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Effect of Oxygen on Lactose Metabolism in Lactic Streptococci

JOHN B. SMART* AND TERENCE D. THOMAS
New Zealand Dairy Research Institute, Palmerston North, New Zealand

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Three strains of *Streptococcus lactis*, two of *Streptococcus cremoris*, and one of *Streptococcus thermophilus* metabolized oxygen in the presence of added carbohydrate primarily via a closely coupled NADH oxidase/NADH peroxidase system. No buildup of the toxic intermediate H_2O_2 was detected with the three *S. lactis* strains. All six strains contained significant superoxide dismutase activity and are clearly aerotolerant. Lactose- or glucose-driven oxygen consumption was biphasic, with a rapid initial rate followed by a slower secondary rate which correlated with factors affecting the in vivo activation of lactate dehydrogenase. The rate of oxygen consumption was rapid under conditions that led to a reduction in lactate dehydrogenase activity (low intracellular fructose 1,6-bisphosphate concentration). These conditions could be achieved with nongrowing cells by adding lactose at a constant but limiting rate. When the rate of lactose fermentation was limited to 5% of its maximum, nongrowing cells of *S. lactis* strains ML3 and ML8 carried out an essentially homoacetic fermentation under aerobic conditions. These same cells carried out the expected homolactic fermentation when presented with excess lactose under anaerobic conditions. Homoacetic fermentation leads to the generation of more energy, by substrate-level phosphorylation via acetate kinase, than the homolactic fermentation. However, it was not observed in growing cells and was restricted to slow fermentation rates with nongrowing cells.

Streptococci are generally considered to be facultative anaerobes, albeit with a preference for anaerobic conditions. However, the group N streptococci (*Streptococcus lactis*, *S. cremoris*, and *S. diacetylactis*) are capable of oxygen consumption (1, 16, 32) and contain flavin-type NADH oxidases (1, 7, 16, 30, 32), NADH peroxidases, and superoxide dismutase (1, 4, 18, 42). Clearly, some strains of lactic streptococci are equipped for growth and survival in aerobic conditions and can remove H_2O_2, the putative toxic agent of aerobic metabolism of these bacteria (40).

Regardless of whether the presence of oxygen has a detrimental effect on growth, it has been shown to influence the carbon metabolism of a wide range of streptococci and other lactic acid bacteria (9, 13, 19, 31, 41). Compared with anaerobic sugar fermentations, Thiel (33) found increased acetate and decreased ethanol production in aerobic conditions with a number of streptococci, including two *S. diacetylactis* strains and one *S. cremoris* strain. Bruhn and Collins (7) found that aeration caused *S. diacetylactis* to divert pyruvate away from lactate to produce acetoin and diacetyl, while Cogan and Condon (8) observed a similar diversion of products when *S. lactis* 712 was grown aerobically on galactose. Increased acetate production occurred in aerobic cultures of *S. diacetylactis* and *S. cremoris* growing on low concentrations of hexose (6). Fordyce et al. (15) showed with nongrowing cells of *S. lactis* strains ML3 and ML8 that oxygen produced major increases in pyruvate and acetate at the expense of lactate, ethanol, and formate. Clearly, the now well-established pathways leading to heterolactic metabolism in the lactic streptococci (36, 38) can be influenced by the presence of oxygen. A likely mechanism for the involvement of oxygen in the carbon metabolism of these bacteria is via the oxidation of NADH by the NADH oxidase/NADH peroxidase system. This would obviate the need for pyruvate reduction to lactate by the NADH-dependent lactate dehydrogenase. The present study examines the role of this mechanism in lactose catabolism by several strains of *S. lactis* and *S. cremoris*. Another homofermentative lactic streptococcus (*Streptococcus thermophilus*) was also examined for comparative purposes.

* Corresponding author.

MATERIALS AND METHODS

Organisms and culture conditions. *S. lactis* ML8, *S. cremoris* 4350, and *S. cremoris* 4365 were from the collection held at the New Zealand Dairy Research Institute. *S. lactis* KB was a lactate dehydrogenase mutant (25) obtained from L. L. McKay, University of Minnesota, St Paul, Minn. *S. thermophilus* TS2 was from the CSIRO Dairy Research Laboratory, Melbourne, Australia. The *S. lactis* strain ML3 used in the present study was from a freeze-dried culture obtained (in 1968) from the National Institute for Research in Dairying, Reading, England. This strain was stable under oxygen stress and was lysed by *S. lactis* ML3 phage, and its metabolic properties were those expected for ML3.

Other ML3 strains held at this institute were found to generate variants when subjected to oxygen stress which showed variation in their metabolic properties. The ML3 strain used in a previous study in this laboratory (15) had been adapted to grow in defined medium. This strain was also found to have some altered properties (data not shown).

All cultures except TS2 were grown at 30°C in T5 complex broth (37) containing 14 mM lactose. TS2 was grown at 42°C in J8 complex broth (35) with 28 mM lactose. Under anaerobic conditions, growth in T5 and J8 broths ceased due to lactose limitation at pH 5.6 and 5.1, respectively.

All cultures were grown in 500 ml of the above media in a 1-liter flat-bottomed Quickfit culture vessel. For "anaerobic" batch cultures the vessel was continuously sparged with sterile O_2-free N_2/CO_2 (95:5) but was otherwise static. For "aerated" batch cultures the vessel was continuously and vigorously sparged with sterile air and again was otherwise static. For "aerobic" batch cultures the vessel was stirred magnetically at 500 rpm and sparged with sterile air such that the dissolved oxygen tension in the medium was maintained at between 90 and 100% that of fully air-saturated

medium. For "oxygenated" batch cultures the air was replaced by 100% oxygen and the stirring rate was increased to 750 rpm. This maintained the dissolved oxygen tension at >100% that of fully air-saturated medium throughout growth. All batch cultures were inoculated with 10 ml of an overnight, static culture of the appropriate strain.

The stirred batch fermentations were performed on a series III fermentor (L.H. Engineering, Stoke Poges, England) with a model 508 dissolved oxygen module. Growth was measured turbidimetrically at 600 nm, using tubes with 12-mm inside diameter (Spectronic 20 spectrometer; Bausch & Lomb, Inc., Rochester, N.Y.).

Preparation of cell suspensions. Cells were harvested by centrifugation and washed twice by resuspension in 50 mM potassium phosphate buffer (pH 7.0) containing 0.12 M NaCl and 1 mM $MgSO_4$. Cells were finally suspended to the desired density in the same buffer and stored on ice until used.

Oxygen consumption by nongrowing cells. Cell suspensions were prepared from the mid-log phase of either anaerobic or aerated batch cultures. Oxygen consumption by these cells was measured at 30°C (42°C for strain TS2) with a Clark oxygen electrode (YSI model 53 oxygen monitor; Yellow Springs Instrument Co., Yellow Springs, Ohio) unless indicated otherwise. The endogenous rate without added substrate was measured and subtracted from the rate obtained after direct addition of lactose, glucose, or galactose to a final concentration of 1 mM. The oxygen electrode was calibrated as described by Misra and Fridovich (26). To determine the effect of slow rates of fermentation on the rate of oxygen consumption, glucose was added continuously at a limiting rate (15), using a syringe attached to a pump (model 341A; Sage Instruments, Cambridge, Mass.).

H_2O_2 accumulation and reduction by nongrowing cells. Cell suspensions were prepared from aerated batch cultures growing exponentially. Suspensions (2.0 ml) were incubated at 30°C (42°C for TS2) under constant, vigorous aeration for 5 min to equilibrate the system. Lactose was then added to 10 mM and the cells were incubated for a further 10 min. The suspension was clarified by centrifugation and the supernatant was assayed for H_2O_2.

Consumption of H_2O_2 was determined in a similar system except that the cells were incubated anaerobically under N_2/CO_2 (95:5). H_2O_2 was added to 5 mM and lactose (when added) to 10 mM, and the suspension was sampled at regular intervals by rapid centrifugation and then cooling on ice. Supernatants were assayed for H_2O_2, and the rate of reduction was determined by linear regression analysis, using at least four time points within 15 min of addition of H_2O_2.

Enzyme assays. Cells were disrupted in 0.05 M bis-tris propane buffer (pH 7.0) containing 0.5 mM dithiothreitol and 20% glycerol by shaking 5 ml of suspension for 2 min at ~4°C with 3 g of glass beads (75 to 150 μm in diameter) in a Mickle disintegrator. Debris was removed by centrifugation at 35,000 × *g* for 10 min to give a cell-free extract. NADH oxidase and NADH peroxidase (EC1.11.1.1) were assayed spectrophotometrically in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.26 mM NADH. NADH peroxidase activity could be determined independently of the oxidase activity by the addition of H_2O_2 to 1.5 mM and by carrying the reaction out anaerobically in sealed cuvettes when the air was replaced by N_2/CO_2 (95:5). However, as one of the products of the oxidase reaction is the substrate for the peroxidase reaction (i.e., H_2O_2) activities described as NADH oxidase may also include NADH peroxidase activity. Superoxide dismutase (EC1.15.1.1) was determined by the cytochrome *c*/xanthine oxidase method (24). One unit of superoxide dismutase activity was defined as that amount causing 50% inhibition of the rate of cytochrome *c* reduction in this assay. The pyruvate dehydrogenase system (EC4.1.1.1, EC2.3.1.12, and EC1.6.4.3) was determined according to the dismutation assay (29) and lactate dehydrogenase (EC1.1.1.27) was determined as previously described (34). All enzyme assays were carried out at 30°C. Apart from superoxide dismutase, 1 U of enzyme activity is defined as that amount which catalyzes the conversion of 1 μmol of substrate to product per min.

Fermentation product analysis. Fermentation end products were measured enzymatically in clarified supernatants according to published methods for acetate (2), ethanol (10), formate (20), L-lactate (17), and pyruvate (15).

Lactose fermentation by non-growing cells. Cell suspensions were prepared from anaerobic or aerated batch cultures growing exponentially. A portion (usually 4.95 ml) was placed in a 50-ml stirred reaction cell, incubated at 30°C, and made anaerobic by constant sparging with O_2-free N_2/CO_2 (95:5) or aerobic by constant sparging with air. After 5 min the fermentation was started by direct injection of lactose (50 μl, 0.2 M) to give the maximum rate of lactose fermentation. Alternatively, the same volume of lactose was pumped into the cell suspension at a constant rate to give 5 ± 2% of the maximum rate of lactose fermentation (15). Suspensions were centrifuged for 10 min after direct injection, or 2 min after pumping had ceased, and supernatants were assayed for products and residual sugar.

Analysis of FBP. The intracellular concentration of fructose 1,6-bisphosphate (FBP) was determined in perchloric acid extracts (15) of cell suspensions actively metabolizing glucose. The FBP was determined with a Perkin-Elmer fluorescence spectrophotometer (22) and an NADH-coupled indicator system (39).

Other procedures. Bacterial density was determined directly by using membrane filters (36). Protein was estimated by the method of Bradford (3). Residual glucose or lactose was determined with Glucostat reagents (Worthington Diagnostics, Freehold, N.J.), using modifications for the lactose estimation described previously (15). Hydrogen peroxide was determined by the leucocrystal violet/horseradish peroxidase method (27).

Materials. All biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., as the grades with highest analytical purity.

RESULTS

Primary aerobic metabolism. (i) Effect of oxygen on growth. In preliminary experiments, 11 strains of *S. lactis*, *S. cremoris*, *S. diacetylactis*, and *S. thermophilus* grew satisfactorily in shake culture on lactose broth media without additives such as catalase or hematin. Subsequent work was confined to the six strains listed in Table 1, using the fermentors described in Materials and Methods. Vigorous aeration had no obvious effect on the growth curves for ML3, KB, and 4350 but did cause a significant reduction in the doubling times for strains ML8, 4365, and TS2 (Table 1). Growth was exponential for all strains except TS2, which became increasingly inhibited.

The effect of higher oxygen concentrations on the growth of ML3 was studied by using aerobic and oxygenated batch cultures. In the aerobic culture the dissolved oxygen tension remained at or above 80% that in fully air-saturated medium, again with no detectable effect on growth. However, in the

TABLE 1. Effect of aeration on growth rates[a]

Strain	Doubling time (min)[b]	
	Anaerobic	Aerated
S. lactis		
ML3	38	39
ML8	39	48
KB	40	40
S. cremoris		
4350	45	46
4365	52	60
S. thermophilus		
TS2	35	69

[a] Anaerobic and aerated batch cultures were the 500-ml lactose broth cultures described in Materials and Methods.

[b] All doubling times, except for the TS2 aerated culture, were determined during the logarithmic growth phase. TS2 was estimated from the initial rate of growth.

oxygenated culture (where the dissolved oxygen tension was greater than that in fully air-saturated medium) growth, although initially exponential, was later increasingly inhibited (data not shown).

(ii) Oxygen consumption by nongrowing cells. Endogenous oxygen consumption was barely detectable, while all strains, grown anaerobically, consumed oxygen with added carbohydrate (Table 2). Aerobic growth made little difference to the oxygen consumption rates with ML3 and TS2 but gave increased activity with the other three strains. With the exception of TS2, lactose and glucose gave comparable rates. With galactose, oxygen uptake rates were markedly lower in all *S. lactis* and *S. cremoris* strains and undetectable with TS2.

With ML3, oxygen consumption with each of the three added sugars was similar when the cells were grown on glucose or galactose instead of lactose (data not shown). The

TABLE 2. Oxygen consumption by nongrowing cells[a]

Strain	Growth conditions	Oxygen consumption rate (nmol of O_2/min per mg [dry wt] of cells)[b]			
		Endogenous	Lactose	Glucose	Galactose
S. lactis					
ML3	Anaerobic	0.6	44	49	15
	Aerobic	1.1	49	29	15
ML8	Anaerobic	0.6	20	19	5.8
	Aerobic	0.8	77	87	9.5
S.cremoris					
4350	Anaerobic	1.1	6.5	4.5	2.2
	Aerobic	1.3	20	17	4.6
4365	Anaerobic	0.5	13	11	7.2
	Aerobic	0.4	86	90	17
S. thermophilus					
TS2	Anaerobic	0.9	19	0.7	ND
	Aerobic	0.7	13	1.0	ND

[a] All cells were grown on 500 ml of lactose broth medium to the mid-log phase in either anaerobic or aerated batch cultures before preparation of washed cell suspensions. Final cell densities ranged from 0.8 to 6.4 mg (dry weight)/ml.

[b] In some cases (see text) oxygen consumption was biphasic. In all cases the data listed here apply to the initial linear phase only. ND, Not detectable.

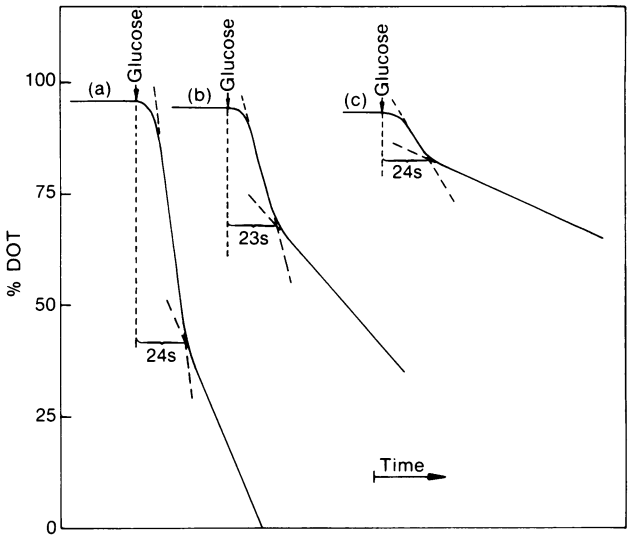

FIG. 1. Glucose-driven oxygen consumption patterns by *S. lactis* ML8 at 30°C and (a) 3.0, (b) 1.5, and (c) 0.6 mg (dry weight) of cells per ml. DOT, dissolved oxygen tension.

only exception was with washed glucose-grown cells, in which the oxidative activity with lactose as energy source was reduced to about 30% and that with galactose was reduced to <10% of the rates with lactose-grown cells. The ability to consume oxygen was strongly dependent on the age of the cells. With ML3, stationary-phase cells (16 to 18 h after cessation of growth) were only able to support low rates of oxygen consumption (<2% that of log-phase cells), regardless of growth substrate or energy supply (data not shown). Pyruvate was also a substrate for oxygen consumption by ML3; however, rates were extremely low at the usual assay pH of 7.0. At pH 5.2 (using 0.1 M MES buffer), rates of 20 nmol of oxygen consumed/min per mg (dry weight) of cells were obtained with 50 mM pyruvate added. Activity with lactose at pH 5.2 was one-third that at pH 7.0. Added lactate (20 mM) gave no detectable oxygen consumption at pH 7.0 or 5.2.

With all *S. lactis* and *S. cremoris* strains the oxygen consumption pattern was biphasic when either lactose or glucose was added, a rapid initial phase being followed by a slower secondary phase (Fig. 1). Both phases were linear and readily distinguished. The rate of the second phase ranged from 24 to 80% of the rate of the initial phase depending on the strain. The data in Table 1 refer to the initial rates only. In the presence of galactose the pattern was different. There was a relatively long lag phase before a single linear phase was reached (data not shown). In contrast, the oxygen consumption pattern with either lactose or glucose added was monophasic for *S. thermophilus* TS2.

(iii) Accumulation and reduction of H_2O_2 by nongrowing cells. The accumulation of H_2O_2 was studied with thick washed cell suspensions (~3.0 mg [dry weight]/ml), provided with lactose and vigorously aerated for 10 min. With this system, none of the three *S. lactis* strains (ML3, ML8, and KB) accumulated any detectable H_2O_2 (<0.01 mM). However, *S. cremoris* strains 4350 and 4365 and *S. thermophilus* TS2 accumulated 0.075, 0.47, and 0.03 mM H_2O_2, respectively.

All strains were able to break down externally added H_2O_2 in washed cell suspensions under anaerobic conditions (Table 3). The rate of H_2O_2 reduction by ML3 and ML8 was

TABLE 3. Reduction of H_2O_2 by washed cells under N_2/CO_2[a]

Strain	H_2O_2 reduction (nmol/min per mg [dry wt] of cells)[b]	
	− Lactose	+ Lactose
S. lactis		
ML3	12.4	22.5
ML8	16.0	54.0
S. cremoris		
4350	28.0	29.7
4365	13.5	14.0
S. thermophilus		
TS2	20.2	21.0

[a] Cells were prepared by growth to mid-log phase in 500 ml of lactose broth medium in aerated batch culture. Final cell densities ranged from 5 to 10 mg (dry weight)/ml.
[b] Initial H_2O_2 concentration was 5 mM and, where added, lactose was 2 mM.

further increased by the addition of lactose, but this had no significant effect with the other strains.

(iv) Oxygen-metabolizing enzymes. NADH-driven oxygen consumption could be demonstrated with a cell extract of ML3 in the oxygen electrode cell (final NADH and protein concentrations, 0.2 mM and 35 μg/ml, respectively). This NADH oxidase activity could also be detected spectrophotometrically (Table 4). There was no evidence for the presence of glucose, lactose, lactate, or pyruvate oxidase activity in this extract.

NADH peroxidase activity could also be detected in crude cell extracts from ML3, using the anaerobic cuvette/ spectrophotometric method (Table 4). As both the NADH oxidase and NADH peroxidase share the common substrate NADH and the likely product of the oxidase reaction (H_2O_2) is the other substrate for the peroxidase, it is not possible to assay the NADH oxidase independently of the NADH peroxidase in the spectrophotometer. Attempts were made to separate the NADH oxidase from the NADH peroxidase in crude cell extracts of ML3. However, both enzymes remained in the soluble fraction after ultracentrifugation (226,000 × *g* for 90 min) and could not be separated by standard purification methods. The methods tried included ammonium sulfate fractionation, a Sephacryl S200 gel permeation column (26 by 900 mm), 10% polyacrylamide nondissociating gel electrophoretograms (identified by activity stains), and finally, a DEAE-sepharose (fast-flow) ion-exchange column (26 by 200 mm) developed with a 0 to 0.5 M KCl gradient (300 × 300 ml) in 0.02 M Tris buffer, pH 7.0, containing 20% glycerol (all above data not shown). It is not possible to determine whether the NADH oxidase/ peroxidase system in ML3 constitutes a single bifunctional enzyme or a tightly bound enzyme couple. Its localization in the soluble fraction suggests that they are flavin-type enzymes (31).

The partially purified ML3 NADH oxidase/peroxidase obtained from the ion-exchange column described above was used for subsequent characterization of the enzymes. The NADH oxidase and NADH peroxidase had specific activities of 2.5 and 1.25 U/mg of protein, respectively (spectrophotometric assays). NADH oxidase activity determined with the oxygen electrode was 1.35 U/mg of protein, which was similar to that by the spectrophotometric assay when the contaminating NADH peroxidase activity was subtracted. Addition of catalase (final concentration, 40 μg/ml) to the oxygen electrode assay had no effect on either the rate of the NADH oxidase reaction when NADH was present in excess or the extent of oxygen consumption when limiting NADH was added. This amount of catalase rapidly liberated oxygen from exogenously added H_2O_2.

Interactions between oxygen and carbon metabolism. (i) Biphasic oxygen consumption patterns. As mentioned earlier, the oxygen consumption pattern of washed cells was

TABLE 4. Effect of aeration on enzyme specific activities[a]

Strain	Growth conditions	Sp act (U/mg)				
		NADH oxidase	NADH peroxidase	Superoxide dismutase	Pyruvate dehydrogenase	Lactate dehydrogenase
S. lactis						
ML3	Anaerobic	0.16	0.04	22	0.09	16
	Aerated	0.39	0.03	38	0.19	10
	Aerobic	0.38	0.08	26	0.12	9
	Oxygenated	0.72	0.05	51	0.17	11
ML8	Anaerobic	0.03	ND	26	ND	11
	Aerated	0.75	0.07	30	0.04	7
KB	Anaerobic	0.17	0.033	26	0.014	ND
	Aerated	0.54	0.034	47	0.065	ND
S. cremoris						
4350	Anaerobic	0.02	0.003	22	ND	10
	Aerated	0.13	0.022	20	0.057	8
4365	Anaerobic	0.02	ND	13	ND	12
	Aerated	0.33	0.055	28	0.043	4
S. thermophilus						
TS2	Anaerobic	0.39	0.062	12	ND	11
	Aerated	0.30	0.29	25	0.031	9

[a] Cell extracts were prepared from cells grown to the mid-log phase in 500 ml of lactose broth medium in anaerobic, aerated, aerobic, or oxygenated batch cultures as indicated. ND, Not detectable.

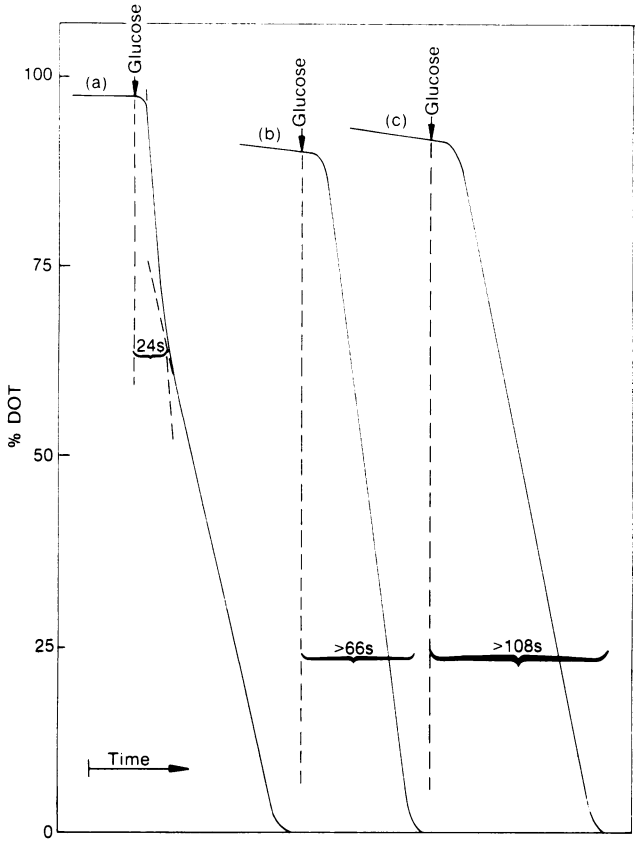

FIG. 2. Effect of fermentation rate on oxygen consumption patterns by *S. lactis* ML8 at 30°C and 4.8 mg (dry weight) of cells per ml. Glucose was added as a single dose (a), at 0.10 mol/min per mg (dry weight) of cells (b), and at 0.07 μmol/min per mg (dry weight) of cells (c). DOT, Dissolved oxygen tension.

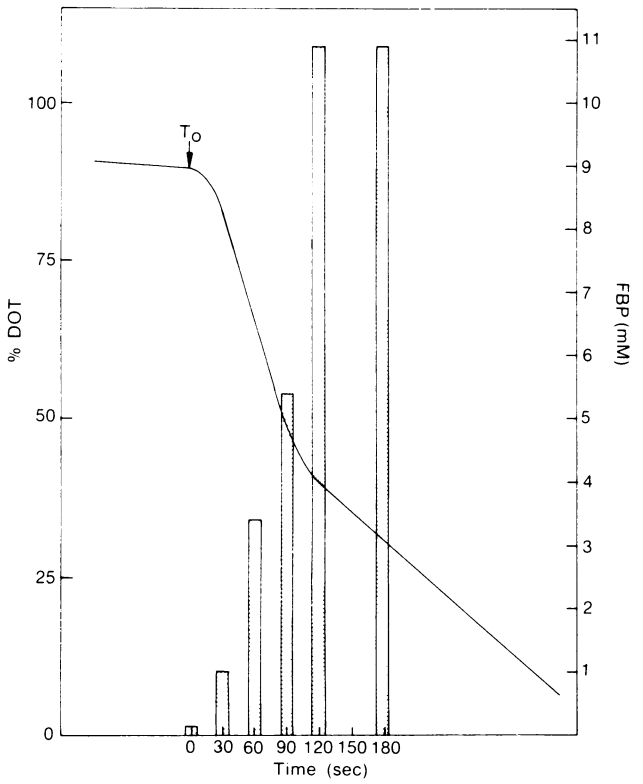

FIG. 3. Increase in intracellular FBP concentrations in *S. lactis* ML8 after addition of glucose (2 mM) as a single dose at time zero. Cell concentration was 7.0 mg (dry weight)/ml, and the experiment was performed in an aerated suspension at 10°C. The corresponding oxygen consumption pattern is also shown. DOT, Dissolved oxygen tension.

biphasic depending upon the carbon source. This phenomenon was studied in further detail in strains ML8, ML3, and 4350. A consistent feature was that the initial rapid phase of oxygen consumption occupied a precise time. This is illustrated in Fig. 1, showing the effect of varying ML8 cell concentration on glucose-driven oxygen consumption. While the rates of both the initial and secondary phases were directly proportional to cell concentration, as would have been expected, the length of the initial phase remained constant at about 24 s. This time was a characteristic of the strain (the comparable times for ML3 and 4350 were 30 and 18s, respectively) and carbon substrate (the initial phase occupied 35 s in ML8 when lactose was substrate and, as stated earlier, there was only a single phase with galactose as substrate).

The biphasic pattern was dependent on the rate of fermentation and could be abolished by adding the glucose at a limiting rate. Thus, pumping the glucose into ML8 cell suspensions in the oxygen electrode chamber at rates of 0.1 and 0.07 nmol of glucose/min per mg (dry weight) of cells gave single linear rates of oxygen consumption until all oxygen in solution was exhausted (Fig. 2). This single phase lasted at least 66 and 108 s, respectively, i.e., over four times the length of the initial phase when glucose was injected rapidly into the cell suspension. Similar data were obtained for ML3 and 4350.

The biphasic pattern was still evident at 10°C, although the length of time occupied by the initial phase was increased by about fivefold for ML8. This slower rate at 10°C allowed measurement of in vivo FBP concentrations during the biphasic oxygen consumption (Fig. 3). The internal FBP level increased steadily with time from a low level (0.1 to 0.2 mM) in starved cells to 11 mM 3 min after the addition of glucose. The buildup of FBP was not instantaneous. Similar data were found for ML3 and 4350.

(ii) Effect of oxygen on enzyme levels and lactose fermentation in growing cells. In ML8, 4350, and 4365 the specific activities of both NADH oxidase and NADH peroxidase were low in anaerobically grown cells, whereas in ML3, KB, and TS2 these constitutive levels were significantly higher (Table 4). Aeration generally increased the specific activity of the primary oxygen-metabolizing enzymes NADH oxidase, NADH peroxidase, and superoxide dismutase. Only in strain TS2 was the NADH oxidase activity higher in anaerobically grown cells. Superoxide dismutase was present at significant levels under anaerobic conditions but, with the exception of ML8 and 4350, increased around two- fold in aerated cultures, consistent with earlier findings for lactic streptococci (4, 12, 23).

Pyruvate dehydrogenase and lactate dehydrogenase were also influenced by growth under aerated conditions (Table 4). With all strains, pyruvate dehydrogenase activity was low in anaerobically grown cells and increased in cells grown in the presence of air. Lactate dehydrogenase was detected in all strains except KB, and specific activities were highest in anaerobically grown cells.

TABLE 5. Effect of aeration on end products in batch cultures[a]

Strain	Growth conditions	% Total carbon end products[b]				
		Lactate	Acetate	Ethanol	Pyruvate	Formate
ML3	Anaerobic	95	ND	3.5	ND	1.5
	Aerated	89	7	ND	ND	ND
ML8	Anaerobic	98	ND	ND	1	1
	Aerated	84	9	ND	3	ND
KB	Anaerobic	67	8	11	2.5	11.5
	Aerated	83	9	0.5	5	ND
4350	Anaerobic	98.5	ND	ND	1	0.5
	Aerated	99	ND	ND	1	ND
4365	Anaerobic	98.5	ND	ND	1	0.5
	Aerated	94	3	ND	2	ND
TS2	Anaerobic	96.5	1	ND	1.5	1
	Aerated	73	10	ND	12	ND

[a] Clarified culture supernatants were obtained during exponential growth in 500 ml of lactose broth medium in anaerobic or aerated batch cultures as indicated.

[b] The proportion of carbon end products was based on the assumption that those compounds measured, plus the CO_2 presumed to be formed from pyruvate dehydrogenase activity, were equal to 100%. ND, Not detectable.

Despite these changes in the levels of pyruvate-metabolizing enzymes, there were generally only minor changes in the fermentation end products in aerated batch cultures which remained essentially homolactic when lactose was the carbon substrate (Table 5). Apart from 4350 and KB, there were slight decreases in the proportion of lactate produced. Interestingly, although lactate dehydrogenase activity could not be detected in strain KB, lactate was still the major fermentation product with this strain, usually accounting for around 50% of the fermented carbon. No attempt was made to determine acetoin, which is another major end product with this strain (26). What changes did occur in the minor products followed a general pattern. With most strains acetate and pyruvate production increased while production of ethanol and formate decreased in aerated cultures compared with anaerobic cultures.

(iii) Effect of oxygen on lactose fermentation by nongrowing cells. When lactose was supplied as a single dose to allow the maximum rates of fermentation by nongrowing cells of ML3, ML8, and 4350, lactate was the major end product under both aerated and anaerobic conditions, although strains ML3 and ML8 were more homolactic in the latter case (Table 6). The balance of the carbon products in these fermentations was almost entirely acetate (and presumably the CO_2 evolved in the production of acetate via pyruvate dehydrogenase), although there was some pyruvate produced. However, when the fermentation rate was limiting, the presence of oxygen had a dramatic effect on the fermentation pattern with ML3 and ML8. Under anaerobic conditions lactate still made up 69% of the total carbon products with ML3 and 84% with ML8. The balance consisted of acetate, pyruvate, ethanol, and formate (and presumably CO_2). When this limited fermentation was carried out in the presence of oxygen, only traces of lactate and no pyruvate, ethanol, or formate were formed. Instead, almost all lactose was fermented to acetate (and presumably the corresponding amount of CO_2 which would be produced in equimolar amounts with acetate). The pattern with 4350, however, was quite different. At the maximum fermentation rates the fermentations were almost totally homolactic regardless of the presence of oxygen. At the limiting fermentation rate lactate still made up 76% of the total carbon products, with the remainder either acetate or pyruvate, and again the presence of oxygen had no effect. Results similar to those given in Table 6 were obtained with all three strains when they were grown anaerobically (data not shown).

DISCUSSION

Although previous reports (1, 16) have highlighted the inhibitory effect of oxygen on growth of group N streptococci, our results are in fact consistent with those earlier studies which show that sensitivity to aeration is extremely variable in these bacteria. O'Leary (J. O'Leary, M.S. thesis, University College, Cork, Ireland, 1970) showed that 17 of 27 strains were inhibited by aeration while growing in milk. With *S. lactis* C10 inhibition was observed with glucose but not with lactose or galactose in aerated broth medium (16).

H_2O_2 is the inhibitory metabolite produced in aerobic broth cultures of *S. lactis* (1, 16; R. C. Grufferty, M.S. thesis, University College, Cork, Ireland, 1981). Our results showed that H_2O_2 was accumulated by some strains when washed cell suspensions were vigorously aerated. All six strains studied degraded exogenously supplied H_2O_2 in anaerobic washed cell suspensions.

NADH oxidase activity was increased by aerobic growth with all strains studied except TS2 and appears to be the primary oxygen-metabolizing enzyme. Similar increases in NADH oxidase activity have been observed in aerated cultures of *S. lactis* (16, 18) and *S. diacetylactis* (7). Unlike oxygen consumption, the reduction of H_2O_2 by washed cells did not require the addition of an energy source, suggesting that cells contain a mechanism for disposing of H_2O_2 that is independent of active metabolism. On the other hand, the rate of H_2O_2 reduction by washed cells of ML3 and ML8 was increased by lactose and, in all strains studied, NADH peroxidase activity was detected in cell-free extracts from aerobically grown cells. Thus, there appear to be at least two systems present for the removal of H_2O_2 in lactic streptococci, one of which is dependent on production of NADH during metabolism and one of which is independent of active metabolism.

A number of different NADH oxidases have been described in lactic acid bacteria, catalyzing a one-, two-, or four-electron reduction of oxygen to superoxide (O_2^-), H_2O_2, or water, respectively (4, 14, 21). Little work has been reported on the stoichiometry of the NADH oxidase reaction in lactic streptococci, although Bruhn and Collins (7) concluded that the oxidase from *S. diacetylactis* reduced oxygen directly to H_2O without producing H_2O_2 as an intermediate. However, their determinations were performed on crude extracts and no account was taken of potential NADH peroxidase activity.

S. lactis ML3 did not accumulate H_2O_2 under the conditions studied here (washed cells and batch culture). With this strain there was a close association between the NADH oxidase and NADH peroxidase activities such that the NADH-dependent oxygen consumption of a partially purified NADH oxidase/peroxidase was totally insensitive to added catalase. While this suggests that H_2O_2 may not be an intermediate of the NADH-dependent reduction of oxygen, it is more likely, in view of the close association of the two activities, that the NADH oxidase is in fact catalyzing a two-electron reduction of oxygen to H_2O_2 and that this is subsequently being converted to H_2O by the NADH perox-

TABLE 6. Effect of oxygen on end products from nongrowing cells[a]

Strain	Fermentation rate[b]	Fermentation conditions	% Total carbon end products[c]				
			Lactate	Acetate	Pyruvate	Ethanol	Formate
S. lactis ML3	Maximum	Aerated	69	21	ND	ND	ND
		N_2/CO_2	88	8	ND	ND	ND
	Limited	Aerated	1.5	66	ND	ND	ND
		N_2/CO_2	69	9.5	15	ND	2
S. lactis ML8	Maximum	Aerated	82	11	2	ND	ND
		N_2/CO_2	96	3	ND	ND	ND
	Limited	Aerated	11	60	ND	ND	ND
		N_2/CO_2	84	6	6	0.5	3
S. cremoris 4350	Maximum	Aerated	99	ND	1	ND	ND
		N_2/CO_2	99	ND	1	ND	ND
	Limited	Aerated	76	1.5	22	ND	ND
		N_2/CO_2	76	1.5	22	ND	ND

[a] Cells were obtained from the mid-log phase of aerated cultures growing in lactose broth medium. Final cell densities ranged from 4 to 11 mg (dry weight)/ml.
[b] The limited fermentation rates were 8.5, 8.6, and 12.2 nmol of lactose added/min per mg (dry weight) of cells for ML3, ML8, and 4350, respectively. This represents 5% of the maximum rate of lactose utilization by these bacteria (15).
[c] The proportion of carbon end products was determined as in Table 5, footnote *b*. In all cases the carbon recovery was >70%. ND, Not detectable.

idase. If this mechanism is general among the group N streptococci, then the variable accumulation of H_2O_2 found can best be explained by an imbalance in the in vivo activities of the coupled oxidase/peroxidase enzymes. With some strains the reduction of oxygen to water is readily accomplished, while with others the toxic intermediate H_2O_2 builds up to autoinhibitory levels. In milk culture, with an intact lactoperoxidase system, the level of H_2O_2 required to cause inhibition of growth would be much less than in broth culture (28).

When nongrowing cells of *S. lactis* were fed a continuous supply of lactose at limiting rates, the resulting heterolactic fermentation was dramatically influenced by oxygen. At approximately 5% of the maximum lactose utilization rate lactate constituted 69% of the measured products of lactose fermentation for ML3 under anaerobic conditions, with the remaining product spread among acetate, pyruvate, and formate. When this experiment was repeated under aerobic conditions, the effect was dramatic. Only traces of lactate were produced, while acetate, and presumably CO_2, were the major products. Thus, the same cells that were capable of normal homolactic fermentation under anaerobic conditions with excess lactose could carry out a homoacetic fermentation under aerobic conditions when lactose was limiting. In this experiment 106% of the carbon was accounted for, ruling out the possibility of other major products. A similar result was found for *S. lactis* ML8 but *S. cremoris* 4350 remained predominantly homolactic under the same conditions. Similar shifts in end-product formation away from lactate production during slow lactose metabolism under aerobic conditions were previously found for *S. lactis* strains ML3 and ML8 (15). However, in those experiments pyruvate as well as acetate was produced in major proportions due apparently to strain variation. Under conditions which result in a homoacetic fermentation, the NADH oxidase/peroxidase system is clearly able to regenerate sufficient NAD^+ to allow a balanced fermentation without the need to produce lactate. In these washed cell experiments the limiting rate of lactose utilization for both ML3 and ML8 was 9 nmol of lactose/min per mg (dry weight) of cells. From the data on oxygen consumption rates in Table 2 it is clear that the NADH oxidase/peroxidase system can readily attain this rate of flux, even accounting for the need for 2 mol of oxygen to be reduced to H_2O to balance the 4 mol of NADH produced in catabolism of 1 mol of lactose to pyruvate.

The pattern of fermentation end products in group N streptococci hinges on the further metabolism of pyruvate. Under anaerobic conditions three enzymes (lactate dehydrogenase [11], pyruvate formate-lyase [38], and pyruvate dehydrogenase [5]) compete for the available pyruvate, with the need to regenerate the appropriate amount of reducing equivalents to balance the fermentation acting as the major constraint. However, in the presence of oxygen the metabolism of pyruvate is complicated by the further competition for NADH by the NADH oxidase/peroxidase system. In aerobically grown cells of the six strains studied here, there was a general trend for an increase in levels of NADH oxidase, NADH peroxidase, and pyruvate dehydrogenase and a decrease in lactate dehydrogenase compared with anaerobically grown cells. In addition, the pyruvate formate-lyase is likely to be inactivated in the presence of oxygen (38, 41). Thus, at the level of enzyme synthesis aerobically grown cells appear to be more suited to the production of acetate, relative to the other end products (lactate, formate, or ethanol), than anaerobically grown cells. Despite these changes, it is not until the rate of lactose metabolism is considerably slowed that a shift in end products from lactate to acetate occurs in the presence of oxygen. The production of acetate instead of lactate under aerobic conditions leads to the generation of more energy by substrate-level phosphorylation via the acetate kinase reaction.

When a dose of lactose or glucose was given to washed cells, the oxygen consumption rate was biphasic, whereas a constant rate was observed when sugar was supplied at a limiting rate. A plausible explanation for this concerns the in vivo regulation of lactate dehydrogenase activity. Fully activated, this enzyme has the potential to utilize NADH and all of the pyruvate to give a balanced homolactic fermentation. The in vivo activity of lactate dehydrogenase is difficult to assess due to its regulation by a number of compounds, notably FBP and tagatose 1,6-bisphosphate (11, 34). The

intracellular concentration of FBP is correlated with shifts between homolactic and heterolactic fermentation (15, 36, 38). The in vivo FBP levels in strains ML3, ML8, and 4350 increased from extremely low levels (<0.2 mM) in aerated starved cells at 10°C to >10 mM a few minutes after addition of glucose. This buildup in internal FBP concentration correlated with the biphasic oxygen consumption patterns such that the point of inflection between the initial rapid phase and the secondary slower phase occurred when the in vivo FBP concentration had reached 5 to 15 mM depending on the strain.

The following hypothesis explains these observations: when conditions are present that lead to a reduction in lactate dehydrogenase activity, such as a lactose- or glucose-limited fermentation, the enzymes pyruvate formate-lyase and pyruvate dehydrogenase compete effectively with the lactate dehydrogenase for pyruvate to give a heterolactic fermentation. In the presence of oxygen, however, the NADH oxidase/peroxidase system in some strains can compete effectively with lactate dehydrogenase, aldehyde dehydrogenase, and alcohol dehydrogenase for the available NADH and, with the concomitant destruction of pyruvate formate-lyase, allow a homoacetic fermentation via the pyruvate dehydrogenase. Fordyce et al. (15) showed that the internal FBP concentration decreased with decreasing rates of glucose fermentation for nongrowing cells of *S. lactis* ML3 and ML8. Thus, the monophasic, relatively rapid rate of oxygen consumption and homoacetic fermentation we find in glucose-limited aerobic washed cell fermentations of ML3 and ML8 can be linked to conditions favoring reduced lactate dehydrogenase activity. The lack of a biphasic galactose-driven oxygen consumption pattern in the five group N streptococci studied here is consistent with the relatively low intracellular FBP or lactate dehydrogenase levels in galactose-grown lactic streptococci (38). The nonappearance of the biphasic oxygen consumption pattern in *S. thermophilus* TS2, regardless of the sugar being metabolized, is consistent with the FBP-independent lactate dehydrogenase in this strain (35).

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