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Glucose and Pyruvate Metabolism of *Spirochaeta litoralis*, an Anaerobic Marine Spirochete

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The pathways of glucose and pyruvate metabolism in *Spirochaeta litoralis*, a free-living, strictly anaerobic marine spirochete, were studied. Addition of 0.2 to 0.4 M NaCl (final concentration) to suspending buffers prevented cell lysis and was necessary for gas evolution from various substrates by cell suspensions. The organism fermented glucose mainly to ethanol, acetate, CO_2, and H_2. Determination of radioactivity in products formed from ^{14}C-labeled glucose and assays of enzymatic activities in cell extracts indicated that *S. litoralis* catabolized glucose via the Embden-Meyerhof pathway. A clostridial-type clastic reaction was utilized by the spirochete to degrade pyruvate to acetyl-coenzyme A, CO_2, and H_2. Formation of acetate from acetyl-coenzyme A was catalyzed by phosphotransacetylase and acetate kinase. Nicotinamide adenine dinucleotide-dependent acetaldehyde and alcohol dehydrogenases converted acetyl-coenzyme A to ethanol. A reversible hydrogenase activity was detected in cell extracts. *S. litoralis* cell extracts contained a rubredoxin similar in spectral properties to other bacterial rubredoxins.

Morphological studies made nearly 60 years ago by Dobell (7) and Zuelzer (29), as well as numerous recent reports on the isolation, cultivation, and physiology of free-living spirochetes (1-6, 10, 24), have demonstrated that these bacteria are ubiquitous in aquatic environments and that considerable metabolic diversity exists among them. The free-living *Leptospira* species require molecular oxygen for growth and catabolize long-chain fatty acids for energy (1, 9). The *Spirochaeta* species are saccharolytic and do not utilize amino acids, fatty acids, other organic acids, or alcohols as energy sources (2-6, 10, 11). The pigmented *S. aurantia* is the only known facultatively anaerobic species (2-4), whereas the other *Spirochaeta* species which have been isolated are strict anaerobes (6, 11).

Within the last decade, there have been a few reports on the isolation of spirochetes from marine environments (11, and references therein). One of these spirochetes, *S. litoralis*, requires both sodium and chloride ions for growth, and ferments glucose, mainly to acetate, ethanol, CO_2, and H_2 (11).

This paper is concerned with the pathways of glucose and pyruvate dissimilation by *S. litoralis* and with the general physiology of this organism. The aim of our studies was to obtain information which would help clarify the natural relationships among spirochetes.

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MATERIALS AND METHODS

Media and growth conditions. *S. litoralis* strain R1 (ATCC 27000) and *S. stenostrepta* strain Z1 (ATCC 25083) were cultivated in complex, glucose-containing media, as previously described (10, 11). Both organisms were harvested by centrifugation during the exponential growth phase, and the cells were used immediately.

Preparation of cell suspensions. Cells of *S. litoralis* become spherical and lyse upon exposure to air. Cell suspensions exhibiting a minimum of lysis and maximal physiological activity were obtained by maintaining anaerobic conditions throughout all manipulations. The appropriate buffer solution (150 ml) used to suspend cells was placed in a 250-ml Erlenmeyer flask and boiled for 5 min. The vessel was then partially submerged in crushed ice, and oxygen-free nitrogen was bubbled through the solution until the latter became cold (5 C). Finally, the contents of the flask were sealed under nitrogen. A reducing agent, Na_2S or dithiothreitol (DTT), was added (0.002 M final concentration) to the anaerobic buffer solution immediately before use.

S. litoralis cell pellets were gently suspended in four times their volume of cold (5 C) buffer, pH 7.0 to 7.5,

containing 0.25 to 0.35 M NaCl. The buffers used were: 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.05 M piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES), or 0.05 M potassium phosphate buffer. Cells were washed twice by centrifugation to remove growth medium constituents and then used immediately.

Cell extracts. *S. litoralis* cells were disrupted at 5 C by passing cell suspensions through a French pressure cell at 8,000 to 10,000 lb/in^2 (5.6×10^6 to 7×10^6 kg/m^2). The treatment resulted in complete cell breakage, as determined with a microscope. Large cellular debris was removed from the disrupted cell suspension by centrifugation (5 C, under argon) at 17,000 $\times$ *g* for 20 min. Subsequent centrifugation of the resulting crude extract at 148,000 $\times$ *g* for 2 h separated the soluble components (supernatant liquid) from the particulate components (pellet). In manometric experiments, the crude suspension of disrupted cells was used directly without centrifugation to minimize the loss of enzymatic activity. All extracts were used immediately after preparation, since enzymatic activities declined rapidly regardless of the method of storage (e.g., −25 C, under argon or other gases).

The method of Stadtman et al. (21) was employed to deplete crude extracts of endogenous coenzyme A (CoA).

Extracts were depleted of rubredoxin by passing them through chromatographic columns of diethylaminoethyl (DEAE)-cellulose (10).

Cell extracts of *S. stenostrepta* were prepared as described previously (10).

Isolation of rubredoxin. Rubredoxin preparations were obtained from *S. litoralis* cell extracts as described by Hespell and Canale-Parola (10). For further purification the rubredoxin preparations were placed on DEAE-cellulose columns (2 by 45 cm), and the rubredoxin was eluted with 0.35 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer solution (pH 7.5) at a flow rate of 15 ml/h until no visible red protein remained on the column. The eluate was then dialyzed against distilled water and finally lyophilyzed to dryness.

Hydrolysis and amino acid analysis of rubredoxin. A 75-μg sample of purified rubredoxin was hydrolyzed in 6.0 N HCl for 12 h at 110 C in an argon atmosphere. Amino acid analysis of the residue was carried out with a model 120 C Beckman amino acid analyzer.

Manometry. Double side-arm Warburg manometer flasks were employed in studies of gaseous and nongaseous products formed from various substrates by whole cells or by cell extracts. Unless otherwise indicated, all experiments were conducted at 30 C with either an argon or nitrogen atmosphere in the flask. Standard manometric techniques were employed (23).

Determination of enzymatic activities. Phosphotransacetylase (EC 2.3.1.8), acetate kinase (EC 2.7.2.1), alcohol dehydrogenase (EC 1.1.1.1), acetaldehyde dehydrogenase (EC 1.2.1.10), and hydrogenase activities were assayed as described previously (10). Acetyl phosphate and acetyl CoA were measured by standard procedures (17).

Glycolytic enzyme activities of cell extracts were assayed by coupling the reactions to pyridine nucleotide oxidation or reduction as described previously (10). Enzyme activity was determined by the rates of absorbancy changes at 340 nm. Specific activity was defined as nanomoles of pyridine nucleotide change per minute per milligram of cell extract protein (10).

Other experimental procedures. Other experimental techniques employed in this study were similar to those used in our previous studies with *Treponema denticola* (12) and free-living anaerobic spirochetes (2, 3, 10, 11). These procedures included determinations of cell and protein concentrations (10), analysis of fermentation products (2, 10, 11), exchange experiments (3, 10), hydrogenase assays (3, 10) and radioactivity determinations (10). All biochemicals and enzymes used were of the highest commercial grade available.

RESULTS

Effects of salts and buffers. Cells of *S. litoralis* suspended in 0.1 M HEPES, 0.1 M PIPES, 0.1 M triethanolamine, or 0.1 M potassium phosphate buffer solutions (pH 6.5 to 7.5) containing 0.3 M NaCl and 0.002 M Na_2S or DTT exhibited virtually no lysis or loss of motility after two washings in the suspending liquid. In contrast, rapid lysis of more than 50% of the cells and complete loss of motility occurred when cells were suspended under similar conditions in solutions including Tris or potassium citrate as the buffering compound. The addition of salts, such as $MgCl_2$ or $CaCl_2$ (0.001 M final concentration), to either of the latter buffer solutions did not decrease the extent of lysis, and the addition of a similar concentration of ethylenediaminetetraacetate resulted in more extensive lysis.

When suspensions of non-proliferating cells were incubated with glucose, the amount of gas evolved varied depending upon the concentration of NaCl present (Fig. 1). When NaCl was not added to the cell suspensions, little or no gas was evolved. At concentrations below 0.2 M or above 0.35 M NaCl, gas evolution was substantially decreased as compared to the optimal gas evolution observed at approximately 0.3 M NaCl. Although the decrease in gas evolution at concentrations lower than 0.2 M NaCl may be ascribed to cell lysis (35% at 0.15 M NaCl), inhibition of gas evolution at NaCl concentrations greater than 0.35 M may not, since no cell lysis occurred (as observed with a microscope).

The NaCl requirement for gas evolution by whole cells was partially replaceable by equimolar concentrations of $LiCl_2$ or $MgCl_2$ (Table 1). However, neither of these compounds, nor any of the other salts tested (Table 1), replaced the NaCl requirement for growth of the organism (11).

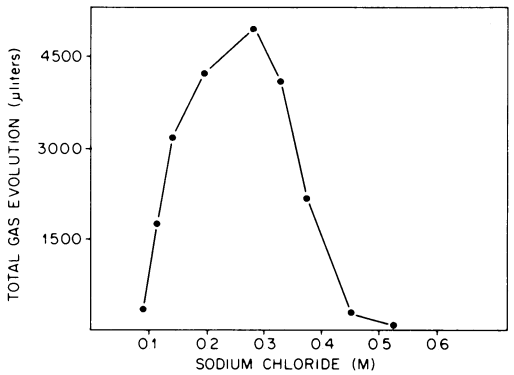

FIG. 1. *Effect of NaCl upon gas evolution from glucose by Spirochaeta litoralis cell suspensions. The following additions were made to each Warburg flask (micromoles): glucose, 100; DTT, 10; potassium phosphate buffer (pH 7.5), 200; NaCl, as indicated; and 9 × 10¹⁰ cells in a final volume of 3.2 ml. Incubation time at 30 C under argon was 2 h. The flask constant used was calculated for CO_2.*

TABLE 1. *Effects of salts on gas evolution from glucose by S. litoralis cell suspensions*[a]

Salt added	Gas evolution (μliters)[b]
None[c]	775
NaCl	3,268
LiCl	2,358
$MgCl_2$	2,268
KCl	640
NaBr	268
KBr	377
NaI	436
KI	497
$NaNO_3$	336
Sodium citrate	762
Potassium citrate	279

[a] The following additions were made to each Warburg vessel (micromoles): glucose, 100; DTT, 10; HEPES buffer (pH 7.5), 100; salt under test, 700; and 1.5 × 10¹¹ cells (in 0.5 ml of 0.1 M HEPES-0.2 M NaCl solution) in a final volume of 3.2 ml. Incubation was at 30 C under argon for 60 min.

[b] The flask constant used was calculated for CO_2.

[c] NaCl was present at 0.031 M final concentration, via NaCl in the cell suspension used.

Fermentation of glucose. When cell suspensions were incubated with glucose-*1*-^{14}C, the majority of the radioactivity present in the products resided in C2 of both acetate and ethanol (Table 2). A small amount of radioactivity was also present in CO_2 and cells. Data obtained from other experiments in which cell suspensions were incubated with glucose-*1*-^{14}C, glucose-*6*-^{14}C, or glucose-*U*-^{14}C showed that the radioactivity in CO_2 derived from glucose-*1*-^{14}C or glucose-*6*-^{14}C was approximately equal and was less than 5% of the radioactivity in CO_2 derived from glucose-*U*-^{14}C.

Glycolytic enzymes. Cell extracts of *S. litoralis* contained enzymes associated with the Embden-Meyerhof pathway (Table 3). The phosphofructokinase activity was maximal in assay mixtures containing 2 × 10⁻⁴ M adenosine triphosphate (ATP), whereas at 5 × 10⁻³ M ATP a 90% inhibition of activity resulted. The inhibition by iodoacetate (90% at 10⁻³ M) and other properties of the glyceraldehydephosphate dehydrogenase were similar to those observed for this enzyme in *S. stenostrepta* (10). In the absence of added substrates, reduced nicotinamide dinucleotide (NADH) and reduced nicotinamide dinucleotide phosphate (NADPH) oxidizing activities were not detected. Although gluconate does not support growth of *S. litoralis* (11), low levels of phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase activities were present in cell extracts (Table 3).

Pyruvate metabolism. Cells and extracts of *S. litoralis* evolved CO_2 and H_2 from pyruvate, but not from lactate or formate. Whole cells or cell extracts of *S. litoralis* catalyzed a CO_2-pyruvate exchange reaction (Table 4). However, no formate-pyruvate exchange reaction was detected under the same conditions. In agreement

TABLE 2. *Fermentation of glucose-1-^{14}C by cell suspensions of S. litoralis*[a]

Products	Amount of products[b]	Distribution of label[c]
CO_2	201.8	22.9
H_2	74.4	
Ethyl alcohol	140.5	1,035.0
Carbon 1		12.3
Carbon 2		845.0
Acetate	57.0	766.0
Carbon 1		11.5
Carbon 2		694.0
Lactate	0.2	
Formate	Trace	
Pyruvate	Trace	

[a] Added per Warburg vessel (micromoles): glucose-*1*-^{14}C, 100; HEPES buffer (pH 7.5), 200; NaCl, 1,000; DTT, 20; and 1.7 × 10¹¹ cells in final volume of 3.2 ml. Incubation time at 30 C under argon was 2 h.

[b] Expressed as micromoles of product formed per 100 μmol of glucose fermented; carbon recovery: 100.7%, including 1.1% glucose carbon incorporated into cell material; oxidation/reduction balance: 1.14.

[c] Expressed as counts per minute per micromole of product. Specific activity of glucose-*1*-^{14}C was 2,488 counts per min per μmol.

TABLE 3. *Enzyme activities in cell extracts of S. litoralis*

Enzyme[a]	Sp act[b]
Hexokinase (EC 2.7.1.1)	30
Glucosephosphate isomerase (EC 5.3.1.9)	1,500
Phosphofructokinase (EC 2.7.1.11)	130
Fructosediphosphate aldolase (EC 4.1.2.13)	250
Triosephosphate isomerase (EC 5.3.1.1)	2,860
Glyceraldehydephosphate dehydrogenase (EC 1.2.1.12)	680
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	6
Phosphogluconate dehydrogenase (EC 1.1.1.43)	9

[a] Not detected: gluconokinase (EC 2.7.1.12), glycerol kinase (EC 2.7.1.30), and glycerolphosphate dehydrogenase (EC 1.1.99.5).

[b] Expressed as nanomoles of pyridine nucleotide change per minute per milligram of cell extract protein.

TABLE 4. *CO_2-pyruvate and HCOOH-pyruvate exchange by whole cells and extracts of S. litoralis*[a]

Exchange	pH	Sp act of residual pyruvate[b]	
		Whole cells	Cell extracts
CO_2-pyruvate	7.0	33.3	169.5
CO_2-pyruvate	8.4	28.4	158.0
HCOOH-pyruvate	7.0	0.9	1.8
HCOOH-pyruvate	8.4	0.8	1.1

[a] Specific activity (counts per minute per micromole): $NaH^{14}CO_3$, 425; $Na^{14}COOH$, 410; 9×10^{10} cells (in 0.5 ml of 0.1 M HEPES-0.2 M NaCl) or 2.5 mg of cell extract protein were added per Warburg vessel. Incubation time at 30 C was 20 min.

[b] Counts per minute per micromole of residual pyruvate.

with observations on the CO_2-pyruvate exchange activity in other bacteria (10, 13, 25, 27), no exchange activity could be demonstrated in *S. litoralis* extracts treated with Dowex resin to remove endogenous CoA. Exchange activity was restored when exogenous CoA was added to CoA-depleted extracts (Fig. 2). Optimal exchange activity occurred at CoA concentrations of 2×10^{-6} to 3×10^{-6} M; higher concentrations were inhibitory (Fig. 2).

Cell extracts catalyzed the formation of acetyl phosphate from pyruvate. Acetyl phosphate formation from pyruvate was stimulated by thiamine pyrophosphate, $MnCl_2$ (or $MgCl_2$), and DTT (or L-cysteine) when these compounds were added individually to extracts to give a final concentration of 2×10^{-3} M. Little or no acetyl phosphate was detected unless catalytic amounts of both CoA and either methyl viologen or benzyl viologen were added to the reaction mixtures (Table 5). The viologen dyes could not be replaced by NAD, NADP, or methylene blue. Acetate kinase and phosphotransacetylase activities detected in cell extracts catalyzed reversible reactions. In the direction of acetate formation, maximal acetate

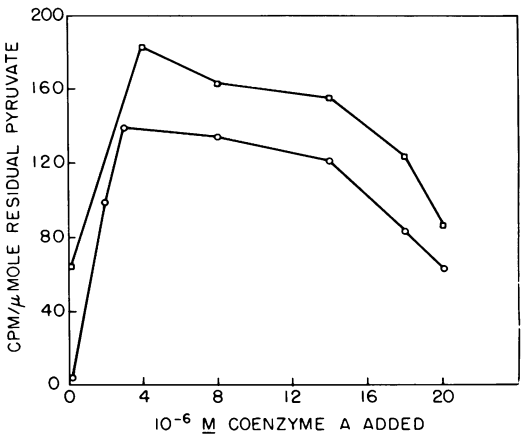

FIG. 2. *Effects of CoA on the CO_2-pyruvate exchange with Spirochaeta litoralis extracts. The assay mixture included either 8 mg of crude cell extract protein (upper curve) or 6 mg of CoA-depleted cell extract protein (lower curve). Specific activity of $NaH^{14}CO_3$ was 425 counts per min per μmol. Symbols: □, crude extract; ○, CoA-depleted extract.*

TABLE 5. *Effects of CoA and methyl viologen on acetyl phosphate formation from pyruvate by S. litoralis cell extracts*[a]

Concn of added methyl viologen or CoA ($M \times 10^{-6}$)	Acetyl phosphate formed (μmol)	
	Methyl viologen[b]	CoA[c]
None	0.35	0.25
5	1.95	0.75
10	2.65	1.45
25	3.25	2.35
40	6.65	2.65
50	6.85	3.10
150	8.30	4.50

[a] The assay mixture contained (micromoles): potassium phosphate buffer (pH 6.8), 100; HEPES buffer, 20; DTT, 10; thiamine pyrophosphate, 5; $MnCl_2$, 5; potassium pyruvate, 50; CoA and methyl viologen, as indicated; and 7.5 mg of cell extract protein in a final volume of 2.0 ml. Incubation was at 30 C for 30 min, under argon.

[b] 2.5×10^{-5} M CoA added to all reaction mixtures.

[c] 2.5×10^{-5} M methyl viologen added to all reaction mixtures.

kinase activity was observed in the presence of adenosine monophosphate (5×10^{-3} M) rather than adenosine diphosphate (ADP) (5×10^{-3} M) as a substrate; both ATP (5×10^{-3} M) and acetate (3×10^{-2} M) were inhibitory.

Formation of ethanol. *S. litoralis* extracts catalyzed ethanol formation and a rapid oxidation of NADH in the presence of pyruvate, acetyl-CoA, acetyl phosphate, or acetaldehyde as substrates. In addition, reduction of NAD occurred when cell extracts were incubated with ethanol. These observations indicated that *S. litoralis* converts acetyl-CoA to ethanol via acetaldehyde and alcohol dehydrogenase activities (EC 1.2.1.10 and EC 1.1.1.1, respectively).

Hydrogenase. Cell extracts of either *S. litoralis* or *S. stenostrepta* evolved hydrogen gas from hydrosulfite-reduced methyl viologen, but not from hydrosulfite-reduced benzyl viologen or methylene blue. The hydrogenases of both organisms did not appear to be membrane-bound and were reversible inasmuch as cell extracts catalyzed an uptake of hydrogen gas when incubated with dye (Table 6). The *S. litoralis* hydrogenase differed from the *S. stenostrepta* hydrogenase in that it did not utilize methylene blue as an electron acceptor (Table 6).

Rubredoxin. When an *S. litoralis* extract was passed through a DEAE-cellulose chromatographic column, a reddish-brown band formed at the top of the column. The reddish-brown material was isolated. It consisted in part of a red protein which exhibited absorption maxima in the 275-, 370- to 375-, and 495- to 500-nm regions (Fig. 3). Reduction of the red protein by sodium borohydride resulted in loss of color and in an altered spectrum (Fig. 3). Analysis of the amino acid content of the red protein showed an abundance of the acidic amino acids aspartic and glutamic, and the absence of histidine and arginine. The spectral properties and amino acid composition of the red protein from *S. litoralis* were remarkably similar to those of rubredoxins isolated from other bacteria (14, 20). It was concluded that the red protein from *S. litoralis* was a rubredoxin.

TABLE 6. *Effects of electron-accepting compounds on H_2 uptake by cell extracts of S. litoralis and S. stenostrepta*[a]

Compound added	H_2 uptake (μmol)	
	S. litoralis	*S. stenostrepta*
Methylene blue	1.2	31.2
Benzyl viologen	27.1	18.2
Methyl viologen	46.3	11.2
Nicotinamide adenine dinucleotide	1.7	4.5
Triphenyltetrazolium chloride	11.6	19.0
None	0	0

[a] Each Warburg vessel contained (micromoles): HEPES buffer (pH 7.0), 100; DTT, 10; NaCl, 1,000; compound under test, 30; and 12 mg (*S. litoralis*) or 20 mg (*S. stenostrepta*) of cell extract protein in a final volume of 3.2 ml. Incubation was at 30 C under H_2 for 20 min.

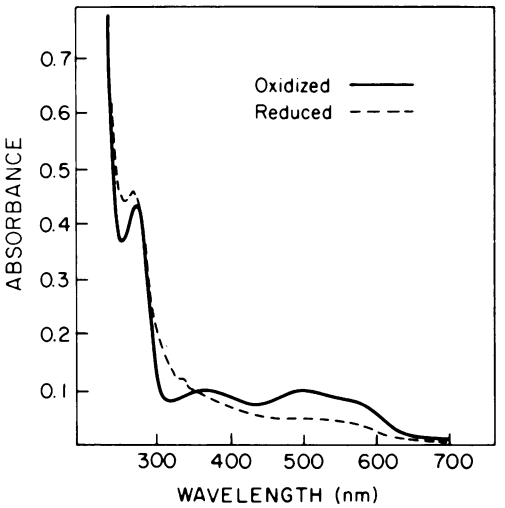

FIG. 3. *Absorption spectra of rubredoxin isolated from Spirochaeta litoralis. The oxidized spectrum (——) of 0.5 mg of purified rubredoxin in 3.0 ml of 0.05 M Tris (pH 7.5) was determined after bubbling air through the solution and the reduced spectrum (-----) after the addition of 1.0 mg of $NaBH_4$ to the solution.*

DISCUSSION

Studies with marine bacteria have shown that high concentrations (0.05 to 0.4 M) of various ions, particularly sodium ions, are necessary for maximal rates of oxidation of substrates by whole cells (19), for activity of certain enzymes (22), for the transport of molecules into cells (8, 28), or to prevent lysis of cells (18). Presumably, *S. litoralis* shares some of these characteristics of marine bacteria. Unlike freshwater *Spirochaeta* species which have been cultivated, *S. litoralis* requires relatively high concentrations (0.2 to 0.4 M) of sodium chloride for growth (11), for maintenance of cell integrity, and probably for the metabolism of substrates by whole cells.

The labeling patterns in products formed by cell suspensions incubated with specifically labeled glucose were consistent with patterns observed with organisms utilizing the Embden-

Meyerhof pathway for glucose catabolism. The presence in cell extracts of enzyme activities associated with the Embden-Meyerhof pathway supports the conclusion that this pathway is the primary route of glucose catabolism in *S. litoralis*. Low levels of both phosphogluconate and glucose-6-phosphate dehydrogenase activities were detected in the cell extracts. This suggests that the organism may use these enzymatic activities to metabolize some of the glucose carbon needed for biosynthetic purposes.

The CoA-dependent formation of acetyl phosphate from pyruvate and the CoA-dependent CO_2-pyruvate exchange activity catalyzed by cell extracts clearly indicate that *S. litoralis* possesses a clostridial-type pyruvate clastic system analogous to that present in other bacteria (13, 15, 16, 25–27).

The dependency in cell extracts of acetyl phosphate formation from pyruvate upon artificial electron-accepting compounds suggests that the electron transport system associated with pyruvate degradation is quite labile. Presumably, in living cells, electrons derived from pyruvate oxidation participate in the formation of hydrogen gas via the hydrogenase system and possibly in the reduction of pyridine nucleotides.

Acetyl-CoA derived from pyruvate cleavage and ADP are converted to acetate and ATP in reactions catalyzed by phosphotransacetylase and acetate kinase. Furthermore, ethanol and CoA are formed from acetyl-CoA via reductions involving acetaldehyde and alcohol dehydrogenases.

As pointed out in a previous publication (11), broken-cell preparations and cell-free extracts of *S. litoralis* are relatively devoid of CoA. Thus, a 3-fold and 18-fold stimulation of CO_2-pyruvate exchange and phosphotransacetylase activities, respectively, were observed when catalytic amounts of CoA were added. Previous studies established that optimal growth of *S. litoralis* occurs in CoA-supplemented media (11). Although pantothenate partially replaces CoA as a growth factor, low cell yields are obtained with CoA-free pantothenate-supplemented media. These data suggest that the biosynthesis of CoA is impaired in *S. litoralis*. The nature of this biosynthetic deficiency remains to be elucidated.

The genus *Spirochaeta* comprises free-living forms which are thought to be phylogenetically distant from spirochetes such as *Leptospira*, *Treponema*, and *Borrelia* species (6). The current study, as well as other studies (2–6, 10, 11), has shown that those *Spirochaeta* which have been investigated possess certain fundamental metabolic characteristics in common. These organisms are exclusively saccharolytic and ferment carbohydrates via the Embden-Meyerhof pathway forming CO_2, H_2, acetate, and ethanol as major products (2–6, 10). Furthermore, they utilize a clostridial-type clastic system for pyruvate degradation, and synthesize the non-heme iron protein rubredoxin (3, 10, 14).

On the other hand, metabolic diversity exists among *Spirochaeta* species. For example, *S. aurantia*, which possesses anaerobic dissimilatory pathways similar to those of strictly anaerobic *Spirochaeta* species (2, 3), has developed the ability to grow aerobically and may obtain energy via oxidative phosphorylation mechanisms (4). *S. litoralis* grows in marine environments and has specific requirements for sodium and chloride ions. Finally, it should be pointed out that *S. zuelzerae* (6, 24) and *Spirochaeta* sp. strain Z-4 (6), whose pathways of carbohydrate fermentation have not been investigated, form dissimilatory products, such as succinate and lactate, which are not produced by or are minor products of other *Spirochaeta* species.

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