Further Studies of the Genus *Pediococcus*

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SUMMARY

The cultural, physiological and serological characters of new *Pediococcus* isolates have been studied. Suggestions are made for the amendment of the definition of the genus *Pediococcus*. The genus is considered to contain the four species *P. cerevisiae*, *P. parvulus*, *P. damnosus* and *P. halophilus*.

INTRODUCTION

The genus *Pediococcus* is now well established by the systematic studies of Mees (1934), Pederson (1949), Pederson, Albury & Breed (1954), Jensen & Seeley (1954), Nakagawa & Kitahara (1959), and Günther & White (1961 *a, b*). There is, however, conflict of opinion among various workers regarding the definition of various species. The description of the type species *P. cerevisiae* Balcke given by Pederson (1949) and followed by Jensen & Seeley and Günther & White is not accepted by Nakagawa & Kitahara who consider that this name is valid only for cultures isolated from beer and apply the name *P. pentosaceus* Mees to strains fitting Pederson's description.

Beer was the original source of Balcke's strain (Balcke, 1884) and many workers have obtained cultures from this habitat. These cultures have been named *Pediococcus cerevisiae* (Nakagawa & Kitahara, 1959); *P. damnosus* (Claussen, 1903) with varieties *salicinaceus* and *perniciosus* (Mees, 1934); *Streptococcus damnosus* with varieties *salicinaceus*, *pentosaceus*, *viscosus* and *limosus* (Shimwell, 1949), and with variety *diastaticus* (Andrews & Gilliland, 1952). Günther & White (1961 *a*) examined three cultures labelled *P. damnosus* (originally from Mees and Claussen) and concluded that they form a distinct physiological group.

Pediococcus halophilus Mees differs from other pediococci in its absolute requirement for sodium chloride for growth (Günther & White, 1961 *a*). The work of Sakaguchi (1959) on bacteria associated with the fermentation process of soya sauce is of interest here. He isolated salt-requiring pediococci which he designated *P. soyae* (Sakaguchi, 1958). Later these isolates were found to resemble closely *P. halophilus* strain TC1 and were renamed *P. halophilus* by Nakagawa & Kitahara (1959).

Other species of pediococci have been proposed by Nakagawa & Kitahara (1959). *Pediococcus acidilactici* Lindner was the name given to strains having an optimum temperature of 40°, and *P. urinae equi* Mees to organisms with the ability to grow in media of pH 9.

Views vary on the taxonomic position of aerococci. Williams, Hirsch & Cowan

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(1953) described a large number of strains and proposed *Aerococcus* as a separate genus, but Deibel & Niven (1960) regarded such strains as pediococci forming the species *Pediococcus homari*. Some of these cultures have been included in the present study and their taxonomic position is further considered.

In their preliminary survey of the serology of the pediococci and allied genera, Günther (1958) and Günther & White (1961 *b*) showed the presence of precipitins not shared with members of the genera *Streptococcus*, *Leuconostoc*, *Staphylococcus* and generally not with aerococci. Separate precipitins characteristic of *Pediococcus cerevisiae* (group I), *P. parvulus* (group II), and *P. damnosus* (group IV) were also demonstrated and could be considered analogous to the 'group' antigens of streptococci. The detection of other precipitins and agglutinins indicated closer serological inter-relationships within the pediococci. However, Günther & White's group III strains did not appear to possess an antigen-antibody system (detectable by the precipitin reaction) in common with *P. cerevisiae*, *P. parvulus*, *P. damnosus*, *P. halophilus* or *Aerococcus viridans*.

The present study extends the work of Günther & White (1961 *a, b*). The examination of the physiological and serological characters of fresh cultures was used to assess the validity of previous classifications. Repetition of Dr Günther's physiological work was avoided except where necessary for comparative purposes or where it became evident that new characteristics might be used to define the genus and species in a more exact manner. The aim of the serological investigation was the more detailed study of *P. parvulus* and group III organisms.

METHODS

Cultures

Sources of cultures. Cultures were freshly isolated from a variety of habitats of both animal and vegetable origin. Tomato juice agar (Oxoid), Rogosa SL agar (DIFCO) and 'Beer' agar (Williamson, 1959) were used as primary selective media. Strains representative of named species were also included. Details of all isolates examined are given in Table 1.

Maintenance of stock cultures and culture media used. The method of Günther & White (1961 *a*) was employed in the maintenance of stock cultures. Tomato juice (TJ) broth or agar with the addition of Tween 80 (0.1 %, v/v), pH adjusted to 6.6, were used in the maintenance, preparation of inocula and all experimental investigation of cultures with the exception of: (*a*) strains labelled *Pediococcus halophilus* where sodium chloride was added to all media, 7 % (w/v) for strains D2, D12, and D8, and 5 % (w/v) for TC1; (*b*) strains BE-121, BE-125, BE-126, BE-127, AG, P1 and PD isolated from beer, in future referred to as the 'brewing strains', which were grown on a beer medium (Williamson, 1959) modified by using bottled pale ale to replace unhopped beer with 'Panmede' liver digest (1 %, w/v) added as liver extract source; (*c*) *Pediococcus homari* FP-2, S-53A, strains CH-3, SF3, KT-1 and KT-2 which were cultured on APT agar or broth (Evans & Niven, 1951).

Media for growth of organisms other than pediococci, used in the serological investigation were as follows: *Aerococcus viridans*, ATP agar or broth; staphylococci, nutrient agar or broth (Günther & White, 1961 *a*); and streptococci, 'Oxoid' cooked meat medium for storage and glucose Yeastrel (GY) agar or broth (Günther & White,

1961a) for routine subculture. Seven strains of aerococci and staphylococci were obtained from the National Collection of Type Cultures, London, and streptococci representing Lancefield's serological groups A to S from Wellcome Research Laboratories, Beckenham, Kent.

Table 1. *Details of cultures examined*

Code numbers	Species name (as received)	Habitat	Source
NZ-1, NZ-3, NZ-6, NZ-R2, NZ-R3, NZ-T1, E-1	—	Cheddar cheese	—
SA-88, SA-90, SA-92, SA-96, SA-100, SA-103	—	Sauerkraut	—
F-85, F-98	—	Faecal material from turkeys	—
TO-111, TO-118, TO-119	—	Tinned tomatoes	—
BE-121, BE-125, BE-126, BE-127	—	Guinness beer	—
BE-1, BE-2	—	Lager beer yeast	(1)
AG	<i>P. damnosus</i>	Beer	(2)
P1	<i>P. limosus</i>	Beer	(2)
PD	<i>P. diastaticus</i>	Beer	(2)
PP	<i>P. pentosaceus</i> (Kitahara & Nakagawa, 1959)	—	(3)
PA	<i>P. acidi lactici</i> (Kitahara & Nakagawa, 1959)	Mash, type not specified	(3)
D2, D12, D8	<i>P. halophilus</i> (Kitahara & Nakagawa, 1959)	Soy sauce brewing mashes	(3)
FP-2	<i>P. homari</i>	Ham curing brine	(4)
S-58A	<i>P. homari</i>	Discoloured bacon	(4)
CH-713, CH-778, CH-797, CH-819, CH-867, CH-85F3	—	Vacuum packed bacon	(5)
KT-1, KT-2	—	Discoloured cooked hams	(6)
NCIB 8559	<i>Streptococcus cremoris</i>	Beer	(7)
L-16, L-20, L-22, L-24, L-92, L-95, L-148, L-171 L-223, L-345, L-347, L-351, L-352, L-354	Group III	Silage	(8)

It may be noted that the species name of some cultures listed above was altered as a result of this investigation.

- (1) Dr D. H. Williamson, The Brewing Research Foundation, Nutfield, Surrey.
- (2) Dr R. B. Gilliland, Arthur Guinness, Son and Co. Ltd., Dublin.
- (3) Dr K. Sakaguchi, Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan.
- (4) Dr C. F. Niven, Jr., A.M.I.F., University of Chicago, Illinois, U.S.A.
- (5) Mr J. J. Cavett, Unilever Research Laboratory, Sharnbrook, Bedford.
- (6) Mr M. G. Read, Kearley and Tonge Ltd., Southall, Mddx.
- (7) National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.
- (8) Dr H. L. Günther, Microbiology Department, Queen Elizabeth College, University of London, W. 8.

Preparation of inocula and conditions of incubation. Transfers for preparing inocula were made every 24 hr except for *Pediococcus halophilus*, BE-1, BE-2 and *Streptococcus cremoris* 8559 where transfers were made every 48 hr; these strains will in future be referred to as the 'slower growing' strains. The 'brewing strains' were subcultured fortnightly. For the examination of most cultural and biochemical characters 0.02 ml. of inoculum/5 ml. of medium was used.

All cultures were incubated aerobically at 30° except strains BE-1, BE-2, and 8559

which were incubated at 22°. The 'brewing strains' were incubated in an atmosphere of 95 % (v/v) hydrogen + 5 % (v/v) carbon dioxide at 22°.

Physiology

In these studies new strains were compared as closely as possible with cultures studied by Günther & White (1961*a, b*), in particular the proposed type strains *Pediococcus cerevisiae* ATCC8081 (Günther & White, 1962) and *P. parvulus* s-182 (Günther, Coster & White, 1962).

Morphological examination. The methods of Günther & White (1961*a*) were used to observe morphology, Gram reaction and motility except that Rowland's method was employed to detect capsules (Rowland, 1914). The slower growing strains were examined after 48 hr and brewing strains after 7 and 14 days of incubation.

Cultural characters. In general the methods of Günther & White (1961*a*) were again used. Brewing cultures were examined after 7 and 14 days of incubation. Colony size was determined by measuring the diameter of ten colonies picked at random. The presence of growth on Rogosa SL agar was observed for all cultures after 24 hr, 48 hr and 7 days of incubation, brewing strains were examined after 7, 14 and 28 days.

Factors affecting growth. Again the methods of Günther & White (1961*a*) were followed except for using pH levels of 4.4 and 8.6, and testing some strains for growth at the 10 % (w/v) sodium chloride level. All strains were grown in the liquid medium used for their routine cultivation and examined after 1, 2 and 7 days of incubation. Brewing strains were examined after 7, 14 and 28 days of incubation.

Biochemical reactions. The methods of Günther & White (1961*a*) were employed for the following tests; utilization of ammonium salts as sole nitrogen source, hydrolysis of gelatin and aesculin; production of carbon dioxide from glucose; final hydrogen-ion concentration in GY broth; production of acetylmethylcarbinol from glucose; haemolytic reaction (brewing strains incubated for 14 days), and production of ammonia from arginine (brewing strains tested after 21 days of incubation). Günther & White's (1961*a*) method was also used to determine the reaction in litmus milk, bearing in mind that not all strains appear to require folic acid for growth (Günther & White, 1961*a*). A fairly large inoculum was used (0.04 ml. of T.B.) so some carry-over may have occurred.

The presence of catalase was observed after cultivation for 24 hr using the method and medium of Günther & White (1961*a*). As some strains grew poorly in this medium all cultures were also tested after subculturing for 24 hr (7 days for brewing strains) on YTG agar (Felton, Evans & Niven, 1953) modified by the addition of Tween 80 (0.1 %, v/v), FeSO₄ (0.04 %, w/v), MgSO₄ (0.08 %, w/v) and MnCl₂ (0.014 %, w/v).

The method devised by Rogosa (1961) was employed to test for the reduction of nitrates. Cultures were grown in the indole nitrite medium (BBL) with MgSO₄, MnSO₄ (0.05 %, w/v) and with and without added glucose (0.01 %, w/v).

The type of lactic acid produced from glucose by pediococci was examined using the medium and method of Camien & Dunn (1954). The medium was modified by the addition of MgSO₄ and MnSO₄ (0.05 %, w/v) and Tween 80 (0.1 %, v/v). Cultures were incubated for 1 month. The optical rotation was determined polarimetrically on a 5 % (w/v) aqueous solution.

The general method of Günther & White (1961*a*) was followed in determining carbohydrate fermentation reactions apart from a modification of the basal medium by the addition of Tween 80 (0.1 %, v/v).

No record of lipolytic enzymes in pediococci was found in the literature. The observation that the presence of Tween 80 stimulated growth (Garvie & Gregory, 1961; Coster, 1963) might indicate the presence of lipolytic enzymes. The method of Sierra (1957) was employed to test for the presence of these enzymes in some pediococcus cultures using TJ agar as the basal medium.

Serology

Organisms used in the preparation of antisera. Antisera were prepared against 14 strains of pediococci, 11 of these being strains already examined physiologically by Günther & White (1961*a*). *Pediococcus cerevisiae* (group I) was represented by strains 8081 and BP-1; *P. parvulus* (group II) by strains SS-101, HY-22s, S-182 and S-344; group III by cultures L-24, L-92, L-345, L-351 and L-352. Strains NZ-R3, F-98 and *P. damnosus* PD were also used to prepare antisera. An antiserum was prepared against *Aerococcus viridans* NCTC8251.

Preparation of antisera. All antisera except antiserum *P. damnosus* PD were prepared by intravenous injection of whole living organisms into rabbits known to be free of naturally occurring antibodies. Cells were grown in 40 ml. glucose Lemco broth containing as %, w/v; glucose, 1.0; lab Lemco, 1.0; peptone (Evans), 1.0; NaCl, 0.5; MgSO₄, 0.05; MnSO₄, 0.05; Tween 80, 0.1 (v/v). The methods of Günther & White (1961*b*) were followed regarding inoculation and incubation of the medium, and preparation of cells for animal injection.

Great difficulty was experienced in preparing antisera against strains L-24 and L-92. Potent antisera were obtained after five courses of injections and antibodies detected for only a week in the animal before loss in potency occurred. Two injections of 2 ml. each were given always a week before these antisera were withdrawn.

Due to the lack of reactivity noted in preliminary precipitin tests of cultures considered to resemble the species *P. damnosus* Mees with *P. cerevisiae* antisera, a disintegrated cell suspension (prepared with the Mickle tissue disintegrator) was used to obtain a potent antiserum against strain PD by the method of Shattock (1949) who found this method successful for refractory Lancefield group D streptococci. Shattock's method was also used by Günther & White (1961*b*). Cells for injection were grown in beer broth for 14 days.

Sera were stored at 4° without preservative.

Commercial antisera of streptococci Lancefield's groups A to Q were obtained from Burroughs Wellcome and Co. Ltd.

Preparation of extracts. The HCl extraction method of Lancefield (1933) as modified by Sharpe (1955*a*) and Günther & White (1961*b*) was employed. Duplicate extracts were prepared of new isolates and of most of the pediococci in Dr Günther's collection (Günther & White, 1961*a*, Table 1), and also of representative aerococcus, staphylococcus, leuconostoc and streptococcus strains.

Precipitin tests. The method of Sharpe (1955*a*) was used, observations being made at intervals up to 1 hr.

Precipitin-absorption tests. The techniques outlined by Sharpe (1955*a*) and Günther & White (1961*b*) were followed.

Tube-agglutination tests. Tests were carried out in Dreyer's agglutination tubes in 0.9 % (w/v) saline using a suspension of washed cells, opacity approximately equivalent to Brown's tube no. 3, grown for 16 hr in TJ broth. The tubes were read after overnight incubation at 50°.

RESULTS

Physiology

All new isolates resembled each other in their morphological arrangement, the cells occurring singly, in pairs, tetrads, regular and irregular clusters and short chains of 4 to 5 cells. All cultures were Gram-positive, non-motile, without spores or capsules. The formation of a turbid growth and slightly viscid sediment in broth was typical of all strains except the strain *Pediococcus limosus* (P1) which could form slime. No strains were able to hydrolyse gelatin, utilize ammonium salts as sole nitrogen source, reduce nitrates to nitrites or nitrogen, or produce carbon dioxide from the fermentation of glucose. Lipolytic enzymes were not detected.

On comparing the characters shown by new isolates with those described by other workers it was evident that all new cultures closely resembled previously defined species. In this section the detailed consideration of characters of new strains is therefore presented under species headings.

Strains fell into two major physiological groups, those resembling pediococci as defined by Pederson (1949), Jensen & Seeley (1954), Günther & White (1961*a, b*); and those similar to *Pediococcus homari* (Deibel & Niven, 1960) and *Aerococcus viridans* (Williams *et al.* 1953). The latter strains have been labelled aerococci in Table 2 which summarizes the physiological characters of the strains studied.

Pediococci. The majority of isolates belonged to this group and resembled strains previously placed in the four species *Pediococcus cerevisiae*, *P. parvulus*, *P. damnosus* and *P. halophilus*.

Pediococcus cerevisiae. Seventeen strains belonged to this species, NZ-1, NZ-3, NZ-6, NZ-R2, NZ-R3, NZ-T1, E-1, TO-111, TO-118, TO-119, CH-713, CH-788, CH-797, CH-819, CH-867, and the two cultures received as *P. pentosaceus* (PP) and *P. acidilactici* (PA). Cultures were easily recognized by their profuse growth in TJ broth and on Rogosa SL agar in 24 hr; surface agar colonies having a diameter between 0.25 mm. and 1.2 mm.; ability to initiate growth at pH 8.6 after incubation of 5 days; the production of ammonia from arginine, a low pH value in GY broth, and the formation of acid, usually with coagulation, in litmus milk. All isolates grew well in the presence of 4% and 6.5% (w/v) sodium chloride in 24 hr except for one strain where growth was delayed at the 6.5% level, only half showed poor growth at the 10% level. Cultures placed in this species showed growth at the widest temperature range, the optimum occurring between 30° and 37°, some strains growing at 45° and 50°. Catalase was present in all strains on first testing (except PA), but could not be detected in one culture (NZ-6) on repeating the test 18 months later. A narrow zone of beta haemolysis was observed in 10 cultures on first isolation. This character was lost in all but 4 cultures on retesting a year later. Lactic acid isolated from one culture (NZ-R3) was optically inactive.

Pediococcus parvulus. Eight cultures SA-88, SA-90, SA-92, SA-96, SA-100, SA-103, F-35, F-98, formed a distinct entity based on the following characters: moderate growth in TJ broth; moderate growth after 48 or 72 hr on Rogosa SL agar; colony

size ranging from 0.25 mm. to 0.7 mm.; inability to grow at pH 8.6; inability to grow at 45° or in the presence of 10 % (w/v) sodium chloride. All organisms, except one, grew in the presence of 4 % (w/v) sodium chloride within 24 hr. Growth was considerably delayed at a concentration of 6.5 %, one strain did not grow at this sodium chloride concentration. Catalase was not detected, ammonia was not produced from arginine nor acetylmethylcarbinol from glucose. Litmus milk showed a weak acid reaction. Lactic acid obtained from two strains (SA-100, F-35) was optically inactive. The two strains isolated from turkey faeces (F-35 and F-98) differed from the other cultures in this group in showing poor growth at 37° and in the presence of 4 % sodium chloride, with no growth at 40°.

Pediococcus damnosus. The ten cultures isolated from beer and beer yeast, BE-1, BE-2, BE-121, BE-125, BE-126, BE-127, NCIB 8559, AG, PD and P1 were characterized by their low optimum temperature of 22°, no growth at 37° nor in the presence of 4 % (w/v) and 6.5 % sodium chloride. Growth in broth cultures was moderate to poor, and it is obvious that if a better growth medium could be devised these results might be modified and the subgrouping amended. Catalase was absent and no ammonia was formed from arginine. (More variation in physiological characters as between strains occurred in this species than in the species *P. cerevisiae* and *P. parvulus*.) Two main divisions were recognized within *P. damnosus*.

Three strains (BE-121, BE-126, AG) were distinct in having transparent colonies on beer agar, some punctiform in shape with a diameter varying from 0.15 mm. to 0.9 mm.; giving poor growth in broth culture; no growth at 10°, 30°, pH 4.4 or 8.6, or in the presence of 4 % (w/v) and 6.5 % sodium chloride. No acetylmethylcarbinol was formed from glucose. Growth was stimulated by anaerobic incubation. These cultures formed group (a).

The remaining strains except strain P1 formed group (b) and showed the off-white circular colonies characteristic of pediococci, and moderate growth in broth culture. Growth took place at 10°, 30°, and pH 4.4. On further examination strains BE-1, BE-2 and NCIB 8559 were found to have a colony size varying between 0.2 mm. and 0.7 mm., they also formed acetylmethylcarbinol from glucose and fermented trehalose. Strains BE-125, BE-127 and PD attained a larger colony size of up to 1.2 mm. The culture received as *P. limosus* P1, although showing the characters shared by group (b) cultures, was distinctive in forming slime in beer and TJ broth under anaerobic conditions, giving good growth in the liquid media used and having a larger colony size of up to 2 mm. diameter.

Pediococcus halophilus. Cultures D2, D12 and D8 were distinctive in their absolute requirement for sodium chloride in all media used during this study. Growth was present but delayed at 1 % (w/v), 2 %, 3 %, 4 %, 15 % and 20 %, maximum growth was obtained after 48 hr in 7 % and 10 % sodium chloride. Temperature growth range was from 10° to 40°, strains were able to initiate growth at pH 8.6 after 5 days of incubation but not at pH 4.4. Catalase was not detected, ammonia was not formed from arginine and no growth occurred on Rogosa SL agar.

Aerococci. Cultures *Pediococcus homari* FP-2, S-53A; KT-1, KT-2, CH-3SF3 were separated from strains considered to be pediococci on the basis of the following characters; inability to grow well in TJ broth, on Rogosa SL agar and at pH 4.4; inhibition of growth under anaerobic incubation and in stab culture, growth occur-

Table 2. Comparison of physiological characters of *pediococci* and *aerococci*

	Pediococci							
	<i>P. cerevisiae</i>	<i>P. parvulus</i>	<i>P. damnosus</i>		P1	Group III*	<i>P. halophilus</i>	Aerococci
			Group (a)	Group (b)				
Number of strains	17	8	3	7	1	14	3	5
Growth in TJB (pH 6.6)	+++	++	+	+	++	++	++	—
Optimum temp. 22°	—	—	+	+	+	—	—	—
Optimum temp. 30°	+	+	—	—	—	+	+	+
Growth at 10°	+	+	—	+	+	+	+	+++
37°	+++	+++	—	—	—	++	++	+++
40°	+	+	(6)	—	—	++	+	++
45°	+	—	.	—	.	—	—	—
Growth in NaCl 4%, w/v	+++	+++	—	—	—	++	++	+++
6.5%	++	+	(7)	—	—	(11)	++	+++
10%	+	—	.	.	.	.	++	+++
Growth at pH 4.4	+++	+++	—	+	+	—	(4/4)	+++
8.6	+++	—	—	—	—	—	(4/4)	+++
Growth on RSLA	+++	++	+	+	+	+	—	—
Slime production	—	—	—	—	+	—	—	—
Catalase reaction	+	—	—	—	—	—	—	(4)
NH ₃ from arginine	+	—	—	—	—	—	+	—
Aesculin hydrolysis	+	+	(4)	+	(3)	+	+	+
AMC from glucose	+	—	—	+	(3)	—	+	—
Final pH in GYB	3.2-4.2	3.74-4.02	5.41-5.94	3.95-6.62	4.13	4.4-5.0	4.52-4.64	4.72-5.26
Litmus milk								
Acid reaction	+	+	(weak)	—	—	+	(8)	—
Dye reduction	—	—	—	—	—	—	—	—
Coagulation	+	(15)	—	—	—	—	—	—
Acid from								
Glucose	+	+	+	(2)	+	(5)	—	+
Xylose	—	(12)	—	—	—	—	(13)	—
Arabinose	—	—	—	—	+	(2)	(13)	—
Fructose	+	+	—	(2)	+	(2)	+	+
Sucrose	—	+	(1)	—	—	—	+	+
Lactose	+	(14)	—	(5)	—	—	(12)	+
Maltose	+	(16)	+	(7)	+	(2)	+	+
Trehalose	+	+	(7)	—	(2)	+	(8)	+
Raffinose	—	—	—	(2)	+	(3)	—	+
Inulin	—	—	—	—	—	—	—	+
Dextrin	—	+	(1)	—	—	—	(2)	+
Glycerol	+	(11)	—	—	—	+	—	(2)
Mannitol	—	—	—	—	—	+	+	+
Sorbitol	—	+	(3)	—	—	—	+	(3)
Salicin	+	—	(7)	—	—	—	+	—
	+	+	—	(2)	—	—	+	+

+++ , Profuse growth; ++ , good growth; + , moderate to poor growth, or a positive reaction; — , no growth or reaction.

TJB = tomato juice broth; RSLA = Rogosa SL agar; GYB = glucose Yeastrel broth; AMC = acetylmethylcarbinol.

Figures in parentheses denote number of strains giving indicated reaction: if expressed as a fraction denotes number of strains giving reaction/number tested.

* Most results taken from table 2, Günther & White (1961*a*).

ring along the upper half only of the inoculation; good growth in the presence of 4 % (w/v), 6.5 % and 10 % sodium chloride but not at 15 % or 20 %; profuse growth at pH 8.6.

Serology

The antiserum prepared against *Pediococcus cerevisiae* 8081 used as a reference serum gave similar results in the precipitin reactions to the antiserum prepared against the same culture by Günther & White (1961*b*) but showed less specificity in the agglutination tests.

Cultures of *Pediococcus damnosus* M-1, 8519, 8520 examined by Günther & White (1961*a*) are included in our *P. damnosus* group (b) of this species. Difficulty was experienced in obtaining potent extracts of some cultures placed in the species *P. damnosus* on physiological evidence which requires further investigation.

No formation of the Tween antibodies noted by Middlebrook & Dubos (1947) for the tubercle bacillus was observed in any of our antisera which thus resembled lactobacilli antisera (Sharpe, 1955*b*).

Precipitin tests. The results are summarized in Table 3. All antisera reacted with their homologous extract and most of the heterologous extracts of the same species or physiological group. Antisera prepared against *Pediococcus cerevisiae* reacted with most extracts of *P. cerevisiae*. Antisera against *P. cerevisiae* 8081 and BP-1 reacted with approximately half the extracts of *P. parvulus* cultures while antiserum NZ-R3 precipitated consistently with more extracts of *P. cerevisiae* and *P. parvulus* strains.

Pediococcus parvulus and *P. damnosus* antisera reacted with the majority of *P. parvulus* and *P. cerevisiae* extracts. *Pediococcus damnosus* antiserum reacted with strains *P. damnosus* BE-125, BE-127 and P1 but not with strains placed in group (a) or other group (b) strains. Extracts of this species showed little reactivity with other antisera, group (a) not reacting with any antisera.

The antisera prepared against group III strains showed many cross-reactions with *Pediococcus cerevisiae*, *P. parvulus* and a few *P. damnosus* extracts. Group III extracts did not react with antisera prepared against strains other than group III, except for a few isolated reactions which proved to be non-specific on absorption of the antiserum with homologous cells. Cross-reactions were also observed between the antiserum prepared against *Aerococcus viridans*, and *Pediococcus cerevisiae* and a few *P. parvulus* extracts, but none occurred between extracts of aerococci and pediococcal antisera except one strain (FP-2) which formed a heavy precipitate.

Extracts of *Pediococcus halophilus* strains did not react with any antisera except that of *P. cerevisiae* NZ-R3; precipitated with one culture (TC 1) with *A. viridans* antiserum.

No cross-reactions were noted between a leuconostoc or staphylococcus except for *Staphylococcus aureus* and the group III antiserum L-851. This antiserum also precipitated with extracts of Lancefield's group B and L streptococci. A cross-reaction was seen between antiserum *P. cerevisiae* NZ-R3 and a Lancefield group D streptococcus. On testing commercial streptococcal antisera against extracts of representative strains of pediococci, positive precipitin reactions were obtained with *P. cerevisiae*, *P. parvulus* and *P. damnosus* (b). Similar results were observed with a group D serum obtained from the Institut Pasteur, Paris. No other reactions occurred between pediococcal antisera and extracts of streptococci or commercial streptococcal antisera and extracts of pediococci.

Table 3. *Results of precipitin tests using pediococcal and aerococcal antisera*

Antisera... ..	<i>P. cerevisiae</i>		<i>P. parvulus</i>			Group III					<i>P. dam-</i> <i>nosus</i>	<i>Aerococcus</i> <i>viridans</i>
	8081*	NZ-R3	F-98	S-182	S-344	L-24	L-92	L-345	L-351	L-352	PD	8251
<i>P. cerevisiae</i> 8081	++	++	+	+	+	+	±	+	++	++	+	+
NZ-R3	-	++	±	-	-	±	±	-	±	-	+	-
Other strains	+30/37	+34/37	+28/35	+32/37	+21/37	+28/35	+26/35	+28/35	+26/35	+28/35	+20/25	+23/34
<i>P. parvulus</i> F-98	-	±	++	+	-	±	-	-	-	±	+	±
S-182	-	-	+	++	+	-	-	-	-	-	+	-
S-344	-	±	±	+	++	±	-	-	-	-	+	-
Other strains	+12/35	+23/35	+28/35	+28/35	+18/35	+18/35	-27/35	-28/35	-29/35	-20/35	+14/17	-31/35
Group III L-24	-	-	-	-	-	+	±	+	±	+	-	-
L-92	-	-	-	-	-	+	+	±	±	+	-	-
L-345	-	-	-	-	-	±	±	++	+	++	-	-
L-351	-	-	-	-	-	±	+	++	++	++	-	-
L-352	-	-	-	-	-	±	±	++	+	++	+	-
Other strains	-9/9	-8/9	-7/9	-8/9	-9/9	+8/9	+8/9	+9/9	+9/9	+7/9	+5/9	-8/9
<i>P. damnosus</i> (b) PD	-	-	-	±	±	-	±	±	±	±	+	-
Other strains	-8/8	-7/8	-4/8	-4/8	-6/8	-8/8	-5/7	-7/8	-6/7	-5/7	-4/8	-8/8
<i>P. damnosus</i> (a)	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3	-3/3
<i>P. damnosus</i> var. <i>limosus</i>	-	+	+	+	+	±	-	+	+	+	+	-
<i>P. halophilus</i>	-4/4	+2/4	-4/4	-4/4	-4/4	-4/4	-4/4	-4/4	-4/4	-4/4	-4/4	+1/4
<i>P. urinae equi</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Aerococcus viridans</i> 8251	-	-	-	-	-	-	-	-	-	-	-	++
Other strains	-10/10	-9/10	-10/10	-10/10	-9/10	-9/9	-10/10	-10/10	-10/10	-9/10	-8/8	+7/10

++, strong precipitate; +, good precipitate; ±, weak precipitate; -, no precipitate formation.

Fractions indicate no. of extracts giving reaction/total no. tested.

* Reference antiserum.

Precipitin-absorption tests. The results of the absorption of three group III antisera with homologous and heterologous cells are shown in Tables 4 and 5.

The presence of separate antigens in group III was shown on absorption of antisera prepared against L-351 and L-352 with cells of *Pediococcus cerevisiae* and *P. parvulus* where precipitin reactions indicated that antibody activity was removed for these two species but not for group III extracts. All antibody activity was lost in a similar experiment with the antiserum prepared against L-24.

When the antiserum against L-352 was absorbed with cells of L-92, the absorbed antiserum did not react with extracts of L-92 and L-24, but did react with homologous and other extracts of group III, indicating the presence of a different antigen.

Table 4. *Absorption of antiserum L-352 (group III) with cells of pediococci and an aerococcus*

Antigen	Unabsorbed	Antiserum absorbed with							
		Group III			<i>P. cerevisiae</i>			<i>P. Aerococcus parvulus viridans</i>	
		L-352	L-92	L-92 + L-352	8081	F-166	NZ-T1	s-188	8251
Group III									
L-352	++	—	+	—	+	+	+	+	+
Other strains	+	—	+	—	+	+	+	+	+
			—*		—*	±*		—*	
<i>P. cerevisiae</i>	+	+	—	—	—	—	—	—	+
<i>P. parvulus</i>	+	(±)	(±)	—	—	(+)	—	—	+
<i>Aerococcus</i> sp.									
FP-2	+	—	—	—	—	—	—	.	—

., Not tested.

* = Result of reaction with extracts L-24 and L-92.

Symbols in parentheses indicate majority of extracts giving reaction, other symbols as Table 3.

Table 5. *Absorption of antisera L-24 and L-351 (group III) with cells of P. cerevisiae, P. parvulus and group III*

Antigen	Unabsorbed	Antiserum						
		L-24			L-351			
		Absorbed with			Absorbed with			
		Group III	<i>P. cerevisiae</i>	<i>P. parvulus</i>	Unabsorbed	Group III	<i>P. cerevisiae</i>	
		L-24	NZ-R3	S-290		L-351	L-345	CH-867
Group III								
L-24	+	—	—	—	±	—	—	—
L-351	±	—	.	.	++	—	—	+
Other strains	+	—	—	—	+	—	—	±
<i>P. cerevisiae</i>	+	±	—	—	+	—	—	—
<i>P. parvulus</i>	±	±	—	—	+	—	—	—
<i>P. damnosus</i> P1	.	.	.	.	+	—	—	—

Symbols as Table 4.

On absorbing the same batch of antiserum with homologous cells to remove antibody activity for all group III extracts, all antibody activity was lost.

The antibodies present in antisera L-24 and L-352 reacting with extracts of *Pediococcus cerevisiae* and *P. parvulus* were not removed on absorption with homologous cells. Absorption might not have been complete.

Table 6. *Results of agglutination tests*

Antigen	Antisera							
	<i>P. cerevisiae</i>		<i>P. parvulus</i>		Group III			
	8081	NZ-R3	F-98	s-182	L-24	L-92	L-345	L-352
<i>P. cerevisiae</i>								
8081	1280	40	20	40	20	—	20	20
NZ-R3	80	640	80	160	80	320	80	40
<i>P. parvulus</i>								
F-98	40	160	160	80	20	20	80	80
s-182	80	160	80	1280	—	20	80	80
Group III								
L-24	—	—	—	—	320	640	—	—
L-92	—	—	—	—	80	160	20	—
L-345	80	160	—	160	80	160	1280	1280
L-352	160	160	·	160	40	160	1280	1280

Figures indicate reciprocals of the highest titres read.
—, no agglutination reaction observed, > 20.

On absorption of antiserum L-352 with an aerococcus no antibodies were removed except those reacting with extracts of strain FP-2 thereby giving additional evidence that this strain resembled aerococci. Extracts of *Pediococcus damnosus* strain P1 showed no reaction with antiserum L-351 absorbed with *P. cerevisiae* and group III cells. It would appear that no separate antigen was involved.

Tube-agglutination tests. The results are summarized in Table 6. Half the antisera gave titres of 1/1280 with homologous cells. Titres did not exceed 1/80 with heterologous cells of the same species except for several group III strains. This group showed some heterologous titres of the same value as homologous titres.

Cross-reactions were present between antisera of *Pediococcus cerevisiae* and cells of *P. parvulus* and group III except strains L-24 and L-92. The same was true for sera prepared against *P. parvulus*.

The group III sera prepared against L-24 and L-92 reacted with all strains of group III as well as some strains of *Pediococcus cerevisiae* and *P. parvulus* to titres of 1/20, 1/80 and one of 1/320. It was noted that a higher titre was obtained between antiserum L-92 and cells of L-24 than with homologous cells. However, the agglutinating titres were proportionally the same as those observed with antiserum L-24 and cells of L-92. Sera prepared against L-345 and L-352 did not react with cells of L-24 of group III, and antiserum L-345 only agglutinated with strain L-92 to a titre of 1/20. These two antisera reacted with all strains of *P. cerevisiae* and *P. parvulus* included in this experiment and would appear to share a common type antigen.

DISCUSSION

In this study most of the *Pediococcus* cultures examined resembled the species *Pediococcus cerevisiae* Pederson and would appear to be more 'typical' of the genus than the cultures obtained from brewing sources.

It will be noted that *Pediococcus cerevisiae* Pederson may be found in a wide variety of habitats and thus may be more suitable as the type species than the less common strains from breweries, although these were the first *Pediococcus* described. The strains the Japanese workers studied were mostly of brewing origin, and although they applied the name *P. cerevisiae*, in our opinion these strains should be classed as *P. damnosus*.

For classification purposes the definition of species in the genus *Pediococcus* given by Nakagawa & Kitahara (1959), based primarily on growth at different pH levels, was found difficult to apply to strains from a variety of sources and we prefer to separate the species by consideration of a number of different characters.

No strains having a high optimum growth temperature were found. The culture received as *Pediococcus acidilactici*, separated from other *Pediococcus* by Nakagawa & Kitahara (1959) on the ability to grow at higher temperatures, was not observed to be different in its temperature growth range from some other strains identified as *P. cerevisiae*. The good general agreement in physiological and serological findings between new isolates from sauerkraut and faecal material, and strains defined as *P. parvulus* Günther & White adds further weight to the acceptance of this new species in the genus *Pediococcus*. There does appear to be some physiological variation within the species, verifying the results of Günther & White (1961*a, b*). New *P. parvulus* cultures showed a greater tolerance to sodium chloride than Günther & White's strains, possibly due to the presence of Tween 80 in the medium thus rendering their criterion 'growth in 4% NaCl' unsatisfactory (Günther & White, 1961*a*, Table 1). It is suggested that the criterion 'growth at pH 8.6' be included to separate *P. cerevisiae* from *P. parvulus*. Colony size was larger in the new strains again possibly due to the incorporation of Tween 80 in the medium. The definition of *P. parvulus* may have to be amended on this point. No marked serological differences were noted.

Organisms isolated from brewing sources emerged as a distinct group. The species name of *Pediococcus damnosus* Mees is accepted as applicable to such cultures. The species may be divided into several groups. The distinctive physiological features of *P. damnosus* group (a) and lack of antibody activity with *P. damnosus* PD antiserum is evidence for the proposal of this group as a variety. In sensitivity toward temperature and in stimulation of growth by anaerobic incubation, group (a) resembles the *P. cerevisiae* strains described by Nakagawa & Kitahara (1959). Their proposed varietal names of this species, *diastaticus*, *salicinaceus* for salicin fermenting isolates, and *mevalovor* for cultures requiring mevalonic acid for growth do not adequately define group (a) strains. We therefore suggest the name *P. damnosus* var. *damnosus*. The description of *Streptococcus damnosus* var. *diastaticus* (Andrews & Gilliland, 1952) the name originally applied to the culture received as *P. diastaticus* fitted the general character of group (b) strains and 'diastaticus' is proposed as the varietal name. Cultures *P. damnosus* M-1, 8519, 8520 examined by Günther & White (1961*a*) resembled group (b) isolates BE-1, BE-2 and NCIB 8559 in colony size, acetylmethyl-

carbinol production and fermentation of trehalose. Further work may show these cultures to be a distinct entity. It should be noted that NCIB 8559 was received as *Streptococcus cremoris*. We consider it to be a *P. damnosus*. The most accurate taxonomic position of the culture *P. limosus*, pending further work with slime-forming strains, is considered to be as a further variety of *P. damnosus*, *P. damnosus* var. *limosus* Shimwell. Thus there may be three variants of *P. damnosus*.

The results obtained with salt-requiring strains would indicate that they can be separated from other pediococci at the species level. The nutritional studies of *Pediococcus halophilus* and other pediococcal strains by Sakaguchi (1960) and Nakagawa & Kitahara (1959) which showed that *P. halophilus* required more vitamins but less amino acids for growth than the other strains, affords additional evidence of specific differences. The ability to grow in high salt concentrations involving the withstanding of high osmotic pressures, and the type of colony growth distinguishes *P. halophilus* from *P. homari* Deibel & Niven and *Aerococci viridans*. Characters shared with aerococci such as sensitivity towards acid conditions, ability to ferment sucrose and mannitol, and formation of optically active lactic acid (Sakaguchi, 1958) places this species in an intermediate position between aerococci and other pediococcal species.

The numerous cross-reactions shown by group III antisera with extracts of other species or serological groups was also experienced by Swartling (1951) and Briggs & Newland (1952) with *Streptococcus cremoris* cultures where absorption experiments were required to verify the possession of the Lancefield group N antigen. Similarly, with our group III antisera, precipitin-absorption experiments indicated the presence of separate antigens for group III, and *Pediococcus cerevisiae* and *P. parvulus*. This evidence together with the ability to form dextrorotatory lactic acid (detected in two cultures, Coster, 1963; Garvie, personal communication) is characteristic of these organisms which thus may be considered as a definite group certainly at variety or perhaps at species level. They resemble *P. urinae equi* in the formation of dextrorotatory lactic acid (Garvie, personal communication) but no serological relationship was found. We had hoped to be able to assign these group III organisms to a new species but did not feel justified in so doing at this stage as no new isolates were obtained during the course of the present work and 14 strains only were available from Dr Günther's collection. Further work, preferably with fresh isolates, may establish the differences at species level. Günther & White (1961*a*) considered that these strains resembled the published description of *Streptococcus damnosus* var. *diastaticus* (Andrews & Gilliland, 1952), but the present work on the species 'damnosus' shows that they are closer to *P. cerevisiae* than to *S. damnosus* var. *diastaticus*.

The position with regard to the culture received as *Pediococcus urinae equi* (PUE) remains obscure. Günther & White (1961*b*) reported serological reactions between PUE extracts and *P. cerevisiae* sera but we were unable to confirm these results—PUE reacted with none of the sera (of whatever group) that we tested. In view of this, we did not feel it reasonable to assign this organism to group III despite the resemblance shown in such characters as the formation of dextrorotatory lactic acid.

The five new isolates from meat sources resembled aerococci and *Pediococcus homari* and formed a distinct group easily distinguished from other strains by their sensitivity towards acid and oxygen tension, tolerance to sodium chloride and lack

of common antigens. Such marked differences can be expressed by division at the specific or generic level. The placing of these strains in the species *P. homari* would entail widening the definition of the genus to include such strains. Much evidence is present on which to base a separation of these organisms from other *pediococci* at generic level, thus retaining *Aerococcus* as a distinct genus.

In the light of recent work, it is suggested that the definition of the genus as given in *Bergey's Manual* (1957) be widened to include the formation by some strains of dextrorotatory lactic acid, and of slime under defined cultural conditions. The requirement of carbon dioxide for good growth (Nakagawa & Kitahara, 1959) by some strains should also be included.

Proposed classification of the genus Pediococcus

Species name	Synonym	Reference
<i>P. cerevisiae</i> Balcke	<i>P. pentosaceus</i>	(1)
	<i>P. acidi lactici</i>	(1)
var. <i>dextrinicus</i>	Group III	(2)
var. <i>urinae equi</i>	<i>P. urinae equi</i>	(1)
<i>P. parvulus</i> Günther & White		(2)
<i>P. damnosus</i> Claussen	<i>P. damnosus</i>	(8)
	<i>P. cerevisiae</i>	(1)
var. <i>diastaticus</i>	<i>Streptococcus damnosus</i>	(4)
	var. <i>diastaticus</i>	
var. <i>damnosus</i>	<i>Streptococcus damnosus</i>	(5)
var. <i>limosus</i>	var. <i>limosus</i>	
<i>P. halophilus</i> Mees	<i>P. soyae</i>	(6)
<i>Aerococcus viridans</i> Williams, Hirsch & Cowan	<i>P. homari</i>	(7)

References

(1) Nakagawa & Kitahara (1959)	(5) Shimwell (1949)
(2) Günther & White (1961 a)	(6) Sakaguchi (1958)
(3) Mees (1934)	(7) Deibel & Niven (1960)
(4) Andrews & Gilliland (1952)	

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