Lipids of *Pediococcus cerevisiae* and some methicillin-resistant substrains

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WILKINSON, B. J., WHITE, D. C. and WHITE, P. J. 1974. Lipids of *Pediococcus cerevisiae* and some methicillin-resistant substrains. Antonie van Leeuwenhoek **40**: 39–47.

The phospholipids of *Pediococcus cerevisiae* were identified as phosphatidyl glycerol, lysylphosphatidyl glycerol, cardiolipin, phosphatidic acid and an unknown. Evidence was obtained for the presence of mono- and diglucosyl diglycerides. The major fatty acids were C18:1, C16:0, and C16:1, with smaller amounts of C14:0, C14:1, and C18:0. The methicillin-resistant strains did not contain more lipid or lipid phosphate than the parent strain when they were grown in the presence of methicillin. The percentages of fatty acids in the organisms were not markedly different. Some variation in the proportions of the phospholipids was noted.

INTRODUCTION

It has been reported that in some instances the lipid composition of bacteria resistant to antibiotics is quantitatively and/or qualitatively different from that of the sensitive strains (Hugo and Stretton, 1966; Dunnick and O'Leary, 1970; Anderes, Sandine and Elliker, 1971; Vaczi, 1966, and Vaczi and Farkas, 1961).

Several methicillin-resistant and -dependent strains of *Pediococcus cerevisiae* were available (Widdowson and White, 1971), the resistance of which was unexplained (Wilkinson and White, 1973). The lipids of these organisms were examined to see if there was any correlation between resistance and lipid composition.

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The lipid composition of *P. cerevisiae* has been examined to some extent (Ikawa, 1963) and there has been a recent report of the fatty acid content of this organism (Uchida and Mogi, 1972). In the present study the phospholipids and glycolipids of the parent and resistant organisms have been examined in more detail and the fatty acid content has been determined. In addition, total lipid, lipid phosphate, and the percentages of neutral, "glyco" and phospholipids have been measured.

MATERIALS AND METHODS

Organisms. Pediococcus cerevisiae ATCC 8081 (sensitive to 10 μg of methicillin/ml) and *P. cerevisiae* CRD (a methicillin-dependent substrain resistant to up to 300 μg of methicillin/ml) were maintained as described by P. J. White (1968). *P. cerevisiae* 8081 R (resistant to up to 150 μg of methicillin/ml) and *P. cerevisiae* MRD2 (methicillin-dependent and -resistant to up to 300 μg of methicillin/ml) were described by Widdowson and White (1971) and were maintained like *P. cerevisiae* CRD.

Medium. The partly defined medium of P. J. White (1968) was used. Methicillin was sterilized by filtration and was included (100 μg/ml) in the medium for growth of the methicillin-resistant organisms.

Culture conditions. The organisms were grown in 1 litre of medium in a 2-litre conical flask and were incubated without shaking at 37 C. When the lipids were to be labelled, 500 μC_i of $H_3{}^{32}PO_4$ were included in the medium. Organisms were always harvested in the exponential phase of growth (after about 8 hr with parent organisms, and about 24 hr with the resistant organisms).

Harvesting and washing of organisms. Organisms were harvested at 6000 *g* for 10 min at 2 C and were washed once by resuspension in cold distilled water.

Determination of dry weight. The washed organisms from 1 litre of culture were resuspended in distilled water (20 ml) and duplicate 1 ml samples were placed in preweighed vials. The vials were heated at 105 C overnight and weighed.

Extraction of lipid. Lipids were extracted by a modification of the Bligh and Dyer method (1959). A suspension of organisms from 1 litre of culture (200–400 mg dry wt) in water was made up to 100 ml with 0.05 M phosphate buffer pH 7.5. Methanol (250 ml) and 125 ml of chloroform were added to the suspension in a 1-litre separatory funnel, shaken vigorously and allowed to stand for two hours. Chloroform (125 ml) and 125 ml of water were added, shaken and allowed to stand overnight. The lower phase (containing the lipid) was passed through anhydrous sodium sulphate and rotary evaporated to dryness. The lipid residue

was resuspended in chloroform and transferred to a preweighed tube. The chloroform was removed with a gentle stream of nitrogen and the tube weighed.

Isolation, methylation and identification of the fatty acids. About 250 mg dry weight of washed organisms were resuspended in 10 ml of 3 N KOH containing 50% (vol/vol) aqueous ethanol and were heated in a sealed tube for 7 hr at 105 C. The fatty acids were extracted and methylated as described by White and Cox (1967). The fatty acid methyl esters were identified and measured by use of a polar (ethylene glycol succinate) column and non-polar column (SE-30) in a model 402 gas chromatograph (F & M Scientific Co., Avondale, Pennsylvania, U.S.A.).

Fractionation of lipid. The combined lipid extracts from three, 1-litre batches of each organism were fractionated on a 1 cm diameter 1.5 g Unisil silicic acid 100–200 mesh column based on the method of Vorbeck and Marinetti (1965*b*). The column was washed with 20 ml of redistilled *n*-heptane, 20 ml of diethyl ether and 20 ml of chloroform. The sample was applied in a small volume of chloroform and was eluted stepwise with the following solvents: 1. chloroform (20 ml), 2. chloroform + acetone (1 + 1 vol/vol, 20 ml), 3. acetone (20 ml), 4. chloroform + methanol (49 + 1 vol/vol, 20 ml), 5. chloroform + methanol (1 + 1 vol/vol, 20 ml) and 6. methanol (20 ml). Fraction 1 was designated "neutral" lipid, fractions, 2, 3, and 4 "glycolipid" and fractions 5 and 6 "phospholipid."

Lipid phosphate was measured as previously described by White and Frerman (1967).

Deacylation of lipids. Lipids were deacylated by the method of R. L. Lester as described by D. C. White (1968). The deacylation of the lipids of *P. cerevisiae* was shown to be complete in 2 hr.

Chromatography. Intact ^{32}P-labelled lipids were chromatographed two-dimensionally on silica-gel-impregnated paper (Whatman SG-81) in solvents 1 and 3 of Wuthier (1966). The intact "glycolipid" fraction was chromatographed ascendingly in one dimension on SG-81 paper in chloroform + methanol + H_2O (65 + 25 + 4 by volume). Deacylated lipids (glycerol phosphate esters) were chromatographed on aminocellulose paper (Whatman AE-81) as described by D. C. White (1968). Glycerol phosphate esters were also separated on thin layer plates (Eastman Chromagrams 6064) in the solvent systems of Short, White and Aleem (1969). Spots were detected by exposure to Kodak No-Screen X-ray film and radioactivity was measured by a liquid scintillation spectrometer (D. C. White, 1968).

RESULTS

Measurement of some lipid parameters in exponential phase organisms. The percentage of the dry weight of the organisms present as lipid varied between 3.6 and 4.6%, lipid phosphate between 11.8 and 19.3 μmoles per gram dry wt of organisms (Table 1). Neither of these parameters were consistently higher or lower in the methicillin-resistant organisms. The total lipid extract of the organisms was made up of 10–20% "neutral" lipid with parent organism apparently having the lowest content of neutral lipid. The "glycolipid" fraction accounted for 47–57% of the total lipid and the "phospholipid" fraction from 28–39% of the total lipid of these organisms (Table 1). There was a good recovery of lipid from the silicic acid column, over 90% of the weight of the lipid or lipid phosphate applied to the column was recovered when the contents of the fractions were summed. There was good agreement between the amount of "phospholipid" indicated by the μmole of lipid P and by the fractionation procedure.

Identification of the phospholipids. When lipid extracts of organisms grown in the presence of ^{32}P were chromatographed on SG-81 paper and radioautographed, phospholipids with the chromatographic mobilities of phosphatidyl glycerol (PG), phosphatidic acid (PA), lysylphosphatidyl glycerol (LPG), cardiolipin (CL) and an unknown, designated X, were detected (Fig. 1b). Deacylation of PG yields glycerol phosphoryl glycerol (GPG), PA yields α glycerol phosphate (αGP), CL yields bis-glycerol phosphoryl glycerol (GPGPG), LPG yields GPG and X presumably yields an unknown glycerol phosphate

Table 1. Measurements of some features of lipids in exponential phase *Pediococcus cerevisiae* strains. Organisms were grown and harvested, and analyses performed as described in Materials and Methods.

	Pediococcus cerevisiae strain			
	8081	CRD	8081R	MRD2
mg dry wt of organisms per ml medium at time of harvest[1]	0.38	0.23	0.21	0.26
% of dry wt of organism as lipid[2]	4.1	3.6	4.6	3.6
μmole lipid phosphate per g dry wt of organism[1]	18.2	15.1	19.3	11.8
Lipid fractionation % (by weight) of total lipid present as				
(1) "neutral lipid"	10.7	19.6	18.2	14.6
(2) "glycolipid"	50.3	49.5	47.0	57.3
(3) "phospholipid"	39.1	30.9	34.8	28.1

[1] Average of at least 6 determinations.
[2] Average of at least 3 determinations.

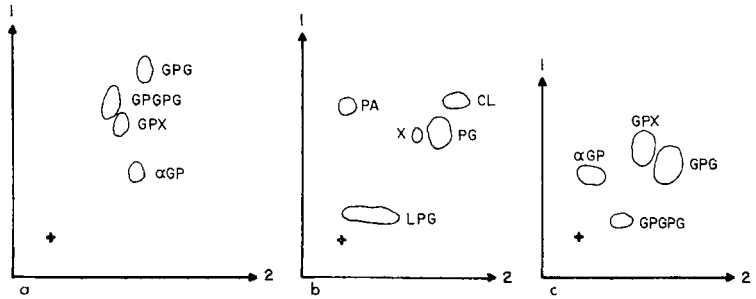

Fig. 1. Composite diagrammatic representations of radioautograms of chromatograms of the phospholipids and glycerol phosphate esters of some strains of *Pediococcus cerevisiae* grown in the presence of $H_3\ ^{32}PO_4$.

In all cases + represents the origin and the ordinates labelled 1 and 2 the extent of the solvent front in each direction.

(a) Radioautogram of glycerol phosphate esters derived from the phospholipids of *P. cerevisiae* grown with ^{32}P after separation on cellulose thin-layer plates.

(b) Radioautogram of *P. cerevisiae* lipids containing ^{32}P after separation on silica gel-impregnated paper.

(c) Radioautogram of glycerol phosphate esters derived from the phospholipids of *P. cerevisiae* grown with ^{32}P after separation on aminocellulose paper.

ester. Portions of the lipid extracts were deacylated by mild alkaline methanolysis and the resultant glycerol phosphate esters were chromatographed on AE-81 paper and Eastman Chromagram plates. Radioautograms of the chromatograms revealed the expected glycerol phosphate esters (Figs. 1a and c) as well as an unidentified glycerol phosphate ester (GPX). That LPG was present was confirmed by a positive ninhydrin reaction on overloaded SG-81 papers and by identification of lysine upon chromatography of the alkaline hydrolysate of the lipid extract. A portion of the deacylated extract was chromatographed on Whatman No. 4 paper with an ascending solvent (ethanol + butan-1-ol + H_2O + dicyclohexylamine, 10 + 10 + 5 + 2 by volume). Only one spot was detected with ninhydrin (0.25% wt/vol in acetone) with a mobility corresponding to authentic lysine. Ikawa (1963) also observed the presence of lysine in lipid extracts of this organism. The percentage of each of the phospholipids in these organisms is given in Table 2. The percentage composition is based on the deacylated glycerol phosphate esters separated on AE-81 papers; LPG yields PG on mild alkaline methanolysis but based on data from SG-81 chromatography was only about 5% or less of the total phospholipid. All three methods gave similar values for the percentage composition. Speculations as to the identity of X are given in the discussion. The parent strain and strain 8081R contained high proportions of PG; PG was the major phospholipid in strains CRD and MRD2 but they had higher proportions of CL and X than the other two strains.

Table 2. Percentage phospholipid composition of some strains of *Pediococcus cerevisiae*

Phospholipid	*Pediococcus cerevisiae* strain			
	8081	CRD	8081R	MRD2
Phosphatidyl glycerol	92.0	57.7	89.4	73.8
Cardiolipin	0.83	24.4	3.3	11.7
Phosphatidic acid	0.65	N.D.	3.0	2.0
Compound X	6.5	17.9	4.2	12.4

The percentage composition was based on percentage of total ^{32}P of glycerol phosphate esters chromatographed on AE-81 paper. LPG is converted to PG on mild alkaline methanolysis and was up to 5% of the total lipid ^{32}P based upon SG-81 chromatography of intact phospholipids. N.D. = not detected.

Glycolipids. When a portion of the "glycolipid" fraction was chromatographed ascendingly on SG-81 paper in one dimension, two major spots were detected with the alkaline silver nitrate spray (Trevelyan, Procter and Harrison, 1950) with R_f values of 0.92 and 0.56. Monoglucosyldiglyceride and diglucosyldiglyceride from *S. aureus* had similar R_f values (White and Frerman, 1967) in this system. Glucose was identified as the only sugar in the "glycolipid" fraction (as it was by Ikawa, 1963) by hydrolysis of a portion of the fraction in 1 ml of 1 N HCl for 2 hr at 105 C. After removing the acid by repeated drying and water treatment of the sample, the residue was taken up in a small volume of water and chromatographed descendingly on Whatman No. 1 paper in *n*-butanol + pyridine + water (6 + 4 + 3 by volume). Comparison with authentic standards revealed two alkaline-$AgNO_3$-positive substances corresponding to glucose and glycerol. These observations suggest the presence of monoglucosyl- and diglucosyldiglyceride in the lipids of these organisms.

Fatty acids. Fatty acids were identified by a James (1960) plot of the logarithm of the retention time versus the number of carbon atoms in the fatty acid when compared with authentic standards chromatographed on EGS and SE-30 columns. There were no qualitative differences in the fatty acid content of the four organisms and no large differences in the percentage composition of the fatty acids (Table 3). In each case C18:1 was the major fatty acid (62–72%) with substantial amounts of C16:0 (21–28%) and C16:1 (4.5–6.1%) fatty acids and smaller amounts of C14:0, C14:1 and C18:0 fatty acids. No evidence for the presence of C17 and C19 cyclopropane fatty acids was found.

Table 3. Percentage fatty acid composition of some strains of *Pediococcus cerevisiae*

Fatty acid	*Pediococcus cerevisiae* strain			
	8081	CRD	8081R	MRD2
14.0	1.0	2.4	1.0	1.9
14.1	0.3	0.7	0.2	Tr
16.0	22.6	27.9	21.5	23.8
16.1	6.1	5.3	4.5	6.0
18.0	1.7	1.3	1.1	0.7
18.1	68.3	62.4	71.7	67.7

Fatty acids were extracted by saponification from exponential phase organisms and were methylated and chromatographed on polar and non-polar columns. The percentages were calculated from the total response after gas chromatography. The data presented is from an SE-30 column; an ethylene glycol succinate column gave similar results.
Tr = trace.

DISCUSSION

The strains of *P. cerevisiae* studied contained about 4% of their dry weight as lipid and about 16 μmoles of lipid phosphate per gram dry wt; these values are not unusual for gram-positive bacteria. The methicillin-resistant strains did not have increased lipid content such as was found for penicillin-resistant *S. aureus* (Hugo and Stretton, 1966; Dunnick and O'Leary, 1970; Vaczi and Farkas, 1961; Vaczi, 1966).

The major phospholipid was PG, with smaller amounts of LPG, PA, CL and an unknown X. This phospholipid composition is qualitatively and quantitatively similar to that reported for other lactic acid bacteria; e.g. *S. faecalis* (Vorbeck and Marinetti, 1965*a*; Dos Santos Mota et al., 1970), and several strains of *Lactobacillus* (Exterkate et al., 1971). Short and White (1970) isolated a lipid from *S. aureus* which they designated as phosphatidylglucose. This lipid had the same mobility in the same three systems used in this study as compound X. Phosphatidylglucose was thought to be present in *Mycoplasma laidlawii* (Smith and Henrikson,1965) but was later shown to have a glyceryl-phosphoryl-diglucosyl diglyceride structure (Shaw, Smith and Verheij, 1970). It seems that compound X may have this structure in view of the occurrence of this compound in closely related organisms (Shaw, 1970; Shaw and Stead, 1972). Strains MRD2 and CRD had lower proportions of PG and higher proportions of CL and X than the parent strain and strain 8081R; it is interesting that strains CRD and MRD2 are methicillin-dependent whereas the parent strain and 8081R are not.

Uchida and Mogi (1972) have made an extensive survey of the fatty acids of species of *Pediococcus* and report a percentage composition of *P. cerevisiae*

ATCC 8081 virtually identical to that found in this study. Small amounts of C20:1 and C17 cyclopropane fatty acids found in stationary-phase organisms by the Japanese workers were not found in this study of exponential phase organisms. The fatty acid content of these strains of *P. cerevisiae* is similar to those of *Lactobacillus* and *Streptococcus* species rather than *Micrococcus* or *Staphylococcus* as was noted by Uchida and Mogi (1972).

It would appear that unlike some other antibiotic-resistant organisms, major changes in lipid content and composition are not associated with the development of resistance to methicillin in strains of *P. cerevisiae*.

Received 3 *May* 1973

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