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Leuconostoc oenos sp.nov.

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SUMMARY

Nineteen strains of Gram-positive cocci isolated from wine and belonging to the genus *Leuconostoc* were examined and are considered to form a new species which is named *Leuconostoc oenos*. These strains differ from other leuconostocs because they grow better in a medium containing tomato juice than in yeast glucose citrate broth normally used in this laboratory for the genus. Furthermore, they grow well in media in which the pH value is too acid for other Leuconostoc species to initiate growth. The cultures of *L. oenos* do not form dextran from sucrose. They form acid from fructose, glucose, trehalose and aesculin, generally from melibiose and salicin. They may form acid from arabinose, xylose, galactose, mannose and cellobiose, but not from lactose, maltose, sucrose, raffinose, dextrin, glycerol, mannitol or sorbitol.

INTRODUCTION

Gram-positive cocci belonging to the genus *Leuconostoc* occur in wine as part of the normal bacteriological flora (Bidan, 1956; Radler, 1958; Ingraham, Vaughn & Cooke, 1960; Peynaud & Domercq, 1961), but none of these reports satisfactorily identifies the Leuconostoc species which they described. Cultures obtained from Drs Radler, Peynaud and Ingraham were therefore examined and compared with strains of other species of the genus *Leuconostoc* held in the National Collection of Dairy Organisms (Shinfield) and described by Garvie (1960).

METHODS

Cultures. The strains of wine leuconostocs examined are listed in Table 1. They were found to require conditions of growth different from those required by other species of the same genus. The media and methods used had therefore to be modified to suit the wine strains.

General media for, and cultivation of, wine leuconostocs. The cultures were grown in acidic tomato broth (ATB) consisting of (w/v): Evans peptone, 1·0 %; Yeastrel, 0·5 %; glucose, 1·0 %; $MgSO_4.7H_2O$, 0·02 %; $MnSO_4.4H_2O$, 0·005 %; and (v/v): tomato juice, 25 %; pH 4·8; autoclaved at 121° for 15 min.; before inoculation 0·5 ml. of 1·0 % (w/v) solution of cysteine hydrochloride sterilized by filtration was added to each 10 ml. of media. The cultures were incubated at 22° for 3–4 days.

Stock cultures. Agar stabs were prepared by adding 1·5 % agar to ATB. After inoculation these were incubated in anaerobic jars, evacuated then filled with H_2+CO_2 (90:10, v/v). The cultures were then kept in cold store for several months.

General media for, and cultivation of, non-wine leuconostocs. These strains were

grown in yeast glucose citrate broth (YGCB) consisting of (w/v): Evans peptone, 1·0 %; Lemco, 1·0 %; Yeastrel, 0·5 %; glucose, 1·0 %; triammonium citrate, 0·5 %; sodium acetate, 0·2 %; $MgSO_4.7H_2O$, 0·02 %; $MnSO_4.4H_2O$, 0·005 %; and (v/v): Tween 80, 0·1 %; pH 6·7; autoclaved 121° for 15 min.

Table 1. *Source of cultures of Leuconostoc examined*

NCDO no.	Designation when received	Donor
1668	Plince 3	E. Peynaud, Station Agronomique et Oenologique, Bordeaux, France
1669	St Caprais 105	
1670	Ducru Beaucaillon 6	
1671	Brane 33	
1672	Fourtet 1	
1673	Peyreau 1	
1674	Baudry 1	
1675	St Caprais 133	
1705	Fourtet 2	
1706	Grand Puy 3	
1707	Palmer 6	
1708	St Caprais 131	
1709	St Caprais 122	
1694	12 A	F. Radler, C.S.I.R.O., Merbein, Australia
1695	16 A	
1696	45 B	
1821	ML 25	R. E. Kunkee, University of California
1822	ML 27	
1823	ML 34	

Cysteine hydrochloride was added before inoculation with *Leuconostoc cremoris* (*Betacoccus cremoris*, Knudsen & Sørensen, 1929) and cultures of strains of this species were incubated at 22° for 48 hr. The same conditions of growth were used for a few group III strains (Garvie, 1960). All other strains were incubated at 30° for 24 hr (without cysteine hydrochloride).

The turbidity (extinction) of cultures was measured in ATB or YGCB with a Lumetron colorimeter model 400A (Photovolt Corporation, N.Y.) and an orange (580 mμ) filter.

Growth at pH 3·7 was tested in citric+malic acid broth (CMB) which consisted of (w/v): Evans peptone, 1·0 %; Yeastrel, 0·5 %; glucose, 1·0 %; citric acid, 0·25 %; DL-malic acid, 0·25 %; KH_2PO_4, 0·25 %; $MgSO_4.7H_2O$, 0·02 %; $MnSO_4.4H_2O$, 0·005 %; and (v/v): Tween 80, 0·1 %; tomato juice, 5·0 %. The medium was divided and portions adjusted to pH 6·7, 4·7 or 3·7, tubed in 5 ml. quantities, and sterilized by autoclaving at 121° for 15 min. (The pH value of the media did not change on sterilization.) Cysteine hydrochloride was added for the strains of *Leuconostoc oenos* and *L. cremoris* and tubes were inoculated with one drop of a culture in ATB or YGCB. All cultures were seeded into CMB at pH 3·7. Control cultures of *L. oenos* were grown in CMB at pH 4·7 and for control cultures of other strains in CMB at pH 6·7.

Growth in the presence of 10 % (v/v) ethanol. CMB was prepared at double strength and adjusted to pH 4·7 for *Leuconostoc oenos*, and pH 6·7 for other species. The medium was tubed in 2·5 ml. quantities and water added to bring the volume to 4·5 ml. (0·25 ml. less for those strains to which cysteine hydrochloride was added). The medium was autoclaved at 121° for 15 min., cooled and 0·5 ml. absolute ethanol

then added. Tubes were inoculated with a drop of culture, closed with a sterile rubber bung and incubated for 4 days for *L. oenos* or 1–2 days for other species.

Growth in litmus milk (LM) and yeast glucose litmus milk (YGLM). Cultures were incubated at 22° for 7 days.

Growth temperatures. Growth was observed in ATB (with cysteine hydrochloride added) after incubation for 3 days at 37·5° and 7 days at 10°.

Production of ammonia from arginine. Cultures were grown at 22° for 7 days in the medium used by Garvie (1960) and in MRSB (de Man, Rogosa & Sharpe, 1960) prepared without ammonium citrate but with 0·3 % arginine hydrochloride. Nessler's solution was used to test for the production of ammonia.

Production of dextran from sucrose. One ml. of a 50 % (w/v) solution of sucrose, sterilized by autoclaving at 121° for 15 min., was added to 10 ml. ATB agar and plates poured and streaked. Since the wine leuconostocs do not grow aerobically the plates were incubated as already described in an atmosphere of H_2+CO_2 for 14 days.

Utilization of citrate. One ml. of a 10 % (w/v) solution of triammonium citrate was added to 10 ml. ATB, cysteine added and the tubes inoculated. After 3 days' incubation at 22° the residual citrate was assayed by using *Streptococcus lactis* var. *diacetilactis* NCDO 1007 (Garvie, 1967*a*).

Type of lactic acid formed. The cultures were incubated for 3 days in dilute tomato broth (Garvie, 1967*b*). The type of lactic acid formed was estimated by using a DPN method for L(+)-lactate and a modification of the method of van den Hamer & Elias (1958) for D(−)-lactate (Garvie, 1967*b*).

'*Carbohydrate*' *fermentation.*The basal medium consisted of (w/v): Evans peptone, 1·5 %; Yeastrel, 0·6 %; NaCl, 0·5 %; agar, 0·5 %; bromcresol green, 0·004 %; pH 5·2. The medium was prepared in 5 ml. quantities in 6 in. × $\frac{5}{8}$ in. test tubes and autoclaved at 121° for 15 min. The tubes were placed in boiling water to melt the agar, cooled to 45° and 0·5 ml. of 2 % (w/v) Seitz filtered 'Carbohydrate' added. The tubes were inoculated with 0·2 ml. of a 3-day culture, allowed to set and incubated for 21 days at 22°.

The production of gas from glucose. The method of Abd-el-Malek & Gibson (1948) was used.

Catalase was detected by emulsifying the growth from agar in a drop of H_2O_2 (20 vol.) and observing gas formation.

RESULTS

All the nineteen strains of wine leuconstocs were Gram-positive, catalase negative, and formed pairs of chains of cocci. They formed gas from glucose, did not form ammonia from arginine and did not change litmus milk. They formed D(−)-lactic acid. These properties are characteristic of bacteria which belong to the genus *Leuconostoc.*

Growth conditions for wine leuconostoc

YGCB did not support the growth of five of the wine leuconostoc strains (Garvie & Mabbitt, 1967). All nineteen strains grew in ATB, but maximum turbidity was obtained only after several days of incubation. A comparison was made between the growth of the wine cocci and thirty-two other leuconostocs, in both YGCB and ATB. Both media were prepared at pH values of 6·7, 6·0, 5·5 and 4·8. (It was found, as with CMB, that the pH values of YGCB and ATB did not change on sterilization.)

The results are summarized in Table 2.

Of the non-wine leuconostocs (groups I–IV) only three strains (*Leuconostoc paramesenteroides*; Garvie, 1967*c*) grew well in YGCB starting at pH 4·8, and of these only two grew in ATB starting at the same pH value. The other twenty-nine cultures did not grow in media at pH 4·8 but they grew in both media starting at pH 6·7, giving a higher extinction in YGCB than ATB. Maximum turbidity was reached in 24 hr for most strains, but 48 hr were required for *L. cremoris* and other strains which grow best at 22°. None of the strains which grew in ATB at initial pH 4·8 grew in CMB at initial pH 4·2 or 3·7. On the other hand, the wine strains grew well in ATB (initial pH 4·8) and of these 14 also grew in YGCB starting at both pH 6·7 and 4·8 but not as well as in ATB. Growth was slow with all strains and incubation was therefore continued for 3 days. All strains grew in CMB at initial pH 3·7 but growth was not as good as at pH 4·2 or 4·7. A few strains gave slight growth in CMB adjusted to pH 3·2. The ability to grow well in CMB (initial pH 4·2) is used to separate *Leuconostoc oenos* from other species of the genus, for these do not grow in this medium. The addition of cysteine to the medium had a greater effect on those strains of *L. oenos* which did not grow in YGCB than on those which grew in this medium. The growth of most strains was improved by the addition of cysteine; for five strains it was essential.

Table 2. *Range of turbidity (extinction) readings obtained on cultures of Leuconostocs grown in yeast glucose citrate broth (YGCB) and acidic tomato broth (ATB)*

		Medium			
		YGCB		ATB	
		Initial pH value			
Species*	No. of strains examined	pH 6·7	pH 4·8	pH 6·7	pH 4·8
		Lumetron readings			
Group I. *L. cremoris*	6	3·1–1·6	0·6–0·0	2·0–1·15	0·0
Group II. *L. lactis*	3	3·5–2·2	0·65–0·4	2·7–1·4	1·1–0·0
Group III. *L. paramesenteroides*	6	5·1–3·2	5·1–0·0	5·8–0·7	3·7–0·5
Group IV } *L. dextranicum*	3	3·1–2·7	0·7–0·2	2·6–1·9	0·5–0·25
Group V } *L. dextranicum*	4	4·0–2·1	1·3–0·0	3·6–1·7	0·7–0·2
Group VI. *L. mesenteroides*	10	4·9–3·6	2·0–0·0	3·8–2·5	1·8–0·7
Group VII a } *L. oenos*	5	1·2–0·3	2·4–0·8	4·8–3·5	3·7–2·6
Group VII b } *L. oenos*	14	4·0–2·0	4·2–1·7	5·4–3·0	4.7–2·2

* Leuconostoc species named according to Garvie (1967*c*).

Characteristics of the wine leuconostocs

Table 3 gives the results obtained with the wine leuconostocs and compares them with the characteristics of other species (Garvie, 1960).

In the earlier work the fermentative properties of strains were examined in a medium adjusted to pH 6·7 and with bromcresol purple (BCP) as indicator, but since the wine strains grew better in media adjusted to a more acid pH value they were examined in a different medium adjusted to pH 5·2 and with bromcresol green as indicator. However, 12 strains (two of each species) previously examined in BCP medium were tested in the low pH medium; the fermentation pattern was found to be

unaffected by the change of medium. The substances fermented are shown in Table 3. Although there was no tomato juice in the medium results were obtained with all strains of *Leuconostoc oenos* by using the techniques described. On a few occasions a smaller inoculum or washed organisms were tried but acid was not formed from any substrate.

Table 3. *The characters of the species of the genus Leuconostoc*

Figures in parentheses are the numbers of strains examined in that particular test. Where no figures in parentheses are given all strains were examined. Other figures give the number of strains giving a positive reaction.

	L. cremoris Group I	*L. lactis* Group II	*L. oenos* Group VII	*L. paramesenteroides* Group III	*L. dextranicum* Group IV	*L. dextranicum* Group V	*L. mesenteroides* Group VI
No. of strains examined ...	10	9	19	17	5	16	31
Growth in YGCB*	+ (6)	+ (3)	14	+ (6)	+ (3)	+ (4)	+ (10)
Growth in ATB* better than YGCB	− (6)	− (3)	+	− (6)	− (3)	− (4)	− (10)
Growth in 10 % (v/v) ethanol in CMB*	− (2)	− (2)	+	− (2)	− (2)	− (2)	− (2)
Growth at pH 4·8 in CMB	− (6)	− (3)	+	2 (6)	− (2)	− (4)	Slight (10)
Growth at pH 3·7 in CMB	− (2)	− (2)	+	− (2)†	− (2)	− (2)	− (2)
Growth at 37·5°	−	+	15	14	4	+	28
YGLM* reaction acid	+	+	10	17	+	+	+
clot	4	2	−	10	+	15	27
reduction	1	2 (slight)	2	2	+	12	26
gas	1	−	−	2	1	7	14
Dextran synthesis	−	−	−	−	+	+	+
Dissimilation of citrate	+ (6)	1 (5)	18	1 (8)	1 (13)		2 (11)
Acid from arabinose	−	1	9	16	−	−	+
xylose	−	−	3	5	−	+	24
fructose	−	7	+	16	+	14	30
glucose	+	+	+	+	+	+	+
galactose	+	+	4	+	3	13	29
mannose	−	8	11	+	+	15	30
cellobiose	−	−	13	7	−	3	19
lactose	+	+	−	5	2	10	16
maltose	−	+	−	16	3	+	29
sucrose	−	8	−	15	4	+	+
trehalose	−	1	+	+	+	+	+
melibiose	−	8	14	+	−	15	25
raffinose	−	3	−	7	−	9	16
dextrin	−	−	−	6	−	−	−
aesculin	−	−	+	7	−	8	29
salicin	−	−	14	−	−	3	24
mannitol	−	−	−	7	−	3	11

† The two strains which grow at pH 4·8.

* YGCB = yeast glucose citrate broth; ATB = acidic tomato broth; CMB = citric-malic acid broth; YGLM = yeast glucose litmus milk.

DISCUSSION

It is suggested that the wine leuconostocs should be separated from other species of the same genus because the wine strains grow in media with a low initial pH value (i.e. 3·7) and grow well at pH 4·2, while other leuconostocs will not grow in media with an initial pH value of 4·2 or less. Acid tolerance is not the only property which

separates the wine strains from all the other species. Failure to ferment sucrose and maltose excludes them from all species except *Leuconostoc cremoris* while failure to ferment lactose and (usually) galactose together with the ability to form acid from fructose, aesculin and (usually) salicin and melibiose excludes them from *L. cremoris.* For reasons given elsewhere (Garvie, 1967*c*) *L. dextranicum* and *L. mesenteroides* are considered to include only strains which form dextran from sucrose. The non-slime forming strains previously included in *L. mesenteroides* are placed in a new species *L. paramesenteroides*. The wine cultures do not form slime, and are therefore excluded from *L. dextranicum* and *L. mesenteroides*. *L. paramesenteroides* is most likely to be confused with the wine strains but acid production from maltose, sucrose, salicin and aesculin should put the majority of strains in the correct species.

Table 4. *The separation of six proposed species of Leuconostoc*

	L. mesenteroides	*L. dextranicum*	*L. paramesenteroides*	*L. lactis*	*L. cremoris*	*L. oenos*
Production of slime from sucrose	+	+	−	−	−	−
Acid formed from arabinose	+	−	u+	u−	−	±
lactose	± (slow)	± (slow)	± (slow)	+	+	−
maltose	u+	u+	u+	+	−	−
sucrose	+	u+	u+	u+	−	−
trehalose	+	+	+	u−	−	+
Hydrolysis of aesculin	u+	u−	±	−	−	+
salicin	u+	u−	−	−	−	u+
Growth at pH 4·8	− or slight	− or slight	±	−	−	+
4·2	−	−	−	−	−	+
3·7	−	−	−	−	−	+

u+ = most strains +, occasional strains only found to be −;
u− = most strains −, occasional strains only found to be +.
± = some strains +, some strains negative

When strains of *Leuconostocs oenos* were first cultured in this laboratory growth was poor. When strains of other Leuconostoc species had been difficult to grow the addition of cysteine hydrochloride to the medium greatly improved growth. This was therefore tried with *L. oenos* and was successful. The Leuconostoc strains encouraged by cysteine had also been found to grow well on agar media when incubated in anaerobic jars evacuated and filled with a mixture of hydrogen and carbon dioxide, while growth of these strains on nutrient agar when incubated aerobically is unreliable. Similarly, *L. oenos* does not grow aerobically on agar media. The addition of cysteine to broth media and incubation of plates in an atmosphere of hydrogen + carbon dioxide has not been found to inhibit the growth of any leuconostocs and is useful for growing all strains of *L. cremoris*, strains received from Dr Whittenbury as representative of his group 1 (Whittenbury, 1966) and also for a few other strains of *L. paramesenteroides* and a few of *L. dextranicum*.

Fornachon (1964) and Malan, Ozino & Gandini (1965) reported sucrose fermentation by strains of leuconostocs they isolated from wine. Although in both these papers the cultures were regarded as belonging either to *Leuconostoc mesenteroides* or *L. dextranicum* the pH value of the media used makes this classification appear unlikely and the cultures described appear to be *L. oenos*. It is possible that some strains or under some conditions acidophilic leuconostocs ferment sucrose (see below). As

might be expected wine leuconostocs are more tolerant to ethanol than are other leuconostocs. It is proposed to call the wine strains *L. oenos*. A suggested differential key is given in Table 4: other differences between the species are shown in Table 3.

Difficulties in naming leuconostocs found in wine have been reported previously. Bidan (1956) was unable to give a species name to one of his strains, the other he called *Leuconostoc gracile*. This name was chosen because Pedersen (*Bergey's Manual*, 1948) had found that one strain of '*Bacterium gracile*' (Muller-Thurgau & Osterwalder, 1913, 1918) which he received from Dr A. Osterwalder was probably a leuconostoc. Neither this strain nor any of the originals are now available and it is not possible to be sure that the original description of '*B. gracile*' referred to a leuconostoc. Since Bidan's culture fermented lactose and raffinose it is doubtful whether it was the same as the present wine strains. Radler (1958) concluded his cultures were like, but not identical with, *L. citrovorum*, and Pilone & Kunkee (1965) use the name *L. citrovorum* for ML 34 (NCDO 1823). Radler's strains and strain ML 34 are included in the present work and are typical of *L. oenos*. Fornachon (1964) described acidophilic cocci found in Australian wine and concluded that they were non-dextran forming varieties of *L. mesenteroides*. Two strains received from Dr J. C. M. Fornachon while this paper was in preparation were found to be *L. oenos*. Similarly, of six strains received from Professor C. E. Malan as *L. mesenteroides*, *L. dextranicum* and *L. citrovorum*, five are *L. oenos* and one has not been satisfactorily identified. Using the methods given none of these eight strains formed acid from sucrose.

The gas-forming cocci isolated from wine and gas-forming cocci isolated from other sources have not always been seen to be different, partly perhaps because leuconostocs have been confused amongst themselves and also with organisms of other genera. *Leuconostoc cremoris* seems to be found only in the field of dairy bacteriology, but other species are more widely distributed. At this time the true taxonomic significance of the ability to tolerate and grow at acid pH values cannot be assessed, but since this property is linked with differences which have classically been used as a means of separation among the lactic-acid bacteria, acid tolerance is an additional argument for defining a new species.

Leuconostoc oenos is not a homogenous group and some strains are considerably easier to grow than others. It is difficult to select a type strain, however, NCDO 1674 is proposed.

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