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Glucose Metabolism and NADH Recycling by *Treponema hyodysenteriae*, the Agent of Swine Dysentery

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Glucose metabolism and the mechanisms of NADH oxidation by *Treponema hyodysenteriae* were studied. Under an N_2 atmosphere, washed cell suspensions of the spirochete consumed glucose and produced acetate, butyrate, H_2, and CO_2. Approximately twice as much H_2 as CO_2 was produced. Determinations of radioactivity in products of [^{14}C]glucose and [^{14}C]pyruvate metabolism and analyses of enzyme activities in cell lysates revealed that glucose was catabolized to pyruvate via the Embden-Meyerhof-Parnas pathway. The results of pyruvate exchange reactions with $NaH^{14}CO_3$ and $Na^{14}COOH$ demonstrated that pyruvate was converted to acetyl coenzyme A (acetyl-CoA), H_2, and CO_2 by a clostridium-type phosphoroclastic mechanism. NADH:ferredoxin oxidoreductase and hydrogenase activities were present in cell lysates and produced H_2 from NADH oxidation. Phosphotransacetylase and acetate kinase catalyzed the formation of acetate from acetyl-CoA. Butyrate was formed from acetyl-CoA via a pathway that involved 3-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, butyryl-CoA dehydrogenase, and butyryl-CoA transferase. *T. hyodysenteriae* cell suspensions generated less H_2 and butyrate under 10% O_2–90% N_2 than under 100% N_2. Cell lysates contained NADH oxidase, NADH peroxidase, and superoxide dismutase activities. These findings indicated there are three major mechanisms that *T. hyodysenteriae* cells use to recycle NADH generated from the Embden-Meyerhof-Parnas pathway—enzymes in the pathway from acetyl-CoA to butyrate, NADH:ferredoxin oxidoreductase, and NADH oxidase. Versatility in methods of NADH oxidation and an ability to metabolize oxygen could benefit *T. hyodysenteriae* cells in the colonization of tissues of the swine large bowel.

Treponema hyodysenteriae is an anaerobic spirochete and the agent of swine dysentery. By and large, the characteristics enabling this bacterium to colonize and damage the mucosal surfaces of the swine cecum and colon are unknown. To successfully colonize, *T. hyodysenteriae* cells must undoubtedly multiply and persist at a mucosal site. One approach to understanding the colonizing ability of this spirochete is to investigate its growth requirements and metabolic traits.

A colleague and I reported the results of investigations of substrate utilization and glucose metabolism by *T. hyodysenteriae* growing cells (33). The spirochete uses various carbohydrates for growth and, when cultured with glucose as the growth substrate, produces acetate, butyrate, CO_2, and H_2. These findings were consistent with those of previous investigators (16, 17, 25). Additionally, two unusual features of the metabolism of *T. hyodysenteriae* were revealed (33). First, under an N_2-O_2 (99:1) atmosphere, cells of the spirochete consume substrate amounts of oxygen (2 μmol/ml of culture). Second, growing cells produce large amounts of H_2 (twice as much H_2 as CO_2). Unfortunately, a detailed study of *T. hyodysenteriae* glucose metabolism could not be carried out with cell cultures (33) because growing cells metabolize unidentified components of the culture medium in addition to glucose, thus complicating investigations of glucose metabolism.

In the present investigation, washed cell suspensions and cell lysates were used to determine pathways of glucose metabolism and mechanisms of NADH oxidation by *T. hyodysenteriae*. Primary objectives were to discover a mechanism for oxygen consumption and to explain the basis for the production of H_2 by this pathogenic spirochete. My ultimate objective is to identify physiological traits contributing to the success of *T. hyodysenteriae* as a mucosal pathogen.

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MATERIALS AND METHODS

Organisms and culture conditions. Cells of *T. hyodysenteriae* B204 were routinely cultured in tubes of BHIS broth (32). For 2-liter cultures, the serum content of BHIS broth was reduced to 5% (vol/vol) and the medium was contained in 3-liter Florence flasks. A flask of prewarmed (37°C) medium was inoculated with approximately 2×10^{10} spirochete cells in the exponential phase of growth and then sealed with a stopper fitted with a rubber dropper bulb with a 1-cm slit opening. The dropper bulb acted as a safety valve to vent gasses produced by the bacteria during growth. The initial culture atmosphere was N_2-O_2 (99:1), and each culture was stirred with a magnetic stirring bar (33). Cultures were incubated for 20 to 26 h at 38°C.

Clostridium butyricum 3266 was supplied by I. M. Robinson, National Animal Disease Center, and was cultured anaerobically in BHIS broth lacking serum.

Preparation of cell suspensions and cell lysates. For preparation of cell suspensions, bacteria from a 2-liter culture (optical density at 620 nm, 1.3; approximately 4×10^8 cells per ml, as determined by direct microscope counts) were harvested by centrifugation (5,000 × *g*, 10 min), washed once with 150 ml of anaerobic phosphate buffer, and resuspended in 20 ml of the same buffer. Anaerobic sodium phosphate buffer (0.05 M, pH 7.0) was deoxygenated by autoclaving (20 min) and cooling under N_2 and then further chemically reduced by adding dithiothreitol (DTT; final

concentration, 0.03% [wt/vol]). From a 2-liter culture, a cell mass of approximately 3.8 g (wet weight) (350 to 400 mg of total cell protein) was harvested.

For preparation of lysates for enzyme assays, cell suspensions (20 ml) were disrupted either with a French pressure cell (7,500 lb/in^2) or by sonication (model 250 Sonifier; Branson Sonic Power Co., Danbury, Conn.; output setting 6; three 10-s bursts). Lysates obtained by sonication are specified in the text. Both disruption methods resulted in lysis of greater than 99.9% of the cells, as determined by microscopy. Lysates to be passed through DEAE columns were centrifuged (30,000 × *g*, 30 min) to remove large debris and unbroken cells. The protein content of cell lysates was determined by the method of Lowry et al. (19) with bovine serum albumin as a standard. At every step in the preparation of cell suspensions and lysates, buffers and cells were kept cold (5°C) and under N_2 or argon.

Metabolism by cell suspensions. Washed cell suspensions in 20-ml serum bottles (Wheaton Industries, Millville, N.J.) were used for metabolic studies. Each bottle contained 100 μmol of glucose, 10 μmol of DTT, and approximately 10^{11} cells in 5 ml of anaerobic phosphate buffer. Bacteria and all solutions were kept under an N_2 atmosphere. Serum bottles were sealed with butyl rubber stoppers (no. 2048-11800; Bellco Glass, Inc., Vineland, N.J.). Bottles containing cell suspensions were incubated at 38°C with constant agitation (model 02156 water bath; American Optical Corp., Buffalo, N.Y.; setting 6). After 4 h of incubation, 12 N H_2SO_4 (0.1 ml) was injected through the stoppers and into assay mixtures to be used for the analysis of metabolic gasses. Other assay mixtures were centrifuged (Microfuge; Beckman Instruments, Inc., Fullerton, Calif.; 12,000 × *g*, 5 min). Of the resulting supernatant, 1 ml was heated (15 min, 95°C) and used for analysis of glucose content. Another 2.5 ml was titrated to pH 10 with NaOH and used for volatile fatty acid analysis. In experiments investigating the influence of oxygen on metabolism, a sample (1.5 ml) of the N_2 atmosphere over the suspension was replaced with the same volume of O_2 to provide an initial atmosphere containing 10% O_2.

In each experiment, control assay mixtures were included and provided data to compensate for zero-time metabolism (H_2SO_4 added before incubation), endogenous metabolism (suspensions without added glucose), and volatile fatty acid recovery (acetate, propionate, and butyrate added to acidified suspensions before incubation).

Analysis of metabolic substrates and products. Substrates (oxygen and glucose) and potential products (alcohols, gasses, and organic acids) of washed cell suspensions were assayed by gas chromatography and other methods used in metabolic studies of growing *T. hyodysenteriae* cells (33). Formate (13) and pyruvate (pyruvate test kit no. 726-UV; Sigma Chemical Co., St. Louis, Mo.) were determined by enzyme-based assays. Acid products were also fractionated and quantitated by high-performance liquid chromatography with an Aminex HPX-87H ion-exchange column (Bio-Rad Laboratories, Richmond, Calif.) and a mobile phase of 0.007 N H_2SO_4–10.8% acetonitrile (11).

Metabolism of [^{14}C]glucose and [^{14}C]pyruvate. Washed cell suspensions were incubated with [1-^{14}C]glucose, [3,4-^{14}C]glucose, or [1-^{14}C]pyruvate in 20-ml serum bottles as described above. Attached to the underside of the rubber stopper sealing each serum bottle was a plastic collection cup (Center Well no. 882320-0000; Kontes Scientific Glassware, Vineland, N.J.) containing 200 μl of 2.5 N NaOH. After incubation and the addition of H_2SO_4, the reaction mixture was stored overnight at 5°C to ensure that released CO_2 was efficiently trapped in the NaOH. The radioactivity of CO_2 was determined by liquid scintillation counting of aliquots of the CO_2 trap solution. The amount of CO_2 produced from glucose or pyruvate was determined by gas chromatography with parallel incubation mixtures without CO_2 traps and containing a nonradioactive substrate.

For recovery of radioactive acetate and butyrate, the acidified incubation mixture was deproteinized by $Zn(OH)_2$ precipitation, and volatile acids were steam distilled from the clarified mixture (21). The condensate was titrated to pH 8 to 9 with 0.01 N NaOH and heated on a steam bath until the liquid had evaporated. The resulting dry residue was dissolved in 0.5 ml of a solution of 0.007 N H_2SO_4 with 10.8% (vol/vol) acetonitrile, and volatile acids in this sample were separated and quantitated by high-performance liquid chromatography (11). Eluted fractions containing acetate or butyrate were collected and assayed for radioactivity.

Aqueous solutions (0.5 to 1.0 ml) containing radioactive compounds were mixed with 10 ml of Aquasol liquid scintillation cocktail (Dupont, NEN Research Products, Boston, Mass.) and counted in a model LS-9800 liquid scintillation counter (Beckman). Counting efficiencies were similar for all samples, as determined through the use of an external standard program of the LS-9800.

Specific radioactivity values for products (counts per minute per micromole) were adjusted for the metabolism of endogenous substrate. These adjustments were based on the determination, in parallel experiments, of the specific radioactivities of products of D-[U-^{14}C]glucose. The lower specific radioactivity of a product relative to that of D-[U-^{14}C]glucose was considered to result from the metabolism of nonradioactive endogenous substrate.

Enzyme assays. Hydrogenase activity in *T. hyodysenteriae* cell lysates was determined by measuring H_2 evolution from chemically reduced dyes (12, 23). Under an argon atmosphere, assay mixtures (5 ml) in 10-ml stoppered serum bottles contained 50 μmol of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) per ml, 5 μmol of DTT per ml, 10 μmol of sodium hydrosulfite per ml, 10 μmol of methyl viologen, benzyl viologen, or methylene blue per ml, and cell lysate (4 mg of protein per ml of assay mixture). Assay mixtures were incubated with shaking in a 38°C water bath. After 60 min, atmosphere samples were assayed by gas chromatography. Hydrogen uptake was tested with mixtures containing oxidized dyes (no hydrosulfite) and incubated under an atmosphere of H_2-argon (20:80).

Glyceraldehyde-phosphate dehydrogenase, fructose-bisphosphate aldolase, phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase activities in lysates of washed *T. hyodysenteriae* cells were assayed as described by Hespell and Canale-Parola (12). Acetate kinase was determined by measuring acyl phosphate produced from acetate by lysates of sonicated cells passed through a DEAE-cellulose column to remove coenzyme A (CoA) (27). Butyryl-CoA dehydrogenase was assayed as described by Engel and Massey (5). DTT was eliminated from the buffer used to wash and suspend bacterial cells for this assay. Butyryl-CoA transferase was assayed by the method of Barker et al. (1). Butyrate kinase and 3-hydroxybutyryl-CoA dehydrogenase were assayed as described by George and Smibert (7). Phosphotransacetylase was measured by monitoring the ability of cell lysates that had been treated with an anion-exchange resin to carry out arsenolysis of acetyl phosphate (31). Superoxide dismutase, catalase, and peroxidase were assayed as described previously (2, 4). All

enzymes producing NADH, for example, glyceraldehyde-phosphate dehydrogenase, were assayed under argon, owing to the presence of NADH oxidase activity.

Oxidase and peroxidase activities coupled to the oxidation of NADH or NADPH were assayed at room temperature (23°C) by monitoring the decrease in the A_{340} of an assay solution (3 ml) containing 50 μmol of anaerobic sodium phosphate buffer (pH 7.0) per ml, 2 μmol of DTT per ml, 0.2 μmol of NADH or NADPH per ml, and cell lysate (2 to 40 μg of protein per ml of assay mixture). Stock solutions of the nucleotides were prepared with HEPES buffer (0.05 M, pH 8.5). Assays were contained in 3-ml anaerobic cuvettes (8493D12 Spectrocell; 10 mm; Thomas Scientific) fitted with injection caps containing Teflon-coated silicone septa (Supelco, Inc., Bellefonte, Pa.). The cuvette atmosphere initially was argon. The oxidase assay was initiated by flushing the cuvette atmosphere with 100% O_2 through the silicone septum and inverting the cuvette two or three times. The peroxidase assay was begun by injecting 0.05 ml of H_2O_2 (0.33% [vol/vol]) into the cuvette. Absorbance measurements were taken and recorded with a Gilford Response II spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, Ohio).

Pyruvate metabolism: exchange reactions and acetyl phosphate production. The ability of *T. hyodysenteriae* whole cells and cell lysates to mediate the exchange of $^{14}CO_2$ or [^{14}C]formate with pyruvate was tested by methods used for other organisms (12, 35). Reaction mixtures (5 ml) in 20-ml stoppered bottles contained 50 μmol of sodium phosphate (pH 7.0) or Tris (pH 8.4) buffer per ml, 5 μmol of $MnCl_2 \cdot 4H_2O$ per ml, 5 μmol of thiamine pyrophosphate (TPP) per ml, 2 μmol of DTT per ml, 0.01 μmol of CoA per ml, 20 μmol of sodium pyruvate per ml, 20 μmol of $NaH^{14}CO_3$ or $Na^{14}COOH$ per ml, and either washed whole cells (2×10^{10} cells per ml) or cell lysates (5 to 8 mg of protein per ml of reaction mixture). Mixtures were incubated with shaking at 38°C for 60 min under argon. Exchange activity was stopped by injecting acid (0.1 ml of 12 N H_2SO_4) into the mixture. Large cell debris was removed by centrifugation (12,000 × *g*, 10 min). Mixtures containing cell lysates were deproteinized by treatment with $Zn(OH)_2$ (21). Pyruvate was recovered from supernatant fluids and quantitated by high-performance liquid chromatography as described above. Radioactivity associated with pyruvate was determined by liquid scintillation techniques as described above.

The formation of acetyl phosphate from pyruvate by cell lysates was measured by previously described techniques (12, 18) (see Table 5 for assay components and conditions).

Production of H_2 from NADH. NADH:ferredoxin oxidoreductase (NFOR) activity was assayed through the coupling of this enzyme with hydrogenase in *T. hyodysenteriae* cell lysates, i.e., by measuring H_2 produced from NADH oxidation (14, 26). For removal of endogenous ferredoxin, lysates (20 ml) of sonicated cells were passed through a DEAE-cellulose column (2 by 5.5 cm) previously equilibrated with anaerobic Tris hydrochloride buffer (0.05 M, pH 7.4). Once the lysate had entered the column, cellular proteins were eluted with Tris buffer (0.05 M, pH 8.0). The first 15 ml of eluate was discarded, and the next 10 ml (DEAE-treated lysate [DTL]) was collected and used for the NFOR assay.

NFOR assay mixtures were contained in 10-ml serum bottles flushed with argon and sealed with butyl rubber stoppers (see Table 6 for components of a standard 1.5-ml assay mixture). Mixtures were incubated for 30 min at 38°C in a shaking water bath, and 0.1 ml of 12 N H_2SO_4 was injected into each bottle. Assay atmospheres were sampled by injecting 3 ml of distilled water into each bottle while simultaneously removing a 2.5-ml sample of the headspace atmosphere with a second syringe fitted with a gastight lock. This sample was analyzed for H_2 by gas chromatography.

TABLE 1. Glucose metabolism by *T. hyodysenteriae* cell suspensions under anaerobic conditions[a]

Expt	Yield[b] of:				Carbon recovery (%)	Oxidation-reduction index
	Acetate	Butyrate	H_2	CO_2		
1	137	27	340	180	94	0.9
2	145	17	308	154	85	0.9

[a] Products assayed for but not detected were propionate, lactate, succinate, isobutyrate, isovalerate, valerate, ethanol, and butanol. Each incubation mixture beneath an N_2 atmosphere contained 10^{11} cells, 100 μmol of glucose, and 10 μmol of DTT in 5 ml of anaerobic sodium phosphate buffer (0.05 M, pH 7). Suspensions were incubated with shaking for 4 h at 38°C.

[b] Expressed as micromoles of product per 100 μmol of glucose consumed (average of two incubation mixtures for acids and three incubation mixtures for gasses). Cell suspensions consumed 70 μmol of glucose in experiment 1 and 58 μmol in experiment 2. Adjustments were made to product yields to account for endogenous metabolism (see the text).

Chemicals and reagents. Ferredoxin (type V, F-7629) from *Clostridium pasteurianum* and other reagents used in enzyme assays were purchased from Sigma. CoA was used in the reduced form (no. C3019; Sigma). Glucose was obtained from Fisher Scientific Co., Chicago, Ill. D-[1-^{14}C]glucose (NEC-043X), D-[3,4-^{14}C]glucose (NEC-353), D-[U-^{14}C]glucose (NEC-042H), sodium [^{14}C]bicarbonate (NEC-086), sodium [1-^{14}C]pyruvate (NEC-255), and sodium [^{14}C]formate (NEC-089) were purchased from Dupont, NEN Research Products.

RESULTS

Glucose metabolism by cell suspensions. Under anaerobic conditions, *T. hyodysenteriae* washed cell suspensions consumed glucose and produced H_2, CO_2, acetate, and butyrate (Table 1). The calculated oxidation-reduction index was 0.9, an indication that the metabolism was essentially balanced for electron flow; i.e., the oxidation of reducing equivalents, such as NADH, could be largely accounted for. As was the case for growing cells (33), substantially more H_2 than CO_2 was produced. In control experiments, washed cell suspensions of *C. butyricum* evolved 145 μmol of CO_2 and 224 μmol of H_2 per 100 μmol of glucose fermented, consistent with the known ability of that bacterium to produce more H_2 than CO_2 (36).

In every metabolism experiment involving cell suspensions, 20 to 33% of the product yield resulted from the metabolism of an unidentified, endogenous substrate. Various attempts to deplete cells of substrate—by using cells harvested at different stages of the growth curve, by repeatedly washing bacteria, and by preincubating cell suspensions for 1 h without added substrate—were unsuccessful. To compensate for the extra products, I subtracted the product yields of control cell suspensions (incubated without added substrate) from the product yields of cell suspensions incubated with substrate.

Conversion of glucose to pyruvate. When washed cell suspensions of *T. hyodysenteriae* were incubated with [^{14}C]pyruvate, essentially all of the radioactivity released from the carboxyl group of pyruvate appeared in CO_2 (Table 2). Radioactive CO_2 was produced from the C-3 and C-4

TABLE 2. Distribution of radiolabel in products of [^{14}C]glucose and [^{14}C]pyruvate metabolism by *T. hyodysenteriae* cell suspensions

Substrate[a]	Specific radioactivity[b] of:		
	CO_2	Acetate	Butyrate
[1-^{14}C]pyruvate	10,360	20	54
[1-^{14}C]glucose	150	7,100	16,760
[3,4-^{14}C]glucose	6,000	140	210

[a] The specific radioactivities of the substrates were as follows: [1-^{14}C] pyruvate, 13,750; [1-^{14}C]glucose, 16,400; and [3,4-^{14}C]glucose, 10,500.

[b] Expressed as counts per minute per micromole of product. Values were adjusted to compensate for products of an unidentified endogenous metabolite(s).

atoms of glucose and not from the C-1 atom (Table 2), a pattern typical of glucose metabolism by the Embden-Meyerhof-Parnas (EMP) pathway (9). The specific radioactivities of acetate and butyrate (Table 2) were also consistent with their production via this pathway.

T. hyodysenteriae cell lysates contained glyceraldehyde-phosphate dehydrogenase and fructose-bisphosphate aldolase activities, typical of the EMP pathway (Table 3). Enzymes characteristic of the Entner-Duodoroff pathway (glucose-6-phosphate dehydrogenase) and pentose phosphate pathway (glucose-6-phosphate and phosphogluconate dehydrogenases) were not detected. Thus, findings from radiolabeling studies and from enzyme analyses indicated that the major pathway for the metabolism of glucose to pyruvate in *T. hyodysenteriae* is the EMP pathway.

Pyruvate breakdown. *T. hyodysenteriae* cell suspensions and lysates exchanged large amounts of radiolabel from CO_2 with pyruvate but only small amounts from formate with pyruvate (Table 4). CoA was necessary for the pyruvate-CO_2 exchange. Cell lysates supplied with pyruvate produced acetyl phosphate (Table 5). Yields of acetyl phosphate were highest when both CoA and phosphate were supplied. Methyl viologen, an artificial electron acceptor, slightly increased acetyl phosphate production. Lysates passed through DEAE columns (DTL), a process known to remove endogenous ferredoxin, produced less acetyl phosphate than did untreated lysates (Table 5). Full capacity for acetyl phosphate production was restored when ferredoxin (100 μg) from *C. pasteurianum* was added to assay mixtures containing DTL (Table 5).

Hydrogen was produced (0.7 to 1.0 μmol per assay) from reduced methyl viologen by cell lysates. Hydrogen was not detected (limit of detection, 0.05 μmol) when boiled (5 min)

TABLE 4. CO_2-pyruvate and formate-pyruvate exchange activities in *T. hyodysenteriae*

Mediator	pH	Exchange activity[a] of:	
		$NaH^{14}CO_3$-pyruvate	$Na^{14}COOH$-pyruvate
Cell suspensions	7.0	4,400	140
	8.4	4,800	150
Cell lysates	7.0	4,240	143
	8.4	3,900	175

[a] Exchange activity is expressed as radioactivity (counts per minute per micromole) of pyruvate recovered after incubation of cell suspensions and cell lysates with nonradioactive pyruvate and either $NaH^{14}CO_3$ (CO_2) (9,200 cpm/μmol) or $Na^{14}COOH$ (10,500 cpm/μmol).

TABLE 3. Enzyme activities in *T. hyodysenteriae* cell lysates

Enzyme[a]	Substrate; cofactor(s)	Source or reference	Sp act[b]
Glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12)	Glyceraldehyde-3-phosphate; NAD	12	900
Fructose-bisphosphate aldolase (EC 4.1.2.13)	Fructose-1,6-bisphosphate	12	6.0
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	Glucose-6-phosphate; NAD and NADP	12	ND
Phosphogluconate dehydrogenase (EC 1.1.1.44)	6-Phosphogluconate; NADP	12	ND
NADH oxidase (EC 1.6.99.3)	Oxygen; NADH Oxygen; NADPH	This study	760 4
NADH peroxidase (EC 1.11.1.1)	H_2O_2; NADH H_2O_2; NADPH	This study	7.0 ND
Phosphotransacetylase (EC 2.3.1.8)	Acetyl phosphate; CoA	31	1.5
Acetate kinase (EC 2.7.2.1)	Acetate; ATP	27	31
3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35)	Acetoacetyl-CoA; NADH Acetoacetyl-CoA; NADPH	7	1.4 ND
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	Butyryl-CoA; PMS-DCPIP[c] Crotonyl-CoA; NADH	5	0.5 ND
Butyrate kinase (EC 2.7.2.7)	Butyrate; ATP	7	ND
Butyryl-CoA transferase	Butyryl-CoA; acetate	1	17

[a] Assays based on NAD(P)H oxidation or NAD(P) reduction were incubated under anaerobic conditions.

[b] For the first six enzymes, specific activities are expressed as nanomoles of pyridine nucleotide per minute per milligram of cell lysate protein. For the next six enzymes, specific activities are expressed as units per milligram of protein (units as defined in the references). ND, Not detected with 100 to 200 μg of lysate protein.

[c] PMS-DCPIP, Phenazine methosulfate-dichlorophenol indophenol.

TABLE 5. Production of acetyl phosphate from pyruvate by *T. hyodysenteriae* cell lysates

Assay mixture contents[a]	Acetyl phosphate yield[b]
Untreated lysate	380
Minus pyruvate	ND
Minus cell lysate	ND
Minus CoA	36
Minus phosphate	145
Plus methyl viologen (1 mM)	420
DEAE-treated lysate	90
Plus ferredoxin[c]	400
Minus DTL, plus ferredoxin[c]	ND

[a] An assay mixture (1 ml) consisted of the following: DTT, 5 μmol; TPP, 5 μmol; $MnCl_2$, 1 μmol; CoA, 0.25 μmol; pyruvate, 100 μmol; potassium phosphate buffer (pH 7.0), 25 μmol; untreated cell lysate (5.5 mg) or DTL (6.7 mg); and HEPES buffer (pH 7.0), 50 μmol. The assay mixtures were incubated under argon for 45 min at 37°C.

[b] Expressed as micromoles per gram of lysate protein. ND, Not detected (<30 μmol/g).

[c] *C. pasteurianum* ferredoxin (100 μg).

TABLE 6. Production of H_2 from NADH by DEAE-cellulose-treated *T. hyodysenteriae* cell lysates

Modification of standard assay mixture[a]	H_2 yield[b]
None (complete)	512
Minus ferredoxin	ND
Minus NADH	3
Minus acetyl-CoA-regenerating system	2.5
Minus cell lysate	ND

[a] The standard assay mixture (1.5 ml) consisted of the following: Tris hydrochloride (pH 7.5), 150 μmol; DTT, 7.5 μmol; flavin adenine dinucleotide, 0.015 μmol; NADH, 4 μmol; *C. pasteurianum* ferredoxin, 0.3 mg; DTL, 8 to 10 mg; alcohol dehydrogenase, 75 U; phosphotransacetylase, 15 U; CoA, 1.5 μmol; acetyl phosphate, 30 μmol; and acetate, 75 μmol. The last four components represent an acetyl-CoA-regenerating system. Ethanol (100%, 0.025 ml) was added to start the reaction. Assay mixtures were incubated under an argon atmosphere at 37°C for 30 min.

[b] Expressed as micromoles of H_2 per gram of cell lysate protein. ND, Not detected (<2 μmol/g).

cell lysates were used or when reduced benzyl viologen or reduced methylene blue replaced methyl viologen. Under an atmosphere containing 20% (vol/vol) H_2, cell lysates mediated the reverse reaction, that is, consumed H_2 (1 to 2 μmol per assay) and reduced methyl viologen. These findings indicated that the spirochete has hydrogenase activity (EC 1.18.3.1).

The exchange of CO_2 with pyruvate by cell suspensions and lysates (Table 4), the biochemical requirements for converting pyruvate to acetyl phosphate (Table 5), and the presence of hydrogenase activity in cell lysates indicated that *T. hyodysenteriae* cells possess a clostridium-like phosphoroclastic reaction for pyruvate breakdown (9).

Hydrogen production from NADH. The phosphoroclastic breakdown of pyruvate produces H_2 and CO_2 in equimolar amounts (9). Although the evidence described above indicated that *T. hyodysenteriae* metabolizes pyruvate by this mechanism, both washed cells (Table 1) and actively dividing cells (33) of the spirochete produced more H_2 than CO_2. The oxidation-reduction index of the metabolism (0.9 [Table 1]) indicated that the oxidation of reducing equivalents generated from the EMP pathway could be accounted for. It therefore seemed possible that *T. hyodysenteriae* cells were directly converting NADH to H_2. Although a pathway directly coupling H_2 production with NADH oxidation has apparently not been reported for any spirochete, such a pathway has been described for various anaerobic bacteria and involves the enzymes NFOR (EC 1.18.1.3) and hydrogenase (14, 26).

Hydrogen gas was evolved when lysates of sonicated cells were incubated in assay mixtures similar to those used to detect NFOR activity in other bacteria (Table 6) (14, 26). H_2 production was drastically reduced when NADH was omitted from the assay. Exogenous ferredoxin was necessary for H_2 production, suggesting the involvement of a *T. hyodysenteriae* ferredoxin in the coupling of NADH oxidation with H_2 production, although alternative electron transfer proteins are possible (24). An acetyl-CoA-regenerating system (or one or more components of that system) was essential for H_2 production. It remains to be determined whether a constant acetyl-CoA level (as supplied by a regenerating system) is essential for activation of the *T. hyodysenteriae* pathway, as is the case for clostridia (14).

Acetate and butyrate formation. Phosphotransacetylase, which converts acetyl-CoA to acetyl phosphate, and acetate kinase, which yields acetate and ATP from acetyl phosphate, were present in *T. hyodysenteriae* cell lysates (Table 3). Enzymes for butyrate formation included 3-hydroxybutyryl-CoA dehydrogenase and butyryl-CoA dehydrogenase (Table 3). Butyryl-CoA dehydrogenase could not be detected by monitoring NADH oxidation during the conversion of crotonyl-CoA to butyryl-CoA and was measured by monitoring the reverse reaction—butyryl-CoA oxidation coupled to the reduction of dichlorophenol-indophenol. This finding suggested that the spirochete resembles certain other bacteria which have an intermediate electron-transferring compound that couples NADH oxidation with the reduction of crotonyl-CoA to butyryl-CoA (7). Butyrate kinase was not detected, but butyryl-CoA transferase, which transfers CoA to acetate, was present. The finding of this activity indicated that the butyrate pathway produces ATP indirectly, i.e., via the pathway to acetate (Fig. 1).

Oxygen metabolism. Washed *T. hyodysenteriae* cells under 10% O_2 consumed substrate amounts of O_2 (Table 7). Oxygen uptake was accompanied by decreased yields of H_2 (7 μmol) and butyrate (approximately 2 μmol) relative to the anaerobic yields of these products (Table 7). On the basis of the established mechanisms for NADH oxidation by butyrate and H_2 formation (Fig. 1), the oxidation of 11 μmol of NADH was apparently accommodated by the consumption of 5 to 6 μmol of O_2 (or 2 μmol of NADH per μmol of O_2).

Cell lysates of the spirochete contained both NADH oxidase and NADH peroxidase activities (Table 3). NADH oxidase activity was appreciable, comparable to that of glyceraldehyde-phosphate dehydrogenase (760 versus 900 [Table 3]), and associated with the supernatant of lysed cells that had been subjected to ultracentrifugation to pellet cell membranes (140,000 × *g*, 2 h). In separate experiments, H_2O_2 production by the oxidase was assayed by adding horseradish peroxidase and 4-aminoantipyrine to the assay mixtures (4). Although over 200 nmol of NADH was oxidized, H_2O_2 was not detected (limit of detection, 20 nmol), suggesting that the product of the oxidase reaction was H_2O and not H_2O_2. NADPH oxidase and NADPH peroxidase activities were not detected.

Peroxidase (EC 1.11.1.7) (with 4-aminoantipyrine as a hydrogen donor) and catalase (EC 1.11.1.6) were not present in cell lysates, as determined by standard assay techniques (4). Superoxide dismutase (EC 1.15.1.1) activity was detected both by a spectrophotometric assay (4) and by activity staining with electrophoretically separated lysate proteins (2).

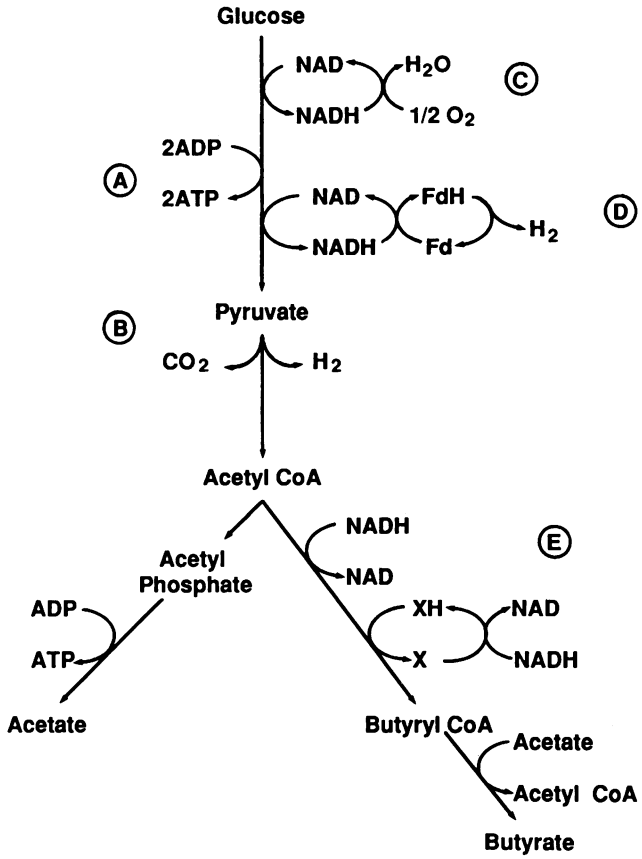

FIG. 1. Glucose metabolism by *T. hyodysenteriae*. (A) EMP pathway. (B) Clostridium-type phosphoroclastic reaction. Major mechanisms of NADH oxidation include NADH oxidase (C), NFOR coupled to hydrogenase (D), and 3-hydroxybutyryl-CoA dehydrogenase plus butyryl-CoA dehydrogenase (E). X, Unidentified electron-transferring compound; FdH, reduced ferredoxin; Fd, ferredoxin.

DISCUSSION

On the basis of the findings reported in this paper, the pathways for glucose dissimilation by the pathogen *T. hyodysenteriae* have been determined and are depicted in Fig. 1. Glucose is converted to pyruvate via the EMP pathway, yielding 2 mol of NADH per mol of glucose metabolized. Pyruvate is broken down to equimolar amounts of acetyl-CoA, CO_2, and H_2 via a clostridium-like phosphoroclastic reaction. Acetyl-CoA is at a branch point for metabolic routes leading either to acetate or butyrate. ATP is generated by the EMP pathway (2 mol of ATP per mol of glucose), by acetate kinase (1 mol of ATP per mol of acetyl-CoA), and by butyryl-CoA transferase (1 mol of ATP per mol of acetyl-CoA used to make butyryl-CoA). NADH generated by the EMP pathway can be recycled by three alternative mechanisms. Two moles of NADH can be oxidized in a pathway yielding 1 mol of butyrate. One mole of NADH can be oxidized in a pathway involving NFOR and yielding 1 mol of H_2. Finally, NADH oxidation can be coupled to the reduction of molecular O_2, mediated by NADH oxidase. This last mechanism apparently uses 2 mol of NADH per mol of O_2, as deduced from the shift in products when cell suspensions consumed O_2 (Table 7). This stoichiometry remains to be confirmed, however, by studies with purified enzyme. Although NADH peroxidase activity was detected (Table 3), the specific activity of the enzyme was low, only 1/100 that of NADH oxidase, suggesting that this enzyme does not represent a major route for NADH oxidation in *T. hyodysenteriae*.

TABLE 7. Influence of O_2 on metabolic products of *T. hyodysenteriae* cell suspensions

Expt	Atmosphere	O_2 (μmol consumed)	Yield[a] of:			
			Acetate	Butyrate	H_2	CO_2
1	N_2		15	2.0	27	12
	N_2-O_2 (90:10)	5.1	14	<0.4	20	13
2	N_2		16	3.2	32	15
	N_2-O_2 (90:10)	6.0	14	1.2	25	16

[a] Products originated from the metabolism of both exogeneous glucose and an unidentified endogenous substrate. Data are expressed as the average yield (micromoles of product per milliliter of assay) from 2 incubation mixtures in each experiment.

The benefits of alternative mechanisms of NADH oxidation and branched routes for acetyl-CoA metabolism for butyric acid-producing clostridia have been described by Thauer et al. (34). NFOR activity in such bacteria enables NADH to be oxidized without the use of intermediates of glucose metabolism. If less acetyl-CoA is needed for the oxidation of NADH in the pathway to butyrate, more acetyl-CoA can be converted to acetate. Since twice as much ATP is produced in the pathway to acetate than in the pathway to butyrate, the clostridia derive a greater ATP yield. Inasmuch as *T. hyodysenteriae* has the same pathways for glucose metabolism, the same benefit is likely to exist for this spirochete.

Alternative pathways for NADH disposal, especially if coordinately regulated in response to environmental conditions, would likely provide *T. hyodysenteriae* with a greater metabolic efficiency (i.e., a greater ATP yield per mole of substrate) than that of intestinal bacteria lacking this versatility. Such versatility could therefore provide a selective advantage in the colonization of the gastrointestinal tract, a process whose success is thought to be influenced by intermicrobial competition for limiting nutrients (6).

The finding that *T. hyodysenteriae* cell suspensions consumed substrate amounts of O_2 (Table 7) is consistent with the observation that cells in culture take up O_2 (33). Glucose was metabolized whether or not the atmosphere over cell suspensions contained O_2 (Tables 1 and 7). Therefore, the oxygen requirement for consistent growth of *T. hyodysenteriae* under specified culture conditions (33) appears unrelated to the ability of the spirochete to metabolize glucose.

The high specific activity of NADH oxidase (760 [Table 3]) indicates that this enzyme is a major, and perhaps the sole, mechanism by which *T. hyodysenteriae* cells consume O_2. The finding that the activity is separate from the membranes of lysed cells is consistent with the results of Harris et al. (R. A. Harris, D. L. Harris, and J. M. Kinyon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D43, p. 58) and indicates that the enzyme has a cytoplasmic or periplasmic location.

The benefits to bacteria of O_2 consumption by soluble NADH oxidases have been considered to include (i) providing a means for NADH oxidation so the organism can use reduced growth substrates such as glycerol, lactate, and mannitol (3, 30); (ii) increasing ATP yield by providing an outlet for NADH oxidation that does not consume metabolic intermediates (10, 20); and (iii) eliminating O_2 from the environment as a means of protection against O_2 toxicity (22,

28). Of these, only the first can be ruled out for *T. hyodysenteriae*. None of three strains could use glycerol, lactate, or mannitol for growth under a 1% O_2 atmosphere (33).

Savage (29) speculated that an important virulence mechanism for O_2-utilizing pathogens of the gastrointestinal tract could be the ability to colonize aerated environments or to obtain O_2 from host tissues. *T. hyodysenteriae* cells colonize the O_2-respiring epithelial surfaces of the swine cecum and colon and in advanced stages of the disease induce lesions through which O_2-laden erythrocytes pass into the environment of the spirochete (8, 15). Physiological traits, such as NADH oxidase, that enable *T. hyodysenteriae* cells to take advantage of or to contend with the O_2 in their environment would likely contribute to successful colonization of intestinal mucosal surfaces.

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