OXYGEN TOXICITY IN *CAMPYLOBACTER JEJUNI*: PHYSIOLOGICAL COMPARISON OF A MICROAEROPHILIC WILD-TYPE STRAIN WITH AN AEROTOLERANT MUTANT

by

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(ABSTRACT)

A comparative study of the microaerophilic *Campylobacter jejuni* strain H840 with an aerotolerant mutant, MC711-01, revealed that catalase and, to a lesser extent, SOD activity is correlated with the aerotolerance and enhanced resistance of MC711-01 to H_2O_2. When cells were cultured under 6% oxygen, the specific activity of catalase was significantly higher in crude extracts of MC711-01 than of H840. The catalase activity of MC711-01 more than doubled when cells were cultured under 21% oxygen, and this activity was 2.6 times greater than that of H840; no corresponding increase was observed in strain H840. There was no significant difference in the mean SOD activity of the two strains when cultured under 6% oxygen or in H840 cells cultured under either 6% or 21% oxygen; however, the SOD activity of MC711-01 increased 1.5 times when cells were cultured under 21% oxygen.

Survival studies revealed that MC711-01 was significantly more resistant to H_2O_2 when cultured under either 6% or 21% oxygen. However, both MC711-01 and H840 were more susceptible to H_2O_2 when grown

under 21% oxygen, indicating that both strains might be more highly stressed when the cells are grown at this oxygen tension.

The present study suggests that in C. jejuni, the level of catalase activity may influence the degree of susceptibility to H_2O_2 and consequently, the degree of aerotolerance.

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INTRODUCTION

All living organisms are susceptible to the toxic effects of oxygen, yet microaerophilic bacteria exemplify a unique paradox: they can use oxygen as a terminal electron acceptor for respiration but fail to grow in the levels of oxygen present in the air atmosphere (21% oxygen). One well known example of a microaerophilic bacterium is Campylobacter jejuni, the major causative agent of bacterial diarrhea in humans. Highly reactive derivatives of oxygen -- hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($^1\Delta gO_2$) -- have been implicated as the causative agents of oxygen toxicity. These toxic derivatives may arise as by-products of cellular metabolism as well as from the spontaneous autooxidation reactions or photochemical reactions in the environment. Microaerophilic bacteria are more susceptible to toxic forms of oxygen in culture media than are other oxygen-dependent bacteria, although the reasons for this are not known in many instances.

A number of theories have been suggested to explain oxygen toxicity including:

i) Insufficient levels or types of protective enzymes such as superoxide dismutase (SOD), catalase, or peroxidase.

ii) The presence of oxygen-labile cell components or essential enzymes.

iii) Low respiratory rates that are incapable of maintaining a reduced cell interior.

Despite these theories, the fundamental nature of oxygen toxicity is still only poorly understood. Probably no single theory will be applicable to all microaerophilic organisms.

Because oxygen is both beneficial and detrimental to microaerophilic bacteria, these organisms are particularly useful for studying the basis of oxygen toxicity. Moreover, a comparison of aerotolerant mutants with their microaerophilic wild types would be an important asset to the investigation of the nature of oxygen toxicity in microaerophiles.

In 1988, our laboratory published the isolation of an aerotolerant mutant of C. jejuni ATCC 29428. Although the mutant strain could grow at higher oxygen levels than the wild type strain, no physiological or biochemical comparison of the two strains was done. Consequently, the purpose of the present research project was to make such a comparison, particularly with regard to the activities of catalase and superoxide dismutase in the two strains.

LITERATURE REVIEW

Characteristics of Campylobacter jejuni

Members of the genus Campylobacter are gram-negative, non-spore-forming, curved rods, approximately 1.5 - 3.5 μm in length and 0.2-0.4 μm in width (Smibert, 1984). All of the species except Campylobacter cryaerophila and Campylobacter nitrofigilis are microaerophilic; the two exceptions can grow aerobically on a complex media such as brucella agar. C. jejuni cells are obligately microaerophilic in nature, growing optimally in an atmosphere containing 6% O_2 and 3-5% CO_2. Metabolism is strictly respiratory,and only oxygen serves as the terminal electron acceptor. Energy is obtained from tricarboxylic acid cycle intermediates or amino acids; carbohydrates are neither fermented nor oxidized. The cells are catalase- and oxidase-positive.

Campylobacter jejuni is the leading cause of bacterial diarrhea in humans and is transmitted via contaminated food, raw milk, and water. Prior to the development of selective culture medium and isolation techniques, C. jejuni had been isolated from human blood infections but were believed to be rare pathogens (Bokkenheuser, 1970). The involvement of C. jejuni in diarrheal illness was not recognized until 1972, when a selective filtration system was developed and coupled with incubation under microaerophilic conditions (Dekeyser, 1972). The filtration system allowed only small bacteria such as C. jejuni to pass through the filter (0.65 μm pore diameter). Since that time, the use of various combinations of antibiotics has eliminated the need for filtration (Skirrow,

1977; Butzler et al., 1979; Blaser et al., 1979; Bolton et al., 1982). The use of selective media together with incubation under microaerobic conditions led to the recognition that C. jejuni is a major cause of diarrhea in many countries. In the United States, Campylobacter isolations are now reported to be more frequent than those of Salmonella and Shigella combined (Centers for Disease Control, 1988).

Microaerophily and Oxygen Toxicity

Overview. Although microaerophilic organisms are capable of using oxygen as a terminal electron acceptor, oxygen is toxic to them. Microaerophiles fail to grow, or grow only poorly, at the level of oxygen (21%) present in the air atmosphere. Highly reactive derivatives of oxygen such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($^1\Delta gO_2$) have been implicated as being responsible for oxygen toxicity. These toxic derivatives may arise as the by-products of cellular metabolism, as well as from spontaneous autooxidation or photochemical generation in the environment. The toxic derivatives of oxygen and their relationships to one another are shown in Figure 1.

Chemical properties of oxygen. From the viewpoint of oxidation-reduction potentials, the oxygen molecule should be a very strong oxidizing agent, because the E'_o of the O_2/H_2O half-cell system is +0.8 V at pH 7. However, spontaneous two-electron reductions of O_2 in the absence of an enzyme or other catalyst occur only at low frequencies at physiological temperatures. This is because the oxygen molecule con-

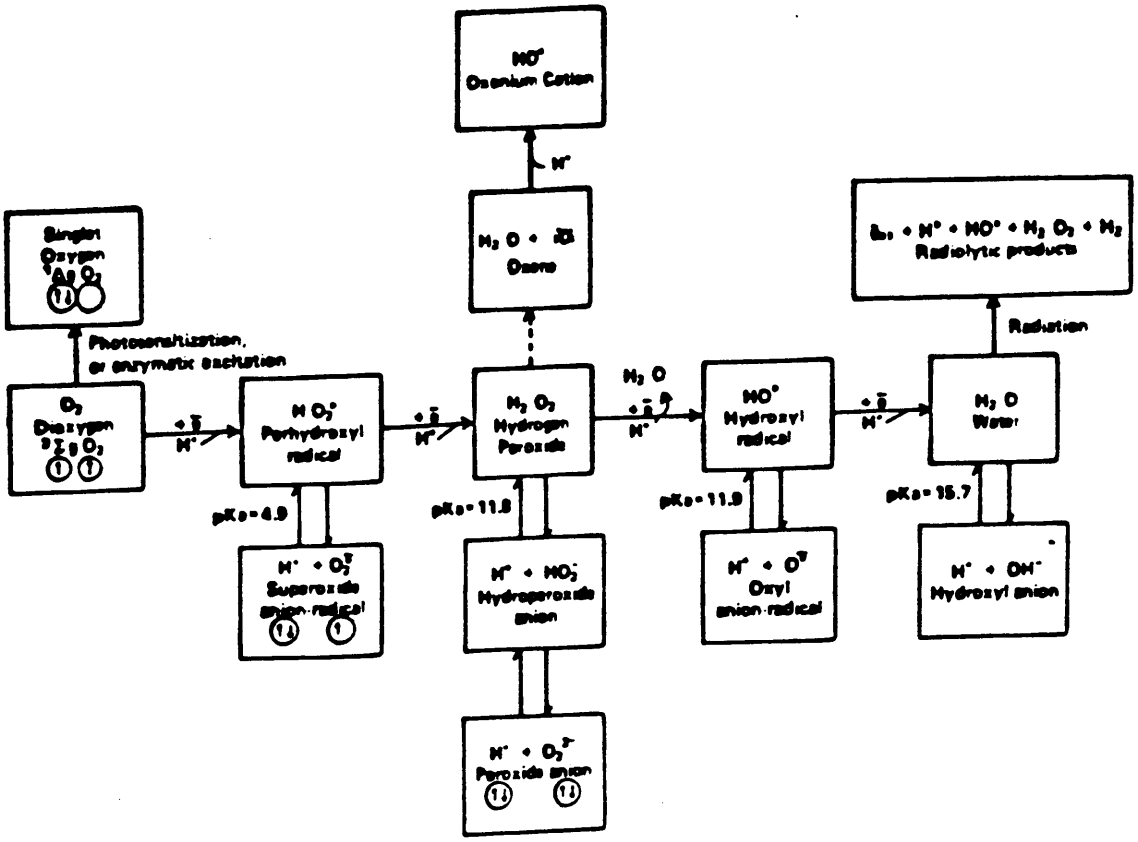

Figure 1. Possible states of oxygen and its stepwise reduction to water (Byczkowski and Gossner, 1988)

tains two unpaired electrons which have a parallel spin and are located in different π^* antibonding molecular orbitals. Because these antibonding electrons have the same spin, the acceptance of two more electrons by O_2 requires that the new electrons must have a spin opposite to that of the antibonding electrons. It is difficult to find an electron donor fulfilling the conditions for two-electron reduction unless high energy of activation is supplied (as by an appropriate enzyme). An alternative to the spontaneous two-electron reduction exists, namely, a one-electron reduction. All that is required here is that the electron donor must supply one electron having a spin opposite to that of the antibonding electrons of the O_2 molecule. For this reason, reactions involving a spontaneous one-electron reduction of O_2 are relatively common.

Superoxide radicals. If the oxygen molecule gains one electron, it will pair with one of the antibonding electrons. The products are superoxide radicals ($O_2^{\cdot-}$) and perhydroxyl radicals ($HO_2^{\cdot}$) which contain one unpaired electron. The overall reaction is as follows:

$$O_2 + H^+ + e^- \longrightarrow HO_2^{\cdot}$$

The perhydroxyl radical will dissociate to H^+ and $O_2^{\cdot-}$ at physiological pH values due to its low pK_a (4.9) as shown in Figure 1 (Byczkowski et al., 1988). Superoxide radicals are more reactive than oxygen because of the unpaired electron and its relative instability in aqueous solutions. The superoxide anion behaves mainly as a reducing agent in biological systems (Frimer, 1982).

Several spectrophotometric methods can be used to detect the presence of superoxide radicals (Clifford et al., 1984). Two common methods are as follows:

i) Cytochrome c reduction. Superoxide radicals will reduce cytochrome c, causing an increase in absorbance at 550 nm.

ii) Nitroblue tetrazolium (NBT) reduction. In the oxidized state, NBT appears soluble and yellow. If superoxide anions reduce NBT it will be converted to a blue-black formazan.

Hydrogen peroxide. A two-electron reduction of oxygen will generate hydrogen peroxide (H_2O_2), hydroperoxide anions (HO_2^-), and peroxide anions (O_2^{2-}) (See Figure 1). The peroxide and hydroperoxide anions have no unpaired electrons and are therefore not free radicals. At physiological pH, any O_2^{2-} or HO_2^- formed will immediately become protonated to form H_2O_2 due to the high pK_a (Figure 1). The overall reaction is as follows:

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

As we noted previously, two-electron reduction of O_2 is difficult unless energy of activation is provided, as by an enzyme. However, H_2O_2 can be generated spontaneously from superoxide radicals, in a reaction termed spontaneous superoxide radical dismutation, in which one $O_2^{\cdot-}$ gives up its electron to another $O_2^{\cdot-}$:

$$O_2^{\cdot-} + O_2^{\cdot-} + H^+ \longrightarrow O_2 + H_2O_2$$

This dismutation occurs most rapidly at a pH of 4.7, the pK_a value for superoxide (at which equal concentrations of $HO_2^{\cdot}$ and $O_2^{\cdot-}$ would exist). At this pH, the rate constant $k = 1.02 \times 10^{-8}\ M^{-1}\ sec^{-1}$. For every unit increase in pH, the rate of reaction decreases by a factor of approximately 10. At high pH values, e.g., pH 13, the half-life is about 160 minutes. This is because of the negative electrical charge of the $O_2^{\cdot-}$, which makes it difficult for collisions between superoxide

radicals to take place. Even at pH 7, a superoxide radical can diffuse much farther than, say, a hydroxyl radical, in a biological system before interacting with another superoxide radical.

Two commonly used methods for the detection of H_2O_2 are as follows (Clifford, et al.):

i) Measurement of oxygen evolution. Because catalase will break down H_2O_2 to H_2O and O_2, the rate of oxygen evolution can be measured and used to determine the H_2O_2 concentration.

ii) Oxidation of scopoletin. When reduced scopoletin is activated by light, it will fluoresce at 460 nm. The oxidation of scopoletin by H_2O_2 and horseradish peroxidase will cause a decrease in fluorescence that is directly proportional to the H_2O_2 concentration.

Singlet Oxygen. If an oxygen molecule is energized, a spin reversal of one of the π^* antibonding electrons occurs, forcing the 2 unpaired electrons of dioxygen to become antiparallel, but still in different orbitals. When this happens, singlet oxygen, $^1\Sigma g^+O_2$, is generated. Singlet $^1\Sigma^+O_2$ rapidly decays to another form of singlet oxygen, $^1\Delta gO_2$, in which the two antibonding electrons occupy the same orbital (See Figure 1). This usually occurs before the $^1\Sigma g^+O_2$ has time to react with any nearby biological molecules (Byczkowski, et al., 1988). The first singlet state, $^1\Delta gO_2$, is unstable with a half life of 2-5 μsec in aqueous solutions (Perez, 1985).

Singlet oxygen is approximately 93 kJ above the triplet ground state and emits low level chemiluminescence, as it decays to the ground state (Candenes et al., 1984). The chemiluminescence provides a means

for measurement of singlet oxygen. Emission takes place at 1270 nm for the single molecular transition and at 634 and 703 for simultaneous bimolecular transitions (Candenes et al., 1985). Singlet oxygen can cause lipid peroxidation by an initial abstraction of a hydrogen from an polyunsaturated lipid, followed by free radical propagation. While singlet oxygen has a very short lifetime in aqueous solution, it lasts longer in hydrophobic environments such as the interior of membranes (Frimer, 1982).

Hydroxyl radicals. A potentially more devastating radical, the hydroxyl radical (OH·) may be produced from the reaction of H_2O_2 with another electron. At physiological pH, the hydroxyl radical exists as a very unstable and reactive oxidant. Due to its high pK_a, the hydroxyl radical will only dissociate in alkaline solutions to form the oxyl anion-radical ($O^- + H^+$), as shown in Figure 1.

The hydroxyl radical (OH·) may be produced by the reaction of hydrogen peroxide with a superoxide anion by the Fenton reaction:

$$H_2O_2 + O_2^{\cdot-} \xrightarrow[Fe^{3+}]{\text{chelated}} O_2 + OH\cdot + OH^-$$

In the absence of $O_2^{\cdot-}$, merely adding ferrous (Fe^{2+}) ions or the reduced ions of other transition metals, such as Ti, Cr, Co,and Cu, to H_2O_2 can generate hydroxyl radicals (Czapski, 1984).

The toxicity of $O_2^{\cdot-}$ has sometimes been attributed to its role in generating OH· by the Fenton reaction. However, the function of $O_2^{\cdot-}$ is, in essence, to reduce the metal ion, i.e., reduce Fe^{3+} to Fe^{2+}. The reduced metal in turn donates its electron to H_2O_2, resulting in the

production of $OH^{\cdot}$ and OH^{-}. Because the role of $O_2^{\cdot-}$ is merely that of a reductant, other reductants might serve as well. In fact, thiols, ascorbate, NADH, NADPH, and various radicals can be used instead of $O_2^{\cdot-}$ as a source of reducing power for the Fenton reaction (Imlay and Linn, 1988).

Hydroxyl radicals are the most reactive of any oxygen derivative and are capable of damaging almost any biological molecule. If hydroxyl radicals are formed at any concentration, they will decay in 10^{-4} sec to submicromolar concentrations (Czapski, 1984).

Two indirect methods are commonly used to identify $OH^{\cdot}$ radicals:

i) Spin trapping is a method in which a solute such as 5,5-dimethyl-1-pyrroline N-oxide (DMPO) reacts with $OH^{\cdot}$ to yield longer-lived free radicals. These free radicals then may be characterized by their electron spin resonance spectra (ESR).

ii) Competition kinetics is a method in which $OH^{\cdot}$ scavengers are added so that the known reaction products can be analyzed and the relative rate constants may be determined. Methane production by the reaction of $OH^{\cdot}$ with dimethyl sulfoxide is one example.

Reaction of Toxic Forms of Oxygen with Biological Material. It is often difficult to determine which of the various toxic forms of oxygen is directly responsible for a toxic effect on living cells. For instance, if a flux of $O_2^{\cdot-}$ causes cell death, and because $O_2^{\cdot-}$ gives rise to H_2O_2, and H_2O_2 plus $O_2^{\cdot-}$ gives rise to $OH^{\cdot}$, cell death might be due to either H_2O_2 or the $OH^{\cdot}$, rather than the $O_2^{\cdot-}$. If we find, for

example, that catalase (which destroys H_2O_2) or superoxide dismutase (which destroys $O_2^{\cdot-}$) can protect cells, it becomes more likely that $OH^{\cdot}$ is the toxic agent, since either enzyme would remove one of the two reactants required for the Fenton reaction. On the other hand, we might discover that catalase protects the cells and superoxide dismutase fails to do so. This would suggest that H_2O_2 is the toxic agent. For example, the toxic effects of cysteine on anaerobic bacteria incubated under aerobic conditions is apparently due to H_2O_2, because catalase, but not superoxide dismutase can protect cells (Carlsson, 1978). However, the identity of the culprit would still not be clear because the H_2O_2, once inside the cells, might be used to generate $OH^{\cdot}$, and the $OH^{\cdot}$ might be the actual damaging substance. Such examples show the difficulty of deciding which toxic form of oxygen is the most important. Moreover, it is likely that the particular culprit may vary with the particular biological system being studied.

Brawn and Fridovich (1980) demonstrated that the aerobic action of xanthine oxidase on xanthine would cause DNA strand scission in ColE1 DNA. Hydroxyl radicals ($OH^{\cdot}$) apparently were the actual DNA-damaging oxidant because either catalase or SOD, as well as hydroxyl radical scavengers such as mannitol and benzoate, prevented DNA strand scission.

Steiner et al. (1984) showed that low doses of H_2O_2 could induce single-strand breakage in the DNA of cells of Treponema pallidum that were exposed to the oxidant. Extensive breakage was caused by 100 μM H_2O_2. Catalase and dithiothreitol protected T. pallidum from all but a low level of breakage. It was not clear that H_2O_2 was the actual DNA-

damaging agent, however, because the H_2O_2 might have given rise to OH· within the cells.

Imlay and Linn (1986) demonstrated a bimodal pattern of killing occurs when E. coli K-12 cells were treated with H_2O_2. Mode 1 killing occurs when E. coli is exposed to low concentrations of H_2O_2 (1.0-2.0 mM) and is caused by DNA damage (Imlay et al., 1988). Mutant strains that lack DNA repair enzymes are especially sensitive to mode 1 killing. A subsequent study (Imlay and Linn, 1988) demonstrated that the single-strand breaks in DNA result from the collapse of the deoxyribose ring after abstraction of an hydrogen atom. Mode 2 killing requires higher concentrations of H_2O_2 (>20 mM) and the site(s) of damage have not been identified (Imlay et al.,1988). Mode 2 killing exhibits a classical multiple-order dose response curve.

Imlay and Linn (1988) have also shown that mode 1 killing only occurs in actively metabolizing cells, and iron as well as reducing equivalents are required. These results indicate that the Fenton reaction might be producing the DNA damaging oxidant. Yet, compounds that quench OH· radicals failed to protect against mode 1 killing (Imlay et al.,1988). Imlay and Linn (1988) proposed that OH· radicals are released from the breakdown of biologically active ferryl radicals which are complexed with DNA. In addition, they proposed that NAD(P)H serves as the reductant for the metal.

Goldstein and Weissman (1977) demonstrated that enzymatically generated superoxide anions are capable of disrupting lipid bilayers. A flux of superoxide anions disrupted membranes sufficiently enough to cause leakage of an impermeable anion from inside of prepared liposomes.

Both catalase and SOD prevented damage to the lipid bilayers. This finding is compatible with the possibility that hydroxyl radicals were the actual membrane-damaging substance because either enzyme would remove a reactant essential for the Fenton reaction.

Kellogg and Fridovich (1975) found that the peroxidation of linolenate could be accomplished by the aerobic action of xanthine oxidase on acetaldehyde. Lipid peroxidation was inhibited by catalase, SOD, and scavengers of singlet oxygen, but not by hydroxyl radical scavengers. They proposed that the Haber-Weiss reaction liberates singlet oxygen which in turn is responsible for lipid peroxidation. In a subsequent study, Kellogg and Fridovich (1977) found that an enzymatic flux of $O_2^{\cdot}$ and H_2O_2 not only causes lipid peroxidation, but it also causes the lysis of human erythrocytes. Catalase, SOD, and scavengers of singlet oxygen prevented both lipid peroxidation and erythrocyte lysis, but hydroxyl radical scavengers had no effect.

Kim et al. (1985) discovered that enzymes could be cleaved by reactive oxygen species that were produced in the presence of metal ions and reducing equivalents. Their results suggest that hydroxyl radicals are generated at specific sites on enzymes to which iron is bound and are responsible for the inactivation and degradation of proteins.

Theories for Explaining Microaerophily

While several theories have been offered to explain the fundamental nature of oxygen toxicity (Krieg and Hoffman, 1986), the physiological basis of microaerophily is only poorly understood. It seems evident

that no one theory will be applicable to all microaerophilic organisms. Several theories for explaining microaerophily will be explained below.

Sensitivity to Toxic Forms of Oxygen in Culture Medium. Hoffman et al. (1979) proposed that microaerophilic bacteria are more sensitive to toxic forms of oxygen in culture medium than other oxygen-dependent bacteria. The agents which greatly enhanced aerotolerance, superoxide dismutase (SOD), and catalase, must have acted on the culture medium because these proteins are too large to penetrate the bacterial cell. Superoxide radicals and peroxide were detected in both illuminated Brucella broth and, to a lower extent, in Brucella broth which had been stored in the dark (Hoffman et al. 1979). The aerotolerance of Campylobacter jejuni was also enhanced by the addition of ferrated norepinephrine and a combination of sodium metabisulfite, sodium pyruvate, and iron salts (FBP mixture; George et al., 1978; Hoffman et al., 1979). The enhanced aerotolerance was attributed to the superoxide-scavenging ability of FBP and the ability of pyruvate to destroy H_2O_2.

Studies with Spirillum volutans provide additional support for Hoffman's proposal (1979) that microaerophilic bacteria are much more sensitive than aerotolerant bacteria to toxic derivatives of oxygen in culture medium. The microaerophilic S. volutans is extremely sensitive to hydrogen peroxide and superoxide radicals, to a lesser extent, in the culture medium (Padgett et al., 1981). Growth of S. volutans was inhibited by as little as 0.29 μm H_2O_2 in the culture medium. The addition of both catalase and SOD acted in a synergistic manner to increase the aerotolerance of S. volutans (Padgett et al., 1981).

Low Respiratory Rates. The possibility exists that organisms which possess low respiratory rates are incapable of maintaining a reduced cell interior. Accumulation of oxygen within the cell would afford little protection to oxygen-labile cell components. Cole and Rittenberg (1971) suggested that low respiratory rates may be responsible for the microaerophilic nature of S. volutans. However, the low respiratory rate they reported for S. volutans was probably an artifact: Caraway and Krieg (1974) found 0.05 M phosphate buffer to be inhibitory to the respiratory rates of S. volutans, and Cole and Rittenberg had used 0.05 M phosphate buffer in their cell suspensions.

Although the respiratory rates of C. jejuni were relatively high and the levels of cytochromes did not vary with oxygen tension, Hoffman et al. (1979) found the respiratory rates did decrease with increasing oxygen tension.

Lack of Protective Enzymes. Insufficient levels or types of protective enzymes, such as superoxide dismutase (SOD), catalase, or peroxidase, might render an organism susceptible to the toxic effects of oxygen.

Superoxide dismutase (SOD) is a metalloprotein that catalyzes the dismutation of superoxide radicals. At a physiological pH of 7, the rate constant for the spontaneous dismutation of superoxide radicals is 1×10^5 M^{-1} sec^{-1}. Under the same conditions, but in the presence of SOD, the rate constant is 2×10^9 M^{-1} sec^{-1} (McCord et al., 1977). Considering the fact that the steady state superoxide radical concentration in the cell is probably at least four or five orders of magnitude lower than that of SOD, the overall gain in the rate of dismutation in vivo

may exceed a factor of 10^9 (McCord et al., 1977). SOD owes its catalytic effect to its elimination of the necessity for two negatively charged superoxide radicals to collide with each other for dismutation. The enzyme serves as an intermediary between the superoxide radicals because it contains a transition metal (either Cu^{2+}/Cu^{+}, Mn^{3+}/Mn^{2+}, or Fe^{3+}/ Fe^{2+}). The action of the enzyme is as follows:

$$\text{Enzyme-Me}_{ox} + O_2^{\cdot-} \longrightarrow \text{Enzyme-Me}_{red} + O_2$$

$$\text{Enzyme-Me}_{red} + O_2^{\cdot-} + 2H^{+} \longrightarrow \text{Enzyme-Me}_{ox} + H_2O_2$$

$$\text{Overall: } O_2^{\cdot-} + O_2^{\cdot-} + H^{+} \longrightarrow O_2 + H_2O_2$$

Fridovich proposed the superoxide theory of oxygen toxicity, which states that the superoxide radical is a major factor in oxygen toxicity and superoxide dismutase constitutes an essential defense against it (Fridovich, 1982). The superoxide radical has been implicated as the causative agent in lipid peroxidation (Kellogg and Fridovich, 1975), lysis of erythrocytes (Kellogg and Fridovich, 1977), and destruction of enzymes (Kim et al., 1985). However, superoxide radicals per se may not be toxic. As mentioned earlier, the apparent toxicity may be due to the fact that they mediate the formation of other toxic forms of oxygen, such as hydrogen peroxide or hydroxyl radicals, which are more destructive oxygen derivatives.

Obligate anaerobes were believed to be devoid of SOD until the discovery of high SOD activity in Clostridium thiosulfatophilum and C. perfringens (Hewitt and Morris, 1975), and the presence of low levels of SOD activity in many other anaerobes (Fridovich, 1982).

Studies of SOD activity in Escherichia coli have provided some of the most convincing evidence for the superoxide theory of oxygen toxicity. Gregory et al. (1973) discovered that E. coli possesses: a) an Fe-containing SOD located in the periplasmic space which is constitutive and apparently constitutes an essential defense against exogenous $O_2^{\cdot-}$, and b) a Mn-containing enzyme which appears to be inducible and serves as an essential defense against endogenous $O_2^{\cdot-}$.

The microaerophile C. jejuni has been found to possess SOD activity as high as the levels present in E. coli (Hoffman et al., 1979). But despite the high levels of SOD activity, C. jejuni fails to grow aerobically. As a result of these findings, Hoffman suggested that the total SOD content may not be as important as the location of the SOD activity in the cells; SOD may not be present at crucial sites in the cell to protect against exogenous superoxide radicals (Hoffman, 1977).

Many microaerophilic bacteria are extremely sensitive to H_2O_2 in their culture media. Catalase constitutes a major cellular defense against H_2O_2. Catalase catalyzes the destruction of H_2O_2 by the following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Padgett et al. (1981) found S. volutans to be as sensitive to as little as 0.029 μm H_2O_2 in the culture medium. The high sensitivity to H_2O_2 is not surprising, because this organism lacks catalase. While the addition of catalase to the culture medium increased the aerotolerance of S. volutans, a combination of catalase and SOD acted in a synergistic manner to increase aerotolerance (Padgett et al. 1981).

Despite the presence of catalase in C. jejuni, growth is inhibited by low levels (35 μM) of H_2O_2 in the culture medium (Hoffman et al., 1979).

Because catalase will not effectively scavenge low levels of H_2O_2 in the culture medium due to its low affinity (high km) for H_2O_2, the presence of peroxidase might be important for microaerophiles. Peroxidase possesses high affinity for H_2O_2 and therefore is more efficient at destroying low levels of H_2O_2 in the medium. Peroxidase catalyzes the reduction of H_2O_2 by the reaction below, where R is an organic cofactor:

$$RH_2 + H_2O_2 \longrightarrow R + 2H_2O$$

Padgett and Krieg (1986) isolated an aerotolerant mutant of S. volutans following a one step treatment with ethyl methane sulfonate, a mutagenic agent. While both the mutant and the microaerophilic wild-type lacked catalase activity, the aerotolerant mutant possessed approximately three times the peroxidase activity of the wild type S. volutans.

Occurrence of Oxygen-Labile Cell Components. Both C. jejuni and S. volutans have been shown to be extremely sensitive to H_2O_2 in culture medium (Hoffman et al., 1979; Padgett et al., 1981). Because the addition of exogenous enzymes, such as SOD and catalase, to the culture medium enhances aerotolerance, and because it is unlikely that such proteins penetrate bacterial cells, the components of the cell surface would be a probable site of attack.

Cytochromes of low redox potential are vulnerable to attack by toxic forms of oxygen. Low redox potential forms of cytochromes b and c have been reported in Campylobacter fetus subsp. intestinalis (Harvey

and Lascelles, 1980). Hoffman reported a cytochrome c-type oxidase in C. jejuni which could be a potential target for inactivation by toxic derivatives of oxygen (Hoffman and Goodman, 1982).

Campylobacter species are sensitive to metronidazole, a chemical known for its "selective" toxicity towards anaerobes. The effectiveness of metronidazole depends upon the presence of ferredoxins or flavodoxins which are strongly electronegative cofactors. Ferredoxins and flavodoxins are readily autooxidizable. Misra and Fridovich (1971) have shown that superoxide radicals may be generated upon the autooxidation of ferredoxins. The presence of ferredoxins or flavodoxins may be a contributing to the oxygen toxicity in Campylobacter species.

Ferredoxin-linked pyruvate and α-ketoglutarate dehydrogenase activity has been reported in Campylobacter fetus (Lascelles and Calder, 1985). These enzymes are oxygen-labile and are therefore another important cell constituent that might be inactivated when exposed to oxygen. Lascelles and Calder (1985) suggested that the inability of the primary dehydrogenases to maintain a flow of electrons would put constraints on the efficiency of the terminal oxidase.

Metabolic Generation of Toxic Derivatives of Oxygen. Some microaerophilic bacteria may contribute to the toxicity of their environment by generating toxic oxygen derivatives as by-products of cellular metabolism. Studies of C. sputorum spp. bubulus revealed that $O_2^{\cdot-}$ was produced when either formate or lactate was oxidized and that H_2O_2 was generated as a by-product of formate oxidation (Niekus et al., 1978). The respiration of C. sputorum is relatively insensitive to cyanide (Stouthamer et al., 1979), and cyanide-insensitive respiration is often

associated with the production of $O_2^{\cdot-}$ (Hassan and Fridovich, 1977). The addition of iron salts, which destroy $O_2^{\cdot-}$, failed to permit the growth of C. sputorum at higher oxygen tensions, probably due to the absence of catalase in this organism (Stouthamer et al., 1979). The microaerophilic nature of C. sputorum may be due to their ability to produce $O_2^{\cdot-}$ and H_2O_2 coupled with the absence or low activities of protective enzymes.

Culture Conditions That Increase Aerotolerance

Media Supplements. The aerotolerance of many microaerophilic bacteria is enhanced by the addition of supplements to culture media that will destroy toxic forms of oxygen. Thompson et al. (1951) discovered that pyruvate was effective in protecting bacteria against H_2O_2 and $OH^{\cdot}$ radicals generated during irradiation. Several explanations for the effectiveness of pyruvate were suggested: i) Pyruvate breaks down H_2O_2; ii) pyruvate lowers the oxygen tension in suspensions by providing a readily oxidizable substrate; and iii) pyruvate protects essential sulfhydryl groups on enzymes by modifying the state of reduction in the cell.

Steiner et al. (1984) found that the addition of catalase (750 U/ml) to the medium offered partial protection against DNA breakage in Treponema pallidum when it was treated with H_2O_2. In the presence of dithiothreitol (DDT), a reducing agent, DNA damage was significantly reduced. Steiner et al. (1984) suggested that the greater protective ca-

pacity of DDT may be due to its ability to chemically repair oxygen-induced damage inside the treponemal cell.

The addition of either ferrated norepinephrine or FBP was effective in enhancing the aerotolerance of C. jejuni (George et al., 1978; Hoffman et al., 1979). These compounds may be effective because of their capacity to quench toxic forms of oxygen. The addition of SOD, and catalase, to a lesser extent, also significantly enhanced the aerotolerance of C. jejuni cells (Hoffman et al., 1979).

Activated charcoals have been found to prevent photochemical oxidation reactions in complex media and to decompose H_2O_2 and O_2^- (Hoffman et al., 1983). Hoffman suggested (1983) that charcoals may prevent photochemical oxidation reactions either by absorbing the reactant compounds, decomposing the reduced forms of oxygen, or by trapping free radicals.

FBP, blood and charcoal are all supplements which are frequently used to enhance aerotolerance in culture media used to grow campylobacters. Studies by Bolton et al. (1984) confirmed that these supplements are not growth enrichment factors but instead detoxify the media by quenching toxic forms of oxygen.

Catalase, sodium dithionite (a powerful reducing agent), and histidine (a hydroxyl radical scavenger), has been shown to enhance the aerotolerance of C. jejuni (Juven and Rosenthal, 1985). These results provide additional support to show that agents which enhance aerotolerance of microaerophilic bacteria are those that have that ability to breakdown toxic forms of oxygen.

Lee et al. (1988) have shown that the 0.01% sodium bisulfite content of unsupplemented brucella medium deteriorates with age and has a significant effect on the aerotolerance of C. jejuni. When commercial dehydrated medium was stored for 2½ months and hydrated medium was stored for 1½ months prior to usage, the degree of aerotolerance greatly decreased. The addition of 0.01% sodium bisulfite reversed the inhibitory effects of the aged medium. Also, in brucella medium prepared from all of the individual ingredients except bisulfite, C. jejuni exhibited a greatly decreased aerotolerance, failing to grow at even 3% oxygen. Sodium bisulfite may act in concert with iron in the medium to destroy $O_2^{\cdot}$ radicals (Hoffman et al., 1979).

Incubation Temperature. Goosens et al. (1984) suggest that plates streaked for the isolation of C. jejuni should be incubated at 42°C. Although C. jejuni will grow at 37°C, the growth of C. jejuni will be enhanced at 42°C. In addition, some of the normal fecal flora will be inhibited at this temperature.

Wang et al. (1983) examined the effect of temperature and atmosphere on the isolation of C. jejuni from human stools. When cells were grown at 42°C, C. jejuni could be isolated from all 16 stool specimens under oxygen atmospheres of 5%, 10%, 15%, and in a candle jar. When plates were incubated at 37°C in 15% oxygen or in a candle jar (>15% O_2), some positive samples were missed. For each atmosphere tested, the colony sizes were significantly larger at 42°C than at 37°C. These results suggest that C. jejuni is more aerotolerant at 42°C than 37°C.

Lee et al (1988) isolated an aerotolerant mutant of C. jejuni ATCC 29428 that was able to grow at 42°C on unsupplemented Brucella agar in

atmospheres up to 26% oxygen. Both the wild type C. jejuni and the mutant were significantly more aerotolerant when grown at 42°C. For example, the wild type grew at 15% oxygen at 42°C but failed to grow at this level when incubated at 37°C. The aerotolerant mutant grew at 26% oxygen when incubated at 42°C, but was unable to grow at this oxygen level when incubated at 37°C.

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MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Campylobacter jejuni ATCC 29438 strain H840 and its aerotolerant mutant MC711-01 isolated by Lee et al. (1988) were used in this study. They were maintained by weekly transfers in brucella semisolid medium. Incubation was under an air atmosphere. Because the aerotolerance of C. jejuni cells is affected by the ageing and storage of brucella medium (Lee et al., 1988), all dehydrated media were stored under a nitrogen atmosphere to minimize oxidation of the sodium bisulfite.

Cells used for survival studies were grown at 37°C in 250-ml side-arm flasks containing 50 ml of brucella broth under an atmosphere of 6% O_2, 3.5% CO_2, and 90.5% N_2. Cells used for enzyme assays were grown in 4.0-L flasks containing 1.0-L of brucella broth. C. jejuni H840 and MC711-01 were cultured simultaneously at 37°C in adjacent shaking water baths at 68 oscillations per minute. For the growth of cells under 6% O_2, a gas mixture consisting of 6% O_2, 3% CO_2, and 91% N_2 was bubbled through the broth at a flow rate of 40 cc/min. For growth of cells under 21% O_2, growth was obtained first under 6% O_2, and when a visible turbidity was reached (NTU 3.0), the gas mixture was replaced by one containing 21% O_2, 3% CO_2, and 76% N_2.

Cell Extracts

Cells from the middle of the logarithmic phase of growth were collected by centrifugation at 9,600 x G for 25 minutes and washed three times with 50 mM potassium phosphate buffer (pH 7.0). Cells were broken by a single passage through the French pressure cell at 20,000 psi and the suspension was centrifuged at 25,400 x g for 30 min. The supernatant (crude extract) was used for the catalase and peroxidase assays. Cells for SOD assays were processed similarly except that they were suspended and washed in 20 mM potassium phosphate buffer (pH 7.4), and the crude extracts were dialyzed against two 1.0-L volumes of 20 mM potassium phosphate buffer (pH 7.4) for 48 h.

Enzyme Assays

All assays of cell extracts were performed at 25°C using a Bausch and Lomb spectrophotometer Model 2000. Except for the SOD which is reported as U/mg/min, specific activity is reported as the micromoles of substrate converted per min per mg protein. Protein determinations were performed according to the method of Lowry et al. (1951). Catalase (EC 1.11.1.6) was assayed by the method of Beers and Sizer (1952). Peroxidase (EC 1.11.1.7) was assayed by the method of Nickel and Cunningham (1969). Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Martin et al. (1987). Boiled cell extracts served as controls.

For the assay of catalase activity in whole cells, cells were harvested by centrifugation at 9,600 X g for 20 minutes and were washed

twice with 50 mM potassium phosphate buffer (pH 7.0). Because the absorbance of whole cells at 240 nm was very high, catalase activity could not be assayed by the Beers Sizer method (1951). Instead, it was assayed by using an Clark-type oxygen electrode to measure the rates of oxygen evolution when H_2O_2 (final concentration = 0.002 M) was injected into cell suspensions. The whole cell catalase activity was determined by comparing the observed rates of oxygen evolution with those of a bovine liver catalase (Sigma) solution of known activity. The activity of the bovine liver catalase solution was determined by the method of Beers and Sizer (1952). Boiled cell suspensions served as controls. The molar concentration of H_2O_2 was determined immediately before usage.

Susceptibility to Hydrogen Peroxide

Experiments were performed to determine the degree of susceptibility of the mutant and wild type C. jejuni cells to H_2O_2. Cells were grown to midlog phase under either 6% O_2 or 21% O_2, as previously described. Appropriate dilutions of reagent-grade 30% H_2O_2 (Fisher Scientific Co.) were added to flasks containing C. jejuni H840 and MC711-01 cells to yield a final H_2O_2 concentration of 2.0 mM H_2O_2 for treatment of cells grown under 6% O_2, or 1.0 mM H_2O_2 for treatment of cells grown under 21% O_2. The molar concentration of H_2O_2 was determined immediately before each assay. Bovine liver catalase (100 U/ml) (Sigma) was added to some flasks as a control. In each assay, 1.0-ml samples of culture were removed periodically from each flask, serially diluted in

brucella broth, and plated in triplicate on brucella agar plates. Plates were incubated at 37°C for 72 hours in an atmosphere containing 6% O_2, 3.5% CO_2, and 90.5% N_2.

Statistical Methods

The data was analyzed using SAS. The model for the analysis was designed as follows:

Catalase: $y_{ijk} = \mu + \alpha_i + \beta_j + \delta_k + \alpha\beta_{ij} + e_{ijk}$

SOD: $y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_k + \alpha\beta_{ij} + e_{ijkl}$

Whole Cell Catalase: $y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_k + \alpha\beta_{ij} + e_{ijkl}$

The meaning of the terms are listed below:

y_{ijk} and y_{ijkl} = observed value
μ = overall mean
α_i = fixed effect for oxygen
β_j = fixed effect for strain
δ_k = fixed effect for day
$\alpha\beta_{ij}$ = strain*oxygen interaction
e_{ijk} and e_{ijkl} = random error associated with each observation

RESULTS

Tolerance to Hydrogen Peroxide

Despite the presence of catalase in C. jejuni, this organism is poisoned by low levels of H_2O_2 in culture media. Because H_2O_2 may play an important role in microaerophily, four separate experiments were performed to determine whether the aerotolerant mutant strain MC711-01 might be more resistant to H_2O_2 than the wild type H840 strain.

Cells grown under 6% O_2 and suspended in brucella broth were exposed to 0.002 M H_2O_2, with samples being withdrawn at various intervals for plating onto brucella agar. Typical results are shown in Figure 2 (see also Figures 4, 5, and 6 in Appendix B). At 0 min there was an immediate reduction in the colony count following addition of the H_2O_2; the counts for both H840 and MC711-01 decreased to a similar extent (50-70%). The decrease occurred consistently in the four experiments. Controls to which catalase had been added showed no such decrease. Despite the initial rapid decrease following the addition of H_2O_2, at the end of 148-160 min the percent survival was much greater for MC711-01 than for H840. The absolute numbers of the survivors varied considerably among the four experiments; however, the difference between the viable counts of the two strains at the end of the exposure period was always at least two orders of magnitude.

When similar experiments were performed with cells grown under 21% oxygen, both H840 and MC711-01 were killed much more rapidly than when they had been grown under 6% oxygen. In fact, the killing rate was so

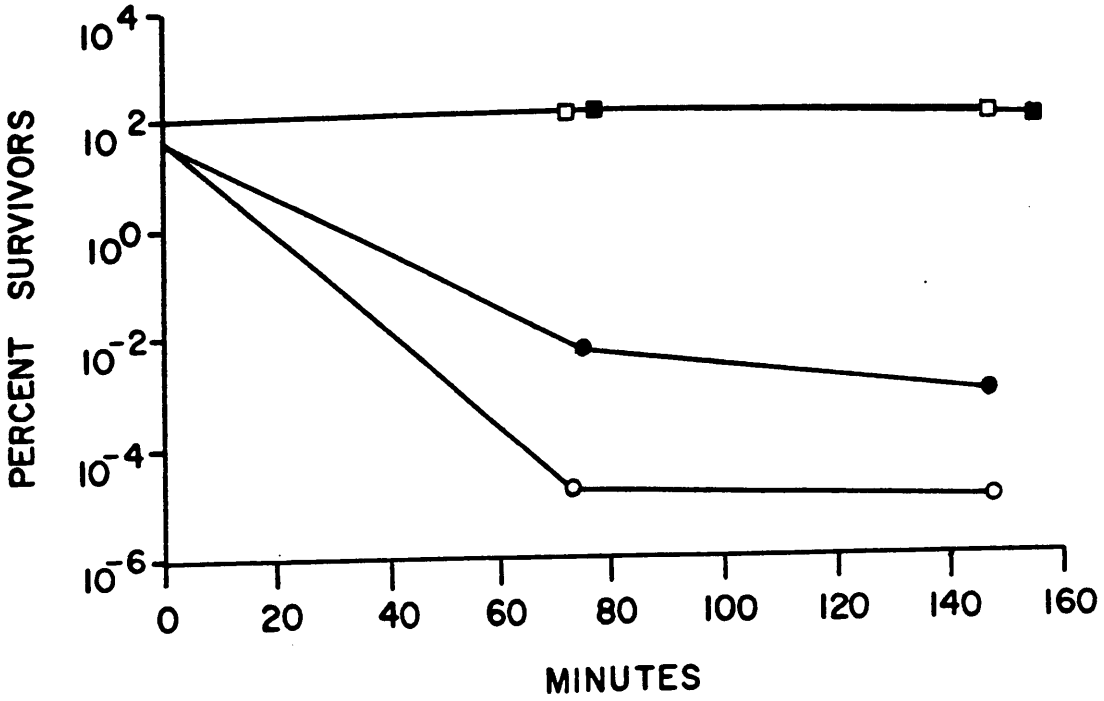

Figure 2. Survival of H840 and MC711-01 cells exposed to 0.002 M H_2O_2 in brucella broth. The cells had been grown under a 6% oxygen atmosphere. Symbols: □, H840 control; ■, MC711-01 control; ○, H840 H_2O_2 added; ●, MC711-01 H_2O_2 added.

rapid that few or no cells remained viable after 1.5 h. Consequently, it was necessary to decrease the H_2O_2 concentration to 0.001 M in order to obtain sufficiently slow rates of killing that could be accurately measured. Typical results are shown in Figure 3 (see also Figures 7 and 8 in Appendix B). The overall pattern of killing was similar to that seen for cells grown under 6% O_2 and exposed to 0.002 M H_2O_2: both strains exhibited an immediate decrease of ca. 30% in plate count at 0 min, and MC711-01 eventually showed a much greater percent survival than did H840.

Enzyme Activities

To determine whether the greater resistance of the mutant cells to H_2O_2 was correlated with activities of enzymes that destroy toxic forms of oxygen, the specific activities of catalase, superoxide dismutase (SOD), and peroxidase were assayed in crude extracts of cells grown under both 6% and 21% oxygen. The results are summarized in Table 1.

When cells were grown under 6% oxygen, catalase was the only enzyme whose mean specific activity was significantly higher ($p= 0.04$) in MC711-01 than H840. In assays of cells from various cultures, the activity was consistently ca. 1.5 times higher in MC711-01. While there was no significant difference in SOD activity, the peroxidase activity was lower in MC711-01 than H840. In fact, the peroxidase activity of MC711-01 extracts was approximately one-third of that in H840.

Changes in the specific activities of these enzymes occurred when cells were grown under 21% oxygen instead of 6% oxygen (Table 1).

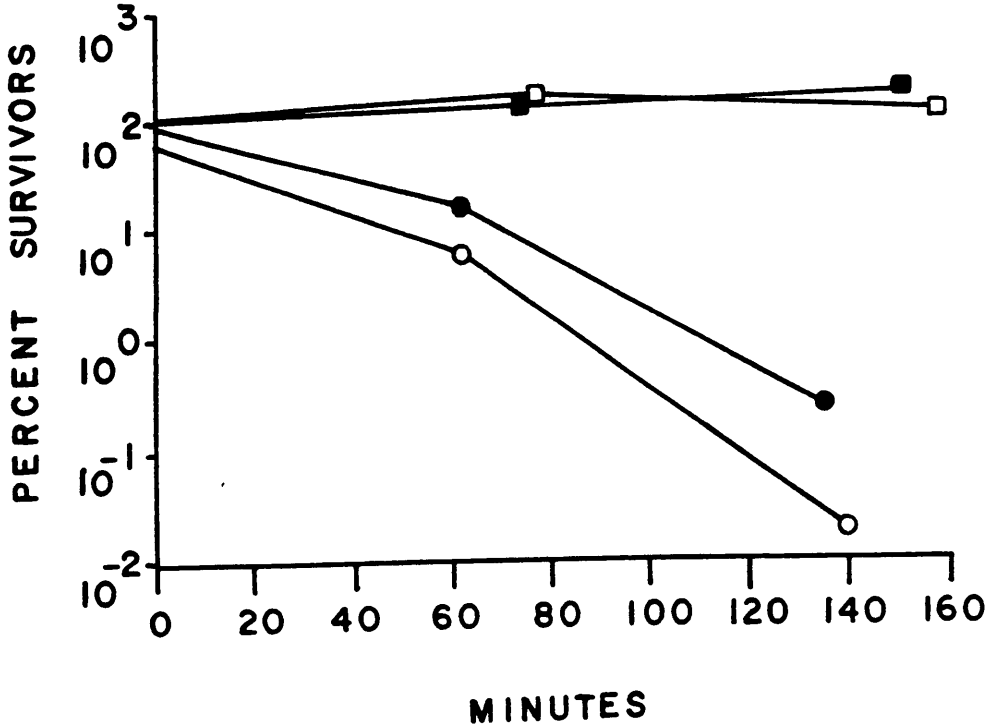

Figure 3. Survival of H840 and MC711-01 cells exposed to 0.001 M H_2O_2 in brucella broth. The cells had been grown under a 21% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added.

TABLE 1. Enzymatic activities of Campylobacter jejuni H840 and mutant MC711-01 cultured at 6% and 21% oxygen.[a]

	Specific activity[b]			
Enzyme	6% O_2 H840	6% O_2 MC711-01	21% O_2 H840	21% O_2 MC711-01
Catalase	2.4 ± 0.94	3.8 ± 0.84	3.3 ± 0.6	8.5 ± 0.56
SOD	4.1 ± 0.76	3.4 ± 1.0	3.7 ± 1.2	5.1 ± 1.1
Peroxidase	0.013 ± 0.006	0.004 ± 0.0009	0.006 ± 0.0004	0.004 ±0.0003

[a] Carbon dioxide (3.5%) was included in the gas mixtures.
[b] Activities are expressed as micromoles of substrate converted per min (international units) per mg protein except for superoxide dismutase, the activity of which is expressed as units per mg protein. Values are the means from at least three experiments.

Although there was no significant increase in the catalase activity of H840, the activity of MC711-01 was 2.2 times higher (p=0.001). This activity was 2.6 times that in H840 (p = 0.001). The mean SOD activity of H840 was not significantly different in cells grown at 21% oxygen, whereas it increased 1.5 times in MC711-01 (p = 0.004) and was 1.4 times higher than that of H840 (p = 0.01). The mean peroxidase activity of H840 decreased by approximately one-half but was still higher than that of MC711-01, which showed no change in activity.

Catalase Activity of Whole Cells

Because extracts of MC711-01 possessed significantly greater catalase activity than H840 when cells were grown under 21% oxygen, and because MC711-01 was more resistant than H840 to H_2O_2, assays of the catalase activity of intact cells were performed. The results are summarized in Table 2.

In contrast to results with cell extracts, there was no significant increase in the mean catalase activity of MC711-01 cells cultured under 21% oxygen. However, the difference in activity between H840 and MC711-01 grown at either 6% O_2 or 21% O_2 was significant (p = 0.03). When grown under 6% O_2 MC711-01 had 1.4 times the activity of H840, and when grown under 21% O_2 had 1.5 times the activity of H840. The catalase activity of both strains was inhibited by 0.5mM potassium cyanide and 0.5 mM sodium azide.

TABLE 2. Whole cell catalase activities of Campylobacter jejuni H840 and MC711-01 cultured at 6% and 21% oxygen.[a]

Strain	Specific activity[b]	
	6% O_2	21% O_2
H840	0.95 ± 0.50	0.76 ± 0.35
MC711-01	1.31 ± 0.35	1.19 ± 0.16

[a] Carbon dioxide (3.5%) was included in the gas mixtures.
[b] Activities are expressed as micromoles of substrate converted per min (international units) per milligram protein. Values are the means from three experiments.

DISCUSSION

The findings of this study suggest that catalase and, to a lesser extent, SOD activity may be correlated with the aerotolerance and greater resistance of MC711-01 cells to H_2O_2. The catalase and SOD activities in crude extracts of MC711-01 increased significantly when cells were cultured under 21% O_2, whereas H840 cells showed no corresponding increase. Interestingly, assays of catalase activity using whole cells of MC711-01 indicated no corresponding increase in activity when the cells were grown under 21% oxygen, suggesting that the catalase might be compartmentalized in some manner within the cell rather than freely dispersed throughout the cytoplasm. Nevertheless, the whole-cell catalase activity of MC711-01 was still 1.5 times greater than that of H840. The greater catalase activity of MC711-01 would be expected to enable these cells to cope with H_2O_2 more efficiently. It may also be related to the greater aerotolerance of this strain: in cultures incubated under an atmosphere containing 21% O_2, it is more likely that toxic forms of oxygen such as H_2O_2 will be generated in the medium than in cultures incubated under 6% O_2. MC711-01 cells also possessed greater SOD activity than did H840 cells when grown under 21% O_2, although the difference was not as great as for catalase activity. Thus SOD might also be an important protective enzyme for MC711-01 cells which are grown under 21% O_2.

The survival studies which were performed to determine the tolerance of C. jejuni strains to H_2O_2 reveal that MC711-01 has greater resistance than H840 cells at the end of the exposure period. Oddly, many

of the cells of both strains are killed immediately following the addition of H_2O_2. This initial brief period of rapid killing might be a period during which the cellular catalase decreases the H_2O_2 concentration to a more tolerable level. The remaining H_2O_2 might not be so readily destroyed by catalase because of the relatively low affinity of this enzyme for H_2O_2; thus the remaining low level of H_2O_2 would kill cells at a slower rate during the approximately 1.5 h period following the addition of H_2O_2. Eventually the H_2O_2 level would become so low as to no longer be toxic, and this might account for the eventual leveling off of the number of survivors. Because the whole-cell catalase activity of MC711-01 is consistently higher that of H840 (regardless of whether the cells are grown under 6% O_2 or 21% O_2), MC711-01 cells may have a greater ability than H840 to detoxify the medium and thus survive. The potential ability of C. jejuni to detoxify the medium containing H_2O_2 is suggested by studies on E. coli reported by Imlay and Linn (1986). These investigators demonstrated that a bimodal pattern of killing occurs when E. coli is exposed to H_2O_2. Mode one killing occurs rapidly, is associated with low concentrations of H_2O_2 (1.0-2.0 mM), and is attributed to DNA damage. Mode two killing requires higher concentrations of H_2O_2 (> 20 mM) and the sites of damage have not been identified. Imlay and Linn (1988) found that at the lower peroxide concentrations the rate of killing eventually subsides, and they suggested that this is due to the detoxification of the media by cellular catalase.

Both H840 and MC711-01 are considerably more susceptible to H_2O_2 if cells have been grown under 21% O_2 instead of 6% O_2. One possible

explanation is that both strains might be more highly stressed when growing under 21% O_2. An O_2 concentration of 21% would certainly be stressful for strain H840, and even though MC711-01 can grow at 21% O_2, this concentration is near the limit of its oxygen tolerance, because the cells usually fail to grow at 26% O_2 (Lee et al. 1988). As shown by Hoffman et al. (1979), H_2O_2 and $O_2^{\cdot-}$ are generated spontaneously in brucella broth, especially when the medium is illuminated. Although MC711-01 is more aerotolerant than H840, both strains would probably suffer some damage due to spontaneously generated H_2O_2 and $O_2^{\cdot-}$ in the culture medium. This damage might render the cells less resistant to subsequent exposure to 0.002 M H_2O_2. Cells grown under 6% O_2 would not be so stressed and would be more resistant to this concentration of H_2O_2.

Although the recovery of MC711-01 survivors was consistently higher than that of H840 at the end of the period of exposure to H_2O_2, there was considerable variation from one experiment to another in the absolute numbers of survivors. This "day-to-day" variation might have been due to slight differences in the total amount of H_2O_2 present in brucella broth in which the cells were suspended just before the addition of H_2O_2. The brucella broth was stored for various periods (no more than 24 h) in the dark prior to use, and some H_2O_2 was probably generated spontaneously during the storage period. This may have increased the total level of H_2O_2 to various extents beyond that of the H_2O_2 that was added to the medium. Moreover, during the survival experiments, the broth was exposed to normal room illumination, and there may have been differences in the degree of illumination from one experiment to another; this may have resulted in the generation of some H_2O_2. In

addition, there may have been slight differences in the concentration of H_2O_2 that was added to the cells suspensions: although the concentration of H_2O_2 was determined prior to each survival experiment, there were some differences in the length of time that elapsed before the H_2O_2 was added to the cell suspension. During this pre-experiment period there might have been some slight breakdown of the H_2O_2 that would have lessened its toxicity.

The level of catalase activity in crude extracts of MC711-01 and H840 was generally higher than that measured in intact cells. This might be attributable to differences in the availability of the enzyme and the substrate. In other words, instead of catalase being dispersed as a solution, as it is in a crude extract, it would be "packaged" within cells, which form a suspension rather than a solution. Thus the H_2O_2 and the enzyme would have a lower probability of colliding with each other when whole cells were tested.

With regard to SOD, previous studies have shown that C. jejuni possesses SOD activities as high as those in E. coli, yet this SOD activity fails to permit the cells to grow under 21% O_2 (Hoffman et al., 1979). In the present study, there was no significant difference in SOD activity between MC711-01 and H840 when the cells were grown under 6% O_2, but the activity was significantly higher in MC711-01 cells than H840 when the cells grown under 21% O_2. Although the difference was not as great as that for the catalase activity, it suggests that under higher (i.e., more stressful) O_2 level an increased SOD activity may contribute to aerotolerance.

Several studies indicate that other microaerophilic bacteria such as Spirillum volutans and Treponema pallidum are highly susceptible to H_2O_2 (Padgett et al., 1981, Steiner et al., 1984). Hoffman et al. (1979) suggested that the basis for microaerophily might be a high susceptibility of microaerophilic organisms to H_2O_2 and other toxic forms of oxygen that are generated spontaneously in culture media. Because these toxic forms of oxygen would be produced more readily in culture media incubated under an air atmosphere than under low levels of O_2, this would restrict the growth of a microaerophile to low oxygen tensions. S. volutans and T. pallidum lack catalase activity, and it is easy to understand why these species are highly susceptible to H_2O_2. On the other hand, C. jejuni contains catalase, making it less easy to see why it should be highly susceptible to H_2O_2. This present study suggests that in C. jejuni, and possibly other catalase-positive campylobacters, the level of catalase activity may be an important factor that influences the degree of susceptibility to H_2O_2 and, consequently, the degree of aerotolerance.

Although the sites of cellular attack by H_2O_2 have not been identified, many cell components could be targets for inactivation, as summarized by Krieg and Hoffman (1986). Potential sites of attack include cell surface components such as outer membrane proteins; low redox-potential cytochromes; ferredoxins and flavodoxins; pyruvate:ferredoxin oxidoreductase; and DNA.

APPENDIX A

REAGENTS, GAS ATMOSPHERES, ENZYME ASSAY, AND RESPIRATION RATE METHODS

Reagents

The following were purchased from Sigma Chemical Co., St. Louis, Missouri: catalase, superoxide dismutase, xanthine oxidase (Grade I), cytochrome c (Type III), hematoxylin, nicotinamide adenine dinucleotide (reduced), formic acid, sodium pyruvate, sodium bisulfite, N-methylphenazonium methosulfate, and metronidazole.

Xanthine was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Reagent grade 30% H_2O_2 , sodium thiosulfate, ferrous sulfate and toluene were purchased from Fisher Scientific Company, Raleigh, North Carolina.

The sodium salt of 2,3',6-trichloroindophenol was obtained from Eastman Kodak Company, Rochester, New York.

Potassium cyanide was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Oxygen Atmospheres. The oxygen concentrations for the gaseous environments used to cultivate C. jejuni H840 and MC711-01 cells were obtained by evacuation of the culture vessel until the desired oxygen level (e.g. 6%) was reached. CO_2 was added to a level of 3.5% and nitrogen was added to complete the filling of the culture vessel. The percentage of

each gas added to the culture vessel was based on manometric measurements.

For the growth of large volumes of C. jejuni H840 and MC711-01, gas mixtures consisting of either 6% O_2, 3.0% CO_2, and 91% N_2, or 21% O_2, 3.0% CO_2, and 76% N_2, were bubbled through the media at a rate of 40 cc/min. The gas mixtures were obtained from Industrial Gas and Supply Company, Bluefield, West, Virginia.

Enzyme Assays: Principles and Procedures

Catalase. Catalase assays were performed according to the method of Beers and Sizer (1952). Hydrogen peroxide absorbs strongly at 240 nm (molar extinction coefficient = 43,600). If a suspension contains catalase, there will be a decrease in absorbance due to the breakdown of H_2O_2 by the following equation:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

The reaction mixtures contained the following:

Potassium phosphate buffer (pH 7.0), 50 mM.....1.5 ml
Cell extract .....0.5 ml
Hydrogen peroxide, 55 mM.....1.0 ml

Each assay was performed in triplicate. The specific activity reported was based upon an average of three assays. Buffer plus cell extract served as the blank. The reaction was initiated by adding the H2O2. Extracts which had been boiled for 15 min served as controls.

Peroxidase. The spectrophotometric assay by Nickel and Cunningham (1969) was used to assay for peroxidase. Peroxidase catalyzes the following reaction where R represents an organic electron donor.

$$RH_2 + H2O2 \longrightarrow R + 2H2O$$

For the spectrophotometric measurements, peroxidase catalyzes the oxidation of (reduced) leuco 2,3',6'-trichloroindophenol (TIP) by H2O2. When leuco TIP is oxidized, a blue chromophore which absorbs strongly at 675 nm is produced. The rate of blue color formation is proportional to the enzyme concentration (Nickel and Cunningham, 1969).

The TIP solution was freshly prepared in 40 mM sodium phosphate buffer (pH 6.0). The TIP solution was chemically reduced with 0.05 M sodium thiosulfate. Following one hour of chemical reduction at room temperature, the solution was filtered through a 2.0 μm (pore size) filter and placed on ice. The substrate, 1.2 mM H2O2, was prepared in 40 mM sodium phosphate buffer (pH 6.0). The reaction mixture consisted of:

TIP, 0.8 mM.....1.0 ml
Hydrogen peroxide, 1.2 mM.....1.9 ml
Cell extract.....0.1 ml

Reaction mixtures were blanked against TIP plus substrate. Cell extract was added to initiate the reaction. Extracts which had been boiled for 20 minutes were used as controls to determine the rate of nonenzymatic oxidation.

SOD Assays. The xanthine oxidase-cytochrome c assay for SOD activity by McCord and Fridovich (1969) was used initially. In this assay superoxide radicals are generated by the action of xanthine oxidase, a flavoprotein. Xanthine oxidase oxidizes xanthine and the reduced flavoprotein subsequently reduces O2 to $O2^{\cdot-}$. The superoxide radicals

($O_2^{\cdot-}$) in turn reduce cytochrome c to the ferrous form which has a red color. This superoxide-dependent reduction of cytochrome c results in an increase in absorbance at 550 nm which can be monitored spectrophotometrically. SOD will inhibit the reduction of cytochrome c. One unit of SOD activity will inhibit the standard assay by 50%. The reaction mixtures consisted of:

Xanthine, 1 mM	0.15 ml
Cytochrome c, horse heart, 0.1 mM	0.30 ml
NaCN, 30 mM	0.005 ml
SOD buffer, pH 7.8	2.5 ml

The SOD buffer consists of 50 mM potassium phosphate buffer (pH 7.8) containing 1.0 mM EDTA. To calibrate the standard assay, sufficient xanthine oxidase was added to the reaction mixture to obtain a rate of cytochrome c reduction of 0.025 A/min. Xanthine oxidase was added to initiate the reaction. The units of SOD activity were determined from a calibration curve which was provided by the courtesy of Dr. E. M. Gregory.

Problems were encountered with this assay method. Cell extracts of both the wild type and mutant C. jejuni cells reduced the cytochrome c in the absence of all of the other reaction components. Increased washing of cells, dialysis for 16 hours, ultracentrifugation at 100,000 x g, and oxidation of cell extracts failed to rid the extracts of the reductant; extracts continued to reduce cytochrome c. Boiled extracts would not reduce cytochrome c. To be assured that the extracts were not respiring with xanthine, it was excluded from the reaction mixture; however, the reduction of cytochrome c continued in the absence of xanthine. In order to eliminate the possibility that the superoxide radi-

cals were being generated in the extracts themselves, SOD (70 U/ml) was added to the reaction mixtures. The rate of cytochrome c reduction was unchanged even in the presence of SOD.

Due to the problems encountered in the xanthine oxidase-cytochrome c assay, it was necessary to use an alternate assay. The hematoxylin assay of Martin et al. (1987) was used to assay for SOD activity. This assay is based upon studies by Martin et al. (1987) which showed that the autooxidation of reduced hematoxylin to the oxidized form at pH 7.4-7.6 is dependent upon superoxide radicals. The superoxide radicals that are required are generated as intermediates in the course of autooxidation. The oxidized form of hematoxylin absorbs light at 550-560 nm. If SOD is present, it will compete for the superoxide radicals, thereby inhibiting the formation of the oxidized form of hematoxylin and decreasing the rate of increase in absorbance at 550 nm. Reaction mixtures consist of:

Potassium phosphate buffer, 50 mM,
containing 1 mM EDTA (pH 7.5).....2.5 ml
Cell extract..... 0.5 ml
Reduced hematoxylin, 10mM in
10 mM potassium phosphate buffer (pH 6.0).....approx. 0.03 ml*

* or a volume sufficient to cause an absorbance increase of 0.02 min^{-1} at 550 nm.

Under these conditions (change in absorbance = 0.02 min^{-1}), the hematoxylin assay is one-half as sensitive as the xanthine oxidase-cytochrome c method. Thus the amount of SOD activity needed to inhibit the rate of increase in absorbance by one-half would be 2 units if measured by the xanthine oxidase-cytochrome c method. When the rate of increase in the absorbance is 0.02 min^{-1}, the relation between percent in-

hibition and the SOD units may be determined from the graph in Fig. 6 of Martin et al. (1987).

Whole cell Assays. For the assay of enzymatic activity of whole cells, the cells were harvested by centrifugation at 9,600 x g for 20 minutes. Cells used in the whole cell peroxidase assays were washed twice with 50 mM potassium phosphate buffer (pH 7.0). Cells used for the whole cell SOD assay were washed twice with 20 mM potassium phosphate buffer (pH 7.4) containing 1.0 mM EDTA. Both toluene-treated and untreated whole cells were assayed for peroxidase and SOD activity. Toluene-treatment of the whole cells involved the addition of 2.0 ml of toluene to 4.0 ml of cells. The suspensions were gently shaken for 1.0 minute. The upper toluene layer was removed and the treated cell suspensions were assayed immediately. Whole cell protein was determined by the method of Lowry et al. (1951). The methods that were used to assay peroxidase and SOD activities in crude cell extracts were also used with the whole cells. Peroxidase was assayed according to the method of Nickel and Cunningham (1969) and whole cell SOD was assayed by the method of Martin et al. (1987). Boiled cell suspensions served as controls.

Respiration Rates

Both the mutant MC711-01 and wild type C. jejuni H840 cells were grown according to the procedure described in the Materials and Methods section for growing large volumes of cells for the enzymes assays, except 750 ml of media was used instead of 1000 ml. Cells in the midlog

phase of growth were collected by centrifugation at 9,600 x g for 20 min, washed twice in 50 mM potassium phosphate buffer (pH 7.0) and suspended in buffer. A Clark-type electrode (YSI oxygen probe) was used to measure respiration rates. A Haake pump circulated water through the Clark cell at a constant temperature of 37°C. After the system was equilibrated with 50 mM potassium phosphate buffer (pH 7.0) saturated with air, the buffer was replaced with cell suspension. Air was bubbled through the suspension until the polarograph (YSI model 53 O_2 monitor) was stable between 80-100% relative oxygen. Then 100 µl of 0.15 M formate (pH 7.0) was injected and the rate of oxygen uptake was recorded (Sargent-Welch recorder, model SRGL). Potassium cyanide (10 mM) was used to inhibit respiration.

The amount of oxygen in the buffer was determined by the method of Robinson and Cooper (1970). This is a direct calibration method using N-methylphenazonium methosulfate (PMS).

Appendix B

Tolerance of Campylobacter jejuni to H_2O_2

Two typical graphs showing the survival of H840 and MC711-01 exposed to H_2O_2 were presented earlier in the Results section of this thesis. The results from additional experiments are shown in Figures 4, 5, and 6.

As indicated in the results section, when cells were grown under 21% oxygen, the addition of 0.002 M H_2O_2 was highly toxic to both the mutant and wild type cells. Because 0.002 M H_2O_2 was the concentration previously added to cells grown under 6% oxygen and because the previous results were obtained one year earlier, the possibility existed that H840 and MC711-01 might have become altered during continual serial transfer of stock cultures. Therefore, it was necessary to determine whether the previous results, obtained one year earlier, were reproducible. However, the results from the survival curves using cells which had been grown under 6% O_2 were reproducible (Figures 2 and 4); therefore, both the mutant and wild type cells appear to be more susceptible to the toxic effects of H_2O_2 when grown the cells are grown under 21% oxygen. The survival assays were subsequently performed using 0.001 M, instead of 0.002M, H_2O_2. The results from additional survival experiments are shown in Figures 7 and 8.

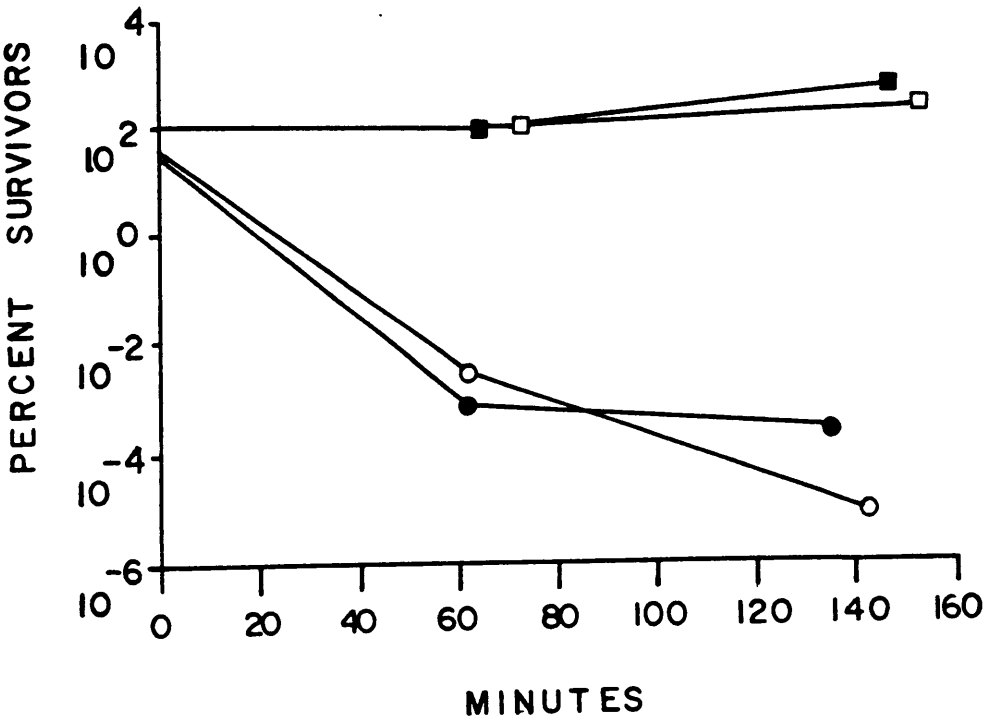

Figure 4. Survival of H840 and MC711-01 cells exposed to 0.002 M H_2O_2 in brucella broth. The cells had been grown under a 6% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added.

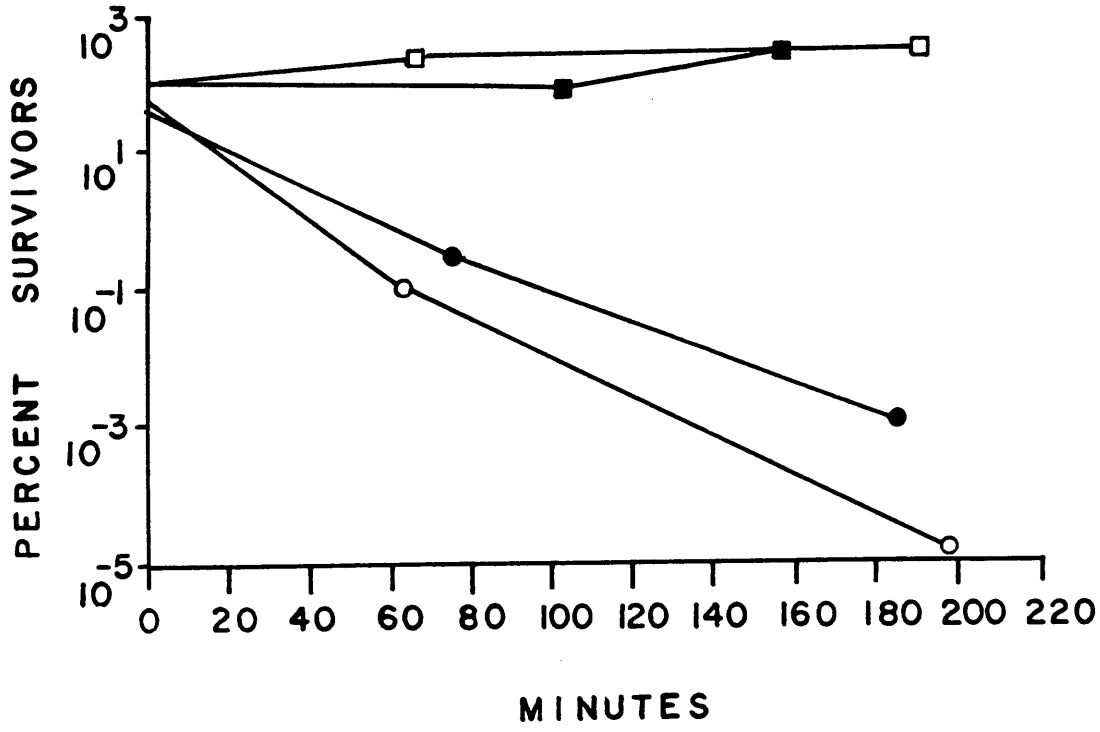

Figure 5. Survival of H840 and MC711-01 cells exposed to 0.002 M H_2O_2 in brucella broth. The cells had been grown under a 6% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added.

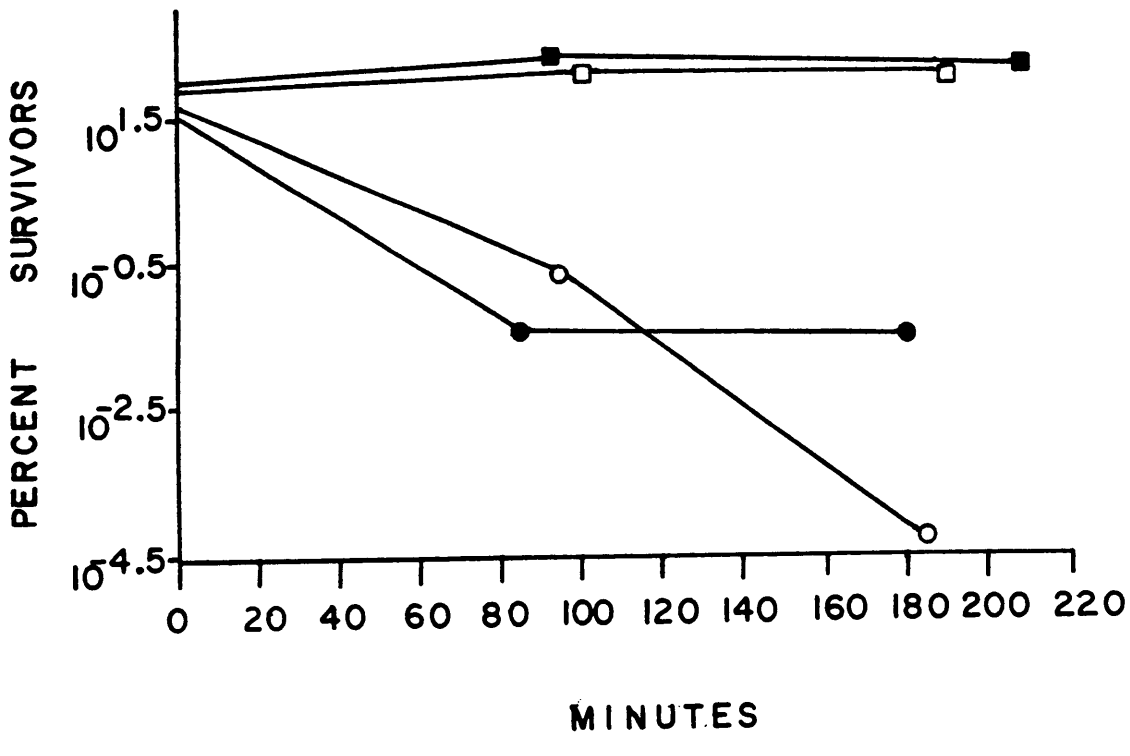

Figure 6. Survival of H840 and MC711-01 cells exposed to 0.002 M H_2O_2 in brucella broth. The cells had been grown under a 6% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added.

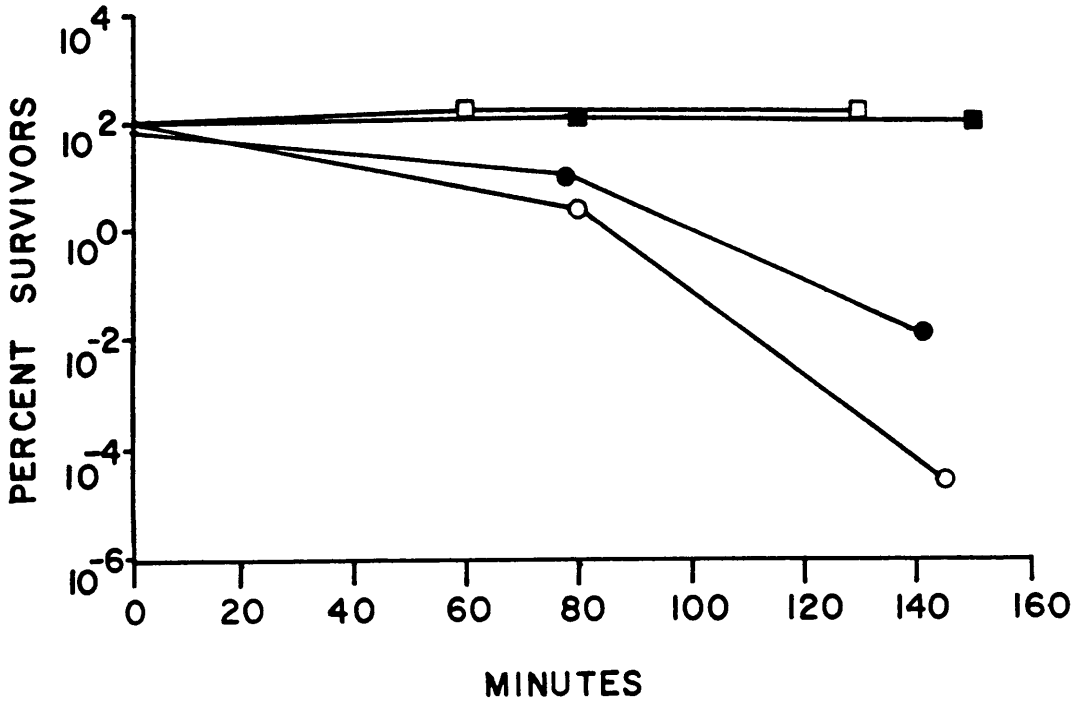

Figure 7. Survival of H840 and MC711-01 cells exposed to 0.001 M H_2O_2 in brucella broth. The cells had been grown under a 21% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added.

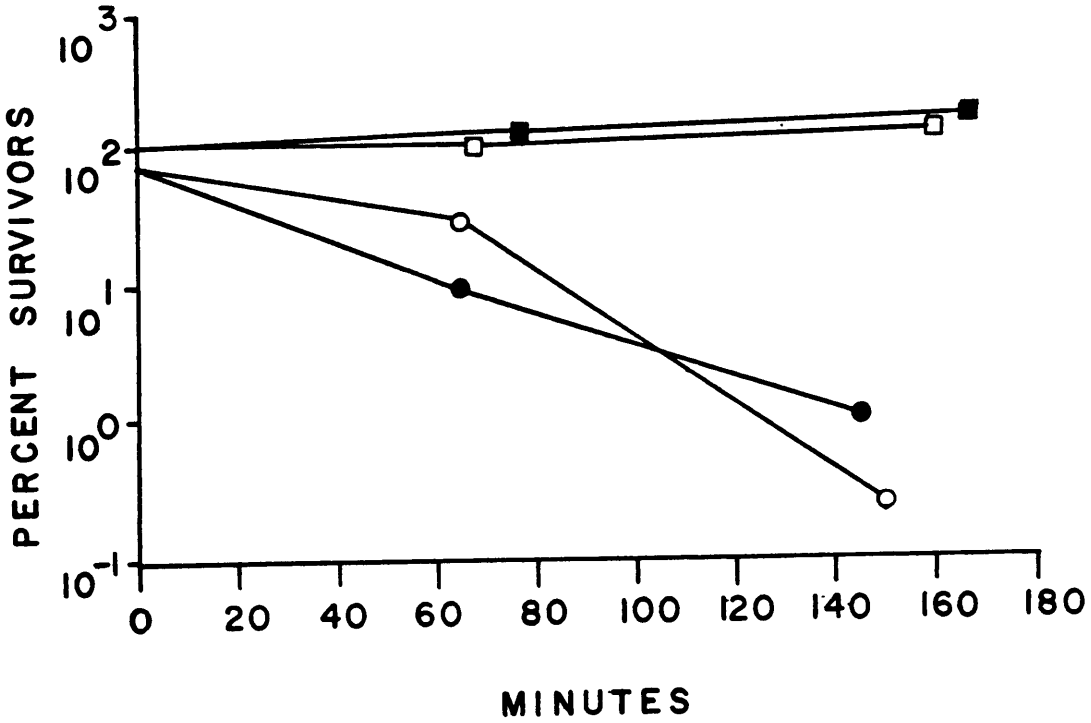

Figure 8. Survival of H840 and MC711-01 cells exposed to 0.001 M H_2O_2 in brucella broth. The cells had been grown under a 21% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added.

Effect of Using MFBP Supplement for Plate Counts

Because both MC711-01 and H840 cells were more susceptible to H_2O_2 when grown under 21% oxygen, MFBP supplement was added to brucella agar to determine whether it would enhance the recovery of C. jejuni. In two of the experiments, cells that had been grown under 21% oxygen were exposed to 0.001 M H_2O_2, were plated on unsupplemented brucella agar and also on brucella agar supplemented with MFBP. The results are shown in Figures 9 and 10. The FBP supplement enhanced the recovery of both mutant and wild type cells by an average of 47% in samples taken at 2.5 hours. It is interesting to note that the MFBP supplement had little or no effect on the recovery of cells from either the control flasks or from samples of the H_2O_2-treated flasks taken during the first 1.5-2.0 hours. Therefore it appears that MFBP is most beneficial for the recovery of the more highly stressed cells (e.g., those which have been subjected to low levels of H_2O_2 for more than two hours).

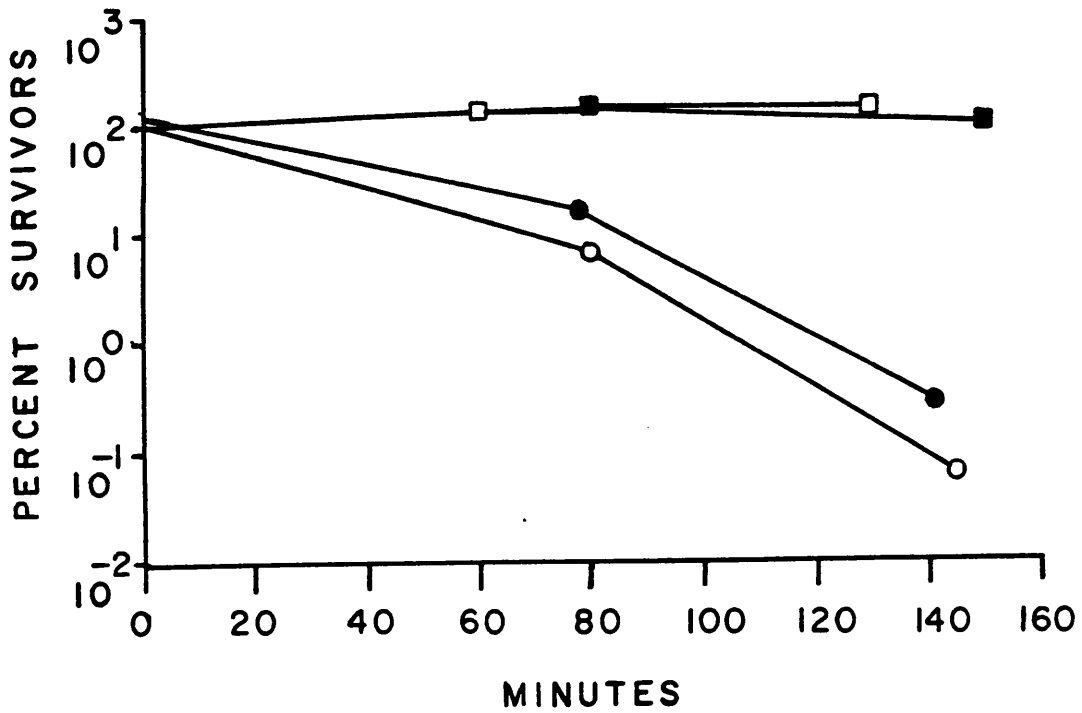

Figure 9. Effect of MFBP supplement on the recovery of H840 and MC711-01 cells exposed to 0.001 M H_2O_2 in brucella broth. The cells had been grown under a 21% oxygen atmosphere. Symbols: □, H840 control; ■, MC711-01 control; ○, H840 H_2O_2 added; ●, MC711-01 H_2O_2 added. This graph corresponds to Figure 7.

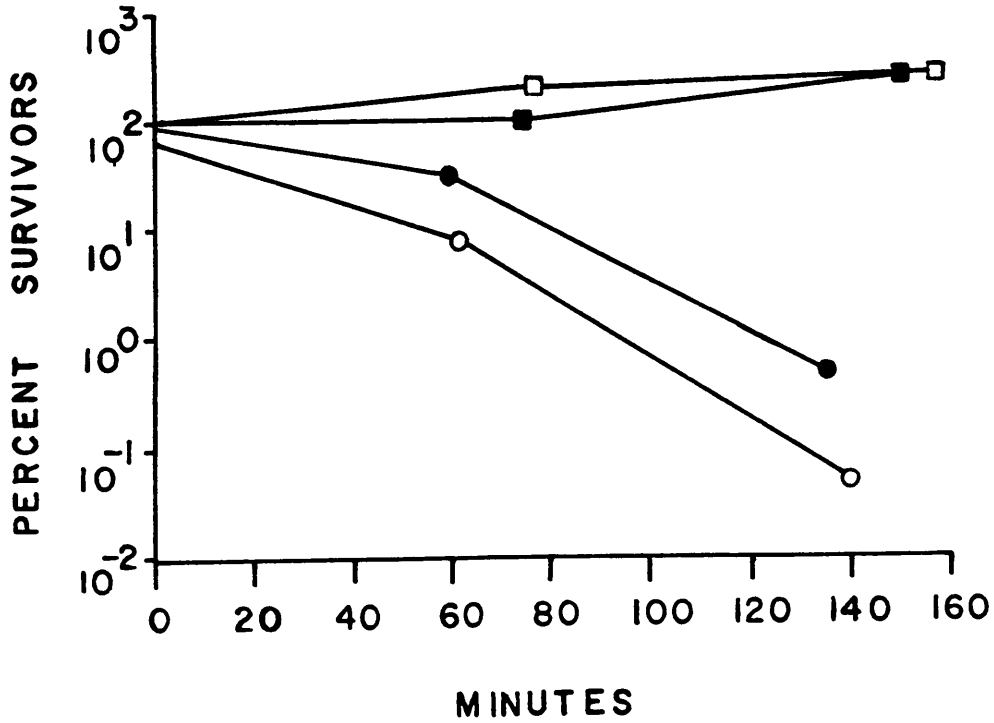

Figure 10. Effect of MFBP supplement on the recovery of H840 and MC711-01 cells exposed to 0.001 M H_2O_2 in brucella broth. The cells had been grown under a 21% oxygen atmosphere. Symbols: □ , H840 control; ■ , MC711-01 control; ○ , H840 H_2O_2 added; ● , MC711-01 H_2O_2 added. This graph corresponds to Figure 3.

Appendix C

Enzyme and Respiration Rate Assays

Factors That Influenced Hematoxylin Assay For SOD. Initial attempts to assay for SOD activity by the hematoxylin (HTH) method failed due to the procedure used for preparing the cell extracts and to the experimental conditions employed. The following factors enabled consistent results to be obtained for the HTH assay:

i) Dialysis was essential for obtaining SOD activity. All extracts were dialyzed for 60 hours in 5/8 inch membrane tubing (MW cutoff = 6-8,000) against two, 1.0-L volumes of 20 mM potassium phosphate buffer (pH 7.4).

ii) All reaction components, except the cell extracts, were stored at room temperature prior to use.

iii) Air was bubbled through the buffer to assure that is was well oxygenated.

iv) The HTH assay was tested with extracts which had been prepared in either 50 mM potassium phosphate buffer (pH 7.8) or 20 mM potassium phosphate buffer (pH 7.4). SOD activity was slightly higher with extracts prepared in 20 mM potassium phosphate buffer (pH 7.4). The SOD activity was also tested using buffer containing 1 mM mercaptoethanol, but the mercaptoethanol had no effect on activity.

Whole-Cell SOD and Peroxidase Assays. Whole-cell SOD and peroxidase assays were performed using both untreated and toluene-treated

cells. The results are summarized in Table 3. These assays were performed with cells that had been grown under 6% O_2, 3.0% CO_2, and 91% N_2. As expected, the peroxidase and SOD activities in the toluene-treated cells were similar to those of the cell extracts. While untreated H840 cells showed a small amount of peroxidase activity, none was detected in the untreated MC711-01 cells. Moreover, no SOD activity was detected in either untreated H840 or MC711-01 cells which suggests that the location of SOD within the cell may be inaccessible to the substrates of the assay.

Respiration Rates. Because the possibility exists that organisms possessing a low respiratory rate might be incapable of maintaining a reduced cell interior, the respiratory rates of C. jejuni H840 and MC711-01 were measured to determine whether any differences exist between the two cell types. Formate was used as the oxidizable substrate because previous studies (Hoffman et al., 1979) showed that the respiratory rates in C. jejuni strain H840 were highest with this substrate. Respiration rates were tested in cells grown under both 6% and 21% oxygen. The QO_2 (µl O_2/mg dry cell weight/hr) for cells oxidizing formate is listed in Table 4.

No decrease in respiratory rate was observed in cell grown at the higher oxygen tensions. Moreover, there was no apparent difference between the respiration rates of the mutant versus those of the wild type cells.

Potassium cyanide (10 mM) was added to cell suspensions to determine whether cyanide-resistant respiration occurred. When 100 µl of 10 mM KCN was added to undiluted cell suspensions (midlog phase cells

TABLE 3. Whole Cell SOD and Peroxidase Activity for Campylobacter jejuni H840 and the mutant MC711-01.

Enzyme	Specific activity[a]			
	H840 Toluene-Treated	H840 Untreated	MC711-01 Toluene-Treated	MC711-01 Untreated
SOD	3.6 ± 0.49	0.0	5.05 ± 2.1	0.0
Peroxidase	0.014 ± 0.005	0.0015 ± 0.0001	0.003 ± 0.003	0.0

[a] Activities are expressed as micromoles of substrate converted per minute (international units) per milligram protein. SOD activity is expressed as units per milligram protein.

Table 4. Respiratory rates of Campylobacter jejuni H840 and MC711-01 cultured under 6% and 21% O_2.

	Q_{O_2} (μl O_2/mg DCW/hr)	
Strain	6% O_2	21% O_2
H840	445 ± 115	398 ± 58
MC711-01	395 ± 187	477 ± 166

harvested from 250 ml of brucella broth and suspended in a total of 40 ml of potassium phosphate buffer, pH 7.0) the rate of respiration decreased but it did not stop. In order to halt respiration completely, it was necessary to dilute the cell suspensions fourfold. In addition, the injection of 100 µl of 5 mM sodium azide had no effect on the rates of oxygen uptake.

Appendix D

Metronidazole Sensitivity

Campylobacter species are susceptible to metronidazole, a chemical known for its selective toxicity towards anaerobes. Freydiere et al.(1984) reported that the in vitro susceptibility of 40 C. jejuni strains to metronidazole ranged from 0.25 to 64 µg per ml. Because aerotolerant Campylobacter species such as C. cryaerophila exhibit a greater resistance to metronidazole than microaerophilic species (Neill et al., 1985), the minimum inhibitory concentration (MIC) of metronidazole for C. jejuni H840 and the aerotolerant mutant MC711-01 was determined to see if MC711-01 might be more resistant.

Tubes of brucella broth containing various levels of metronidazole were prepared in triplicate and inoculated with 0.1 ml of a culture in the midlog phase of growth. Tubes containing no metronidazole served as a positive control. The tubes were incubated at 37°C for 48 hours in an atmosphere containing 6% O_2, 3.5% CO_2, and 90.5% N_2. The lowest concentration of metronidazole that completely inhibits growth, as indicated by lack of visible turbidity was recorded as the minimum inhibitory concentration (MIC).

The MIC of metronidazole was similar for both H840 and MC711-01, 10 µg/ml. The lack of any apparent difference in the susceptibility of the mutant and the wild type suggests that there is no difference between the two strains with respect to the presence of autooxidizable ferredoxins or flavodoxins.

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