OCCURRENCE OF PYRUVATE:FERREDOXIN OXIDOREDUCTASE IN *CAMPYLOBACTER*, *WOLINELLA*, *HELICOBACTER* AND *ARCOBACTER* SPECIES

by

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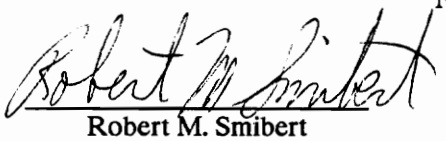
APPROVED:

Noel R. Krieg, Chairman



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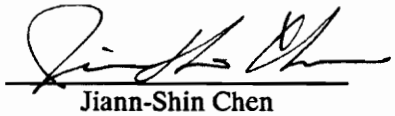

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(ABSTRACT)

Pyruvate:ferredoxin oxidoreductase activity was demonstrated in microaerophilic members of *Campylobacter, Wolinella,* and *Helicobacter. Arcobacter cryaerophila* (sic) and *Arcobacter nitrofigilis*, two aerobic species, lacked any detectable activity. Under anaerobic conditions crude extracts of *Campylobacter*, *Helicobacter* and *Wolinella* were capable of reducing the electron carriers benzyl viologen and metronidazole in the presence of pyruvate. Addition of *Clostridium pasteurianum* ferredoxin to the metronidazole-linked reaction enhanced metronidazole-reducing activity, suggesting that electron transport to artificial electron carriers is facilitated by ferredoxin. All species exhibited varying degrees of sensitivity to metronidazole (MIC = <0.8 to 25 μg/ml) except *Arcobacter cryaerophila*, which was resistant to >100 μg/ml. This further supports the theory that these organisms possess ferredoxin-linked reactions. The presence of the oxygen-labile enzyme pyruvate:ferredoxin oxidoreductase may be related to the inability of these microaerophilic bacteria to grow in normal atmospheric levels of oxygen.

Under aerobic conditions crude extracts of the organisms were also capable of reducing NAD in the presence of pyruvate. This might be accounted for by an NAD-linked pyruvate dehydrogenase; alternatively, it might be due to an enzymatic reduction of NAD by electrons from the reduced ferredoxin generated during the ferredoxin-linked pyruvate oxidoreductase reaction.

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Introduction

Members of the genera *Campylobacter*, *Wolinella*, *Arcobacter* and *Helicobacter* are of interest to clinical and veterinary microbiologists because they are responsible for a variety of diseases in both humans and animals. They are of special interest to microbial physiologists because they are microaerophilic bacteria.

Microaerophiles are organisms that are capable of oxygen-dependent growth and can use oxygen as a terminal electron acceptor, but they either cannot grow, or can grow only poorly, at the concentration of oxygen present in air (21% O_2, vol/vol). They require microaerobic conditions (less than 21% O_2, the optimum oxygen level depending on the species and strain).

Several theories have been proposed to explain the microaerophilic nature of organisms such as *Campylobacter*, *Wolinella*, *Arcobacter* and *Helicobacter*. One theory suggests that these species may possess essential cell constituents which are inactivated by oxygen, or toxic forms of oxygen. One candidate for such an oxygen-labile cell constituent would be pyruvate:ferredoxin oxidoreductase, an enzyme that occurs characteristically in anaerobic bacteria and anaerobic protozoa but not in aerobic or facultatively anaerobic bacteria. In anaerobic bacteria, pyruvate is oxidized by the ferredoxin-linked enzyme. In aerobes and facultative anaerobes, it is oxidized by an NAD-linked pyruvate dehydrogenase. Which enzyme microaerophilic bacteria have is of interest, because if they rely on pyruvate:ferredoxin oxidoreductase, this might explain the inability of microaerophilic bacteria to grow in normal atmospheric levels of oxygen. Pyruvate:ferredoxin oxidoreductase might be a key target for inactivation by oxygen because it is highly unstable in the presence of oxygen and can be assayed only under anaerobic conditions.

The specific objectives of this research were to determine whether or not pyruvate:ferredoxin oxidoreductase occurred in the species of *Campylobacter*, *Wolinella*, *Arcobacter* and *Helicobacter*, and, if present, to measure its specific activity.

Literature Review

The Genus *Campylobacter*

The genus name *Campylobacter* is derived from the Greek word *Campylo* meaning "curved". The genus was first proposed in 1963 by Sebald and Vèron (1963) to include microaerophilic vibrio shaped bacteria that were different from *Vibrio cholerae* and other members of the genus *Vibrio* in a number of respects. The genus has recently been described by Vandamme et al. (1991) as follows: Slender, gram negative vibrioid cells, with the exception of *Campylobacter rectus* which is a straight rod. Cell are 0.2-0.5 μm wide and 0.5-5.0 μm long. The rods may have one or more helical turns and can be as long as 8 μm. They also appear S-shaped and gull-winged when two cells form short chains. Cells are nonsporeforming and may form spherical or coccoid bodies in old cultures. It has been suggested that these coccoid bodies may be viable but nonculturable, whereas others believe they are degenerative forms and not viable. Cells are motile with a characteristic corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell. Campylobacters are microaerophilic. They possess a strictly respiratory type of metabolism and, in the absence of other terminal electron acceptors, they require an oxygen concentration of 3 to 15% (vol/vol) and a carbon dioxide concentration of 3 to 5%. Some species can grow under anaerobic conditions with either fumarate, formate + fumarate, or H_2 + fumarate in the medium. Fumarate serves as the terminal electron acceptor and formate and H_2 as the electron donor. Some species can use nitrate or trimethylamine oxide as a terminal electron acceptor for anaerobic respiration. Campylobacters are chemoorganotrophic. They neither ferment nor oxidize carbohydrates and instead obtain carbon and energy form amino acids, tricarboxylic acid cycle intermediates, or other organic acids. In some species the oxidation of H_2 or formate can serve as a major source of energy. Neither blood nor serum is required for growth. Methyl red, Voges-Proskauer, gelatin hydrolysis and lipase activity are all negative. Cells are oxidase-positive and urease-negative, except for some strains of *C. lari* which are urease-positive. Pigments are not produced. Menaquinone-6 and methyl-substituted menaquinone-6 are the major cellular quinones. Some species are pathogenic

for humans and animals; others seem to be part of the normal flora found in the reproductive organs, intestinal tract and oral cavity of humans and animals. The mol% G + C of the DNA ranges from 30 to 38. The type species is *Campylobacter fetus*.

At present, the genus contains the following species: *Campylobacter fetus, Campylobacter hyointestinalis, Campylobacter mucosalis, Campylobacter concisus, Campylobacter sputorum, Campylobacter jejuni, Campylobacter coli, Campylobacter lari, "Campylobacter upsaliensis," Campylobacter curvus, and Campylobacter rectus.*

The Genus *Wolinella*

The genus *Wolinella* has been described by Tanner and Socransky (1984) as follows: Cells are slender, nonsporeforming, gram-negative, helical or curved. They are 0.5-1.0 by 2-6 μm, with tapered ends. Cells show a rapid, darting motility by means of a single polar flagellum. Cells are H_2- and formate-requiring microaerophiles capable of respiring with oxygen. In the absence of alternative terminal electron acceptors such as fumarate, they exhibit O_2-dependent growth with low levels of oxygen and do not grow anaerobically or aerobically (Wolin and Jacobs 1961). Fumarate and nitrate are used as electron acceptors for anaerobic respiration. Chemoorganotrophic. Carbohydrates are neither fermented or oxidized, nor do they support growth. Oxidase-positive and catalase-negative. The organisms were originally isolated from bovine rumen fluid (Wolin et al. 1961) but have also been isolated from humans with gingivitis, periodontitis pockets, and lesions in alveolar bone (Wolin et al. 1961; van Palenstein Helderman et al. 1976; Smibert and Holdeman 1976; Tanner et al. 1981). The type (and only) species is *Wolinella succinogenes*.

The Genus *Helicobacter*

The genus *Helicobacter* as described by Goodwin et al. (1986) contains helical, curved, or straight unbranched gram-negative cells that are 0.3 to 1.0 μm wide and 1.5 to 5.0 μm long and have rounded ends and spiral periodicity. Non-sporeforming. Cells in old cultures may form spherical or coccoid bodies. A darting type of motility occurs by means of a single polar flagellum (*Helicobacter cinaedi* and *Helicobacter fennelliae*) or multiple unipolar or bipolar and lateral flagella (*Helicobacter pylori* and *Helicobacter mustelae*). The flagella are sheathed (Goodwin et al. 1989; Han et al. 1989). Microaerophilic with a respiratory type of metabolism. Chemoorganotrophs. Carbohydrates are neither oxidized nor fermented. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates but not from carbohydrates. Optimal growth occurs at 37°C in a humid atmosphere on agar media; no growth occurs at 25°C. Hydrogen is required or stimulates growth. No growth occurs in the presence of 3.5% NaCl. Growth occurs in the presence of 0.5% glycine and 0.04% triphenyltetrazolium chloride. Catalase-positive. Oxidase-positive. No pigment is produced. No H_2S production occurs in triple sugar iron agar when tested by the method of Roop et al. (1984). No hydrolysis of hippurate occurs. Susceptible to ampicillin, gentamicin, rifampin, and tetracycline; resistant to trimethroprim. Variable resistance to nalidixic acid, cephalothin, metronidazole, and polymyxin. The G+C content of the DNA ranges form 35 to 44 mol%. Isolated from gastric mucosa of humans and animals, from blood and feces of homosexual males, and from intestines of hamsters. Some organisms in the genus may be associated with gastritis and peptic ulceration. The type species is *Helicobacter pylori*.

The genus contains the following species: *Helicobacter pylori, Helicobacter cinaedi, Helicobacter fennelliae* and *Helicobacter mustelae*.

The Genus *Arcobacter*

The genus Arcobacter has been described by Vandamme et al. (1991) as follows: Cells are gram-negative nonsporeforming rods 0.2 to 0.9 μm wide and 1.0 to 3.0 μm long. Cells usually appear curved, S shaped, or helical. Motile (with a darting, corkscrewlike motion) by means of a single polar unsheathed flagellum. Growth occurs at 15, 30 and 37°C; no growth occurs at 42°C. Growth occurs in an air atmosphere but optimal growth occurs under microaerobic conditions (3 to 10% O_2). Hydrogen is not required for microaerophilic growth. Growth occurs in the presence of 1 and 2% NaCl and 1% pteridine vibriostatic compound O/129. No growth occurs in the presence of 1% glycine and 0.1% triphenyltetrazolium chloride. Catalase-positive. Oxidase-positive. Nitrate is reduced. Methyl red and Voges Proskauer tests are negative. Indole is not produced. Carbohydrates are neither fermented nor oxidized. Organic and amino acids are utilized as carbon sources. Hippurate, esculin, starch, and DNA are not hydrolyzed; gelatin is not liquefied. Nonhemolytic. Menaquinone 6 and a second atypical menaquinone 6, the identity of which remains to be established, are the major respiratory quinones. Strains have been isolated form root-associated sediments and roots of salt marsh plants, from aborted fetuses of several species of farm animals, and from various other animal and human sources. Pathogenicity is unknown. The DNA base composition ranges from 28 to 31 mol% G+C. the type species is *Arcobacter nitrofigilis*.

The genus contains the following organisms: *Arcobacter nitrofigilis* and *Arcobacter cryaerophila* (*sic*).

Theories Explaining Microaerophily

Microaerophiles are organisms for which oxygen is both beneficial (they can use it for energy-yielding respiration) and detrimental (they are poisoned by high levels of oxygen and its

derivatives). By definition, a microaerophile is an organism that is capable of O_2-dependent growth and can use O_2 as a terminal electron acceptor; however, it either cannot grow, or grows very poorly, at the concentration of O_2 present (21%) in air (Krieg and Hoffman 1986). Some microaerophiles may be able to grow anaerobically by using alternative terminal electron acceptors or fermentative pathways. However, their requirement for low levels of O_2, when O_2 is used as a terminal electron acceptor, is what distinguishes them from anaerobes that can merely tolerate low levels of O_2, and from aerobes or facultative anaerobes.

Several theories have been proposed to explain the microaerophilic nature of organisms such as *Campylobacter, Helicobacter*, and *Wolinella*. These theories are given below.

High Susceptibility to Toxic Forms of Oxygen in Culture Media. Hoffman, George et al. (1979) proposed that microaerophilic bacteria are more susceptible to toxic forms of oxygen in culture media. Compounds that quench toxic forms of oxygen include superoxide dismutase (SOD), catalase, and a combination of sodium or potassium ferrous sulfate, metabisulfite, and pyruvate (FBP mixture) (Blakemore et al., 1979; Bolton and Coates 1983; Burton and Morita 1964; George et al. 1978; Hoffman, George et al. 1979; Jones et al. 1970; Padgett et al. 1982; Steiner et al. 1984). These compounds act to enhance the aerotolerance of various microaerophilic organisms when added to the culture media. Since it is unlikely that enzymes such as SOD and catalase can penetrate the bacterial cells, they most likely act on toxic forms of oxygen that are external to the cell's cytoplasm.

Under microaerobic conditions, the generation of toxic forms of oxygen in the culture media would necessarily be lower than under aerobic conditions, thus allowing a more favorable environment for microaerophiles to grow.

Excessive Metabolic Generation of Toxic Forms of Oxygen. It is possible that microaerophiles may produce and excrete toxic forms of oxygen themselves. Niekus et al. (1977) demonstrated that *C. sputorum bv. bubulus* generates superoxide radicals ($O_2^{-\cdot}$) during formate

and lactate oxidation, and hydrogen peroxide (H_2O_2) during formate oxidation via a H_2O_2-producing formate oxidase. It is the latter enzyme that Niekus et al. have suggested may contribute to the microaerophilic nature of *C. sputorum*. Growth was slowed in experiments in which the oxygen tension in cultures was raised from 2 to 15 kPa (2 to 15%), and this was correlated with a loss of lactate and formate dehydrogenase activity. The latter enzyme was shown to be H_2O_2-sensitive. Therefore, it was proposed that the inhibition of growth and oxygen consumption was due to the sensitivity of formate dehydrogenase to the H_2O_2 generated by formate oxidase. However, even when *C. sputorum* is cultivated in the absence of formate (as, for example, in Brucella medium), it is still microaerophilic (George et al. 1978). In addition, George et al. (1978) found that FBP mixture—a medium supplement that acts to remove H_2O_2—had little effect on enhancing growth of *C. sputorum*. Thus the sensitivity of formate dehydrogenase to H_2O_2 is not the only factor responsible for the microaerophilic nature of the organism.

Low Rates of Oxygen Uptake. It has been suggested that microaerophiles may possess unusually low respiratory rates in comparison to aerobes or facultative anaerobes. If respiratory rates are low, oxygen that fails to become reduced at the cell surface could reach the cell interior and irreversibly inactivate oxygen-labile cell components such as iron-sulfur proteins. However studies with *C. jejuni* (Hoffman et al. 1979) and *Spirillum volutans* (Caraway and Krieg 1974) showed these organisms to have reasonably high respiratory rates. This suggests that low respiratory rates are probably not responsible for the microaerophilic nature of these organism.

High Rates of Oxygen Uptake. High rates of oxygen uptake may lead to the generation of toxic forms of oxygen as a result of the increased level of activity of various respiratory chain components. NADH dehydrogenase and ubisemiquinone, two components of the mitochondrial respiratory chain, are examples redox systems known to generate $O_2^{-\cdot}$ (Turrens and Boveris 1980; Turrens *et al.* 1982, 1985). The $O_2^{-\cdot}$ can in turn react with H_2O_2 in the presence of iron or copper to yield $OH\cdot$. Also, $O_2^{-\cdot}$ itself can directly inactivate several enzymes such as catalase

and glutathione peroxidase; since the latter two enzymes function to eliminate H_2O_2, their inactivation could lead to a decreased oxygen tolerance (Kono and Fridovich 1982, Blum and Fridovich 1985). Several other flavoenzymes have also been shown to produce toxic forms of oxygen. NAD(P)H-dependent flavoenzymes glutathione reductase, lipoyl dehydrogenase and ferredoxin-$NADP^+$ oxidoreductase can catalyze one-electron reduction of metal ions such as chromium(VI) and vanadium(V) and simultaneously reduce O_2 to generate H_2O_2 and $OH\cdot$ (Shi and Dalal, 1990). Under high respiratory rates, increased levels of activity of these enzymes could lead to the production of excessive amounts of toxic forms of oxygen, thereby resulting in damage to various biological components.

Lack of Protective Enzymes. Enzymes such as catalase, peroxidase and superoxide dismutase act to remove toxic derivatives of oxygen. It is possible that microaerophiles might lack sufficient levels of these protective enzymes. *S. volutans* (Padgett et al. 1982), *A. magnetotacticum* (Maratea and Blakemore 1981), *Agromyces ramosus* (Jones et al. 1970), *B. hermsii* (Austin et al. 1981), *Beggiatoa* spp. (Burton and Morita 1964), and several species of *Campylobacter*, including *C. sputorum* bv. *sputorum* and *bubulus* (Roop et al. 1985) lack catalase activity. Interestingly, several of the catalase-negative organism do possess SOD activity [*S. volutans* (Padgett et al. 1982); *B. hermsii* (Burton and Morita 1964); and *C. sputorum* bv. *sputorum* and *bubulus* (Niekus et al. 1978)], suggesting the lack of catalase may be more important than the lack of SOD for cell survival.

Peroxidase, which acts to protect cells by removing H_2O_2, may also play an important role in aerotolerance. Studies with *Spirillum volutans* indicated that the wild type strain is extremely sensitive to hydrogen peroxide with growth inhibition in modified peptone-succinate-salts (MPSS) broth under microaerobic conditions occurring with as little as 3×10^{-6} percent H_2O_2 (Padgett et al. 1982). In comparison, an aerotolerant mutant of *S. volutans* was shown to possess twice the tolerance to H_2O_2 than the wild type (Padgett et al. 1982). In order to determine the source of this difference, Padgett et al. assayed the cell-free extracts of the wild type and mutant for their specific

activities of SOD, catalase, *o*-dianisidine peroxidase, cytochrome *c* peroxidase and NADH-peroxidase. The only enzyme activity that differed significantly between the wild type and mutant was *o*-dianisidine peroxidase. The level of this activity was 13 times higher in the mutant. This suggests that the aerotolerance of mutant strain may be due to *o*-dianisidine peroxidase's ability to remove H_2O_2.

Although the absence or unusually low levels of protective enzymes may be a significant factor affecting aerotolerance, several microaerophiles possess the enzymes catalase, along with peroxidase and SOD, yet they remain microaerophilic. The specific activity for catalase, SOD and peroxidase in *Campylobacter jejuni* [expressed as micromoles of substrate converted per min (international units) per mg protein] is 2.4, 4.1, and 0.013, respectively (Vercellone et al., 1990). Microaerophily in these organisms may be due to the ineffectiveness of intracellular catalase, peroxidase and SOD to provide protection against exogenous H_2O_2 and O_2^- in the culture media, as indicated by the enhancement of aerotolerance that occurs when catalase and SOD are added to the media (Hoffman et al. 1979).

Occurrence of Unusually Oxygen-Sensitive Cell Constituents. It has been suggested that *Campylobacter*, *Wolinella*, *Helicobacter* and *Arcobacter* species contain several cell components which may serve as the site of attack by toxic forms of oxygen. Possible targets include proteins associated with the outer membrane, redox active components of the cytoplasmic membrane and enzymes in the periplasmic space. Low-potential cytochromes and cytochrome oxidase represent important components of the cell membrane that may be sensitive to the damaging effects of oxygen. Hoffman et al. (1979) reported a low-redox potential cytochrome-*c* in *C. jejuni*, and a similar cytochrome has been reported in other species of *Campylobacter* (Niekus et al. 1980, Harvey and Lascelles, 1980). Upon partial purification, Hoffman et al. demonstrated that the cytochrome was inactivated by excessive amounts of H_2O_2 or $O_2^{\cdot -}$, suggesting these cytochromes may be possible targets for inactivation.

Campylobacter, *Helicobacter*, *Wolinella* and *Arcobacter* may also contain flavoproteins and iron-sulfur proteins such as ferredoxin which may be sensitive to toxic oxygen derivatives. Flavodoxins and ferredoxins have a very electronegative E_h and are easily autooxidizable; thus in the presence of strong oxidizing agents such as O_2 or H_2O_2 they could be inactivated. Flavoproteins have been demonstrated in *C. jejuni* (P. S. Hoffman, unpublished results), but their function remains unknown. It is possible that flavodoxins or ferredoxins may replace nicotinamide adenine dinucleotide as the principal carrier of reducing power within the cell. A ferredoxin has been shown to occur in *T. pallidum*, as indicated by the ability of extracts of the treponeme to reduce metronidazole and by the occurrence of pyruvate:ferredoxin oxidoreductase (H. A. George, R. M. Smibert, J. N. Miller, personal communication). *C. fetus* probably contains a ferredoxin or flavodoxin, as indicated by the ability of the pyruvate and α-ketoglutarate dehydrogenase systems in crude extracts to reduce metronidazole (Lascelles and Calder 1985). Since it is likely that toxic derivatives of oxygen (i.e., H_2O_2, $OH\cdot$, $O_2^{-\cdot}$) exert their damaging effects external to the cell's cytoplasm, the presence of these oxygen-labile redox compounds might be a factor in explaining the microaerophilic nature of these organisms.

Mechanisms of Pyruvate Oxidation

The oxidative decarboxylation of 2-oxoacids such as pyruvate is a key reaction of intermediary metabolism. Most organisms exploit the high reducing power of these substrates for the reduction of a low-E'_0 electron carrier and the concomitant formation of an energy-rich thioester linkage between coenzyme A and the resulting carboxylic acid (e.g., acetic acid or succinic acid) (Kerscher and Oesterhelt 1977).

During aerobic respiration, most bacteria link the electron acceptor NAD^+ to a 2-oxoacid dehydrogenase multienzyme complex. The enzyme complex is made up of large aggregates of three

different enzymes, each of which catalyses one of the three partial reactions. Under anaerobic conditions, NAD^+ would tend to act as an electron trap, which has only a limited capacity because the E'_0 of the NAD^+/NADH oxidation/reduction (O/R) system is not low enough for many reduction reactions.

Anaerobic bacteria rely on alternative mechanisms for pyruvate utilization. Pyruvate:ferredoxin oxidoreductase is an example of such an alternative pathway. This enzyme uses low-potential electron carriers such as flavodoxin, ferredoxin, or rubredoxin for pyruvate oxidation; thus the high reducing power of pyruvate can be used for reactions requiring stronger reductants than NADH. The overall reaction catalyzed by this enzyme is as follows:

$$\text{Pyruvate} + \text{CoASH} + NAD^+ \rightarrow \text{Acetyl-S-CoA} + CO_2 + \text{NADH} + H^+$$

Pyruvate Oxidation Under Aerobic Conditions. The oxidation of pyruvate by *Escherichia coli* under aerobic conditions is carried out by a well characterized pyruvate dehydrogenase multienzyme complex. This complex, as seen in Figure 1, consists of three enzymes: 24 molecules each of pyruvate dehydrogenase (E1) and dihydrolipoate transacetylase (E2) and 12 molecules of dihydrolipoate dehydrogenase (E3). The core of the enzyme complex is formed by the E3 molecules while the E1 and E2 molecules are bound to the periphery. The first step in the oxidative decarboxylation is the addition of pyruvate to C-2 of the thiazolium ring of thiamine pyrophosphate (TPP) contained within E1. This results in the formation of lactyl-TPP-E1. The lactyl-TPP-E1 is next decarboxylated yielding hydroxyethyl-TPP-E1. The hydroxyethyl moiety is then transferred from TPP to the lipoate group of E2, and the sulfide bond of lipoate is reduced concomitantly. The acetyl group thus formed is then released as acetyl-CoA, and under catalysis of E3 the sulfhydryl form of lipoate is oxidized by NAD^+ to the disulfide form. The enzyme complex is then ready for the oxidation of another molecule of pyruvate to acetyl-CoA.

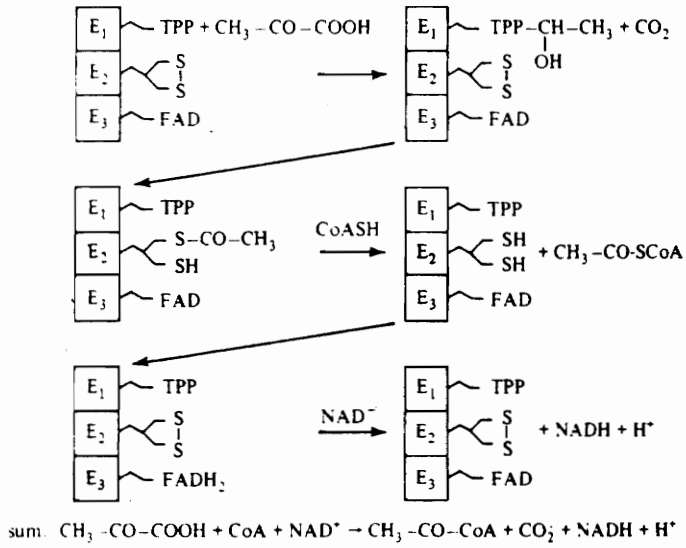

Figure 1. Reactions catalyzed by the pyruvate dehydrogenase complex. E_1, pyruvate dehydrogenase; E_2, dihydrolipoate transacetylase; E_3, dihydrolipoate dehydrogenase; TPP, thiamine pyrophosphate; the disulfide compound linked to E_2 is the oxidized form of lipoate. (From Gottschalk 1986).

Pyruvate Oxidation Under Anaerobic Conditions. One possible mechanism for the oxidation of pyruvate under anaerobic conditions involves the enzyme pyruvate:ferredoxin oxidoreductase. The overall reaction catalyzed by this enzyme is as follows:

$$\text{Pyruvate} + \text{CoASH} + \text{Fd}_{(ox)} \rightleftharpoons \text{Acetyl-S-CoA} + \text{CO}_2 + \text{Fd}_{(red)}$$

Unlike the 2-oxacid dehydrogenase complex this enzyme lacks lipoic acid and has a relatively simple structure comprising only two types of subunits suggesting fundamental differences between the catalytic mechanism for both classes of enzyme. The proposed catalytic mechanism for pyruvate:ferredoxin oxidoreductase, described by Kerscher and Oesterhelt (1981), is illustrated in Figure 2. The first step is the binding of the 2-oxoacid to TPP, followed by decarboxylation to yield hydroxyalkyl-TPP. This intermediate then donates one electron to the iron-sulfur cluster (and further to ferredoxin) to form a stable free radical. This complex is stable until reaction with coenzyme A causes the transfer of the second electron to an enzyme-bound iron-sulfur cluster. The resulting acetyl group is transferred concomitantly to coenzyme A. Reoxidation of the iron-sulfur cluster by ferredoxin completes the cycle. Thus the catalytic cycle of pyruvate:ferredoxin oxidoreductase consists of four steps. Two steps of one-electron transfer are catalyzed by iron-sulfur clusters whereas the conversion of pyruvate to acetyl CoA is performed at a single active center by the action of a single prosthetic group, TPP.

METRONIDAZOLE

Metronidazole [Flagyl; 1-(2-hydroxylethyl)-2methyl-5-nitroimidazole] is used extensively to treat infections due to anaerobic protozoa and bacteria. It is believed that the compound exerts its toxic effects after reduction of the nitro group has taken place. The basis for the selective toxicity of metronidazole towards anaerobes lies in the reduction potential at which the nitro group is reduced. The E'_0 of such a reaction is about -450 mV, which is thermodynamically compatible with that of

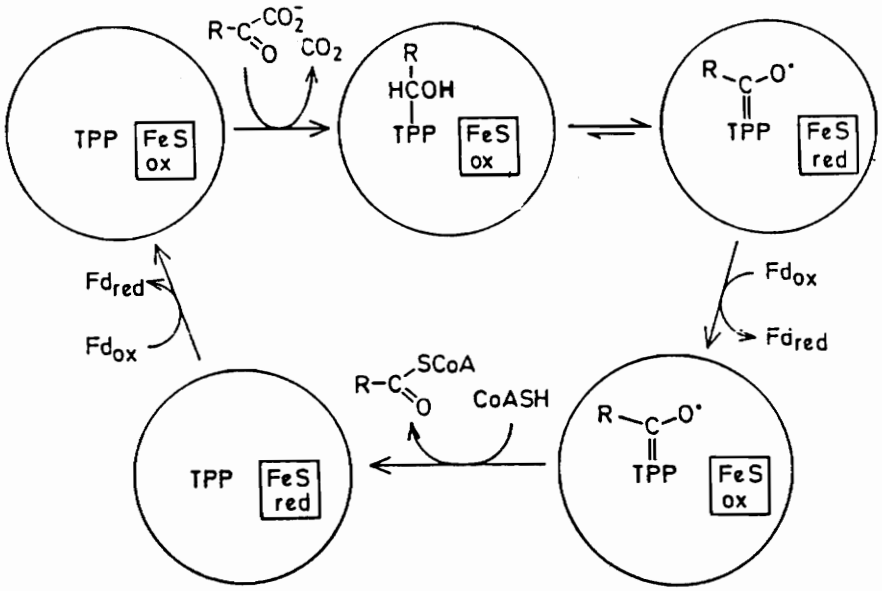

Figure 2. The proposed mechanism of ferredoxin-linked pyruvate oxidoreductase. (From Kerscher and Oesterhelt 1981.)

low-potential electron carriers such as ferredoxin molecules (approximately -430 to -460 mV) (Edwards 1980). Because the lowest E'_0 of the O/R systems produced by aerobes is approximately -350 mV, it is unlikely that aerobes are able to reduce metronidazole. Once reduction of metronidazole takes place a favorable concentration gradient is established in which unreduced drug enters the cell relatively rapidly as the internal concentration is decreased due to reduction (Edwards 1980). Although the reduction of metronidazole does cause temporary inhibition of ferredoxin-linked reactions (e.g., pyruvate:ferredoxin oxidoreductase), these reactions return to normal once the compound is completely reduced. The reduced form of metronidazole is then able to exert its toxic effect on the cell. Reduced metronidazole has been shown to act primarily on DNA, causing loss of DNA helix content, single- and double-strand breaks in DNA, and impairment of the functioning of DNA as an enzyme template (Edwards 1979).

Many microaerophilic bacteria have been shown to be sensitive to metronidazole (Andreasen 1987; Benjamin et al. 1983; Gebhart 1985; Morgan 1987). This strongly suggests that ferrredoxin or flavodoxin linked reactions occur in these bacteria. In anaerobic bacteria one source of electrons for reduction of ferredoxin is pyruvate. The oxidation of pyruvate is catalyzed by a pyruvate:ferredoxin oxidoreductase such as those described for *Treponema phagedenis* (George and Smibert 1982) and *Clostridium acidiurici* (Uyeda and Rabinowitz 1971). Pyruvate:ferredoxin oxidoreductase, if present in microaerophilic bacteria, could be a key target for inactivation by oxygen since the enzyme is unstable in the presence of oxygen and can be assayed only under anaerobic conditions.

Due to the oxygen sensitivity of pyruvate:ferredoxin oxidoreductase, the location of the enzyme within the bacterial cell may play an important role in determining the stability of the enzyme. It is possible that toxic derivatives of oxygen exert their damaging effects as they penetrate the envelope of bacterial cells, damaging respiratory components and enzymes associated with the cytoplasmic membrane. Further damage to cell components may be averted if the cells contain

catalase, peroxidase or SOD. These enzymes will react with toxic forms of oxygen, converting them to less reactive or nonreactive forms. In the absence of these protective enzymes, reactive oxygen species will continue to exert their damaging effects. If pyruvate:ferredoxin oxidoreductase is a membrane-bound enzyme it might be readily susceptible to damage by toxic oxygen derivatives as they penetrate the cell. As a membrane-bound molecule, pyruvate:ferredoxin oxidoreductase would receive little protection from catalase, peroxidase and SOD. This is due to inability of these enzymes to destroy oxygen radicals prior to their entry into the cell. Alternatively, if the pyruvate:ferredoxin oxidoreductase is located within the cytoplasm of the cell it would not only avoid the damaging affects of oxygen radicals as they enter the cell, it would also be protected by enzymes capable of removing the toxic oxygen derivative after they are inside the cell. Determining the location of pyruvate:ferredoxin oxidoreductase within the bacterial cell could lead to a greater understanding of the importance of this enzyme as it relates to the microaerophilic nature of organisms such as *Campylobacter*, *Wolinella*, *Helicobacter* and *Arcobacter*.

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Occurrence of pyruvate:ferredoxin oxidoreductase in *Campylobacter, Wolinella, Helicobacter,* and *Arcobacter* species

Abstract

Pyruvate:ferredoxin oxidoreductase activity was demonstrated in microaerophilic members of *Campylobacter, Wolinella,* and *Helicobacter*. *Arcobacter cryaerophila* (sic) and *Arcobacter nitrofigilis*, two aerobic species, lacked any detectable activity. Under anaerobic conditions crude extracts of *Campylobacter*, *Helicobacter* and *Wolinella* were capable of reducing the electron carriers benzyl viologen and metronidazole in the presence of pyruvate. Addition of *Clostridium pasteurianum* ferredoxin to the metronidazole-linked reaction enhanced metronidazole-reducing activity, suggesting that electron transport to artificial electron carriers is facilitated by ferredoxin. All species exhibited varying degrees of sensitivity to metronidazole (MIC = <0.8 to 25 μg/ml) except *Arcobacter cryaerophila*, which was resistant to >100 μg/ml. This further supports the theory that these organisms possess ferredoxin-linked reactions. The presence of the oxygen-labile enzyme pyruvate:ferredoxin oxidoreductase may be related to the inability of these microaerophilic bacteria to grow in normal atmospheric levels of oxygen.

Introduction

Members of the genera *Campylobacter, Wolinella, Arcobacter, and Helicobacter* are associated with a variety of human and animal diseases. Physiologically, these organisms are microaerophiles: they can respire with oxygen under reduced O_2 concentrations (3-6%) but a normal atmospheric concentration of oxygen (21%) is toxic. Several theories have been proposed to explain the microaerophilic nature of these genera. One theory is that microaerophiles such as *Campylobacter, Wolinella, Arcobacter*, and *Helicobacter* possess essential cell constituents that may be inactivated by molecular oxygen or by toxic forms of oxygen such hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{-\cdot}$), or hydroxyl radicals ($OH\cdot$) (Krieg and Hoffman 1986). One candidate for such an oxygen-labile cell constituent would be pyruvate:ferredoxin oxidoreductase, a key enzyme in bacterial metabolism that occurs characteristically in anaerobic bacteria, such as *Treponema phagedenis* (George and Smibert 1982), *Clostridium acidiurici* (Uyeda and Rabinowitz 1971).

In anaerobic bacteria, pyruvate is oxidized to acetyl-coenzyme A (acetyl-CoA) and CO_2 by the ferredoxin-linked pyruvate oxidoreductase, whereas in aerobes and facultative anaerobes pyruvate is oxidized by an NAD-linked pyruvate dehydrogenase. It is interesting that two microaerophilic bacteria—*Treponema pallidum* and *Campylobacter fetus* subsp. *fetus*—have been shown to possess a pyruvate:ferredoxin oxidoreductase even though they respire with oxygen (H. A. George, R. M. Smibert, and J. N. Miller, personal communication; Lascelles and Calder 1985). Pyruvate:ferredoxin oxidoreductase might be a key target for inactivation by oxygen because it is highly unstable in the presence of oxygen and can be assayed only under anaerobic conditions. The presence of this enzyme might be related to the inability of microaerophilic bacteria to grow in normal atmospheric levels of oxygen. We report here the results of a survey conducted on members

of the genus *Campylobacter*, *Wolinella*, *Helicobacter*, and *Arcobacter* for the presence of pyruvate:ferredoxin oxidoreductase.

Materials and Methods

Bacterial strains and culture conditions

The *Campylobacter*, *Wolinella*, *Helicobacter* and *Arcobacter* strains used in this study are listed in Table 1. With the exception of *C. upsaliensis*, all the strains are the type strains of their respective species. Stock cultures of *C. coli*, *C. jejuni*, *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C hyointestinalis*, *C. lari*, *C. sputorum* and *C. upsaliensis* were grown under an air atmosphere at 37°C in Brucella semisolid medium containing 0.16% agar. *Arcobacter cryaerophila* (sic) and *Arcobacter nitrofigilis* were grown similarly but at 30°C. The medium for *A. nitrofigilis* was supplemented with 1.0% NaCl. *C. mucosalis*, *C. concisus*, *C. curv*us, *C. rectus*, *H. cinaedi*, *H. fennelliae*, and *W. succinogenes* were grown in Brucella semisolid medium at 37°C under an atmosphere containing 6% O_2, 3% CO_2 and 91% H_2. Stock cultures were transferred at weekly intervals.

For each enzyme assay, the top 2 or 3 ml of growth from a 48-h-old culture in semisolid Brucella medium was removed and inoculated into a Roux bottle containing the diphasic culture system described by Roop et al. (1984). The cultures were incubated at 37°C for 24-48 h under atmospheric conditions similar to those used for the stock cultures. An average of 15 to 20 Roux bottles were routinely used for each assay.

Cell disruption and preparation of crude extract

Cells were harvested by centrifugation (8,000 $\times g$ for 10 min) and washed twice with sucrose buffer [50 mM Tris/HCl, pH 7.3; 0.25 M sucrose; 1 mM $MgCl_2$; 1 mM dithioerythritol (DTE)]. After the final washing, cell pellets were suspended in the same buffer without DTE (1-2 ml buffer per gram wet weight packed cells). Cells were disrupted by a sonic dismembrator (4 charges at maximum output for 30 sec each). The cell suspension was pre-cooled to 0-5°C and flushed with O_2-free N_2 (N_2 was passed over a hot copper catalyst and bubbled through a solution of 0.3 mM

dithionite and 0.1 mM methyl viologen) prior to each charge. Cell debris and unbroken cells were removed by centrifugation at 37,000 × g for 20 min at 4°C. The supernatant, defined as crude extract, was decanted and immediately equilibrated with O_2-free N_2 by evacuating and refilling the vessel 12 times. The crude extract was stored on ice until the time of assay. Protein was estimated by the Bio-Rad dye-binding assay with bovine serum albumin as the protein standard (Bio-Rad Laboratories, Richmond Calif.).

Enzyme assays

Anaerobic assays were performed under a nitrogen atmosphere. Anaerobic conditions were achieved by using 1.5-ml, 1-cm cuvets fitted with rubber serum stoppers. Oxygen-free N_2 was passed through the liquid in the cuvets with a 1.5 in., 23-gauge needle that pierced the rubber stopper. The exhaust N_2 exited through a second 23-gauge needle. The needles were removed prior to the addition of the crude extract or other degassed reagents. Under these conditions 0.3 mM benzyl viologen reduced with ca. 0.2 mM dithionite showed a decrease in absorbance at 546 nm of less than 0.002/min. Degassed reagents and cell extract were added to the cuvet by a gas-tight syringe flushed with N_2 between additions.

Absorbance measurements were made with a Bausch and Lomb Model 2000 spectrophotometer equipped with recorder and water-jacketed cuvet chamber through which water at 37°C was flowing. One I.U. of enzyme activity was defined as the utilization of 1 μmole of substrate per min at 37°C.

Two methods were employed for assaying pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1). Both methods were performed under anaerobic conditions. The first method was a modification of the method of Wahl and Orme-Johnson (1986). Benzyl viologen was used as an electron acceptor instead of Fd because it provided a color change that could be measured readily. The mM extinction coefficient is 9.75 at 546 nm. The reaction mixture contained 50 mM potassium phosphate buffer

(pH 7.3), 0.1 mM coenzyme A (CoA), 20.0 mM sodium pyruvate, 1.0 mM benzyl viologen, 5.0 μM thiamine pyrophosphate (TPP), and 1.0 mM $MgCl_2$.

In order to demonstrate that pyruvate:ferredoxin oxidoreductase activity was dependent on low-potential electron carriers such as ferredoxin, rubredoxin, or flavodoxins, a sensitive spectrophotometric assay, similar to the hydrogenase-linked metronidazole reduction assay developed by Chen and Blanchard (1978), was used. The method is based on the fact that, of the reductants likely to be found at physiological concentrations in living cells, only ferredoxins (or flavodoxins and rubredoxins) will reduce metronidazole. The reaction mixture contained 50.0 mM potassium phosphate (pH 7.3), 0.1 mM CoA, 20.0 mM sodium pyruvate, 0.1 mM metronidazole and ferredoxin. Metronidazole reduction was followed spectrophotometrically at 320 nm (the millimolar extinction coefficient is 9.3). The rate of metronidazole reduction, which can be continuously monitored spectrophotometrically, correlates with the amount of ferredoxin (Fd) present in the reaction mixture.

Minimum inhibitory concentration (MIC) for metronidazole.

Brucella broth containing serial two-fold dilutions of metronidazole were prepared in triplicate and inoculated with 0.1 ml of a 48-h-old broth culture of the test organism. Tubes containing no metronidazole served as a positive control. The cultures were incubated at 30°C for *C. nitrofigilis* and *C. cryophila* and 37°C for the remaining organism under atmospheric conditions similar to those used for the stock cultures. The lowest concentration of metronidazole that completely inhibited growth, as indicated by lack of visible turbidity, was recorded as the minimum inhibitory concentration (MIC).

Results

Benzyl viologen-linked pyruvate:ferredoxin oxidoreductase activity.

Benzyl viologen-linked pyruvate:ferredoxin oxidoreductase activity was demonstrated in the cell-free crude extract of 13 species of *Campylobacter*, one species of *Wolinella*, and two species of *Helicobacter* (Table 1). *A. cryaerophila* and *A. nitrofigilis*, the two aerobic species, lacked any detectable activity. Benzyl viologen-linked activity in crude extracts was oxygen-labile; complete loss of activity occurred after 12-18 h of incubation at 4°C under aerobic conditions.

Metronidazole-linked pyruvate:ferredoxin oxidoreductase activity.

Table 1 lists the specific activity of pyruvate:ferredoxin oxidoreductase using metronidazole as the electron acceptor. Under anaerobic conditions, crude extracts of *Campylobacter*, *Wolinella* and *Helicobacter* species were capable of reducing metronidazole in the presence of pyruvate. The addition of *Clostridium pasteurianum* ferredoxin to the reaction enhanced metronidazole-reducing activity.

Minimum Inhibitory Concentrations of Metronidazole

To determine whether a correlation exists between activity of pyruvate:ferredoxin oxidoreductase and susceptibility to metronidazole the minimum inhibitory concentration (MIC) of metronidazole was determined. As shown in Table 2, all species of *Campylobacter*, *Wolinella*, *Helicobacter* and *Arcobacter* exhibited varying degrees of sensitivity to metronidazole with the exception of *Arcobacter cryaerophila*. Resistance in *Arcobacter cryaerophila* exceeded 100 μg/ml.

Table 1. Specific activity of pyruvate:ferredoxin oxidoreductase using benzyl viologen and metronidazole as electron acceptors

Species[a]	Specific activity (I.U./mg protein)		
	Benzyl viologen	Metronidazole	
		No added ferredoxin	Ferredoxin added[b]
C. coli ATTC 33559^T	1.70	0.010	0.027[c]
C. jejuni ATCC 33560^T	4.77	0.060	0.170[c]
C. lari NCTC 11352^T	4.04	0.032	0.066[c]
C. upsaliensis CG-1	1.02	0.000	0.007[c]
C. fetus subsp. fetus ATCC 27374^T	2.20	0.029	0.070[c]
C. fetus subsp. venerealis ATCC 19438^T	0.47	0.026	0.040[c]
C. hyointestinalis ATCC 35217^T	1.35	0.054	0.110[c]
C. concisus ATCC 33237^T	0.10	0.005	0.015[d]
C. mucosalis NCTC 11000^T	0.12	0.004	0.010[d]
C. sputorum ATCC 35980^T	1.42	0.030	0.052[d]
C. curvus ATCC 35224^T	0.21	0.006	0.013[d]
C. rectus ATCC 33238^T	0.26	0.005	0.020[d]
H. cinaedi ATCC 35683^T	0.23	0.006	0.015[d]
H. fennelliae ATCC 35684^T	1.47	0.020	0.034[c]
W. succinogenes ATCC 29543^T	3.00	0.014	0.052[d]
A. cryaerophila NCTC 11885^T	<0.01	ND[e]	
A. nitrofigilis ATCC 33309^T	<0.01	ND	

[a] ATCC, American Type Culture Collection, Rockville, MD, U.S.A.; NCTC, National Collection of Type Cultures, London, England; ^{T}, type strain of species.

[b] Fd, ferredoxin from *Clostrdium pasteurianum*.

[c] 40 μg/ml ferredoxin added to the reaction mixture.

[d] 50 μg/ml ferredoxin added to the reaction mixture.

[e] ND, not determined.

Table 2. Minimum inhibitory concentrations (M.I.C.) of metronidazole

Species	M.I.C. (μg/ml)
C. coli	12.5
C. jejuni	12.5
C. lari	12.5
C. upsaliensis	3.1
C. fetus subsp. fetus	6.2
C. fetus subsp. venerealis	6.2
C. hyointestinalis	12.5
C. concisus	3.1
C. mucosalis	25.0
C. sputorum	1.6
C. curvus	6.2
C. rectus	12.5
H. cinaedi	12.5
H. fennelliae	<0.8
W. succinogenes	3.1
A. cryaerophila	>100.0
A. nitrofigilis	25.0

Discussion

Initially, enzyme activity was measured with the benzyl viologen-linked assay for pyruvate:ferredoxin oxidoreductase activity. The results suggested that the enzyme activity was present in all species tested except *Arcobacter cryaerophila* and *Arcobacter nitrofigilis*.

The metronidazole-linked assay method was used to demonstrate that the enzyme reaction was dependent on ferredoxin, rubredoxin or flavodoxin. Metronidazole is reduced by reduced ferredoxin (or flavodoxin or rubredoxin) but not by the enzyme directly (Lindmark and Mueller 1976). Also, it is not reduced by NADH (Coombs and Rabin 1974; Chen and Blanchard 1979). Therefore a correlation can be drawn between the amount of reduced ferredoxin and the rate of metronidazole reduction. As the results in Table 1 indicate, all species of *Campylobacter*, *Helicobacter* and *Wolinella* tested are able to reduce metronidazole. Furthermore, the stimulation of activity caused by addition of ferredoxin to the reaction mixture provides further evidence that the reaction is dependent on ferredoxin.

The specific activities listed in Table 1 for the two enzyme assay methods indicate that that specific activities were much higher when estimated by the benzyl viologen-linked assay method than by the metronidazole-linked assay method. This could be due to the requirement for ferredoxin as an intermediate carrier of electrons from the enzyme to metronidazole. In the benzyl viologen-linked assay method, the indicator dye is directly reduced by the enzyme.

The lack of enzyme activity in *Arcobacter cryaerophila* and *A. nitrofigilis* suggests that these two species most likely depend on the aerobic enzyme pyruvate dehydrogenase. The presence of this enzyme is indicated by the ability of crude extracts to catalyze the lipoic acid-mediated oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 (unpublished observation).

The inhibition of most species of *Campylobacter*, *Helicobacter* and *Wolinella* by <12.5 μg/ml of metronidazole (Table 2) also supports the theory that these organisms possess ferredoxin-linked

reactions. The aerobic *Arcobacter cryaerophila* and *Arcobacter nitrofigilis* displayed a high resistance to metronidazole in comparison to most of the microaerophilic genera, which is in accord with the lack of detectable pyruvate:ferredoxin oxidoreductase activity (Table 1). However, *C. mucosalis* displayed a level of resistance as high as that of *A. nitrofigilis*. Possible reasons for this unexpected resistance include low permeability of the drug, enzymatic modification of the drug, or a paucity of low-potential electron carriers in the cell.

Determining the effects of superoxide radicals and hydrogen peroxide on the activity of pyruvate:ferredoxin oxidoreductase, and also determining the cellular location of this enzyme, could lead to a greater understanding of the importance of this enzyme to microaerophilic organisms. If the enzyme is a membrane-bound enzyme, toxic derivatives of oxygen such as superoxide radicals or hydrogen peroxide in the culture medium might be able to damage it as they penetrate the cell envelope. Cytoplasmic catalase, peroxidase or superoxide dismutase would be unable to prevent this damage because of their inability to destroy toxic forms of oxygen prior to their entry into the cell. This could explain how some microaerophiles such as *C. jejuni* are poisoned by oxygen even though they possess superoxide dismutase and catalase (Hoffman et al. 1979). On the other hand, if pyruvate:ferredoxin oxidoreductase is a cytoplasmic enzyme it might be protected by cytoplasmic superoxide dismutase and catalase.

Due to the inability of microaerophiles such as *Campylobacter* species to utilize carbohydrates, it is possible that pyruvate:ferredoxin oxidoreductase may act to fix CO_2 and synthesize pyruvate. Pyruvate could then be reduced further by reversal of the Embden-Meyerhof pathway. This process, termed gloconeogenesis, could supply the cells with glucose, ribose, and other carbohdyrates which, in turn, would be used for cell wall and nucleic acid biosynthesis. Moreover, CO_2 fixation by pyruvate-ferredoxin oxidoreductase might explain why campylobacters require a CO_2-enriched atmosphere for growth.

In conclusion, the results presented in this report indicate that the enzyme pyruvate:ferredoxin oxidoreductase is present in members of the microaerophilic genera *Campylobacter*, *Helicobacter* and *Wolinella*, and absent in the genus *Arcobacter*, whose members are aerobic. The presence of this enzyme supports the theory that the occurrence of this oxygen-labile enzyme may be a contributing factor to the microaerophilic nature of these organisms.

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Appendix A

Additional Enzyme Assay Results

NAD-linked Pyruvate Dehydrogenase Activity

NAD-linked pyruvate dehydrogenase (E.C.1.2.4.1., E.C.2.3.1.12., and E.C.1.6.4.3.) catalyses the lipoic acid-mediated oxidative decarboxylation of pyruvate to acetyl-coenzyme A and CO_2. The enzyme was assayed under aerobic conditions. The overall reaction is most easily monitored by measuring the reduction of NAD spectrophotometrically at 338 nm (millimolar extinction coefficient = 6.22 at 338 nm). A modification of the procedure employed by Amarasingham and Davis (1965) for assaying the α-ketoglutarate dehydrogenase system in extracts of *E. coli* was used, with sodium pyruvate substituted for α-ketoglutarate. The reaction mixture contained (in a total volume of 3.0 ml): 1.0 ml of 500 mM Tris/HCl (pH 8.5), 0.1 ml of 2.6 mM coenzyme A, 0.1 ml of 78 mM L-cysteine, 0.1 ml of 10 mM TPP, 0.1 ml of 10 mM $MgCl_2$, 0.1 ml of 250 mM sodium pyruvate, and 0.1 ml of 60 mM NAD^+. All components except NAD^+ were preincubated for 3 min. The reaction was initiated by addition of NAD^+ to the cuvet and the rate of increase in absorbance at 338 nm was recorded. NAD^+ reduction was linear over a period of 1 min. The results for pyruvate dehydrogenase activity in *Campylobacter*, *Helicobacter*, *Arcobacter* and *Wolinella* are listed in Table 1.

Although the assay for pyruvate dehydrogenase was performed under aerobic conditions, it is possible that low-potential electron carriers present in the crude extract may interfere with the reaction. Reduced ferredoxin from a ferredoxin-linked pyruvate oxidoreductase-catalyzed reaction might reduce NAD to NADH, which would be misinterpreted as pyruvate dehydrogenase activity. Passage of the crude extract through an anionic exchange column would remove low-potential electron carriers such as ferredoxin. This would minimize interference of ferredoxin-linked reactions in the overall oxidation of pyruvate by pyruvate dehydrogenase.

The presence of an NADH oxidase system may also interfere in assaying pyruvate dehydrogenase activity: the oxidase might reoxidize NADH that was formed in the pyruvate dehydrogenase-catalyzed reaction to NAD^+, resulting in a falsely low activity for the

dehydrogenase. Performing the assay under anaerobic conditions would prevent this from happening.

Table 1. The specific activity of pyruvate dehydrogenase in campylobacters and related organisms

Species	Specific activity (I.U./mg protein)
C. coli	0.280
C. jejuni	0.030
C. lari	0.050
C. upsaliensis	0.010
C. fetus subsp. fetus	0.020
C. fetus subsp. venerealis	0.010
C. hyointestinalis	0.030
C. concisus	0.010
C. mucosalis	0.020
C. sputorum	0.030
C. curvus	0.006
C. rectus	0.005
H. cinaedi	<0.001
H. fennelliae	<0.001
W. succinogenes	<0.001
A. cyraerophila	0.040
A. nitrofigilis	0.030
E. coli	0.100

NAD-oxidase activity

NADH-oxidase activity was measured in *Campylobacter curvus* and *Campylobacter rectus* in order to determine if the apparent low levels of NAD-linked pyruvate dehydrogenase activity seen in these organisms might have been the result of the oxidation of the NADH that was generated during the conversion of pyruvate. In *C. curvus* the specific activity was 0.008 I.U./mg protein, and in *C. rectus* the activity was 0.006 I.U./mg protein. From Table 1, the specific activities of pyruvate dehydrogenase were in a similar range, viz., 0.006 and 0.005 I.U./mg protein, respectively. Thus these activities would probably have been higher if the pyruvate dehydrogenase assays had been performed under conditions which inhibit NADH oxidase, i.e., anaerobic conditions.

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Appendix B

Additional Comments on Methodology for Enzyme Assays

General Comments

1. Coenzyme-A is a critical component for both the metronidazole-linked and the benzyl viologen-linked assay for pyruvate:ferredoxin oxidoreductase. Prepare stock solution in O_2-free distilled water. Dispense 1-ml portions into tubes and store at -20°C under anaerobic conditions until time of assay. Other components for enzyme assays can be prepared and stored aerobically until the time of assay.
2. Sonication of whole cells is best performed using a glass vessel. The use of plastic or other non-glass items will diminish the efficiency of cell breakage.
3. Following cell breakage and removal of unbroken cells, the crude extract can be placed in a 50-ml side-arm flask fitted with a #3 rubber stopper. To ease the removal of crude extract from this vessel with a syringe, approximately ½ inch can be cut off from the bottom of the stopper.
4. Small volumes of crude extract and other reagents can be stored in 5-ml serum vials (Wheaton 223738) fitted with 20-mm sleeve stoppers (Wheaton 224094) for use in enzyme reactions.
5. Rubber serum vial stoppers for 1.5-ml, 1-cm cuvettes can be made by boring a flanged-type stopper (Thomas 1780-K30) with a #5 cork borer.
6. A brass manifold system was used for degassing reagents and cuvets. The manifold system has six ports, each fitted with rubber tubing and a glass stopcock to regulate the gas flow.
7. Degassed reagents and cell extract can be added to the cuvet by means of a gas-tight syringe (Hamilton 100-μl or 250-μl capacity).
8. Crude extract to be stored for protein assay can be stored under anaerobic conditions in 5-ml serum vials fitted with rubber stoppers wrapped in parafilm.

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