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Physiology and Evolution of Spirochetes

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INTRODUCTION

Since the discovery of the microbial world, morphological characteristics frequently have been assigned primary significance in the interpretation of "natural" or phylogenetic relationships among microorganisms. Thus, attempts to construct classifications based on phylogeny, or to define microbial groups or taxa, have relied heavily upon morphological criteria.

The group of bacteria called spirochetes (order *Spirochaetales*) may be considered an example of a taxon that was proposed as a result of the tendency to attribute phylogenetic or taxonomic value to morphological features. According to modern concepts, spirochetes are helically shaped, heterotrophic bacteria (33). The outermost structure of the spirochetal cell is a thin, three-layered membrane called "outer sheath" or "outer cell envelope" (Fig. 1), possibly corresponding to the "outer membrane" of gram-negative bacteria. This outer sheath completely surrounds the body of the cell or protoplasmic cylinder, which consists of the cytoplasmic and nuclear regions enveloped by the cell membrane and the cell wall. The helical protoplasmic cylinder is wound together with a number of filamentous structures, called "axial fibrils" or "axial filaments," and both the protoplasmic cylinder and the axial fibrils are enclosed by the outer sheath (Fig. 1). The number of axial fibrils present per cell ranges from 2 to more than 100, depending on the kind of spirochete. One end of each axial fibril is inserted near one pole of the protoplasmic cylinder, whereas the other end is not inserted. Since individual axial fibrils extend for most of the length of the cell, axial fibrils inserted at opposite poles of the protoplasmic cylinder overlap in the central region of the cell.

In accordance with present taxonomic criteria, all bacteria that possess the morphological features described above are spirochetes, or members of the order *Spirochaetales* (33). Probably as a result of their unique cellular architecture, spirochetes possess a type of movement not observed in other bacteria. Thus, the spirochetal cell, which has no exoflagella, can locomote or swim in liquid environments without being in contact with solid surfaces. Furthermore, spirochetes locomote by "creeping" or "crawling" on solid surfaces (23, 45). Because of their ultrastructural and chemical resemblances to bacterial flagella, it has been suggested that axial fibrils play a role in the motility of spirochetes (26).

Although spirochetes have certain basic morphological characteristics in common, they exhibit extreme phenotypic diversity. For example, they vary greatly in size, ranging from very small cells (e.g., 0.1 by 6 μm) to large forms 0.75 to 3 μm thick and 100 μm in length or longer (33). Moreover, pronounced physiological differences exist among spirochetes. Thus, aerobic, facultatively anaerobic, as well as obligately anaerobic, species have been described. Almost total specialization in energy-yielding mechanisms has been achieved by some spiro-

FIG. 1. *Schematic representation of a spirochete. The broken line indicates the outer sheath (outer cell envelope). The area delimited by the thick solid line, adjacent to the broken line, represents the protoplasmic cylinder. The circles near the ends of the protoplasmic cylinder indicate the insertion points of the axial fibrils. The solid thin lines, wound around the protoplasmic cylinder, are the axial fibrils.*

chetes, which are limited to dissimilating only certain long-chain fatty acids and a few long-chain alcohols (98). Other spirochetes derive energy exclusively by fermenting sugars (37, 38, 77, 78, 80), whereas others possess remarkable metabolic versatility, being able to catabolize a variety of amino acids and carbohydrates (79; E. Canale-Parola and R. P. Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, K52, p. 155). Furthermore, spirochetes are present in a wide spectrum of natural habitats. Many occur, grow, and persist as free-living forms in bodies of marine and fresh water and in mud (33, 36). Others are part of the normal microflora present on or in eucaryotic hosts. Many of these host-associated spirochetes have become adapted to life within specialized habitats, such as the crystalline style of molluscs (26), the gingival crevice of humans (26), the colon of mammals, where they are attached to epithelial cells (127, 164), or the body surface of protozoa (24, 26, 42, 153). A relatively small number of host-associated spirochetes have the property of pathogenicity and are causative agents of diseases, such as relapsing fever, leptospirosis, syphilis, and other treponematoses (47).

The multifarious phenotypic manifestations of spirochetes reflect marked differences in the genotypes of these bacteria. In fact, it has been found that spirochetes differ greatly in deoxyribonucleic acid base composition, with guanine plus cytosine contents ranging from 36 to 66 mol% (33). Physiological differences, as well as ecological, morphological, and other considerations, have been used in classifying the spirochetes into five genera: *Spirochaeta, Cristispira, Treponema, Borrelia,* and *Leptospira* (33). Table 1, included for the purpose of clarifying the nomenclature used in this article, summarizes some of the characteristics of these five genera.

My main objective in writing this review article is to examine and discuss recent literature on the physiology of spirochetes, although less recent publications pertinent to the presentation are also considered. Also, I shall review and analyze published data that seem to have significance with regard to the evolutionary history of spirochetes. My ultimate intention is to stimulate the interest of microbiologists in this diverse and intriguing group of bacteria.

METABOLISM OF SPIROCHETES

Cultivation of Spirochetes

There have been relatively few extensive studies on the metabolism of spirochetes. This lack of effort may be ascribed to the fact that for many years after their discovery (50), spirochetes have been an ill-defined group of microorganisms, attracting the attention of a limited number of microbial physiologists. Furthermore, many spirochetes, especially many of the anaerobic host-associated forms, are not readily mass cultured inasmuch as they are nutritionally fastidious. Generally, the cultivable anaerobic host-associated spirochetes are grown in complex media supplemented with blood serum, serum components, ascitic fluid, or rumen fluid. Chemically defined growth media, some of which contain over 50 medium components, have been described for a few host-associated anaerobic spirochetes (151, 152).

Growth yields of spirochetes vary considerably. The oral spirochete *Treponema denticola* grows in complex media to a density of 5×10^8 cells per ml and has doubling times of 12 to 14 h (79). *T. vincentii,* another oral spirochete, has been grown to a density of 5×10^7 cells per ml in media containing ascitic fluid (129). Among the pathogens, the leptospires reach densities of 10^8 to 4×10^8 cells per ml in media to which serum or an albumin-fatty acid supplement has been added (6). Cultivation techniques by which high yields of virulent treponemes or borreliae may be obtained have not been developed. A culture medium suitable for the growth of several species of relapsing fever borreliae has been described (106, 137), but this medium supports maximum cell yields of only 5×10^7 cells per ml after 7 days of incubation (106). Similar or somewhat higher yields are obtained when *T. hyodysenteriae,* the primary etiological agent of swine dysentery (85), is grown in broth cultures (110). Various other virulent treponemes and borreliae have not been grown in pure culture in vitro. Among these is *T. palli-*

TABLE 1. *Summary of spirochete classification and nomenclature*[a]

Classification	Characteristics
Spirochaeta	Free-living in aquatic environments. The genus includes three obligately anaerobic species (*S. stenostrepta, S. zuelzerae, S. litoralis*), one facultatively anaerobic species (*S. aurantia*), and a species (*S. plicatilis*) that has not been isolated. A red-pigmented halophilic, facultative anaerobe, referred to as spirochete RS1 (67), is a member of this genus and the name *S. halophila* RS1 has been proposed for it (Greenberg and Canale-Parola, in press). Spirochete RS1 was isolated from a solar lake and requires 0.75 M NaCl, 0.2 M $MgSO_4$, and 0.01 M $CaCl_2$ for optimum growth. Species of *Spirochaeta* have a G + C content of DNA ranging from 50 to 66 mol% (buoyant density) (33).
Cristispira	Large spirochetes usually found in the digestive tract of many marine and freshwater molluscs (33). Not grown in pure culture. See review by Breznak (26).
Treponema	Present in the mouth, intestinal tract, and genital areas of humans and animals. Many are members of the normal microflora of the healthy human and animal body. Some species (e.g., *T. pallidum, T. pertenue, T. carateum*) are pathogenic. Species that have been cultivated in pure culture in vitro are obligate anaerobes. Certain nonvirulent treponemes reportedly isolated from syphilitic lesions or other pathological processes are known by the trivial names of Reiter treponeme, Kazan treponeme, Nichols nonpathogenic treponeme, and Noguchi treponeme (174). In *Bergey's Manual* (33) the Reiter and Kazan treponemes are designated *T. phagedenis*, whereas the Nichols nonpathogenic and the Noguchi treponemes are designated *T. refringens*. Some workers (71) do not believe that the available evidence is sufficient to justify the use of the name *T. phagedenis* for the Reiter treponeme. In this review I refer to the four above-named treponemes by their trivial names. Species of *Treponema* have a G + C content of the DNA ranging from 37 to 46 mol% (33, 38).
Borrelia	Cause relapsing fever in humans and similar diseases in animals (107, 151). Transmitted by lice or ticks. Anaerobic (33) or microaerophilic (107).
Leptospira	Small, obligately aerobic spirochetes, generally with one or both ends of the cells bent or hooked. Found free-living in surface waters or soil and in association with animals and humans (33, 76, 166). Host-associated leptospires can cause disease (leptospirosis) in humans and other mammals. The G + C content of the DNA is 36 to 39 mol% (33).

[a] See *Bergey's Manual* (33) for detailed classification and for micrographs illustrating the overall morphology and size of representative spirochetes. G + C, guanine plus cytosine; DNA, deoxyribonucleic acid.

dum, the causative agent of syphilis, which is generally maintained in a reproducing state by inoculation in living animals, i.e., rabbits. Recently, a report has been published describing the cultivation of virulent *T. pallidum* in cultures containing baby hamster kidney tissue cells (99). In vitro cultivation has not been achieved with certain relapsing fever borreliae (70), such as *Borrelia duttonii*, which has been grown in experimental animals by investigators who studied its metabolism (60). Many spirochetes indigenous to healthy humans or animals have never been cultivated. These include various spirochetes that are inhabitants of the gut of termites, the crystalline style of molluscs, and the intestine of humans.

In contrast to the host-associated forms, free-living spirochetes grow abundantly in serum-free, readily prepared media, reaching high cell densities which, in some cases, approach 10^{10} cells per ml (36, 77). Thus, these organisms serve as useful tools in investigations for which large amounts of cell material are desirable. As a consequence, much of our present knowledge on the metabolism of spirochetes has been acquired through the study of free-living forms.

Various spirochetes have been isolated from natural habitats using selective enrichment methods. Such methods, which have been described in detail elsewhere (36), are based on the ability of spirochetes to pass through filters of small pore diameter (0.2 to 0.45 μm, average diameter) and on the property of spirochetes to migrate more readily than many other bacteria through agar gels. These enrichment techniques have been used successfully for the isolation of thin spirochetes (0.5 μm or less in diameter). Selective isolation methods for spirochetes of larger cell diameter have not been developed.

Anaerobic Energy-Yielding Metabolism

All strains of anaerobic and facultatively anaerobic free-living spirochetes that have been

isolated are carbohydrate fermenters. None has been shown to ferment amino acids, but it is possible that such strains will be obtained by using appropriate selective enrichment techniques.

Under anaerobic conditions, all fermentative free-living spirochetes tested form acetate, CO_2, and H_2 as major products of carbohydrate dissimilation (Table 2). Ethyl alcohol is another major product, except that one species (*Spirochaeta zuelzerae*) does not produce it and spirochete Z4 only forms small amounts of it (Table 2). The latter two spirochetes maintain charge balance not only by producing H_2, but also by reducing greater amounts of pyruvate to lactate than those spirochetes that form ethyl alcohol as a major product. Furthermore, both *S. zuelzerae* and spirochete Z4 produce succinate (Table 2).

The pathways of carbohydrate fermentation of *S. aurantia*, *S. litoralis*, and *S. stenostrepta* have been investigated by me and my co-workers (27, 28, 37, 38, 77, 78, 80, 91). Assays of enzymatic activities in cell extracts and determinations of radioactivity distribution in products formed from ^{14}C-labeled substrates showed that these spirochetes ferment glucose via the Embden-Meyerhof (EM) pathway. Cells or extracts of *S. aurantia* and *S. stenostrepta* ferment pyruvate to the same major products formed from glucose, except that acetoin is produced by cell suspensions or cell extracts of both spirochetes and diacetyl is produced by *S. aurantia* extracts. Whole cells or cell extracts of *S. aurantia*, *S. litoralis*, and *S. stenostrepta* exhibit a coenzyme A-dependent CO_2-pyruvate exchange. No formate-pyruvate exchange was detected in whole cells or extracts, and the data showed that free formate is not involved in CO_2 and H_2 production. It was concluded that the three organisms utilize a clostridial-type clastic system to metabolize pyruvate to acetyl-coenzyme A, CO_2, and H_2 (Fig. 2). Enzymatic assays of cell extracts showed that acetyl-coenzyme A is converted to ethyl alcohol by nicotinamide adenine dinucleotide-dependent acetaldehyde and alcohol dehydrogenase activities (EC 1.2.1.10 and EC 1.1.1.1, respectively). Furthermore, phosphotransacetylase (EC 2.3.1.8), acetate kinase (EC 2.7.2.1), lactate dehydrogenase (EC 1.1.1.27), and hydrogenase activities were

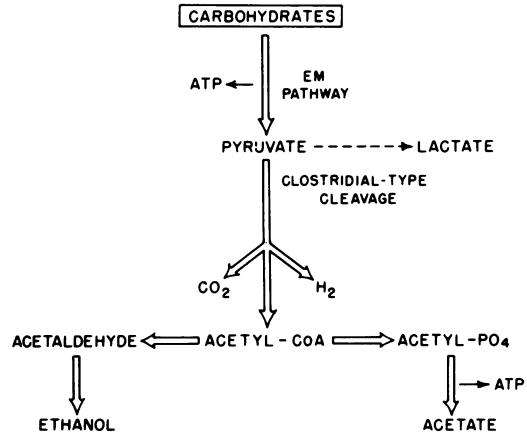

FIG. 2. *Pathways for anaerobic dissimilation of carbohydrates by free-living spirochetes. The broken arrow indicates a minor pathway.*

TABLE 2. *Fermentation products of free-living spirochetes*

Products[b]	Amt of products (μmol/100 μmol of glucose fermented)[a]								
	S. aurantia		*S. litoralis*		*S. stenostrepta*		*S. zuelzerae* (1)	Spirochete Z4 (1)	Spirochete RS1 (1)
	1	2	1	2	1	2			
Acetate	69.2	50.3	37.5	57.0	93	20.4	82	94.8	52.4
Ethyl alcohol	151.0	78.4	109.5	140.5	84	146.2	ND	10.5	132.0
CO_2	165.3	128.2	127.5	201.8	140	187.5	68	72.7	176.1
H_2	107.2	79.5	74.0	74.4	180	27.2	164	186.9	130.3
Lactate	1.0	17.2	6.5	Trace	10	8.2	87	56.8	1.8
Formate	5.2	NR	2.8	Trace	Trace	NR	ND	10.7	ND
Pyruvate	NR	3.1	0.3	Trace	NR	NR	NR	NR	ND
Succinate	ND	NR	ND	ND	ND	ND	13	26.3	ND
Glycerol	NR	4.4	NR	NR	NR	NR	ND	NR	NR
Acetoin, diacetyl	Trace	NR	ND	ND	ND	NR	ND	ND	ND

[a] 1, Products of growing cells; 2, products of cell suspensions. ND, Not detected; NR, not reported.

[b] Data for *S. aurantia* are from Breznak and Canale-Parola (27, 28), for *S. litoralis* from Hespell and Canale-Parola (78), for *S. stenostrepta* from Canale-Parola et al. (37) and Hespell and Canale-Parola (77), for *S. zuelzerae* from Veldkamp (171), for spirochete Z4 from Canale-Parola et al. (38), and for spirochete RS1 from Greenberg and Canale-Parola (in press).

detected in cell extracts of all three species. The data indicated that in *S. aurantia, S. litoralis,* and *S. stenostrepta* the acetyl-coenzyme A formed via the pyruvate clastic system is converted to acetate in reactions catalyzed by phosphotransacetylase and acetate kinase and to ethanol through a double reduction involving aldehyde and alcohol dehydrogenase activities (Fig. 2). A small proportion of pyruvate is not cleaved but is reduced to lactate (Fig. 2). The proposed pathways (Fig. 2) indicate that in addition to the net yield of 2 mol of adenosine triphosphate (ATP) per mol of glucose metabolized via the EM pathway, the spirochetes derive additional ATP through reactions leading to acetate formation from pyruvate.

Anaerobic molar growth yield determinations were conducted with spirochete RS1, which has fermentation pathways indistinguishable from those of *S. aurantia, S. litoralis,* and *S. stenostrepta* (E. P. Greenberg and E. Canale-Parola, Arch. Microbiol., in press). Results of these studies indicated that the ATP yield predicted by the proposed pathways (Fig. 2) would support anaerobic molar growth yields of the magnitude observed. This finding, as well as studies with the three species of *Spirochaeta* previously discussed (28, 77, 80), indicate that the pathways outlined in Fig. 2 constitute the major anaerobic energy-yielding mechanisms utilized by these free-living spirochetes.

In nature, obligately anaerobic species of *Spirochaeta* commonly occur in sulfide-containing mud or water. Thus, it was thought possible that these spirochetes carried out anaerobic respiration, using sulfate as the electron acceptor. However, experiments conducted with *S. stenostrepta* (77) showed that H_2S was not evolved by this spirochete growing in various media to which were added ammonium sulfate and possible electron donors such as pyruvate, lactate, malate, or glucose. A small amount of H_2S was evolved when the organism was cultured in media including sodium thioglycolate, but the gas probably originated from the sulfhydryl group of this compound.

Iron-sulfur proteins, such as rubredoxins and ferredoxins, participate in the metabolic activities of many anaerobic bacteria. Ferredoxins function as electron carriers in various oxidation-reduction reactions (32). Rubredoxins of anaerobic bacteria are known to participate in electron transfer (119), but the specific physiological role(s) of these proteins has not been found. A rubredoxin isolated from the aerobic *Pseudomonas oleovorans* is required for the hydroxylation of fatty acids and hydrocarbons (135, 136). Since iron-sulfur proteins are commonly present in H_2-producing organisms possessing a clostridial-type pyruvate clastic system, investigators have searched for these proteins in *S. aurantia, S. stenostrepta,* and *S. litoralis* (28, 77, 80, 91). Rubredoxins were isolated from cell extracts of these three species. Johnson and Canale-Parola (91) reported that rubredoxins from *S. aurantia* and *S. stenostrepta* contained one atom of iron per molecule and no inorganic sulfide. The proteins' molecular weight (ca. 6,000), spectral properties, and amino acid composition were similar to those of rubredoxins obtained from other anaerobic bacteria. The same authors (91) isolated an unstable ferredoxin from cell extracts of anaerobically grown *S. aurantia.* This ferredoxin had spectral characteristics and amino acid composition typical of other bacterial ferredoxins. It contained four atoms of iron and four acid-labile sulfide residues per molecule, and its molecular weight was near 6,000. Purified spirochetal ferredoxin stimulated acetyl phosphate formation from pyruvate by clostridial extracts depleted of iron-sulfur proteins by passage through diethylaminoethyl-cellulose columns (91). Stimulation of acetyl phosphate formation from pyruvate was also observed when *Clostridium butyricum* ferredoxin was added to diethylaminoethyl-cellulose-treated *S. stenostrepta* extracts (77). These results indicate that ferredoxin participates in the anaerobic pyruvate metabolism of spirochetes. Apparently, spirochetal rubredoxin has a different role, since it did not stimulate acetyl phosphate formation from pyruvate by diethylaminoethyl-cellulose-treated *C. butyricum* extracts (91).

Johnson and Canale-Parola (91) reported that rubredoxin was present in both aerobically and anaerobically grown cells of *S. aurantia,* whereas ferredoxin was not detected in aerobically grown cells. Thus, ferredoxin does not take part in the metabolism of *S. aurantia* cells growing in air. Rubredoxin preparations from aerobically or anaerobically grown *S. aurantia* had identical spectral characteristics, molecular weight, and iron content. These findings show that rubredoxin from aerobically grown *S. aurantia* differs from *P. oleovorans* rubredoxin, which has a molecular weight of 19,000 and binds either one or two atoms of iron (168). It would be interesting to determine whether the rubredoxin occurring in aerobically grown *S. aurantia* functions in hydroxylation mechanisms similar to those present in *P. oleovorans.*

Quantitative information has been reported on the anaerobic dissimilatory pathways of a few host-associated spirochetes. In one of these studies, *B. duttonii* cells were separated from

the blood of heavily infected rats by a method involving differential centrifugation (60). Suspensions of *B. duttonii* cells incubated in the presence of glucose converted this sugar mainly to lactate, without CO_2 evolution or O_2 uptake. The molar ratio of glucose utilized to lactate formed was close to 1:2. Fluoride and iodoacetate inhibited acid production from glucose, whereas azide had little or no effect. Finally, enzymes of the EM pathway were detected in cell extracts (154–156). The results indicate that *B. duttonii* obtains energy by a homolactic fermentation in which carbohydrate is metabolized to pyruvate via the EM pathway; pyruvate acts as the terminal electron acceptor and is reduced to lactate. Aerobic respiration was not detected in the organism.

Another series of studies dealt with *T. denticola*, an anaerobic spirochete that is a common inhabitant of the gingival sulcus and gingival crevice regions of the human mouth. The sulcus-crevice regions, which include the furrow and fissure present between the gingiva and the tooth enamel (34), are inhabited by an enormous variety and number of microorganisms (157), many of which have not been cultivated. Anaerobes and facultative anaerobes are prevalent in these regions (54, 157), with the latter organisms probably serving as scavengers of molecular oxygen to generate anaerobiosis. The physiological interactions between the sulcus-crevice microorganisms and the host, and among members of the microflora residing in this habitat, constitute complex but fascinating problems of ecology. However, understanding of such interactions cannot be achieved until extensive information becomes available on the physiology of microorganisms residing in the sulcus-crevice regions. Such information was sought by investigators who studied the metabolism of *T. denticola* (79; Canale-Parola and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, K52, p. 155; unpublished data). This organism was grown in complex media containing serum, yeast extract, Trypticase, glucose, thiamine pyrophosphate, and other components. To identify the substrates dissimilated by *T. denticola*, ^{14}C-labeled compounds were added to these media, and the amount of radioactivity in the fermentation products was measured. Furthermore, amino acid analyses of growth medium supernatant liquid were carried out before, during, and at the end of growth of the treponemes. These experiments were complemented by amino acid analyses of *T. denticola* cells, by determination of products formed from various substrates by cell suspensions, and by measurements of growth stimulation by different compounds added to media. The results of the experiments indicated that *T. denticola* catabolized L-cysteine, glycine, L-serine, L-alanine, L-arginine, L-citrulline, and L-histidine, as well as carbohydrates. The fermentation products included acetate, lactate, succinate, formate, pyruvate, ethanol, CO_2, H_2S, and NH_3. Acetate was the major nongaseous fermentation product recovered. Under the growth conditions used, the products formed from glucose constituted a small portion of the total products that accumulated in cultures. Amino acid analyses of culture supernatant fluid showed that growing cells of *T. denticola* dissimilated significant amounts of glutamate and aspartate as well. This observation was in apparent contradiction with results of previous experiments, which indicated that cell suspensions did not catabolize either of these two amino acids and that only very low levels of radioactivity were recovered in fermentation products of growing cells when L-[^{14}C]glutamate was added to complex culture media. However, further experimentation involving amino acid analyses indicated that glutamate was dissimilated when it was added to cell suspensions in the bound form, that is, as a component of peptides or proteins. A possible explanation for these results is that *T. denticola* cells do not have a transport system for free glutamate but are able to transfer glutamate-containing peptides across the cell membrane. Aspartate may be transported in a similar way. This behavior would be analogous to that of other bacteria, such as *Bacteroides ruminicola* (139) and *Fusiformis necrophorus* (173), which do not have the ability to transport into the cell certain free amino acids but apparently transport peptides, which function as carriers of amino acids.

Assays of enzymatic activities in *T. denticola* cell extracts indicated that this spirochete degrades glucose via the EM pathway (79). Like the anaerobic free-living spirochetes previously mentioned, *T. denticola* possesses a coenzyme A-dependent CO_2-pyruvate exchange activity associated with a clostridial-type clastic system for pyruvate metabolism (Fig. 3). Pyruvate is decarboxylated via this system to acetyl-coenzyme A, which is converted to acetate through the action of phosphotransacetylase and acetate kinase. Hydrogen gas is not formed by growing cells, and hydrogenase activity has not been detected in cell extracts. Mechanisms to maintain charge balance include the formation of ethanol, succinate, and lactate and the conversion of glycine to acetate. A rubredoxin or rubredoxin-like protein was detected in cell ex-

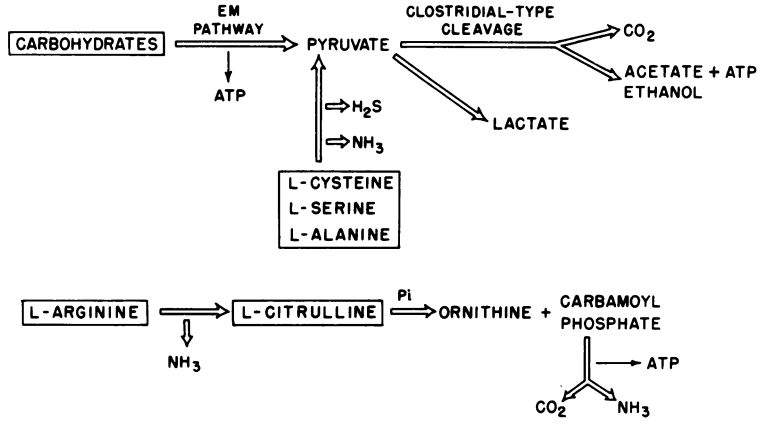

FIG. 3. *Energy-yielding pathways of T. denticola. In addition to those indicated in this figure, other amino acids are catabolized (see text).*

tracts of *T. denticola* (91).

T. denticola ferments some amino acids, such as L-cysteine, L-serine, and L-alanine, by pathways that involve pyruvate as an intermediate (Fig. 3). The pyruvate formed is decarboxylated, with formation mainly of acetate. Electrons generated during pyruvate cleavage are used to form lactate and ethanol. Other amino acids are dissimilated by pathways that do not involve pyruvate as an intermediate. Thus, *T. denticola* catabolizes L-arginine by a pathway similar to that present in other bacteria (48; Fig. 3). This pathway involves conversion of L-arginine to citrulline, which is then cleaved to ornithine and carbamoyl phosphate. The latter is then degraded to NH_3 and CO_2, with formation of ATP. Exogenously supplied L-citrulline is dissimilated through the same pathway (Canale-Parola and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, K52, p. 155).

The picture that emerges from these metabolic studies with *T. denticola* provides a biochemical interpretation for some of the ecological properties of a treponeme, which is a common inhabitant of the human mouth. This anaerobe possesses an abundance of diverse dissimilatory pathways that enable it to derive energy for growth from a relatively wide spectrum of substrates. The metabolic flexibility that *T. denticola* possesses is probably one of the factors that enables it to overcome competition by faster-growing microorganisms in its natural habitat and allows it to survive and thrive in an environment where different growth substrates may become available or unavailable in rapid succession to the indigenous microflora.

The fermentation of glucose by an anaerobic spirochete isolated from bovine rumen contents was studied by Bryant (31). Products of this fermentation were CO_2, ethanol, succinic, lactic, acetic, and formic acids. The amount of succinic acid formed accounted for 41% of the carbon in the fermented glucose. H_2 was not produced. Another rumen spirochete produced formic, acetic, butyric, lactic, and succinic acids from glucose (59). Ziolecki et al. (182) found that succinic and acetic acids were major products of glucose fermentation by eight strains of spirochetes isolated from the bovine rumen.

Allen et al. (7) reported experiments indicating that glucose is a major carbon and energy source for the Reiter treponeme and suggesting that arginine, histidine, serine, threonine, and glutamic acid may serve as energy sources for this treponeme.

Other reports dealing with the dissimilatory metabolism of anaerobic spirochetes describe the demonstration of various enzymes in treponemes and *S. zuelzerae* (5, 8, 143, 165), the deamination of amino acids and transamination reactions in the Reiter treponeme (8, 9), gas evolution by treponemes and *S. zuelzerae* (114), cytochromes in the Reiter treponeme (105), and criteria for the biochemical differentiation of certain oral spirochetes (158).

Aerobic Dissimilatory Metabolism

S. aurantia and the halophilic spirochete RS1 (67), which are facultative anaerobes, and the leptospires, which are obligate aerobes, couple oxidation of substrates with reduction of molecular oxygen. Breznak and Canale-Parola (27, 29) reported that cells of *S. aurantia* growing in the presence of air performed an incomplete oxidation of carbohydrates, producing primarily CO_2, acetate, and pyruvate from glucose. From 20 to 30% of the total sugar carbon used was evolved as CO_2, and approximately 50% was incorporated into cell material.

Slightly more than one-third of the assimilated glucose carbon was used for the synthesis of cell lipids, which constituted 29 to 36% of the dry weight of *S. aurantia*. Apparently, a relatively large proportion of the acetyl-coenzyme A derived from carbohydrate oxidation was utilized for lipogenesis. A tricarboxylic acid cycle either was not present or served in a minor catabolic capacity.

Aerobic and anaerobic molar growth yield determinations showed that *S. aurantia* derived more energy from the aerobic oxidation of sugars than from their fermentation and strongly suggested the presence of oxidative phosphorylation mechanisms in this bacterium (29). A search for cytochromes revealed the presence of cytochrome b_{558} and cytochrome *o*, which were associated primarily with a particulate fraction of *S. aurantia* cell extracts. This fraction also contained O_2-dependent reduced nicotinamide adenine dinucleotide ($NADH_2$) dehydrogenase activity. Cytochromes of the *a* or *c* type were not found in this spirochete. Protoheme, but not heme *a* or mesoheme, was detected. A scheme for terminal electron transport in *S. aurantia* was suggested (29):

$$NADH_2 \rightarrow \text{flavoprotein} \rightarrow [\text{cytochrome } b_{558} \rightarrow \text{cytochrome } o] \rightarrow O_2$$

The brackets indicate that cytochrome b_{558} and cytochrome *o* may be one and the same hemoprotein, as has been observed in other bacteria (22). According to this scheme, the aerobic electron transport in *S. aurantia* is a relatively primitive one and may constitute an early evolutionary attempt by spirochetes at synthesizing functional hemoproteins (29). The aerobic energy-yielding metabolism of halophilic spirochete RS1 is similar to that of *S. aurantia* (Greenberg and Canale-Parola, in press).

The leptospires, which, as previously mentioned, are obligate aerobes, are quite distinct metabolically from spirochetes capable of anaerobic growth. Early attempts at studying the metabolism of leptospires established that respiration of whole cells was stimulated by rabbit blood serum or crude phospholipid preparations, but not by carbohydrates or amino acids (61, 123). It was suggested that the blood serum, which was usually included in growth media for leptospires, contained a constituent(s) that served as respiratory substrate or otherwise stimulated respiration of leptospires (61, 123). It was later shown that long-chain fatty acids present in blood serum were responsible for the observed stimulation of respiratory activity (73). Furthermore, serum protein served as a "detoxifier" by removing inhibitory effects caused by the fatty acids (73). As a result of other studies (10, 51, 52, 74, 89, 93, 95, 98, 148, 160, 161, 170, 172, 178), it was established that fatty acids such as palmitate, stearate, or oleate serve as major carbon and energy sources for leptospires. With the exception of long-chain fatty alcohols (e.g., palmityl alcohol), no other readily utilizable energy and carbon sources are known for these spirochetes (98). Saturated or unsaturated fatty acids, frequently of chain lengths ranging from 15 to 18 carbons, have been used to grow saprophytic or parasitic leptospires. Thus, these aerobic spirochetes utilize a rather limited range of oxidizable substrates for growth. Paradoxically, free long-chain fatty acids, even at very low concentrations, usually inhibit the growth of leptospires, especially the pathogenic strains. Therefore, in culture media it is customary to use fatty acids in bound form, either as serum components or as fatty acid esters, e.g., Tween 80 (polyoxyethylene sorbitan monooleate), in order to minimize the inhibitory or lethal effects. It has been suggested that, in pathogenic leptospires, these effects may be caused by auto-oxidation products of fatty acids (161). Growth of a water leptospire was observed, after a long lag, in a medium containing acetate and L-histidine as the only possible sources of carbon and/or energy (74).

The isolation of aquatic leptospires capable of growing abundantly in serum-free media (12) has facilitated the study of leptospiral metabolism. It was found that growing cells of the aquatic leptospire B16 oxidized oleate to CO_2 and acetate. The amount of CO_2 and acetate produced accounted for 55.8 and 8.5%, respectively, of the oleate utilized. The cell material formed represented 26.5% of the oleate consumed (74). Studies with an aquatic and a pathogenic strain of *Leptospira* indicated that these organisms use beta-oxidation for the degradation of long-chain fatty acids (75). Furthermore, enzymatic assays of cell extracts of a water leptospire and of two pathogenic leptospires indicated that enzyme activities of the tricarboxylic acid cycle and of the EM and pentose pathways were present (10). It was concluded that leptospires oxidize long-chain fatty acids by beta-oxidation and utilize the resulting two-carbon fragments via the tricarboxylic acid cycle (10, 75). Label distribution patterns in cellular amino acids of leptospires grown in the presence of $^{14}CO_2$ were consistent with the occurrence of the tricarboxylic acid cycle (40). Enzymatic activities of the EM and pentose pathways may be utilized by leptospires mainly for biosynthesis.

Cytochromes of the *a*, *c*, c_1, and *o* types were found in pathogenic and aquatic strains of *Leptospira* (11, 61). Carbon monoxide difference spectra suggested that a cytochrome *a* oxidase, possibly a_1 or a_3, and a pigment with absorption characteristics different from those of previously described cytochromes were present in two pathogenic strains, but not in an aquatic strain of *Leptospira* (11). Another significant difference betwen aquatic and pathogenic strains of leptospires is that the former are catalase negative, whereas the latter possess catalase activity (11, 55, 141).

Representatives of different pathogenic leptospiral serotypes were found to utilize urea as a nitrogen source and to possess urease activity (102). These findings may be useful in interpreting the localization of certain leptospires in the kidney during leptospirosis.

Other reports on the dissimilatory metabolism of leptospires describe studies on lipase activity (16, 17, 41, 53, 94, 104, 108, 133, 134), transamination reactions (122), and various enzymatic activities (35, 63, 65, 66).

Physiology of *T. pallidum*

Cox and Barber (44) reported that suspensions of *T. pallidum* cells, extracted from infected rabbit testicles, consumed O_2. Oxygen uptake was inhibited by 10 μM cyanide or 28 mM azide. However, uptake of O_2 by growing cells was not observed inasmuch as *T. pallidum* has not been cultivated in pure culture in vitro. It has been widely believed that *T. pallidum* is an anaerobe, mainly because of reports that the cells retain their motility for longer periods of time under anaerobic conditions than in the presence of air (33, 145). Retention of motility and virulence by *T. pallidum* cells extracted from rabbit testicles is prolonged when the treponemes are suspended in maintenance media that have an electro-negative redox potential (64, 124).

Suspensions of *T. pallidum* cells extracted from rabbit testicles were reported to incorporate amino acids into protein optimally in atmospheres containing 10 to 20% O_2 (13) and to degrade glucose to CO_2, acetate, pyruvate, and lactate (13, 131). Under anaerobic conditions, pyruvate and lactate were major products of glucose degradation, whereas aerobically, greater amounts of CO_2 and acetate were formed (13).

Fitzgerald et al. (58) added *T. pallidum* to cultures of rabbit testicular tissue cells under aerobic conditions. They reported that some of the treponemes attached themselves to and penetrated into the cultured animal cells. Attached and/or intracellular *T. pallidum* cells retained virulence for longer periods of time than cells incubated in the absence of cultured animal cells. Furthermore, the presence of animal cell monolayers extended the persistence of treponemal motility. It was suggested that superoxide ions may be responsible for the toxic effects of air on *T. pallidum* cells extracted from rabbit testicles and that within infected testicles superoxide dismutase present in the mammalian tissue may prevent oxygen toxicity, thus allowing growth of the treponemes (44, 58).

Interpretation of results obtained with *T. pallidum* cells extracted from rabbit testicles is complicated by the possibility that such treponemal populations may be heterogeneous. This was suggested by a report of Baseman et al. (14), who found that *T. pallidum* populations from infected rabbit testicles, when subjected to velocity sedimentation in discontinuous gradients of Hypaque, separated into two distinct bands. The authors suggested that two interacting types of treponemes may exist in tissue during the infectious process. Other authors (82) reported that electron microscopy revealed the presence of rabbit cells and tissue debris in *T. pallidum* preparations. These preparations were obtained from infected rabbit testicles by procedures similar to those used by previous investigators to harvest *T. pallidum* cells for metabolic studies.

A recent article by Jones et al. (99) describes the cultivation of *T. pallidum* in a tissue culture system. *T. pallidum* cells freshly harvested from infected rabbit testicles, and suspended in culture medium, were added to monolayers of baby hamster kidney cells. The mixed cultures were incubated in an atmosphere of 7% CO_2 in air. Subculturing was carried out by transferring treponemes to fresh hamster cell cultures. The authors reported that *T. pallidum* grew through nine subcultivations (ca. three cell generations per subculture) and that virulent cells were demonstrated in subcultures. The investigators emphasized that frequent subculturing (every 24 h) and the use of a treponeme inoculum from a 43-day-old testicular infection were important factors in achieving continued cultivation of virulent *T. pallidum* cells in the system they used. Treponemes from less prolonged rabbit testicular infections caused the cultured tissue cells to lyse, presumably because of the presence of a cytotoxic factor in the treponemal preparations.

Lipids and Fatty Acids

Cells of spirochetes contain a relatively large

amount of lipids. The lipid content of *Spirochaeta* species ranges from 24 to 36% of the cell dry weight (29, 100), the lipid content of treponemes from 18 to 20% (96, 116), and that of leptospires from 18 to 26% (97). The lipid composition of a variety of spirochetes has been investigated (43, 56, 96, 97, 100, 113, 116–118, 126, 163, 169).

Johnson et al. (97) and Livermore and Johnson (117) compared the lipid composition of *Leptospira, Treponema,* and *Spirochaeta* species. Phospholipid constituted 60 to 70% of the total lipid of leptospires, the remaining lipid being free fatty acids. The major phospholipid in leptospires was phosphatidyl ethanolamine. Phosphatidyl choline, which was absent from leptospires, was a major phospholipid in various *Treponema* species. Furthermore, the glycolipid monogalactosyl diglyceride, not found in leptospires, was a major component of lipids in treponemes. Among the *Spirochaeta* species, *S. stenostrepta* contained the monogalactosyl diglyceride, and other species of *Spirochaeta* contained the glucosyl or mannosyl analogue of this lipid. Phosphatidyl choline was not detected in species of *Spirochaeta*.

The cellular fatty acid composition of spirochetes reflects the fatty acid composition of the culture medium (96, 97, 163). For example, it was reported that the Kazan 5 treponeme contained saturated and unsaturated fatty acids ranging from 14 to 18 carbon atoms, depending upon the fatty acids added to the growth medium (96).

With some exceptions, host-associated spirochetes that have been cultivated require an exogenous supply of long-chain fatty acids for growth. Thus, culture media for host-associated spirochetes usually are supplemented with serum or fatty acids complexed with albumin to provide these required substances (118). Both the Reiter and Kazan 5 treponemes require a saturated fatty acid with a chain length of at least 14 carbon atoms and an unsaturated fatty acid with one, two, or three double bonds and a chain length of 15 or more carbon atoms (92, 132). The two fatty acids can be replaced by a single 18-carbon monounsaturated fatty acid with a *trans* configuration rather than the naturally occurring *cis* configuration (92). Requirements for exogenous long-chain fatty acids have been reported for oral treponemes, such as *T. vincentii, T. denticola,* and *T. scoliodontum* (117, 129). Leptospires utilize long-chain fatty acids as their major energy and carbon source, and strains that were studied usually required long-chain fatty acids for growth. These strains cannot elongate the carbon chain of fatty acids available to them (97) and thus require fatty acids with chain lengths of 15 carbons or more (98). *B. hermsii* has a requirement for exogenous long-chain fatty acids (137).

Short-chain fatty acids are known to be required by a number of spirochetes. Some oral treponemes require isobutyrate (72, 159), and a rumen spirochete requires branched and straight, short-chain fatty acids (176). The latter organism apparently uses the short-chain fatty acids present in the growth medium for the synthesis of long-chain fatty acids.

Two spirochetes isolated from the human mouth and two others isolated from pig feces are unusual because they grow in media lacking long-chain fatty acids but containing isobutyrate and valerate (118). Presumably, isobutyrate and valerate serve as precursors for the synthesis of long-chain fatty acids.

Studies conducted with several host-associated spirochetes have shown that the requirement for long-chain fatty acids results from the organisms' inability to manufacture these compounds, which are needed for cellular lipid biosynthesis. For example, the Kazan 5 treponeme cannot synthesize, modify the chain length of, reduce, or desaturate long-chain fatty acids (96). Similar findings have been reported for the Reiter treponeme (126). Leptospires, growing in media including long-chain fatty acids and ^{14}C-labeled acetate, incorporated only a small amount of label into cellular fatty acids (163). Desaturation of fatty acids was accomplished by leptospires in the presence of molecular oxygen (97, 163).

Among the free-living spirochetes, the ability to synthesize long-chain fatty acids is not uncommon. *S. zuelzerae* synthesizes its cellular fatty acids via a pathway originally discovered in *C. butyricum* (125, 146). Furthermore, since water leptospire B16 (74) and species of *Spirochaeta* (27, 78) grow in media devoid of long-chain fatty acids, it can be inferred that these free-living spirochetes are able to synthesize all fatty acids required for their cellular lipids. The ability to synthesize long-chain fatty acids is probably widespread among free-living spirochetes other than leptospires.

A rumen spirochete was shown to hydrogenate linoleic acid to octadec-*trans*-11-enoate (144). The hydrogenation occurred in two steps. First, the conjugated fatty acid octadeca-*cis*-9,*trans*-11-dienoate was formed from linoleic acid through the action of an isomerase. Subsequently, the conjugated fatty acid was reduced to octadec-*trans*-11-enoate (179). The isomerase was associated with a protein- and lipid-rich particulate fraction possibly derived from a lip-

oprotein layer of the protoplasmic cylinder (179). Ferredoxin may be involved in this hydrogenation process (180).

Pickett and Kelly reported a study on the metabolism of lipids in three species of relapsing fever borreliae (137). Thin-layer chromatography of chloroform-methanol extracts of culture supernatant liquid indicated that lysolecithin was removed from complex growth media by the growing spirochetes, whereas lecithin, sphingomyelin, triglycerides, and cholesterol esters were not affected by the growth of the borreliae. Enzymatic assays of sonic cell extracts showed that lysolecithinase (EC 3.1.1.5), glycerophosphorylcholine diesterase (EC 3.1.4.2), and acid phosphatase (EC 3.1.3.2) activities were present. The authors concluded that the borreliae sequentially metabolize lysolecithin to fatty acids, choline, inorganic phosphate, and glycerol. Enzymes of lecithin catabolism were not detected, a finding in agreement with the observation that borreliae fail to utilize lecithin as the sole lipid substrate in culture media.

Substantial amounts of cholesterol were detected in cell preparations of the Reiter treponeme (126). This sterol was not synthesized by the organisms but was taken up by the cells from the growth medium, where it was present as a serum constituent. It has also been reported that relapsing fever borreliae selectively remove cholesterol from the culture medium during growth (137). Although cholesterol apparently has a nutritional role for a strain of *T. refringens* (140), it is not known whether this sterol serves an essential function in the physiology of treponemes and borreliae. Pickett and Kelly (137) pointed out that nonspecific adsorption of cholesterol to the cells may be responsible for the removal of this compound from culture media during growth of spirochetes. Various free-living spirochetes have been grown in media from which cholesterol was absent and obviously do not have a requirement for it (27, 74, 78). Meyer and Meyer (126) reported that *S. zuelzerae* neither synthesizes nor requires sterols.

Carotenoid Pigments

S. aurantia and spirochete RS1, the only known free-living, facultatively anaerobic spirochetes, produce carotenoid pigments (27, 67). Aerobically grown colonies of *S. aurantia* are yellow-orange, whereas those of spirochete RS1 are red. Anaerobically grown colonies are white.

The molecular structure of the major pigments of spirochete RS1 and *S. aurantia* strain J1 was determined by analytical procedures involving mass spectrometry, infrared spectroscopy, chromatographic analysis, hydride reduction, and acetylation and silylation experiments (67). It was found that the major pigment of spirochete RS1 was 4-keto-1',2'-dihydro-1'-hydroxytorulene, also called deoxyflexixanthin (Fig. 4). This pigment accounted for at least 90% of the total pigment content of spirochete RS1. The major pigment from *S. aurantia* was 1',2'-dihydro-1'-hydroxytorulene (Fig. 4), differing from deoxyflexixanthin only in a substitution in the cyclohexene ring. Chromatographic and spectrophotometric evidence indicated that 1',2'-dihydro-1'-hydroxytorulene was also present, as a minor carotenoid component, in spirochete RS1.

The two major carotenoid pigments from *S. aurantia* and spirochete RS1 (67) had been previously detected almost exclusively in gliding bacteria, such as species of *Flexibacter* (2), *Stigmatella* (112), and *Myxococcus* (142). The possible evolutionary significance of the occurrence of these pigments in both spirochetes and gliding bacteria is discussed in a subsequent section of this review. Saproxanthin, a carotenoid pigment remarkably similar in chemical structure (Fig. 4) to the identified pigments of spirochete RS1 and *S. aurantia,* is the major carotenoid of *Saprospira grandis* (1). It is noteworthy that *S. grandis* is a gliding bacterium previously believed to be a spirochete (25, 33).

Motility and Chemotaxis

Spirochetes perform locomotory, rotatory, and flexing movements. Movements involving flexing of the cell are quite varied and include lashing, looping, and bending motions, vibrations, undulations, formation of helical waves traveling along the body, and production of planar waves (87, 177). Because of these manifold and incessant movements, spirochetes are often described as being "extremely flexible" (e.g., see 150). Movement occurs in liquid media and agar gels and is not dependent upon contact with a solid surface. However, as previ-

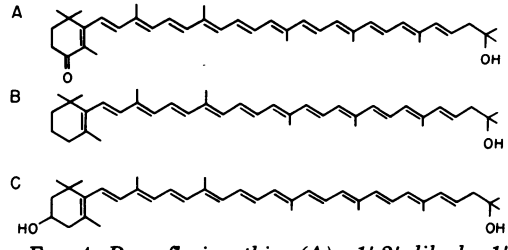

FIG. 4. *Deoxyflexixanthin (A), 1',2'-dihydro-1'-hydroxytorulene (B), and saproxanthin (C).*

ously mentioned, creeping or crawling movement on solid surfaces has been observed (23, 45).

It is widely believed or assumed that axial fibrils play a role in the movements of spirochetes. This belief is held mainly because authors who studied these organelles found them to be strikingly similar in fine structure and chemical characteristics to bacterial flagella. Individual axial fibrils consist of three main morphological components structurally similar to those of bacterial flagella: (i) an insertion apparatus including a number of basal disks; (ii) a proximal hook near the insertion apparatus; and (iii) a shaft or filamentous portion (18, 19, 23, 81, 84, 86, 90, 101, 115, 128). In negatively stained preparations examined by electron microscopy, the filamentous portion appears to consist of a core enveloped by a nonstriated sheath or coat (23, 81, 84, 86, 101, 128). Axial fibrils consist of one or more species of protein strikingly similar in overall amino acid composition to bacterial flagella protein (18, 20, 101, 128). Furthermore, the response of purified axial fibrils to chemicals or enzymes was found to be largely similar to that reported for bacterial flagella (18, 20, 101, 128).

Even though direct evidence is lacking, it seems reasonable to infer that axial fibrils and bacterial flagella may serve similar or analogous functions because they so closely resemble each other morphologically and chemically. It should be noted, however, that axial fibrils, unlike bacterial flagella, are completely endocellular organelles. Thus, even though axial fibrils probably play a role in motility, their mode of action may be expected to be different from that of bacterial flagella.

Antisera against axial fibrils were used to determine whether these organelles are involved in motility of spirochetes (21). Since antisera to spirochetes are capable of immobilizing them, it seemed possible that the immobilizing factor in antisera was directed toward the axial fibrils. It was reasoned that if antisera to purified axial fibrils immobilized the organisms, this would constitute evidence implicating axial fibrils in motility. However, antisera against axial fibrils purified from *S. zuelzerae* did not immobilize this spirochete. In addition, it was found that the immobilizing factor present in antisera to whole cells was directed toward a cell component other than axial fibrils. It was suggested that the spirochetes were not immobilized by anti-axial fibril antibodies because the latter did not have access to the axial fibrils, due to the endocellular location of these organelles.

Other workers reported that anticell sera produced against whole leptospires had immobilizing activity, which apparently resulted from damage to, and degradation of, the cell structure (39). Anti-axial fibril serum had some, but not strong, immobilizing activity against leptospiral cells.

Attempts have been made to interpret the mechanisms of spirochetal movement (15, 23, 45, 88, 103, 175). The movements of *Cristispira* and of large free-living spirochetes were recorded by cinematography and time-exposure, single-frame photographs by Jahn and Landman (88). According to these authors, the spirochetes they studied are driven by irrotational helical waves which travel along the flexible body of the organisms. The organisms move with almost no slippage; that is, as the spirochetal cell advances through a liquid, the rear coils follow the path of the anterior tip almost perfectly. The spirochetes swim with high hydrodynamic efficiency and have no means of resisting the torque generated by the traveling helical waves. In a subsequent publication, Wang and Jahn (175) presented a theory that assumes that rotation about a local body axis occurs and that it cancels the torque produced by the traveling helical waves.

Blakemore and Canale-Parola (23) observed and photographed moving cells of the large, free-living *S. plicatilis*. The cells translated both in contact and with no contact with solid surfaces. During the latter type of motility, *S. plicatilis* cells appeared to rotate rapidly about their longitudinal axis, and wide waves moved along the length of the organisms. Furthermore, the cells vigorously flexed, looped, and darted through the liquid environment. Cells translating in contact with glass surfaces "crept" foward through a fixed pattern of coils, virtually without slippage.

Observations of wet-mount preparations revealed that cells of *S. aurantia* M1 usually swim in straight lines or nearly straight lines and, as they travel in the liquid environment, appear to spin about their longitudinal axis (E. P. Greenberg and E. Canale-Parola, unpublished data). Occasionally a cell stops its locomoting and spinning, flexes its body, and then resumes travel in a new direction. Frequently, after flexing, the cell end that was the anterior end becomes the posterior end.

Cox and Twigg (45) reported that cells of *L. interrogans* serotype *icterohaemorrhagiae*, when moving freely in liquids, do not rotate about their axes. According to these authors, translating leptospires are propelled by helical waves, which travel for a short distance from

the anterior cell end toward the trailing cell end. Whereas the anterior end is not hooked, the trailing end forms a broad semicircular hook. This hook waves in an approximately circular motion in the opposite direction to the helical waves, thus providing stability and preventing rotation of the cell body. In nontranslational movement, both cell ends are hooked and wave in opposite directions, their rapid movement giving the illusion of spinning (45).

Drugs such as procaine-hydrochloride, serotonin creatinine sulfate, atropine sulfate, and others, in a concentration range of 10^{-3} to 10^{-6} M, affect the motility patterns of flagellated bacteria (57, 138). It was found that these drugs also inhibit or interfere with the movements of leptospires (138). These observations suggest that a common mechanism may operate in the motor control of motility in flagellated bacteria and in spirochetes (138).

As discussed in more detail below, Kaiser and Doetsch (103) reported that translational movement of leptospires was strikingly enhanced in viscous environments. Both the number of leptospiral cells exhibiting translational movement and the velocity of the cells were markedly increased in viscous solutions.

A comprehensive model for propulsion of spirochetes has been proposed recently by Berg (15). The model is based on the assumptions that the protoplasmic cylinder is semirigid, that the outer sheath is flexible, and that the axial fibrils rotate in a manner similar to that of bacterial flagella. According to the model, the rotation of the axial fibrils causes the protoplasmic cylinder to rotate within the outer sheath. When the axial fibrils rotate in the same direction, the protoplasmic cylinder rotates in the opposite direction. A change in the rotational direction of the fibrils results in a corresponding change in the rotational direction of the protoplasmic cylinder. In a spirochete that has two axial fibrils, when only one of the fibrils changes direction of rotation, the protoplasmic cylinder stops rotating. If the outer sheath is free, it would be expected to rotate in a direction opposite to that of the protoplasmic cylinder. Thus, the outer sheath behaves like the tread of a tank, rolling about the protoplasmic cylinder. When the protoplasmic cylinder is planar, it rotates in place. When the protoplasmic cylinder is helical, the cell rotates about its longitudinal axis and has translational movement. According to Berg's (15) model, thrust may be generated "in two opposing ways: by the circumferential slip of the helix through the medium" and "by the imbalance in the longitudinal viscous forces due to the roll of the sheath at the outer and inner surfaces of the helix." To interpret the creeping motility of *S. plicatilis* (23), Berg proposed that the long and irregularly shaped protoplasmic cylinder of this spirochete, when near a solid surface, may not be free to rotate. In such a situation "the roll of the sheath will cause the cell to slide in a direction nearly parallel to the local helical axis" (15). Berg (15) suggested that evidence indicating whether the axial fibrils rotate could be yielded by experiments similar to those carried out by Silverman and Simon (149) to determine rotation of *Escherichia coli* flagella. Thus, the axial fibrils could be linked to glass or to polystyrene latex beads with antibodies, after rupturing the spirochetes' outer sheath. Similar experiments with latex beads would be useful in monitoring the motion of the outer sheath (15).

As spirochetes grow in agar media, they usually move through the agar gel, forming so-called growth "veils" (36). Thus, colonies in media containing as much as 1% (or even more) agar tend to spread or diffuse. When cells of *S. aurantia* are inoculated in the center of agar (0.5%) medium plates, they migrate through the agar gel toward the periphery of the plates, forming characteristic concentric growth rings (30). The rings of growing cells originate from the inoculum in the center of the plates, increase in diameter during incubation, and may reach the edge of the plates. In growth media containing glucose (0.02 to 0.1%, wt/vol), the cells in the outermost ring utilize all glucose available as they migrate toward the periphery of the plate. Thus, no glucose is left in the portion of the agar plate circumscribed by this ring. Measurements of ring diameter during incubation showed that the rate of migration of glucose-utilizing rings is greatest at low glucose concentrations (30). This behavior was interpreted as follows. As *S. aurantia* cells in these rings metabolize glucose, a glucose concentration gradient is formed in the agar gel, and the population continuously migrates toward zones of higher glucose concentration within the concentration gradient. There they continue to dissimilate glucose and, as a result, the glucose gradient shifts toward the periphery of the plate. At low glucose concentrations this shift is more rapid and, consequently, the rate of population migration is greater. It was concluded that migration of cell populations present in these rings was due to a chemotactic response toward glucose (30).

More recently, a quantitative assay was developed to investigate the chemotactic behavior of *S. aurantia* M1 (Greenberg and Canale-Par-

ola, Abstr. Annu. Meeting Am. Soc. Microbiol., 1976, I122, p. 131; unpublished data). The assay, similar to that used by Adler (3) to study chemotaxis in *E. coli*, was conducted as follows. The open end of a microcapillary tube containing an attractant in a phosphate buffer-cysteine solution was immersed into a suspension of *S. aurantia* cells. After 1 h, the number of spirochetes that entered the microcapillary tube was determined by plate counts and was compared with the number of spirochetes that entered an identical microcapillary tube filled with phosphate buffer-cysteine solution lacking the attractant. The presence of L-cysteine in the solution enhanced the chemotactic response, for reasons not yet determined. It was found that a variety of sugars, which served as energy sources for the growth of *S. aurantia* M1 (D-mannose, D-fructose, D-galactose, D-glucose, D-xylose, maltose, and cellobiose), also served as attractants. On the other hand, D-mannitol, used by *S. aurantia* M1 as an energy source, did not serve as an attractant. Some sugars that were not used as energy sources (D-glucosamine, D-fucose, 2-deoxy-D-glucose, α-methyl-D-glucoside) attracted *S. aurantia* M1. This indicated that metabolism, and probably transport of the attractant, were not required for chemotaxis. Finally, other sugars not used as energy sources (L-glucose, 6-deoxy-D-glucose, D-ribose, D-sorbitol) did not elicit a positive chemotactic response. The threshold concentrations for attractants, that is, the lowest concentrations at which a response was observed, were as low as 2×10^{-7}. Maximum accumulation of spirochetes in the capillary occurred at attractant concentrations of 10^{-4} to 10^{-1} M. Amino acids such as L-serine, glycine, L-aspartate, L-methionine, and others did not attract *S. aurantia* M1. These studies also showed that galactose taxis in *S. aurantia* M1 is inducible, inasmuch as it occurs in cells grown in the presence of D-galactose or of D-galactose and D-glucose together, but not in cells cultured with D-glucose, as energy source.

EVOLUTION OF SPIROCHETES

In attempting to interpret the evolutionary history of spirochetes, answers to two fundamental questions must be sought: (i) what evolutionary processes were responsible for the unique cellular architecture present in such a heterogeneous assemblage of bacteria?; (ii) what evolutionary steps led to the physiological and ecological diversity we observe in present-day spirochetes?

In this section, I shall set forth two hypotheses that may be used to answer, in part, the two fundamental questions. Furthermore, the hypotheses may serve as preliminary and incomplete interpretations of the evolutionary development of spirochetes.

The Protospirochete Hypothesis

Presumably the first cells evolved over 3 billion years ago, and during the Precambrian era ancestral procaryotic cells were selected out over their competitors (109, 120, 121). It is considered probable that these primitive procaryotes, existing in the O_2-free environment of ancient Earth, were anaerobic fermentative heterotrophs, which eventually developed the ability to generate ATP by metabolizing carbohydrates via the EM pathway (68, 83, 109, 120, 121).

An obligately anaerobic, carbohydrate-fermenting, free-living protospirochete may have evolved from these primitive procaryotes through mutations leading to morphological differentiation. Such differentiation conferred upon the developing protospirochete the traits of "spirocheteness," that is, a helical shape, axial fibrils, and an outer sheath, as well as the unique motility mechanisms associated with this type of cellular configuration. Spirocheteness persisted and was retained by the ancestral spirochetes because it offered them selective advantages. Possible advantages of spirocheteness are discussed in the following subsection of this article.

According to the protospirochete hypothesis, all spirochetes that exist today are descendants of the protospirochete. Among modern spirochetes, the closest relatives of the protospirochete may be the free-living, obligate anaerobes that ferment carbohydrates via the EM pathway, e.g., certain species of *Spirochaeta* (see section, Anaerobic energy-yielding metabolism).

It is believed that the emergence of organisms capable of using water as an electron donor for photosynthesis resulted in the appearance of molecular oxygen in the Earth's atmosphere, an event that made possible the development of aerobic energy-yielding mechanisms in living cells (109, 120, 121). At this stage of evolution, some of the free-living, anaerobic spirochetes, as well as many other anaerobic bacteria, may have acquired the ability to respire aerobically. Present-day, free-living, facultatively anaerobic spirochetes (e.g., *S. aurantia*) may be the descendants of these first spirochetes capable of generating ATP by electron transport to molecular oxygen. In time, selective pressures may have led to the development of free-living, obligately aerobic spirochetes,

e.g., strains of *Leptospira*.

A plethora of new habitats became available to bacteria after the appearance of animals on Earth. On or within the body of these highly complex organisms, spirochetes established themselves in a variety of habitats ranging from the outer surface of protozoa (24, 42, 153) to the epithelial layer of the human colon (127, 164). In these habitats the spirochetes persisted and thrived, developing complex physiological interactions with the host cells and with other resident microorganisms. Most likely, from these ancient host-associated spirochetes evolved those present-day spirochetes that are indigenous to healthy animals, such as species of *Treponema* and *Cristispira*. Survival of the ancestral host-associated spirochetes depended on their being transmitted from host to host. Probably transmittal took place by mechanisms similar to those responsible for propagation of modern host-associated spirochetes (26, 33, 46, 47, 62, 69, 166, 167). Thus, animals were colonized by spirochetes through direct contact with a host, or through contact with the host's secretions or excretions in which the spirochetes temporarily survived. Some of the host-associated spirochetes released by their host into aquatic environments remained viable for relatively short periods of time in the free state and swam to new hosts, which they colonized. Furthermore, arthropod vectors may have been responsible for transmittal. It is possible that spirochetes capable of producing disease evolved from ancient symbiotic or commensal spirochetes that gained the ability to overcome the natural defenses of the host.

Convergent Evolution Hypothesis

The convergent evolution hypothesis states that in the course of evolution, different procaryotes developed the characteristics of spirocheteness. Accordingly, the various kinds of spirochetes we observe today did not evolve from a common protospirochete ancestor but derived from procaryotes that had already become diversified physiologically and ecologically. These procaryotes acquired spirocheteness through independent, but converging, morphological evolutionary processes.

It is likely that selective advantages were gained by procaryotes that attained spirocheteness. Apparently, an important advantage that at least some of them acquired was a marked increase in velocity of translation during movement through highly viscous environments. Kaiser and Doetsch have shown that spirochetes of the genus *Leptospira* exhibit maximum velocity (30 μm/s) when swimming in environments with viscosities exceeding 300 centipoises (103). In contrast, certain flagellated bacteria, such as *Spirillum serpens*, which translate at a maximum velocity of 38.5 μm/s at 2.5 centipoises, show a rapid decrease in velocity at higher viscosities (147). Thus, leptospires and possibly other spirochetes may move rapidly through viscous natural habitats. Viscous natural habitats of spirochetes include the crystalline style of molluscs, fluids of the gingival crevice, intracellular and intercellular regions of animal hosts, and mucosal surfaces, as well as microbial slimes and viscid mud present in aquatic environments inhabited by free-living spirochetes.

Possession of an outer sheath may also confer a selective advantage to spirochetes. This structure may act as a permeability barrier, which protects the axial fibrils from disruptive or damaging environmental agents such as pH extremes or the action of enzymes secreted by other microorganisms. As previously mentioned, Bharier and Rittenberg reported that whereas antisera against whole cells immobilize *S. zuelzerae* cells, antisera against axial fibrils do not (21). In light of this report, it seems possible that the outer sheath serves as a shield against external agents that may affect the functioning of the axial fibrils. If, indeed, axial fibrils play a role in motility, as is suspected, the benefits of this type of protection to the spirochetes are evident.

The traits of spirocheteness, acquired by cells in the course of evolution, have persisted, and present-day procaryotes that possess such traits are quite successful in terms of competition with other microorganisms. Thus, it seems probable that in addition to the selective advantages suggested above, spirocheteness imparts to cells advantages that are not obvious to us at present.

Presently available experimental evidence does not contradict the convergent evolution hypothesis or the protospirochete hypothesis, but it is too meager to favor either hypothesis over the other. The finding that a wide range of guanine plus cytosine contents occurs in the deoxyribonucleic acid of spirochetes (Table 1) does not necessarily support the convergent evolution hypothesis. On the contrary, it may indicate that the protospirochete originated early in the evolutionary history of procaryotes and changes in the deoxyribonucleic acid occurred subsequently.

Energy-Yielding Pathways of Spirochetes

As pointed out by Hall (68), a useful approach toward the construction of a phylogenetic tree

for procaryotes is the comparison of physiological characteristics of existing microorganisms. Thus, analysis of available information on the energy-yielding pathways of spirochetes may serve to uncover fragments of the developmental history of these organisms and to suggest possible evolutionary processes.

It is considered likely that the EM pathway was the first, or among the first, of the presently known fermentative pathways to evolve (83). The EM pathway is present in all free-living and host-associated spirochetes that have been assayed for it. Anaerobic and facultatively anaerobic spirochetes ferment carbohydrates via this pathway. As previously mentioned, in free-living anaerobes and facultative anaerobes, such as species of *Spirochaeta*, the EM pathway is coupled to a clostridial-type pyruvate clastic reaction (Fig. 2). Through this reaction *Spirochaeta* species cleave pyruvate to CO_2, a two-carbon fragment used for acetate and ethanol production, and electrons, which are transferred to the hydrogenase system (Fig. 2). Furthermore, at least some anaerobic host-associated spirochetes, e.g., *T. denticola*, use a clostridial-type cleavage to metabolize pyruvate derived from carbohydrates via the EM pathway or from amino acids (Fig. 3). Thus, the evidence indicates that ATP generation through the EM pathway coupled to a clostridial-type cleavage of pyruvate is a common characteristic among anaerobic and facultatively anaerobic spirochetes. In view of its widespread occurrence, it may be speculated that this was the earliest or most primordial type of energy-yielding metabolism available to spirochetes.

Investigations of the energy-yielding pathways in the host-associated *T. denticola* indicated that whereas this spirochete has conserved the ancestral properties of fermenting carbohydrates via the EM pathway and of metabolizing pyruvate through a clostridial clastic system, it has acquired the ability to catabolize a variety of amino acids. Some of these, e.g., cysteine or serine, are fermented through pathways in which pyruvate is a key intermediate (Fig. 3). Other amino acids, such as arginine or citrulline, are dissimilated via pathways that do not involve pyruvate (Fig. 3). Thus, *T. denticola* has a more complex system of energy-yielding pathways than the free-living anaerobic spirochetes. Competition for substrates in the gingival crevice must be acute, considering that the microbial population in this region of the human body averages 1.3×10^{11} organisms per g (wet weight) of gingival debris, as determined by microscopic counts (157). The metabolic versatility of *T. denticola* offers to this organism a distinct selective advantage in this densely populated habitat.

On the basis of the limited available information, a possible developmental pattern may be suggested for the energy-yielding pathways of anaerobic spirochetes. It appears that the free-living anaerobic spirochetes have not significantly modified their ATP-generating mechanisms since primordial times. However, at least some of the anaerobic spirochetes that have become adapted to life in specialized host-associated habitats have evolved additional ATP-yielding pathways, probably as a result of selective pressures. As mentioned in a preceding section, the anaerobic dissimilatory pathways of facultatively anaerobic spirochetes are essentially identical to those of the free-living obligate anaerobes (Fig. 2). In air, the facultative anaerobe *S. aurantia* performs an incomplete oxidation of carbohydrates mainly to CO_2 and acetate. It does not possess a tricarboxylic acid cycle, but it has developed mechanisms of oxidative phosphorylation and a rudimentary electron transport system, involving one or two cytochromes. In contrast, the leptospires, which are the only known obligately aerobic spirochetes, do not dissimilate carbohydrates. These spirochetes generate ATP by oxidizing long-chain fatty acids to two-carbon fragments, which are channeled into the tricarboxylic acid cycle. The leptospires have enzymes of the EM pathway but apparently use this pathway only in a biosynthetic direction. Furthermore, the electron transport of leptospires, which includes cytochromes of the *a*, *c*, c_1, and *o* types, is more complex than that of *S. aurantia*. Inasmuch as the facultatively anaerobic spirochetes have developed aerobic electron transport systems that enable them to derive energy through oxidative phosphorylation, it may be inferred that they represent an evolutionary form more advanced than the obligately anaerobic free-living forms. On the other hand, the lack of a tricarboxylic acid cycle and the possession of a rudimentary electron transport system apparently places the facultatively anaerobic spirochetes on a lower evolutionary level than the leptospires, which have more efficient aerobic ATP-generating systems.

It cannot be excluded that at least some of the existing free-living spirochetes were derived from host-associated spirochetal ancestors, which, after being released from their animal hosts, succeeded in becoming adapted to life in the free state. It seems unlikely, however, that this is or was a major evolutionary route for spirochetes, inasmuch as experimental evi-

dence has shown that host-associated spirochetes are defective in various biosynthetic abilities. As discussed in the next section, growth and survival of these "defective" spirochetes depends largely on metabolites or growth factors readily available to them in their host-associated environment. The defective forms, separated from their host, would not be expected to compete successfully with microorganisms well adapted to a free-living existence.

Development of Associations with Hosts

Loss of the ability to synthesize a compound required for growth does not prevent microorganisms from multiplying, as long as they can obtain that particular compound from the environment. When such a condition is met, these auxotrophic or defective mutants, lacking a biosynthetic function, may have a selective advantage over parental strains requiring a greater number of energy-consuming biosynthetic steps for growth (181). Thus, it is not surprising that spirochetes indigenous to nutrient-rich habitats within higher organisms are defective in certain biosynthetic abilities. For example, as mentioned above, many spirochetes associated with humans and animals are unable to synthesize long-chain fatty acids, which they require to manufacture cellular lipids. In the environments inhabited by these spirochetes, fatty acids are available, being produced by the host's metabolism and by other resident microorganisms. In contrast, free-living spirochetes, such as species of *Spirochaeta*, retain the ability to synthesize long-chain fatty acids, presumably because these compounds are not readily available to them in their habitats. Among other required substances not synthesized by host-associated spirochetes are thiamine pyrophosphate and *N*-acetylglucosamine (106, 129, 130, 162).

An enzymatic activity absent from many host-associated spirochetes, probably because of mutations resulting in evolutionary selection, is the production of H_2 (31, 60, 79, 114). On the other hand, all known free-living anaerobic spirochetes synthesize hydrogenase systems and produce H_2 (see section, Anaerobic energy-yielding metabolism). Unless anaerobic host-associated spirochetes compensate for the lack of hydrogenase by manufacturing other electron-accepting systems, they may require and depend on electron acceptors present in their natural habitats or growth media.

In conclusion, it is likely that the loss of certain biosynthetic abilities is one of the factors responsible for the development of spirochetes restricted to life within specialized, host-associated habitats. This loss is followed by evolutionary selection favoring the auxotrophic mutants over the parental strains.

Some spirochetes have developed special cellular structures by which they are attached to eucaryotic cells. Thus, spirochetes are anchored to the surface of protozoa by a "rootlet" or "nose-like specialization" of their proximal cell end (24, 153). The intimate physical association between these spirochetes, or intestinal spirochetes (127, 164), and host cells strongly suggests the existence of symbiotic interactions between the animals and the attached microbes. Spirochetes attached to eucaryotic cells may be dependent on these interactions for survival.

Possible Relationship with Gliding Bacteria

Some recent reports suggest that the phylogenetic relationship between spirochetes and gliding bacteria may be closer than previously believed. Greenberg and Canale-Parola (67) determined the molecular structure of the major carotenoid pigments of the facultative anaerobes *S. aurantia* and spirochete RS1 (Fig. 4), the only known pigmented spirochetes. Except for their presence in the spirochetes, and in an unidentified microorganism (4), these carotenoids have been detected only in gliding bacteria (see section, Carotenoid pigments).

Another characteristic common to the facultatively anaerobic spirochetes and gliding bacteria is that their growth is strongly inhibited by actinomycin D (49, 67). Since gliders and spirochetes are both gram negative, this is an unusual response, inasmuch as gram-negative bacteria generally are not appreciably inhibited by actinomycin D (49). Possibly, the sensitivity of gliding bacteria and spirochetes to this antibiotic reflects similarities in cell surface composition or in specific physiological processes.

Studies of motility mechanisms may reveal additional similarities between spirochetes and gliding bacteria. As previously mentioned, spirochetes not only swim free-floating in liquids, but also "creep" or "crawl" on solid surfaces (23, 45). The mechanism responsible for the latter type of movement may prove to be identical or similar to that which propels gliding bacteria.

Spirochetes, Eucaryotic Flagella, and Cilia

It has been suggested that spirochetes, or spirochete-like organisms, might have been the precursors of eucaryotic flagella and cilia. This suggestion is part of a theory interpreting the evolutionary origin of eucaryotic cells in terms

of a series of symbioses (120). According to this theory, primitive amoebae were formed as the result of a symbiotic association between an anaerobic pleomorphic microbe and a smaller aerobic procaryote. The anaerobic organism served as the host, contributing nuclear material and cytoplasm to the association, and it harbored the aerobe (the endosymbiont), which later evolved into mitochondria. Still according to the theory, the next major step in the formation of present-day eucaryotic cells occurred when free-living, motile, spirochete-like organisms became attached to the surface of the mitochondria-containing amoebae. The ectosymbiotic spirochete-like organisms, which were attached by one of their cell poles to the ancestral amoebae, conferred motility to the complex, presumably by undulating in a coordinated manner. The symbiotic theory states that the eucaryotic flagellum and cilium, with its 9 + 2 fibrillar arrangement, evolved from these spirochete-like organisms.

The participation of spirochetes in evolutionary processes leading to the formation of eucaryotic cells has been suggested by the fact that associations analogous to that postulated between spirochete-like organisms and the ancestral amoebae occur among modern microorganisms. Spirochetes are present on the surface of certain protozoa, to which they adhere by an extremity of their cells (24, 42, 111, 153). For example, spirochetes occur on the surface of *Mixotricha paradoxa*, a large protozoon found in the gut of the termite *Mastotermes darwiniensis*. The coordinated undulations of thousands of spirochetes attached to each individual *Mixotricha* cell propel the protozoon uninterruptedly and at constant speed (42).

Concluding Remarks

It is apparent that the available information on the evolutionary history of spirochetes is meager. Some of the reports mentioned in this article suggest various evolutionary possibilities, but conclusions cannot be reached at present. This is not surprising, inasmuch as we know little about the biology of spirochetes. Although these bacteria are widespread, only a small fraction of the many kinds of spirochetes observed in natural habitats has been cultivated. Even among those spirochetes that have been cultivated, relatively few have been studied extensively.

Interpretable fossil records of spirochetes have not been found. Thus, investigations on the evolution of spirochetes must proceed in other directions. One possible approach is the study of the biochemical and physiological characteristics of different kinds of spirochetes. For example, phylogenetic relationships may be clarified through comparative studies on metabolic pathways, on motility and chemotaxis mechanisms, and on amino acid sequences of spirochetal proteins such as rubredoxin and ferredoxin (91).

Summary

Spirochetes are bacteria with unique morphology and motility mechanisms. However, they do not constitute a homogeneous bacterial group but exhibit extreme physiological and ecological diversity. All existing spirochetes may have evolved from an ancestral protospirochete whose descendants underwent extensive physiological differentiation. Possibly, among present-day spirochetes, the free-living, carbohydrate-fermenting, obligate anaerobes are the most direct descendants of the protospirochete, whereas free-living aerobic and facultatively anaerobic forms, and spirochetes indigenous to animals and humans, developed through further evolutionary processes.

According to another evolutionary hypothesis, the multifarious types of modern spirochetes did not evolve from a common protospirochete ancestor but from a number of procaryotes different physiologically and ecologically from one another. These diverse procaryotes acquired the characteristics of spirocheteness, which conferred to them selective advantages.

Analysis of published information on the physiology of spirochetes suggests that ATP generation from carbohydrate dissimilation via the EM pathway, coupled to a clostridial-type cleavage of pyruvate, was the earliest kind of energy-yielding metabolism available to all or many of these bacteria. Subsequently, in response to selective pressures, some spirochetes acquired additional ATP-yielding mechanisms, such as amino acid catabolism or oxidative phosphorylation. Other spirochetes either diverged radically from the ancestral type of ATP-yielding metabolism or never possessed it, their only energy source being respiration coupled to oxidation of a few fatty acids and alcohols.

Spirochetes restricted to life within specialized host-associated habitats may have developed from the free-living forms as a result of evolutionary selection after losing certain energy-consuming biosynthetic functions, e.g., the ability to synthesize long-chain fatty acids, other cellular constituents, and metabolites. Recent reports suggest that at least some spirochetes and gliding bacteria may be phylogenetically closer than previously believed.

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