Oxygen Consumption by *Pediococcus halophilus*

ChiYuki KANBE and Kinji UCHIDA

Soy Sauce Research Laboratories, Kikkoman Corp.,
Noda 278, Japan

Received April 2, 1985

The behavior toward oxygen of several strains of *Pediococcus halophilus* was studied. Although these organisms are generally regarded as facultative anaerobes, this investigation showed that resting cells of *P. halophilus* consumed oxygen at the expense of D-glucose or L-lactate as substrate.

The oxygen consuming activities among strains of soy pediococci varied from 7.06 to 11.63 (nmol/min/mg dry cells) with glucose and 5.52 to 6.59 with L-lactate, respectively. Oxidative metabolism of glucose increased acetate production with a corresponding decrease in lactate formation. Lactate oxidation with O_2 led to the formation of acetate. The oxygen consuming activity was not inhibited by any of the respiratory inhibitors tested such as KCN or NaN_3 .

NADH oxidase activity was found in cell-free extracts of *P. halophilus* No. 51, which is capable of lowering the redox potential of the growth medium. A direct correlation between the abilities to consume oxygen and to reduce the redox potential has not been found so far, but this enzyme is considered to be involved in the aerobic metabolism.

Soy pediococci (*Pediococcus halophilus*) play a vital role in the brewing of soy sauce which contains 16~18% of sodium chloride. Their primary function is to convert glucose to lactic acid at an early stage of the *moromi* (soy sauce mash) fermenting process.^{1,2)} Recently, we reported that some strains of soy pediococci decreased the environmental redox potential during their growth, and that the reducing activities differed greatly among the strains.³⁾ Lactic acid bacteria are generally considered to be facultative anaerobes and there is little information available on the behavior toward oxygen of the genus *Pediococcus*.⁴⁾ On the other hand, there have been several reports on oxygen consumption by some lactobacilli.^{5~10)} Yousten *et al.*⁷⁾ found that *L. plantarum* can utilize oxygen and Götz *et al.*⁸⁾ found the same result. Iwamoto *et al.*⁹⁾ reported that most lactobacilli grown aerobically consumed oxygen markedly in the presence of mannitol. Furthermore, several strains of heterofermentation lactic acid bacteria have been shown to consume oxygen.¹¹⁾

In this report, we report the finding that several strains of *P. halophilus* consume a

significant amount of oxygen in the presence of D-glucose or L-lactate, and also some characteristics of their oxygen consuming activity.

MATERIALS AND METHODS

Organisms and culture conditions. *P. halophilus* strains used including the type strain (Mees' strain, IAM 1676) are stock cultures in this laboratory. *Lactobacillus plantarum* ATCC 8014 and *L. casei* ATCC 7469 were obtained from the Noda Institute for Scientific Research.

P. halophilus was cultured in YPG [% w/v: yeast extract (Difco), 0.3; polypepton (Daigo Eiyo Chemical Ltd., Osaka, Japan), 1.0; glucose, 1.0; K_2HPO_4 , 1.0; Na-thioglycolate, 0.1; NaCl, 10; pH 7.2, sterilized at 1.0 kg/cm² for 10 min] or in MJ medium. MJ medium was prepared as follows: the soy sauce mash before starting lactic acid fermentation was filtered through cloth and then through a bed of Standard Super-Cel to remove solid materials. The filtrate was diluted with water to bring the NaCl concentration to 10% (w/v) and the pH was adjusted to 7.0. This solution was sterilized by passage through a Millipore filter system (pore size, 0.22 μ m). *Pediococci* were precultured in test tubes containing 2.5 ml of YPG at 30°C for 2 days, and then inoculated into 1 liter of YPG or MJ medium and cultured at 30°C stationarily. *Lactobacilli* were cultured in MRS medium¹²⁾ under the same conditions. The cells were harvested in the late-log phase by centrifugation (12,000 g, 10 min) at 4°C.

Preparation of resting cells and cell-free extracts. Harvested cells were washed twice with 0.1 M potassium phosphate buffer (pH 7.0) and then the suspensions were kept for 90 min at 30°C. After starvation, the cells were washed and resuspended in the same buffer (resting cells). The washed cells (dry weight, 350 mg) were suspended in 4 ml of the above buffer and then disrupted with a Branson Sonifer Cell Disrupter 200-P (20 Kc) for 10 min at 0°C. After centrifugation (45,000 g, 30 min), the supernatant fluids were used as crude cell-free extracts.

Assay of oxygen consumption by resting cells. Oxygen consumption was measured with a Beckman oxygen electrode as the rate of the decrease in dissolved O₂ in 0.1 M potassium phosphate buffer (pH 7.0) at 30°C or with a conventional manometric apparatus as described in the legends to Fig. 1 and Table II.

Assay of NADH oxidase. NADH oxidase activity was measured with a Shimadzu Model UV-240 spectrophotometer by monitoring the oxidation rate of NADH in the reaction mixture at 340 nm. Thunberg cuvettes of 1 cm light path were used and the activity of the enzyme was calculated using the absorption coefficient of NADH ($\epsilon = 6.22 \text{ cm}^{-1} \text{ mmol}^{-1}$). Specific activity was expressed as nmol substrate oxidized $\text{min}^{-1} (\text{mg protein})^{-1}$ at 25°C.

Determination of dry weight of cells and protein content. Dry weights were determined with washed cells after heating at 110°C for 20 hr. Protein was determined by the method of Lowery *et al.*¹³⁾ using bovine serum albumin solution as a standard.

Determination of carboxylic acids and sugars. Carboxylic acids were determined with a carboxylic acid-analyzer¹⁴⁾ (S-303; Seishin Pharmaceutical Co., Ltd., Tokyo). D-Glucose was determined enzymatically by the GOD-POD method.¹⁵⁾ Fructose was assayed by HPLC according to the method of Mopper *et al.*¹⁶⁾

Chemicals. NADH and NADPH were obtained from Boehringer Mannheim Yamanouchi Co., Ltd., and L- and D-lactate (Li-salt) were from Sigma Chemical Company. Other chemicals were of reagent grade.

RESULTS

Effect of oxygen on dissimilation of hexose by resting cells of P. halophilus

The effect of oxygen on dissimilation of hexose by resting cells of *P. halophilus* No. 51 was studied. Aerobic dissimilation was carried out in 25-ml test tubes containing reaction mixtures on a reciprocating shaker operated at 300 strokes (2 cm) per min, and anaerobic dissimilation was carried out in 30-ml Thunberg tubes *in vacuo*, respectively. The results of analysis of supernatants obtained after 120 min incubation are shown in Table I. Resting cells of *P. halophilus* were found to dissimilate sugars aerobically, as well as anaerobically. There was no significant difference in the amounts of substrates consumed between the two conditions. The product under anaerobic conditions was only L-lactate, and about 1.75 mol of lactate was formed per 1 mol of hexose fermented.

In contrast, marked production of acetate with a corresponding decrease in lactate formation was observed under aerobic conditions. A little pyruvate was also found, and the molar ratio of lactate to acetate was about 1:1.

TABLE I. EFFECT OF OXYGEN ON DISSIMILATION OF HEXOSE BY RESTING CELLS OF *Pediococcus halophilus* No. 51

The cells were cultured in YPG medium. Each test tube contained 170 μmol of glucose or fructose, 3 ml of 0.1 M phosphate buffer (pH 7.0) and 30 mg (dry weight) of resting cells. Reactions were carried out at 30°C for 120 min.

Substrate	Atmospheric conditions ^a	Consumed sugar ($\mu\text{mol/ml}$)	Acid produced (mol/mol consumed sugar)				
			Lactic	Acetic	Formic	Pyruvic	Total
Glucose	Anaerobic	24.4	1.70	0	0	0	1.70
Glucose	Aerobic	23.3	0.75	0.81	0	0.14	1.70
Fructose	Anaerobic	23.7	1.81	0	0	0	1.81
Fructose	Aerobic	24.4	0.85	0.86	0	0	1.71

^a Atmospheric conditions are described in the text.

FIG. 1. Oxygen Consumption by Resting Cells of *P. halophilus*.

P. halophilus No. 51 was cultured in YPG or MJ medium at 30°C stationarily and harvested in the late-log phase. Oxygen consumption by the cells was measured in a vessel equipped with an oxygen electrode and a stirring rod at 30°C. The reaction mixture (4.3 ml) contained 0.1 M potassium phosphate buffer (pH 7.0), 10 mg cells (dry weight) and 5 mM glucose. The substrate solution (0.1 ml) was added to the cell suspension (4.2 ml) saturated with air after the endogenous O_2 uptake had reached a low level. The electrode was calibrated by exposing it to air (e.g., 21%).

○—○, MJ grown cells; △—△, YPG grown cells; ●—●, MJ grown cells (no substrate in reaction mixture).

Oxygen consumption by resting cells

The above observation suggested that soy pediococci utilized O_2 as an electron acceptor. As shown in Fig. 1, resting cells of *P. halophilus* No. 51 consumed a significant amount of oxygen at the expense of glucose as substrate. The cells showed very low endogenous oxygen consumption without glucose. The cells harvested from MJ medium showed 4~5 fold higher activities than those from YPG medium, although both types of cells were harvested in the same late-log phase. The initial rates of oxygen consumption with various substrates are shown in Table II. It was found that oxygen was consumed with glucose, L-lactate or puruvate, but not with any of the other substrates tested including

TABLE II. THE RATE OF O_2 -CONSUMPTION BY RESTING CELLS OF *P. halophilus* No. 51 IN THE PRESENCE OF VARIOUS ELECTRON DONORS

The cells were cultured in MJ medium. Oxygen consumption was measured by the manometric method at 30°C. The main compartment contained 2.5 ml of cell suspension (ca. 15 mg dry weight). The side arm of the flask contained 0.5 ml of substrate solution, and a filter paper soaked with 0.2 ml of 20% KOH was placed in the central well. After thermoequilibration, the substrate solution was poured into the main part of the flask and the manometer was read at 10 min intervals.

Electron Donors	Concentration (mM)	Initial rate of oxygen consumption ^a
Glucose	25.0	10.08
L-Lactate	25.0	5.59
D-Lactate	25.0	0
Pyruvate	25.0	1.34
Galactose	25.0	0
Arabinose	25.0	0
Xylose	25.0	0
Mannitol	25.0	0
Sorbitol	25.0	0
Glycerol	25.0	0
NADH	10.0	0
NADPH	10.0	0

^a Values are expressed as nmol/min/mg dry cells. The rate of endogenous respiration was 0.67.

NADH and NADPH. It has been shown that this strain can grow on galactose, sorbitol and mannitol, but the resting cells from MJ medium could not use them as substrates for oxygen consumption. The rates of O_2 uptake at the expense of glucose were approximately 2 and 7 times higher than those of L-lactate and puruvate, respectively.

The effect of pH on the oxygen consumption is shown in Fig. 2. The most rapid initial rate of oxygen consumption occurred at pH 6.5~8.5 with glucose as a substrate, and 7.0 with L-lactate, respectively.

Effects of potassium cyanide and sodium azide on the oxygen consumption

The response of the glucose and L-lactate oxidation systems to potassium cyanide or sodium azide was examined (Table III). No significant inhibition of the oxidation was ob-

FIG. 2. pH-Dependency of the O_2 -Consumption by Resting Cells of *P. halophilus* No. 51.

The cells were cultured in MJ medium and oxygen consumption was measured with an oxygen electrode in the presence of 5 mM glucose (●—●) or 10 mM L-lactate (○---○).

Buffers: 0.2 M phthalate buffer (pH 5~6), 0.2 M phosphate buffer (pH 6~9), 0.1 M glycine buffer (pH 9~10).

TABLE III. EFFECTS OF RESPIRATORY INHIBITORS ON THE O_2 -CONSUMPTION BY *P. halophilus*

P. halophilus No. 51 was grown in MJ medium. Oxygen consumption was measured with an oxygen electrode as described in Fig. 1. Values are expressed as nmol/min/mg dry cells.

Inhibitors	Oxygen consumption in the presence of	
	Glucose	L-Lactate
Control	8.88	5.13
KCN 10^{-3} M	8.52	5.22
NaN ₃ 10^{-3} M	9.46	5.23

served when these agents were added to the buffer at 1×10^{-3} M concentration. Addition of 1×10^{-4} M cyanide or azide generally caused complete inhibition of typical cytochrome oxidases, catalases and other sensitive heme-linked terminal oxidases.¹⁷⁾

Oxygen consumption by some strains of pediococci and lactobacilli

Oxygen consuming activities of several strains of soy pediococci and two lactobacilli

TABLE IV. OXYGEN CONSUMPTION BY RESTING CELLS OF VARIOUS STRAINS OF *P. halophilus* AND TWO SPECIES OF *Lactobacillus*

P. halophilus and the lactobacilli were grown in MJ and MRS medium, respectively. Oxygen consumption was measured by the manometric method described in Table 2. Values are expressed as nmol/min/mg dry cells.

Strains	Oxygen consumption in the presence of	
	Glucose	L-Lactate
<i>P. halophilus</i> 24	11.63	6.59
34	10.08	5.59
51	10.04	5.70
325	7.26	5.52
901	7.06	5.56
<i>L. plantarum</i> ATCC 8014	6.10	ND ^a
<i>L. casei</i> ATCC 7469	4.50	ND

^a ND=not determined.

were assayed. The pediococci, by the way, were classified into two types as to their reducing activity. Strains Nos. 34 and 51 can lower the environmental rH of moromi-cultures to below 6, whereas the other soy pediococci, such as Nos. 24, 325 and 901, cannot control the rH of moromi cultures and cannot reduce it to below 8.5.³⁾ The Mees' strain isolated originally from anchovy pickles (1934)¹⁸⁾ could not grow in MJ medium.

As shown in Table IV, five strains of soy pediococci consumed oxygen with either glucose or L-lactate, their activities varying from 7.06 to 11.63 (nmol/min/mg dry cell) with glucose and 5.52 to 6.59 with L-lactate, respectively. There was no significant difference in the abilities to consume oxygen between the two types. The oxygen consumption with glucose by *L. plantarum* ATCC 8014 and *L. casei* ATCC 7469, which were both grown in MRS medium stationarily, were 6.1 and 4.5 (nmol/min/mg dry cell), respectively. The oxygen utilizing activities of soy pediococci, especially of those grown in MJ medium, were found to be comparable to or higher than those of the lactobacilli grown in MRS medium stationarily.

FIG. 3. Oxidative Metabolism of L-Lactate by *P. halophilus*.

P. halophilus No. 51 was grown in MJ medium. The reaction was carried out in Warburg flasks as in Table II except that the L-lactate concentration was 10 mM.

FIG. 4. NADH Oxidase Activity of Cell-free Extracts of *P. halophilus*.

P. halophilus No. 51 was grown in MJ medium. Cell-free extracts were prepared as described under METHODS. The assay was performed in Thunberg cuvettes containing 3.0 ml of reactant. Each cuvette contained 0.1 M phosphate buffer (pH 7.0), cell-free extract and 0.2 mM NADH (tipped from the side arm to start the reaction). (A) was evacuated with a vacuum pump and (B) was not evacuated. At the arrow, (A) was returned to atmospheric pressure with air under shaking.

Oxidative metabolism of L-lactate by *P. halophilus* No. 51

Resting cells of *P. halophilus* No. 51 metabolized L-lactate aerobically and produced acetate as a main product. Accumulation of pyruvate was not detected throughout the

reaction. When 1 mol of L-lactate was used as substrate, equimolar O_2 consumption and acetate production were observed (Fig. 3).

Therefore, this organism was supposed to possess the ability to metabolize L-lactate according to the following equation:

NADH oxidase activity in extracts

Cell-free extracts of *P. halophilus* No. 51 were assayed for oxygen uptake. Substrates which supported O_2 uptake were NADH and L-lactate, and no oxygen uptake was observed with glucose.

Since the involvement of a flavin-linked NADH oxidase in oxidative glucose metabolism by some other lactic acid bacteria was previously reported,^{8,10,19-27} the presence of NADH oxidase in cell-free extracts of *P. halophilus* No. 51 was examined. As shown in Fig. 4, the presence of an enzyme catalyzing the oxidation of NADH in crude extracts of strain No. 51 cells was observed. The specific activity of the cells grown in MJ medium was 48.3 (nmol/min/mg protein) and that of the cells grown in YPG medium was 10.9 (nmol/min/mg protein). The optimum pH was the same, 7.0. NADPH was not oxidized at all by this enzyme preparation.

DISCUSSION

The soy pediococci are regarded as homofermentative lactic acid bacteria. The products formed by resting cells from hexoses under anaerobic conditions in this work coincide with this view. Soy pediococci have been thought to be devoid of a catalase or cytochrome system. In the present study, however, it was observed that the hexose utilization of this bacterium was not inhibited by the presence of oxygen but the molar ratio of acids produced from hexose was greatly changed in the presence of oxygen. These facts suggested that *P. halophilus* had a sort

of protection system against the toxicity of oxygen.

We found that soy pediococci were capable of consuming oxygen with glucose, L-lactate or pyruvate as substrate, and the oxidation of lactate showed stereospecificity for L-lactate. This behavior toward oxygen had not been demonstrated previously for pediococci.

The inhibitor studies indicated that the oxygen consumption of *P. halophilus* occurred through a cytochrome-independent electron transport system.

To elucidate the differences in oxygen consuming activities among strains and to investigate the possible relationship between oxygen consumption ability and reducing activity, O₂ uptake by several strains of *P. halophilus* isolated from *moromi* was assayed. The data (Table IV) showed that all strains were capable of consuming oxygen but no direct correlation between the abilities to consume oxygen and to reduce the redox potential was observed.

Although the reason has not well been elucidated, oxygen consumption of cells grown in MJ medium was 4~5 times higher than that of cells grown in YPG medium, and the activity of NADH oxidase in extracts from the former cells was also 4~5 times higher than that of the latter cells. It is possible to deduce that NADH oxidase may play an important role in aerobic glucose dissimilation. NADH oxidase activity has so far been reported in several lactic acid bacteria. However, the authors are unaware of reports of a NADH oxidase or L-lactate oxidation system in pediococci.

The resting cells consumed oxygen with glucose, but in the cell-free extract experiments, none of the carbohydrate tested was utilized as a substrate for oxygen uptake. L-Lactate and NADH were the only substrates which caused O₂ consumption by cell-free extracts. This may indicate that *P. halophilus* No. 51 does not possess a glucose oxidase which utilizes glucose as a direct electron acceptor. It was also suggested that in the resting cells glucose promoted oxygen consumption by generating NADH, the substrate

for NADH oxidase, but that in cell-free extracts the NADH-generating system which needs a complex set of many enzymes was inactivated. A similar observation was reported for cell-free extracts of aerobically grown *Streptococcus lactis* C10 by Grufferty and Condon.¹⁰⁾ On the other hand, NADH did not act as a substrate for oxygen consumption by resting cells, possibly because NADH cannot pass through the cell membrane of *P. halophilus*.

P. halophilus metabolizes glucose principally to lactate via the EMP pathway under anaerobic conditions. The NADH produced there is reoxidized immediately by lactate dehydrogenase. It is assumed that, under aerobic conditions, NADH is reoxidized by both lactate dehydrogenase and NADH oxidase. Consequently, lactate is not the only major metabolic end-product and pyruvate is split into other end products. Examination of the end products of aerobic glucose dissimilation supported the above assumption that glucose was metabolized to pyruvate, followed by certain reactions resulting in the accumulation of lactate and acetate.

Acknowledgments. The authors thank Mr. H. Koyama for his kind advice and Mr. H. Toriumi for providing the strains of lactobacilli. We also wish to acknowledge Drs. F. Yoshida and T. Mizunuma for their support. We also thank Mrs. K. Shiraishi for her technical assistance.

REFERENCES

- 1) N. Iguchi and K. Sakaguchi, *Shokuhin-Kogyo*, **11B**, 56 (1964).
- 2) K. Nakamura, *J. Soc. Brew. Jpn.*, **66**, 226 (1971).
- 3) C. Kanbe and K. Uchida, *Nippon Nôgeikagaku Kaishi*, **58**, 487 (1984).
- 4) K. Kitahara, "Bergey's Manual of Determinative Bacteriology," 8th Ed., ed. by R. E. Buchanan and N. E. Gibbons, The Williams and Wilkins Company, Baltimore, 1974, p. 513.
- 5) C. F. Strittmatter, *J. Biol. Chem.*, **234**, 2789 (1959).
- 6) J. P. Brown and P. J. Vandemark, *Can. J. Microbiol.*, **125**, 209 (1968).
- 7) A. A. Yousten, J. L. Johnson and M. Salin, *J. Bacteriol.*, **123**, 242 (1975).
- 8) F. Götz, B. Sedewitz and E. F. Elstner, *Arch. Microbiol.*, **125**, 209 (1980).
- 9) Y. Iwamoto, K. Baba and I. Mifuchi, *Yakugaku*

- Zasshi*, **99**, 354 (1979).
- 10) R. C. Grufferty and S. Condon, *J. Dairy Res.*, **50**, 481 (1983).
 - 11) S. Yashima, K. Kawai, Y. Okami and Y. Sasaki, *J. Gen. Appl. Microbiol.*, **16**, 543 (1970).
 - 12) J. C. DE Man, M. Rogosa and M. E. Sharpe, *J. Appl. Bacteriol.*, **23**, 130 (1960).
 - 13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
 - 14) M. Nakajima, Y. Ozawa, T. Tanimura and Z. Tamura, *J. Chromatogr.*, **123**, 129 (1976).
 - 15) C. Kanbe, S. Ushijima and K. Uchida, *Nippon Shokuhin Kôgyo Gakkaishi*, **28**, 528 (1981).
 - 16) K. Mopper and M. Gindler, *Anal. Chem.*, **56**, 440 (1973).
 - 17) J. N. Stannard and B. L. Horecker, *J. Biol. Chem.*, **172**, 599 (1948).
 - 18) A. Nakagawa and K. Kitahara, *J. Gen. Appl. Microbiol.*, **5**, 95 (1959).
 - 19) M. I. Dolin, *Arch. Biochem. Biophys.*, **46**, 483 (1953).
 - 20) S. Fukui, *Agric. Biol. Chem.*, **25**, 876 (1961).
 - 21) D. D. Hoskins, H. R. Whiteley and B. Mackler, *J. Biol. Chem.*, **237**, 2647 (1962).
 - 22) G. A. Walker and G. L. Kilgour, *Arch. Biochem. Biophys.*, **111**, 534 (1965).
 - 23) K. Kawai, S. Yashima, Y. Okami and Y. Sasaki, *J. Gen. Appl. Microbiol.*, **17**, 51 (1971).
 - 24) Y. Iwamoto, K. Baba and I. Mifuchi, *Yakugaku Zasshi*, **99**, 794 (1979).
 - 25) W. Zitzelsberger, F. Götz and K. H. Schleifer, *FEMS Microbiol. Lett.*, **21**, 243 (1984).
 - 26) M. Higuchi, *J. General. Microbiol.*, **130**, 1819 (1984).
 - 27) L. Hansson and M. H. Haggstrom, *Current Microbiol.*, **10**, 345 (1984).
-