tubes receiving whole heads only.

gram of fresh plant material was 23, comparable to the population level of the lactobacilli, but far below that of the streptococci. No pediococci were obtained from 30 samples of soil, many of which were taken adjacent to plants from which pediococci had been obtained. Both samples of corn yielding pediococci were damaged by insects.

It has been observed (18) that pediococci grow on liberated plant juices. In this study, increases in numbers were noted where cell juices became available, as on the surfaces of harvesting and processing equipment, in silage, and on vegetables passing over or through equipment.

Identification of the plant pediococci. Two-dimensional tetrads were common when pediococci were grown in MRS broth (Fig. 1), but in other media the cells may occur singly, in pairs, short chains, and in clusters (Fig. 2). They frequently are indistinguishable from streptococci and leuconostocs which also lie as packets through random deposition on the slide. In some instances, Dyar's cell wall stain (4) reveals the common envelope of the packet reported for *P. cerevisiae* by Ettlinger et al. (5), but the procedure is laborious and the results are not infallible. The terminal *p*H, frequently reported to be 3.6, varied from this to 4.3 among 201 cultures, with an average *p*H of 4.0 to 4.1.

The plant pediococci are the only spherical bacteria able to initiate growth in acetate broth at *p*H 5.0. Therefore, this became the criterion for the designation of newly isolated strains as pedio-

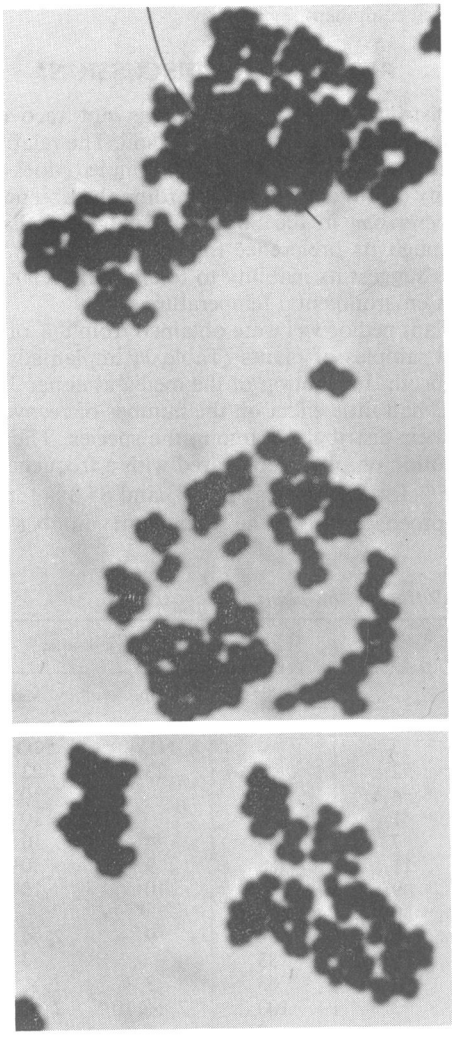

FIG. 1. *Pediococcus acidilactici; Gram stain of 24-hr-old culture grown in MRS broth.* ×970.

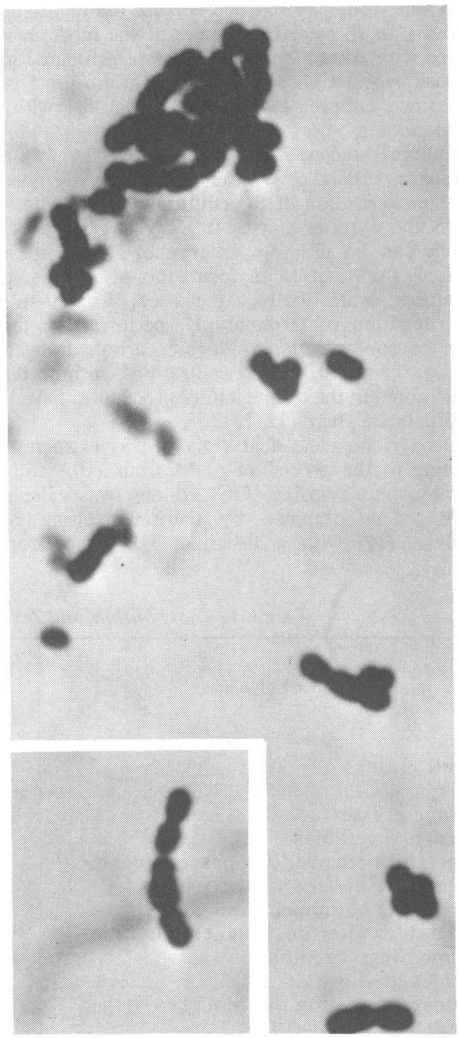

FIG. 2. *Pediococcus acidilactici; Gram stain of 24-hr-old culture grown in acetate (SL) broth.* ×970.

cocci; however, 61 of 513 cultures initiated growth in the medium at *p*H 5.4, but not at *p*H 5.0. Subgeneric properties confirming the identity as pediococci include: the ability to initiate growth in broth at 45 C and in 6.5% NaCl broth adjusted to *p*H 5.8 and 6.5, the dissimilation of malate, the production of ammonia from arginine, and the fermentation of arabinose.

Properties of the species. The majority of the cultures of *P. acidilactici* failed to initate growth at 10 C or in broth adjusted to *p*H 8.0. None produced acid-sensitive catalase, and few fermented maltose. All or nearly all cultures of *P. pentosaceus* initiated growth at 10 C, and in broth adjusted to *p*H 8.0 they produced acid-sensitive catalase and fermented maltose (Table 2). None of the cultures initiated growth at 50 C. Many produced catalase transiently on heated blood medium, becoming negative after 24 hr of incubation. The ability to ferment trehalose, rhamnose, lactose, and salicin has little value in separating the species.

No combinations of properties emerge which suggest either new species or major subgroups within the species. Whittenbury (19) suggested two subgroups within the species *P. acidilactici* on the basis of heme-requiring catalase production and the fermentation of trehalose. His data indicate the use of a limited number of cultures of this species; thus, he may have been unaware of strains having the characteristics of both groups. The unity within the species appears to be confirmed by the GC content, in which strains 146 and 217-11 represent his groups I and II, respectively; the other strains represent cultures between these (Table 3). The few cultures failing to initiate growth in SL broth at *p*H 5.0 have the properties of *P. pentosaceus*.

GC content of several strains of plant pediococci, of reference strains of enterococci, and the values for enterococci reported as being obtained through melting point procedures are presented in Table 3. The values obtained with the plant pediococci are within the range generally accepted for the homofermentative lactic acid-producing bacteria. The GC content for *P. pentosaceus*, 35.1% ± 1.2, is reasonably close to that of *S. faecium*, to which Whittenbury has related the species. The higher value for *P. acidilactici*, 38.5% ± 0.8, tends to confirm the division of the plant pediococci into two species, a subject upon which Whittenbury (19) has commented.

All attempts to disrupt the cells of *P. cerevisiae* failed, despite treatment with the pressure cell and incubation with lysozyme, trypsin, or sodium lauryl sulfate, either singly or in various combinations. The 44.4% GC content is taken from Bóhacek et al. (1); it is included for the purpose of comparison.

Thus, the plant pediococci appear to be a quite homogeneous group of two species of spherical, gram-positive cells occurring as mixtures of single cells, pairs, short chains, and clusters, with the numbers of two-dimensional tetrads varying with the medium in which they are grown. They initiate

TABLE 2. *Properties of the species of the plant pediococci*

Property	*P. acidilactici*		*P. pentosaceus*	
	No. of cultures	Per cent positive	No. of cultures	Per cent positive
Initiation of growth				
At 10 C	68	17.0	148	99.5
At 45 C	296	100.0	217	96.5
At 50 C	268	100.0	132	0
In broth (*pH* 5.0)	296	100.0	217	92.6
In broth (*pH* 8.0)	68	26.5	132	74.3
In broth (*pH* 9.0)	68	19.1	132	12.1
Acid-sensitive-catalase	228	0	69	93.0
Heme-requiring catalase	296	93.0	201[a]	1.0
Ammonia from arginine	296	100.0	217	100.0
Dissimilation of malate	68	98.5	132	100.0
Fermentation of				
Maltose	296	6.9	201	94.5
Trehalose	296	52.3	201	84.5
Rhamnose	228	29.0	69	38.2
Sucrose	228	2.0	69	24.8
Arabinose	68	100.0	132	98.5
Lactose	68	7.3	132	66.7
Salicin	68	53.0	132	95.5

[a] Many strains were transiently positive, becoming negative with continued incubation.

TABLE 3. *Guanine plus cytosine content of the DNA of the enteric streptococci, the plant pediococci, P. cerevisiae, and Aerococcus viridans*

Species	Determined[a]	Reported
S. faecalis H27a	35.4	34–36(11)
S. faecalis var. *liquefaciens* R64a	35.1	34–36(11)
S. faecium R169a	33.7	34–36(11)
P. acidilactici 146	38.8	
P. acidilactici 135	37.8	
P. acidilactici I 170-1	39.0	
P. acidilactici II 217-11	39.3	
P. pentosaceus 20	33.9	
P. pentosaceus 183-1	36.3	
P. cerevisiae		44.4(1)
A. viridans	37.6	37–41 [b]

[a] Each ratio represents the average of six determinations obtained from three determinations on each of two separately extracted samples of DNA.

[b] J. B. Evans (*personal communication*).

growth in media containing acetate at low *p*H, a property shared only with the lactobacilli. They differ from *P. cerevisiae* in the ability to grow at relatively high mesophilic temperatures in the absence of CO_2, and from *P. halophilus* in the ability to thrive in media containing minimal quantities of NaCl.

The pediococci employed in this study were isolated by use of different media: a nearly neutral MRS agar and the acidified acetate broth. Therefore, it appears probable that no additional species exists within the plant subgroup. The taxonomically disputed (3, 9) bacterium, *Aerococcus viridans*, was recovered frequently during studies with the modified MRS medium (12), but the high *p*H at which growth is terminated precludes its isolation by use of the acidified broth. Should it be assigned to the genus *Pediococcus*, the plant group will consist of three rather than two species. The GC content of this bacterium is recorded (Table 3) with no further comment.

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