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INTRODUCTION

Microaerophiles are organisms that can, and in some instances must, respire with oxygen (O_2) but cannot tolerate the levels of O_2 in air (21%, v/v). These organisms cannot defend themselves against reactive oxygen intermediates (ROIs) such as superoxide radicals ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$). These ROIs are formed from O_2 , and the higher the level of O_2 in the atmosphere under which the organisms are cultured the higher the level of reactive oxygen intermediates that are formed. The gigantic bacterium *S. volutans* is a good example of a microaerophile. Although first described in 1830, it resisted isolation for many years because it was vastly outnumbered by contaminants in mixed cultures and colonies would not develop on solid media. Microbiologists finally isolated it in 1962 by using a capillary method by which the bacterium was able to outswim contaminants. Even then it could only be grown in dialysis sacs suspended in a culture of other bacteria. It was originally thought that the other bacteria provided *S. volutans* with some unknown growth factor, but later work showed that they were merely using up some of the dissolved oxygen, creating the necessary microaerobic conditions. Although the physiological, genetic, and biochemical basis of microaerophily in *S. volutans* is not yet known, *S. volutans* does have an inordinately high sensitivity to $O_2\cdot^-$ and H_2O_2 which can be formed as an unavoidable consequence of oxidase reactions and spontaneously by various autooxidation reactions in culture media. Addition of supplements that remove or “quench” these ROIs can enhance the oxygen tolerance of the organisms. Cells of *S. volutans* may be sensitive to

ROIs because they are catalase negative and have only very low levels of peroxidase. There are other hypotheses why these cells are microaerophilic:

(i) The cells lack key DNA repair enzymes.

(ii) They rely on enzymes that are easily inactivated by oxygen.

S. volutans can be grown statically under an air atmosphere in special broth media containing various supplements which destroy ROIs. Growth on solid media is much more difficult, however, and it is difficult to obtain reproducible colony counts even under decreased levels of O_2. This makes studies, such as quantifying survival of cells that are exposed to heat and H_2O_2 difficult. Little is known about the response of *S. volutans* to oxidative stress because genetic characterization studies have not yet been done. Two-dimensional polyacrylamide electrophoresis is a powerful technique in the study of bacterial stress proteins, but this methodology has not yet been applied to *S. volutans* or to oxygen-tolerant mutants of this organism. The goal of the present study is to study the physiological response of the microaerophilic *Spirillum volutans* to oxidative stress, particularly exposure to H_2O_2. The specific objectives are as follows:

(i) To develop a reliable method of obtaining colony counts of *S. volutans* so that susceptibility to stress agents such as heat shock and hydrogen peroxide can be accurately quantified.

(ii) To isolate mutants that are resistant to H_2O_2 to test the idea that such a mutant should exhibit an increased tolerance to O_2.

(iii) To compare the protein profiles and enzyme activities of a H_2O_2-resistant mutant to the wild type cells in an attempt to identify key proteins/genes involved in the peroxide stress response of this microaerophile.

LITERATURE REVIEW

OXYGEN TOXICITY

The appearance of atmospheric O_2 on Earth, presumably due to the evolutionary development of cyanobacteria, offered other biota the opportunity to utilize O_2 as a terminal oxidant in respiration in order to gain energetic advantages over fermentation and respiratory pathways that rely on other oxidants (Imlay and Linn 1988). Even though O_2 can be used by microaerophiles and aerobes as a terminal electron acceptor for respiration, it also can be toxic to these organisms. The incomplete reduction of molecular oxygen during cellular metabolism or spontaneously by autooxidation reactions in the environment can result in formation of reactive oxygen intermediates (ROIs) such as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) (Fridovich 1978). These ROIs can damage cell components such as DNA, RNA, protein, and lipids.

Molecular oxygen

Theoretically, O_2 should be an excellent terminal electron acceptor because the E'_o of the O_2/H_2O half-cell system is very high (+0.8 V at pH 7.0). Oxygen in its ground state is a non-toxic triplet inorganic molecule which has one unpaired electron in each of its two π^* outer antibonding orbitals. However, due to the parallel directions of spin of these electrons, molecular oxygen cannot always accept two electrons readily from a reduced molecule. O_2 must accept a pair of electrons having a spin direction opposite to that of the two unpaired electrons of the O_2 molecule, thus obeying the Pauli exclusion principle

(Martinez-Cayuela 1995). This requirement restricts the range of compounds oxidized by oxygen (Farr and Kogoma 1991). The alternative to spontaneous two-electron reduction is a one-electron reduction that leads to formation of $O_2^{\cdot -}$. The reduction of O_2 to H_2O as the terminal reaction of an electron transport system requires four electrons ($O_2 + 4e^- + 4H^+ \Rightarrow 2\ H_2O$) and does not generate $O_2^{\cdot -}$. However, partial reduction of O_2 can generate ROIs, as indicated below (Salin and Brown-Peterson 1993):

$$O_2 + 1e^- \Rightarrow O_2^{\cdot -}$$
$$O_2 + 2e^- + 2H^+ \Rightarrow H_2O_2$$
$$O_2 + 3e^- + 3H^+ \Rightarrow OH^{\cdot} + H_2O$$

Singlet oxygen ($^1O_2$)

Singlet oxygen is an energized form of O_2 in which the direction of spin of one unpaired electron of ground-state dioxygen is reversed by an input of energy. This can give rise to either of two forms of singlet oxygen: O_2 ($^1\Sigma_g$), in which the two electrons continue to occupy separate orbitals, and O_2 ($^1\Delta_g$), in which the two electrons occupy one orbital and neither occupies the other orbital. Singlet oxygen is not a radical therefore does not possess unpaired electrons. Singlet oxygen is highly reactive because the spin restriction associated with ground state O_2 has been removed. It can subsequently oxidize a large variety of biological molecules such as lipids, proteins, and DNA and is responsible for cell destruction caused by light and some photosensitizers (Weters 1987; Krinsky 1979; Sies and Menck 1992). $^1O_2$ can be formed in a number of chemical, photochemical, and biochemical systems involving photooxidations, free radicals and lipid peroxides (Murray 1979).

The superoxide radical ($O_2\cdot^-$)

The univalent reduction of molecular oxygen produces the superoxide radical, which has one unpaired electron. Superoxide radicals exhibit moderate reactivity towards biomolecules in an aqueous environment compared to other ROIs and are capable of acting as either a reductant or oxidant. This moderate activity allows $O_2\cdot^-$ to diffuse for relatively long distances in biologic systems and thus can be generated at sites distant to the site at which it eventually causes toxicity (Miller and Britigan 1995). Superoxide can be generated enzymatically by certain flavoprotein dehydrogenases or non-enzymatically through the autooxidation of molecules such as ferredoxins, hydroquinones, and thiols (Fridovich 1978; Salin and Brown-Peterson 1993). The superoxide radical has been reported to exert a direct effect on certain enzymes such as catalase (Kono and Fridovich 1982), aconitase (Gardner and Fridovich 1992), and glutathione peroxidase (Blum and Fridovich 1985); however its main role in oxygen toxicity is probably due to its dismutation to form H_2O_2 or its interaction with H_2O_2 in an iron catalyzed Haber-Weiss reaction which can produce reactive hydroxyl radicals (Salin and Brown-Peterson 1993).

The dismutation to H_2O_2 occurs when one $O_2\cdot^-$ gives up its electron to another $O_2\cdot^-$ as follows:

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \Rightarrow H_2O_2 + O_2$$

Superoxide radicals will dismutate spontaneously but the reaction is limited by the electrostatic repulsion of the two anions (Fridovich 1978). At pH 13 superoxide radicals have a half-life of about 160 min whereas at pH 7 it is approximately a millisecond. The

half-life is about 100 times less at pH 4.8, which is the pK value, where equal concentrations of the ionized and nonionized forms are present ($O_2 \cdot^-$ and HO_2):

$$HO_2 + O_2 \cdot^- \Rightarrow HO_2^- + O_2$$

At pH 4.8 there is no charge repulsion, and dismutation takes place faster. The rate actually decreases from pH 4.8 to pH 2 and then remains constant below pH 2. The decrease occurs because the reaction

$$HO_2 + HO_2 \Rightarrow H_2O_2 + O_2$$

is slower than

$$HO_2 + O_2 \cdot^- \Rightarrow HO_2^- + O_2$$

The iron catalyzed Haber-Weiss reaction occurs in two steps as follows:

$$\begin{array}{rcl} O_2 \cdot^- + Fe^{+3}\ chelate & \Rightarrow & O_2 + Fe^{+2} \\ Fe^{+2}\ chelate + H_2O_2 & \Rightarrow & Fe^{+3}\ chelate + OH\cdot + OH^- \\ \hline O_2 \cdot^- + H_2O_2 & \Rightarrow & OH\cdot + OH^- + O_2 \end{array}$$

In this series of reactions, $O_2 \cdot^-$ acts as a reducing agent for the iron in Fe^{+3} chelate. Other reducing agents can accomplish the same reduction and thus superoxide radicals are not absolutely necessary for the generation of hydroxyl radicals. The second step, in which ferrous ions (produced from ferric ions by whatever mechanism) reduce H_2O_2 is called the Fenton reaction.

Hydrogen peroxide (H_2O_2)

The most stable of the oxygen intermediates is hydrogen peroxide, which is not a free radical. It results from the addition of two electrons to O_2 or as a product of dismutation of superoxide radicals. H_2O_2 is a more reactive oxidant than $O_2 \cdot^-$ and, being

uncharged and soluble in organic solvents, it readily crosses biological membranes. The reactions of H_2O_2 with organic molecules remain unclear, partly because it reacts quickly in the presence of contaminating metals to form other ROIs which obscure its own role in oxidation reactions (Farr and Kogoma 1991). It can act as a weak oxidizing agent and can damage DNA (Steiner et al. 1984), lipids (Kellogg and Fridovich 1977), and can attack thiol groups of proteins or reduced glutathione. It can also react directly with some keto acids (Halliwell and Gutteridge 1990; Wefers and Sies 1983). Most importantly, H_2O_2 will react with reduced iron or copper ions to generate hydroxyl radicals (OH·) in the Fenton reaction (Cadenas 1989).

Certain reactions catalyzed by flavoproteins such as xanthine oxidase or NADPH oxidase generate H_2O_2 by forming $O_2\cdot^-$ as an intermediate, which can then dismutate. The $O_2\cdot^-$ is generated when the reduced prosthetic group, $FADH_2$ reacts spontaneously with two molecules of O_2 :

$$FADH_2 + 2O_2 \Rightarrow 2O_2\cdot^- + FAD$$

The 2 $O_2\cdot^-$ then undergo dismutation to yield O_2 and H_2O_2. In other oxidase reactions, however, H_2O_2 can be generated directly by a two-electron reduction of O_2 without formation of $O_2\cdot^-$ as an intermediate (Salin and Brown-Peterson 1993):

$$FADH_2 + O_2 \Rightarrow H_2O_2 + FAD$$

Both $O_2\cdot^-$and H_2O_2 can also be generated nonenzymatically during the autooxidation of various reduced flavins, quinones, thiols, and iron/sulfur proteins (Fridovich 1978; Misra and Fridovich 1971).

The hydroxyl radical (OH·)

Hydroxyl radicals result from the univalent reduction of H_2O_2. Hydroxyl radicals are extremely powerful oxidants (the $E'_{o\ (pH\ 7)}$ of the reaction $OH\cdot + e^- \Rightarrow OH^-$ is +2.33 V) and have the potential to cause oxidative damage to almost any cell component. Hydroxyl radicals have a short half-life in solution since they react with other molecules at nearly diffusion controlled rates. The main source of hydroxyl radicals is the metal-catalyzed Haber-Weiss reaction as described above (Martinez-Cayuela 1995).

MECHANISMS FOR PROTECTING BACTERIA AGAINST ROIs

Peroxidases

Peroxidases are a class of iron-heme proteins that can catalyze the destruction of H_2O_2 by the overall reaction: $RH_2 + H_2O_2 \Rightarrow ROH + H_2O$

One type of peroxidase, donor:H_2O_2 oxidoreductase, is nonspecific where R can be any of a variety of organic compounds. The usual assay substrate for this peroxidase is some nonbiological chromogenic substrate such as *o*-dianisidine. Other peroxidases such as cytochrome *c* peroxidase, NADH peroxidase, glutathione peroxidase, and ascorbate-specific peroxidase, have a requirement for a specific reduced substrate. In some studies

the occurrence of donor:H_2O_2 oxidoreductase has been correlated with aerotolerance (Rolfe et al.1978; Padgett and Krieg 1986).

Catalase

Catalase is an iron-heme protein that catalyzes the destruction of H_2O_2 by the following reaction: $2H_2O_2 \Rightarrow 2H_2O + O_2$

Catalase can effectively remove high levels of H_2O_2 from the cell before excessive damage occurs but it is relatively ineffective at removing low doses because of its low affinity (high K_m) for this oxidant. The importance of this enzyme has been demonstrated directly by mutant strains of *S. typhimurium* and *E. coli* that lack catalase and peroxidase (Levine 1977; Loewen 1984). However, some organisms that possess catalase such as *Campylobacter jejuni* are intolerant of O_2 levels found in air (Vercellone et al. 1990). Catalase was once thought only to occur in bacteria that could metabolize oxygen, but some obligate anaerobes do induce synthesis of this enzyme when exposed to air.

Superoxide dismutase (SOD)

Superoxide dismutases belong to a class of metalloenzymes that effectively scavenge superoxide radicals (Fridovich 1974; Fridovich 1975). They catalyze the dismutation of superoxide radicals:

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \Rightarrow H_2O_2 + O_2$$

SOD can increase the rate of dismutation by a factor of 10^9 at physiological pH values (Fridovich 1978) because the enzyme acts as an intermediary that makes collisions between two superoxide radicals unnecessary. For instance, with Fe-SOD:

$$O_2\cdot^- + Fe^{3+}\text{-SOD} \Rightarrow O_2 + Fe^{2+}\text{-SOD}$$
$$Fe^{2+}\text{-SOD} + O_2\cdot^- + 2H^+ \Rightarrow H_2O_2 + Fe^{3+}\text{-SOD}$$
$$\overline{O_2\cdot^- + O_2\cdot^- + 2H^+ \Rightarrow H_2O_2 + O_2}$$

SOD was once thought to only occur in bacteria that can metabolize oxygen, but have since been found in obligately anaerobic bacteria as well (Gregory et al. 1978). There are superoxide dismutases with either copper and zinc, manganese or iron at the active site, but they all catalyze superoxide dismutation with comparable efficiency (Fridovich 1978). Some bacteria possess one, two, or all of these enzymes while some bacteria have no SOD. MnSOD and FeSOD can occur in the cytosol of bacteria and CuZnSOD can occur in the cytosol and/or the periplasmic space of bacteria.

Alkylhydroperoxide reductases

Alkylhydroperoxide reductases can reduce organic peroxides such as cumene hydroperoxides and *t*-butyl hydroperoxide. Christman et al. (1985) found that a hydrogen-peroxide resistant mutant (*oxyR1*) of *E. coli* contained 20-fold levels of this enzyme.

Glutathione

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly). It is an important antioxidant and is synthesized by glutathione synthetase. High intracellular concentrations of GSH in *E. coli* provide a highly reducing environment (Loewen 1979). GSH can be oxidized by $O_2\cdot^-$ and H_2O_2 to GSSG and glutathione reductase can reduce the GSSG back to GSH. Christman et al. (1985) observed that cell extracts of peroxide-resistant mutants of *E. coli* contain 4 times higher levels of glutathione reductase than do wild type extracts. Reduced glutathione is probably important in preventing excessive damage from ROIs (Gardner and Fridovich 1993; Morse and Dahl 1978), and its coregulation with superoxide dismutase and catalase supports this proposed function (Christman et al. 1985). Another important function of glutathione is to reduce disulfide bridges of proteins caused by oxidative stress.

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase can produce reducing equivalents (i.e., NADPH) needed for other oxidative stress enzymes such as glutathione reductase and alkyl hydroperoxide reductase (Christman et al. 1985).

DNA repair enzymes

Cells have evolved extremely sophisticated DNA repair mechanisms to ensure the stability of their genetic information. Despite the presence of enzymes and other agents that destroy ROIs, inevitably some ROIs escape destruction and oxidative damage to DNA can occur. Some of these DNA repair enzymes are AP endonuclease, endonuclease III, DNA polymerase, excision nuclease, and exonuclease. Although tolerance to H_2O_2

has been related to DNA repair mechanisms in bacteria (e.g., Zirkle and Krieg 1996), to what extent oxygen tolerance is related to DNA repair mechanisms in bacteria is unknown.

Use of host's protective enzymes

It is possible that some bacteria such as *T. pallidum* and *Mycoplasma pneumoniae* which lack key protective enzymes such as SOD, catalase, and/or peroxidase may depend on the host animal's defense mechanisms to protect them from ROIs (Steiner et al. 1984; Lynch and Cole 1980).

Excretion of reactive oxygen intermediates from the cell

Some bacteria such as *Bacillus popilliae*, *Streptococcus pneumoniae*, some mycoplasmas, and some cyanobacteria appear to excrete hydrogen peroxide from the cell in an attempt to avoid oxidative damage (Halliwell 1979).

Respiration rates

Some organisms that depend on oxygen labile enzymes such as nitrogenase must have some way to protect these cell components. An example of this is *Azotobacter chroococcum* which protects its oxygen-labile nitrogenase by maintaining a high rate of respiration which keeps the cell interior relatively anaerobic (Dalton and Postgate 1969).

Carotenoids

Carotenoids not only act as accessory pigments in some bacteria but also can effectively scavenge singlet oxygen (Krinsky 1979).

Thick cell walls and slime

Some cyanobacteria can protect oxygen-labile enzymes such as nitrogenase by formation of thick-walled cells called heterocysts. The heterocysts also lack Photosystem II and thus do not evolve O_2 like the vegetative cells. In addition, the nitrogen-fixer *Derxia gummosa* surrounds its cells with a great deal of slime in which oxygen diffuses slowly.

MULTIGENE OXIDATIVE STRESS RESPONSES OF BACTERIA

Virtually all aerobic organisms have evolved complex defense and repair mechanisms to mitigate the damaging effects of ROIs (McCord and Fridovich 1988; McCord et al. 1971). Many bacteria appear to encode multigene responses to oxidative stress (Farr and Kogoma 1991). There has been tremendous progress in the study of the physiological and genetic responses of bacteria to oxidative stress over the last twenty years, in part because of the discovery of powerful genetic research techniques. The oxidative stress response of bacteria can be categorized according to the type of oxidant involved because each oxidant can have dramatically different effects on gene expression (Dowds 1994). Research on the effect of oxidative damage on stress protein induction has been done mainly with the facultatively anaerobes *E. coli* and *Salmonella typhimurium*.

Peroxide stress response

When bacterial cells of *S. typhimurium* and *E. coli* are treated with low doses of H_2O_2 and other organic peroxides such as cumene hydroperoxide, the synthesis of at least 30 proteins is induced and the cells become more resistant to subsequent doses of peroxide that would otherwise be lethal (Christman et al. 1985; Morgan et al. 1986). Some of these proteins include HPI catalase, alkylhydroperoxide reductase, DnaK, RecA, and Dps (Farr and Kogoma 1991; Altuvia et al. 1994). The genes that encode these proteins are members of the OxyR regulon. A regulon is a gene regulatory system involving genes or operons that are scattered in the genome but that respond to a common regulatory signal or protein. The OxyR regulon is controlled by the OxyR protein that can act as both a sensor of oxidative stress and a transcriptional activator of genes in the regulon. The reduced form of the protein can become oxidized when cells are exposed to a flux of peroxide. Both oxidized and reduced forms of the protein can bind to specific sites in the DNA but only the oxidized form serves as an activator of the genes in the OxyR regulon (Farr and Kogoma 1991).

Superoxide stress response

The genetic and physiological response of cells to superoxide seem to be separate and distinct from the response of cells to peroxide stress. *E. coli* cells treated with paraquat and other superoxide-generating compounds induce the synthesis of at least 33

proteins that are not induced by H_2O_2 (Greenburg and Demple 1989) such as MnSOD, endonuclease IV, GroES, and glucose-6-phosphate dehydrogenase. Some of the proteins induced by superoxide stress, such as HPI catalase and alkylhydroperoxide reductase, are also induced by peroxide, probably because superoxide is unstable and rapidly dismutates to form H_2O_2.

In *E. coli* the *soxR* and *soxS* genes control the superoxide response regulon, *soxRS*, which is a two-component system (Wu and Weiss 1992). In a two-component system one protein is often a sensor while another protein is the response regulator (Ronson et al. 1987). In the *soxRS* regulon, SoxR protein is a superoxide sensor which when activated can stimulate the transcription of a second protein, SoxS. SoxS protein can subsequently activate the transcription of other genes that encode superoxide stress proteins (Wu and Weiss 1992).

Oxidative stress response overlaps with other stress responses

Oxidative stress can induce a number of proteins that are also induced by other stress agents such as heat shock and DNA damage (Christman et al. 1985; Greenburg and Demple 1989; Morgan et al. 1986; VanBogelen et al. 1987; Walkup et al. 1989). In *E. coli*, for example, proteins GroEL and GroES are induced by heat stress, DNA damage, carbon starvation and oxidative stress (superoxide and peroxide) (Morgan et al. 1986; VanBogelen et al. 1987; Greenburg and Demple 1989). In addition, RecA protein, which is involved in the DNA repair, is also induced by oxidative stress (Greenburg and Demple

1989). The induction of these proteins reflects the fact that ROIs can cause damage to a wide range of biomolecules.

MICROAEROPHILES

Microaerophiles are organisms that are capable of oxygen-dependent respiration yet cannot grow at levels of oxygen present in an air atmosphere (21 % O_2 v/v) or grow poorly (Krieg and Hoffman 1986). Microaerophiles are prime examples of the "oxygen paradox": i.e. they can— and in some instances must—respire with O_2 as the terminal electron acceptor, yet oxygen is inherently dangerous to their existence. According to Krieg and Hoffman (1986) the level of oxygen preferred by a microaerophilic bacterium varies with species and strain. Some microaerophiles can use an alternative terminal electron acceptor and some can grow by fermentation, but the preference for low O_2 concentrations when using O_2 as a terminal electron acceptor distinguishes these bacteria from anaerobic, facultatively anaerobic, and aerobic bacteria.

The habitats in which microaerophiles occur are as fascinating as the physiological diversity they possess. For example, *Gallionella ferruginea*, a chemolithoautotrophic microaerophile is found in iron-rich, aquatic environments and uses ferrous iron as its only electron donor and can fix CO_2 via the Calvin cycle (Hanert 1992). *Aquaspirillum magnetotactum*, a chemoorganotrophic microaerophile found in

freshwater swamp sediments is capable of manufacturing magnetosomes (enveloped inclusion bodies containing Fe_3O_4) that govern tactic responses to magnetic fields (Maratea and Blakemore 1981). Some microaerophiles are important human pathogens such as *Campylobacter jejuni*, *Treponema pallidum*, and *Helicobacter pylori*. Others are nitrogen fixers such as *Azospirillum brasilense*. Microaerophiles seem to have evolved to occupy specific ecological niches giving them a competitive advantage over other aerobic, facultatively anaerobic, and anaerobic bacteria. The physiology of microaerophiles is intriguing because they are similar to anaerobes in some ways but similar to aerobes in other ways. Aerobic organisms catalyze the oxidation of pyruvate by an NAD-linked pyruvate dehydrogenase multienzyme complex yet nearly all microaerophiles tested to date possess the enzyme pyruvate:ferredoxin/flavodoxin oxidoreductase (Krieg 1996) once believed to occur only in anaerobes. Microaerophiles that have been reported to contain this enzyme include many *Campylobacter* species (Daucher and Krieg 1995; Lascelles and Calder 1985), *H. pylori* (Hughes et al. 1995) and *T. pallidum* (cited in Krieg and Hoffman 1986). There is also circumstantial evidence (i.e. a high sensitivity to metronidazole) that this enzyme occurs in *S. volutans* (Padgett and Krieg 1986).

THE MICROAEROPHILE *SPIRILLUM VOLUTANS*

Taxonomy

Spirillum volutans is the only species presently contained in the genus *Spirillu*m, which is a member of the β division of the Proteobacteria. Based on 16*S* rRNA sequencing, the genus is most closely related to *Thiobacillus thioparus* (Maidak et al. 1997).

Habitat

S. volutans is widely distributed in many stagnant freshwater environments (Krieg 1992). Only two strains have been isolated in pure culture, one from hay infusion prepared with water from a pond in Virginia (Wells and Krieg 1965) and the other from the cooling water of a sugar beet refinery in England (Rittenberg and Rittenberg 1962).

Enrichment and Isolation

Mixed cultures of the organism occurring in hay infusions or other sources can be enriched by inoculating Pringsheim's soil medium (Rittenberg and Rittenberg 1962). Isolation is difficult even with enrichment because *S. volutans* is greatly outnumbered by other organisms and no selective medium has been described. The only method for isolation is a capillary tube method first described by Geisberger (1936). Rittenberg and Rittenberg (1962) successfully used this method to isolate the first strain of *S. volutans* in pure culture. This method made use of the rapid swimming motility of *S. volutans*, which enabled the organism to outswim the contaminants. However, the Rittenbergs were able to maintain the organism in pure culture only by growing it in a dialysis sac suspended in a culture of other bacteria. It was believed at the time that the presence of the other

bacteria supplied some growth factor for *S. volutans*. The organism was not grown in the absence of other bacteria until its microaerophilic nature was recognized by Wells and Krieg (1965), who indicated that the main function of the mixed culture outside the dialysis sac was to decrease the levels of dissolved oxygen in the medium. In retrospect, it seems possible that the organisms outside of the dialysis sac also might have destroyed toxic ROIs in the medium.

Morphology and Physiology

Morphologically, cells of *S. volutans* are described (Hylemon et al 1973) as large, rigid, and helical 1.4 to 1.7 µm in diameter. They have the form of left-handed, or counterclockwise helices (Swan 1985). The cells are 14-60 µm in length and have from less than one to a maximum of five turns with a wavelength of 16 to 28 µm and a helix diameter of 5-8 µm. The cells are aerotactic (Caraway and Krieg 1974) and are motile by means of bipolar fascicles of flagella which contain nonsheathed flagella. Cells of *S. volutans* are gram negative and often contain many prominent granules of poly-β-hydroxybutyrate.

In regard to physiological properties, *S. volutans* is catalase negative and possesses a strictly respiratory-type metabolism with oxygen as the only known terminal electron acceptor (Krieg 1992). Padgett et al. (1982) found that growth is inhibited by very low levels of ROIs (superoxide radicals and hydrogen peroxide) and that the inclusion of agents which destroy these ROIs such as catalase, superoxide dismutase, potassium metabisulfite, and norepinephrine in the growth medium enhance its

aerotolerance. The organism does not catabolize carbohydrates and NaCl levels greater than 0.02 % and phosphate levels greater than 0.01 M are inhibitory to growth (Krieg 1994). The optimal growth temperature for *S. volutans* is 30-36^{o}C and the optimal pH is 7.0-8.2 (Moore 1984).

Cultivation

Although cells are microaerophilic in dilute liquid media such as nutrient broth and sterile hay infusion (and grow poorly) it is possible to grow cells under aerobic conditions. The first way cells can be cultured in an air atmosphere (21 % O_2) is by using semisolid media. Growth occurs in a semisolid medium as a thin band at a point beneath the surface of the medium where the rate at which the cells use O_2 is equal to the rate at which O_2 is diffusing to the cells. As the cell numbers increase the band enlarges and often settles at a point just below the surface of the semisolid medium within 48 hrs (Krieg 1992). It is also possible to obtain aerobic growth in special broth media such as vitamin-free, salt-free casein-hydrolysate-succinate-salts (CHSS) of Padgett et al. (1982). The development of colonies of *S. volutans* on the surface of a solid medium is much more difficult and depends on addition of bisulfite, catalase, or superoxide dismutase, protection of the medium from illumination, incubation in a humid atmosphere, and incubation under atmospheres containing 12% oxygen or less (Padgett et al. 1982). Even if these precautions are taken, colony counts are highly erratic and vary from 22 to 72% of the direct microscopic count (Padgett et al. 1986).

WHY ARE MICROAEROPHILES MICROAEROPHILIC ?

Lack of key protective enzymes

Since ROIs are an inescapable biproduct of an aerobic lifestyle, aerobic organisms must be able to successfully scavenge and destroy ROIs. Most aerotolerant organisms have evolved effective defense mechanisms against the threat of oxidative damage. Aerotolerant cells such as *E. coli* contain enzymes such as SOD, catalase, and alkylhydroperoxide reductase which destroy dangerous ROIs. Many microaerophiles lack one or all of these important enzymes (Krieg and Hoffman 1986). For example, *S. volutans* (Padgett et al. 1982), *A. magnetotacticum* (Maratea and Blakemore 1981), *Beggiatoa* spp. (Burton and Morita 1964), and several species of *Campylobacter* (Roop et al. 1985) are catalase negative. Some microaerophiles like *T. pallidum* lack both catalase and SOD. Microaerophiles that lack these important enzymes often exhibit a high sensitivity to peroxide and superoxide radicals (Padgett and Krieg 1986; Hoffman et al. 1979; Steiner et al. 1984).

ROIs have also been shown to cause damage to DNA (Imlay and Linn 1988; Imlay et al. 1988). Aerotolerant organisms have evolved DNA repair systems for oxidatively damaged DNA (Imlay and Linn 1988). Little is known about DNA repair in microaerophiles and it is possible that they lack these key DNA repair enzymes. Repair of different types of DNA damage is very important to a cell. Studies by Steiner et al. (1984) which compared DNA repair in *E. coli* to the microaerophilic *T. pallidum* showed that *T. pallidum* was deficient in DNA repair and allowed extensive DNA damage even at low

levels of H_2O_2 (100 μM). This seems to suggest that microaerophiles may be microaerophilic because they are not capable of repairing DNA under high oxygen tensions. However, no studies have been done comparing the oxygen tolerance of mutant bacteria that lack DNA repair mechanisms with that of the parent types that do possess such mechanisms.

High sensitivity to reactive oxygen intermediates (ROIs)

Hoffman et al. (1979) proposed that microaerophiles may be microaerophilic largely due to their high sensitivity to H_2O_2 and $O_2\cdot^-$ occurring in culture media. Exogenous ROIs in culture media presumably could effectively damage the cell membrane(s) and ROIs such as H_2O_2 can cross membranes and oxidize intracellular or periplasmic cell components before they encounter any cell defenses. H_2O_2 could also generate intracellular OH· by means of the Fenton reaction. Growing under reduced oxygen tensions would prevent an excess of ROIs. This proposal is supported by the fact that supplementation of culture media with compounds that destroy H_2O_2 and $O_2\cdot^-$ have been shown to increase the aerotolerance of several microaerophiles (Hoffman et al. 1979; Padgett et al. 1986; Blakemore et al. 1979; see Table 1 in Krieg and Hoffman, 1986). Supplements which destroy H_2O_2 include catalase, peroxidase, MnO_2 (Jones et al. 1970), and pyruvate (Thompson et al.1951). Charcoal can destroy both OH· and H_2O_2 (Hoffman et al. 1983). A high sensitivity to OH· may also play a part in microaerophilic behavior because supplements such as mannitol and histidine which destroy OH· (Brawn

and Fridovich 1981) can also enhance the aerotolerance of microaerophiles (Steiner et al. 1984).

Occurrence of Oxygen-Sensitive Cell Constituents

Microaerophiles may contain cell components that are sensitive to and possibly inactivated by oxygen. Proteins that could be effected by an excess of oxygen are porins, cytochromes, flavoproteins, and iron/sulfur proteins. In addition, pyruvate:ferredoxin/ flavodoxin oxidoreductase has been reported in many microaerophiles and could be a key target for inactivation by oxygen since it is unstable in the presence of oxygen and must be assayed under anaerobic conditions (Krieg and Hoffman 1986).

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Chapter 1

Improved method for colony counts of the microaerophile *Spirillum volutans*

Abstract

Studies of adaptive responses of *Spirillum volutans* to various stresses such as heat shock have been hampered by an inability to obtain reliable colony counts of the organism by the spread plate method, due in part to differences among various lots of the casein hydrolysate component of the medium. Colony counts approaching direct microscopic counts (DMCs) were obtained by inoculating culture dilutions into a semisolid version of the medium and using this to overlay a thicker layer of sterile medium, and by supplementing the medium with pyruvate, which destroys hydrogen peroxide. Both the pyruvate and the overlay were necessary for optimal results. Use of the new overlay/pyruvate method for colony counts revealed that exposure of *S. volutans* to 40°C for 100 min results in a greater survival at 45°C compared with cells having no prior exposure to 40°C.

Microaerophiles illustrate the mixed blessings of a dependence on oxygen in that the organisms can exhibit oxygen-dependent growth but cannot tolerate the level of oxygen present in air (21%, v/v). The gigantic freshwater spirillum *Spirillum volutans* is a classic example. Although first described in 1830, microbiologists failed to isolate it until 1962 because contaminants vastly outnumbered it in mixed cultures and because colonies would not develop on solid media. It was eventually isolated by letting it "outswim" contaminants in a long capillary tube (Rittenberg and Rittenberg 1962), but pure cultures could be maintained only in dialysis sacs suspended in cultures of other bacteria. The latter were presumed to supply an unknown growth factor, but Wells and Krieg (1965) discovered that they were merely using up some of the dissolved oxygen in the culture, and that the organism could be grown alone under gaseous atmospheres containing 1 to 9 % O_2. Although *S. volutans* has a strictly respiratory type of metabolism with O_2 as the sole terminal electron acceptor (Krieg 1992), it does not grow on solid media at oxygen levels above 12 % (although some variants have been reported that can tolerate 21% O_2). The organism can grow aerobically in certain liquid media but, as Krieg and Hoffman (1986) noted, it is much more difficult to grow microaerophiles on solid media than in liquid media. Padgett et al. (1982) were able to obtain colonies of *S. volutans* on the surface of a solid medium by a spread plating method, providing that the following requirements were met: addition of bisulfite, catalase, or superoxide dismutase, protection of the medium from illumination, incubation in a humid atmosphere, and incubation under atmospheres containing 12 % oxygen or less. They also reported that a

new lot of the "vitamin-free, salt-free" acid hydrolyzed casein (CH) component of the medium failed to support growth because it contained less NaCl than a previous lot. This led to the discovery that growth fails to occur with NaCl concentrations less than 0.01 % or greater than 0.02 %. Even with appropriate NaCl levels, colony counts were highly erratic and varied from 22 to 72 % of the direct microscopic count (DMC). The purpose of the present research was to develop an improved method for obtaining colony counts of the organism.

Spirillum volutans ATCC strain 19554 was maintained by aerobic incubation in 5.0-mL portions of casein hydrolysate-succinate-salts-bisulfite (CHSS) broth as described by Padgett and Krieg (1986). The "vitamin-free, salt free" CH (ICN Biochemicals, Aurora, OH) was from lot no. 11523—the same lot used previously by Padgett et al. (1982). When supplies of this lot became low, two new lots were purchased (no. 60023 and no. 69278); however, growth was variable and large inocula (at least 0.6 mL of a previous 24-h-old culture to 5 mL of broth) were required. At first this was attributed to differences in the NaCl content of the "vitamin-free, salt-free" CH, which varied among different lots from 0.46 to 1.1% of the dry powder, but varying the NaCl concentration did not alleviate the problem. The reason for the differences among the various lots is not known but may be related to variations in the manufacturing process.

The inability of *S. volutans* to grow under an air atmosphere has been attributed in part to the organism's lack of catalase and to a consequent susceptibility to very low levels of hydrogen peroxide formed spontaneously in culture media, especially under illumination. Addition of catalase to culture media increases the oxygen tolerance of *S.*

volutans (Padgett et al. 1982), presumably by destroying H_2O_2. Pyruvate is also known to destroy H_2O_2 (Thompson et al. 1951) but had never been used in media for *S. volutans*. It has been reported to enhance the oxygen tolerance of microaerophiles such as *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, and *Campylobacter fetus* subsp. *fetus* (Hodge 1994). It has also been reported to increase the detection of physiologically stressed or injured cells of *Escherichia coli*, *Salmonella* species, and coliform bacteria (Calabrese and Bissonnette 1990; Brewer et al. 1977; Lee and Hartman 1989). Thus it seemed likely that pyruvate might be helpful in overcoming the problems associated with the new lots of CH. We found that the addition of 0.03 % sodium pyruvate to the medium allowed aerobic growth from inoculum volumes of 0.05 mL or less regardless of the particular lot of CH used. This suggested that pyruvate might also be useful in obtaining reliable colony counts of *S. volutans* on solid media.

Another factor favoring colony development by *S. volutans* is high humidity (Padgett et al. 1982), achieved by lining culture vessels with moistened filter paper. It seemed possible that this requirement could be eliminated by inoculating the cells into a medium containing 0.7 % agar (which prevents the swimming motility of *S. volutans*) and using this to overlay a thicker layer of sterile medium.

The colony count method that was finally developed made use of pyruvate-supplemented medium and an overlay. The colony count medium (CCM) was a modification of CHSS medium and had the following composition (per L of distilled water): 2.6 g vitamin-free, salt-free CH (ICN Biochemicals Inc., Aurora, Ohio), 1.0 g succinic acid (free acid), 1.0 g $(NH_4)_2SO_4$, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.12 g KH_2PO_4, 0.04 g

NaCl, 0.3 g sodium pyruvate, 0.002 g $FeCl_3 \cdot 6H_2O$, and 0.002 g $MnSO_4 \cdot H_2O$. The pH was adjusted to 7.3 with KOH, 0.05 g potassium metabisulfite was added, and the pH was readjusted to 7.3. Agar (7 g/L) was added and dissolved by boiling, and the medium was sterilized by autoclaving. The pH of the cooled medium after autoclaving was 6.8.

Cells to be used for colony counts were grown in 5.0 mL of CCM broth contained in slanted 20 x 120-mm screw-cap tubes incubated aerobically for 24 h at 30°C. Direct microscopic counts were made with a hemocytometer and decimal dilutions were prepared in CCM. For the new overlay/pyruvate method, 15-mL volumes of sterile semisolid medium (CCM plus 0.7 % agar) were dispensed into petri dishes and allowed to gel for 30 min. A 0.1-mL volume of dilution of the inoculum was inoculated into 10 mL of semisolid CCM at 45°C and was poured onto the plates as an overlay. After this had gelled, the plates were incubated at 30°C in an atmosphere of 6% O_2 and 94% N_2 for 3-4 days. For the spread plate method, solid CCM (containing 15 g agar per L) was used and the culture conditions specified by Padgett et al. (1982) were followed. For both methods, percent recovery was defined as the mean number of colonies on plates inoculated with 0.1 mL of a $1:10^3$ dilution of culture ÷ the number of colonies expected based on a DMC of the culture x 100.

Table 1 provides a comparison of the spread plate method and the new overlay/pyruvate method. The spread plate method gave percent recovery values of 2 to 24%, depending on the lot used, and the presence or absence of pyruvate in the CCM made little difference. The overlay method using CCM supplemented with pyruvate gave high percent recovery values (87 to 95%), regardless of the particular lot of CH used.

The use of an overlay in the absence of pyruvate was ineffective when two new lots of CH were used; however, with the lot used previously by Padgett et al. (1982) the percent recovery values were similar regardless of the presence or absence of pyruvate. These results indicated that in order to eliminate dependence on a particular lot of CH, a combination of the overlay plus the pyruvate was necessary. Colony sizes were similar with both the spread plate method and the overlay method, ranging from pinpoint to 1 mm in diameter.

The reproducibility of the overlay/pyruvate method is shown in Table 2. Percent recovery values ranged from 64 to 144% and the particular lot of CH made little difference. The omission of pyruvate from the semisolid CCM, however, made a great difference: with the two new lots of CH (no. 60023 and no. 69278), colony counts on semisolid CCM lacking pyruvate were 2 % or less of the DMC, and with lot no. 11523, used previously by Padgett et al. (1982), mean colony counts were erratic and varied from 33 to 92% of the DMC (data not shown).

The beneficial effect of pyruvate on the growth of *S. volutans* in CCM broth was also measured. The broth was prepared with the new lots of CH—the lots which, as mentioned earlier, had given unsatisfactory growth in CHSS broth. With an inoculum of 1.6×10^4 CFU/mL (by the new overlay/pyruvate method), the CFU/mL in CCM broth lacking pyruvate either remained constant or declined during 30 h incubation at 30°C. With pyruvate, however, after a lag period of 5 h growth increased exponentially to 2.9×10^6 CFU/mL by 25 h. Interestingly, the use of a larger inoculum (6×10^4 CFU/mL) resulted in exponential multiplication to 3.3×10^6 CFU/mL in 22 h regardless of the

presence or absence of pyruvate; moreover, the generation times were similar (2.9 h). These results indicate that pyruvate is a necessary component of CCM broth for growth of small inocula but not for growth of large inocula. The beneficial effect of pyruvate in CCM is most likely due to its ability to destroy hydrogen peroxide as opposed to functioning as an additional carbon source for the cells, because the succinate component of CCM is oxidized far more readily than pyruvate (Caraway and Krieg 1974).

Many organisms have the ability to better resist the damaging effects of deleterious agents when first exposed to a lower dose of those agents (Watson, 1990). In nature, *Spirillum volutans* occurs in stagnant, freshwater environments where temperatures can fluctuate widely. Whether or not it can survive unfavorable temperature changes by an adaptive response has not been studied, mainly because of the difficulty of estimating survival by a reliable colony count method. Consequently, we used our new colony count medium to study the response of *S. volutans* to heat stress. A log phase culture grown in CCM broth at 30°C was divided into two portions. One portion continued to be incubated in a water bath at 30°C while the other was incubated at 40°C, which is a sublethal temperature (>99% survival occurred after exposure for 130 min). After 100 min both portions were placed in a water bath at 45°C. Samples were removed at various times, diluted into CCM broth, and plated. As shown in Fig. 1, the cells pre-exposed to 40°C died less rapidly at 45°C than those that had been maintained at 30°C. This indicates that *S. volutans* can acquire thermotolerance as a result of heat shock, which is a highly conserved phenomenon among bacteria (Neidhardt and VanBogelen 1987; Watson 1990).

S. volutans may be capable of adaptive responses to other stresses, such as oxidant stress, pH changes, or exposure to heavy metals. The development of the new overlay/pyruvate method for obtaining colony counts now makes these and other studies possible.

Table 1. Colony counts of *Spirillum volutans* with and without pyruvate on solid CCM agar by the Padgett and Krieg spread plate method and on semisolid CCM agar by the overlay method.

Lot no. of the vitamin-free, salt-free casein hydrolysate component of CCM[a]	Pyruvate present	Mean no. of colonies on plates inoculated with 0.1 mL of a $1:10^3$ dilution of culture[b]		Mean no. of colonies expected from a direct microscopic count of the culture[c]	% Recovery[d]	
		Spread plate method	Overlay method		Spread plate method	Overlay method
69278	-	13 ± 10	1 ± 2	450	3	<1
	+	7 ± 5	428 ± 36	450	2	95
60023	-	26 ± 15	11 ± 10	434	6	3
	+	13 ± 8	378 ± 22	434	3	87
11523	-	87 ± 11	302 ± 14	356	24	84
	+	40 ± 14	317 ± 4	356	11	89

[a] Lots 69278 and 60023 were newly obtained in 1995. Lot 11523 was the remainder of that used by Padgett et al. (1982).

[b] Values represent the mean colony count and standard deviation from triplicate plates.

[c] Each value is the mean of two direct hemocytometer counts per mL x 10^{-4} performed on the corresponding undiluted culture.

[d] % Recovery = Mean colony count ÷ colony count expected from the direct microscopic count x 100.

Table 2. Colony counts of *Spirillum volutans* in semisolid CCM by the overlay method compared to colony counts expected from direct microscopic count.

Lot no. of the vitamin-free, salt-free casein hydrolysate component of CCM	Mean no. of colonies on plates inoculated with 0.1 mL of a $1:10^3$ dilution of culture[a]	Mean no. of colonies expected from a direct microscopic count of the culture[b]	% Recovery[c]
69278	682 ± 70	625	109
	440 ± 40	306	144
	270 ± 66	421	64
	617 ± 48	550	112
	520 ± 80	446	117
	432 ± 117	407	106
	551 ± 39	540	102
60023	327 ± 81	446	73
	341 ± 44	407	84
	537 ± 79	515	104
	474 ± 108	513	92
11523	573 ± 102	515	111
	465 ± 68	513	91
	506 ± 29	540	94

[a] Values are the mean colony counts and standard deviations from different experiments, with duplicate or triplicate plates being used in each experiment.

[b] See footnote *c* in Table 1.

[c] See footnote *d* in Table 1.

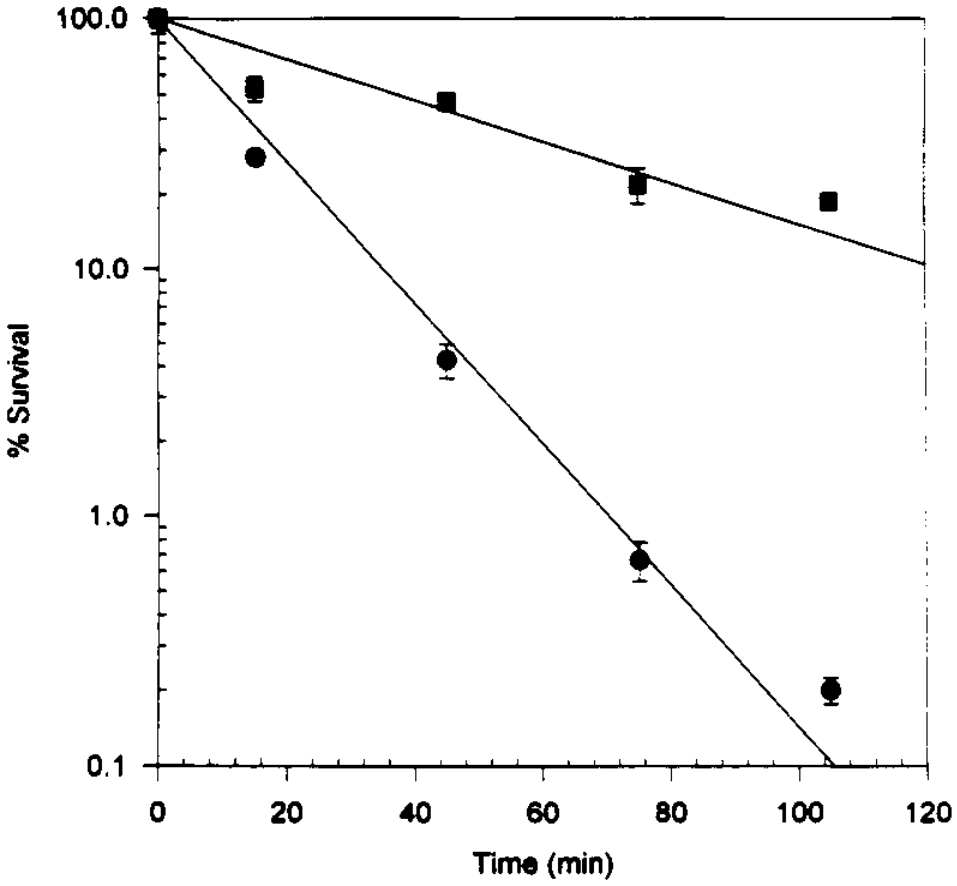

Fig. 1. Survival of *Spirillum volutans* at 45°C with prior exposure to 40°C for 100 min (■) and without prior exposure (●). Each data point represents the mean percent survival and standard deviation from three different experiments, with triplicate plates being used in each experiment.

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Chapter 2

A hydrogen peroxide-resistant mutant of *Spirillum volutans* has NADH peroxidase activity but no increased oxygen tolerance

Abstract

The catalase-negative microaerophile *Spirillum volutans* is killed rapidly by levels of H_2O_2 greater than 10 µM. A mutant isolated by single step mutagenesis with diethyl sulfate was able to survive and grow after exposure to 40 µM H_2O_2 and was effective in eliminating H_2O_2 added to the medium. Nevertheless, the mutant was no more tolerant to O_2 than the wild type. The only apparent phenotypic difference between the wild type and the mutant was that the mutant had high NADH peroxidase activity (0.072 I.U. mg^{-1}) whereas the wild type had no detectable activity (<0.0002 I.U. mg^{-1}). NADH peroxidase has not previously been reported in gram-negative bacteria or in bacteria having a strictly respiratory type of metabolism.

Spirillum volutans is a large, catalase-negative, microaerophilic spirillum and is widely distributed in stagnant pond water. Although it has a strictly respiratory type of metabolism with O_2 as the only known terminal electron acceptor it does not grow on solid media at O_2 levels above 12% (v/v). This has been attributed in part to the organism's lack of catalase which presumably results in susceptibility to very low levels of H_2O_2 formed spontaneously in aerobic culture media, especially under illumination. Growth fails to occur in the presence of approximately 0.29 µM H_2O_2 (Padgett et al. 1982). The addition of catalase to culture media causes an increased O_2 tolerance (Padgett et al. 1982). Moreover, aerotolerant variants have been isolated and found to have increased levels of *o*-dianisidine peroxidase, an enzyme whose activity is barely detectable in the wild type cells (Padgett & Krieg 1986). The resistance of these variants to H_2O_2 could not be compared accurately with that of the wild type, however, because of the lack of a reliable colony count method.

A recently developed colony count method (Alban and Krieg 1996) now makes it possible to quantify the susceptibility of *S. volutans* to peroxide more accurately than by the growth inhibition method used by Padgett et al. (1982). The present study was done to compare the sensitivity of a peroxide-resistant mutant with that of the wild type by using the new plating method, to find the probable basis for the increased peroxide resistance, and to test the idea that such a mutant should exhibit an increased O_2 tolerance.

Spirillum volutans ATCC 19554 and an H_2O_2-resistant mutant derived from it (see below) were cultured in 5.0-mL portions of colony count medium (CCM; Alban and Krieg 1996) minus agar and pyruvate, contained in slanted 20 x 120-mm tubes with loose screw caps. The tubes were incubated at 30°C with daily transfer. The inoculum for each culture consisted of 0.2 mL of the previous day's culture.

In all experiments, media were prepared and stored in the dark and all cultures were incubated in the dark to prevent generation of H_2O_2 and superoxide caused by illumination (Hoffman et al. 1979b, Padgett et al. 1982).

The concentration of H_2O_2 in stock solutions was measured spectrophotometrically at 240 nm ($\varepsilon = 43.6$). The H_2O_2 remaining at various times after addition to cultures was measured with a PeroXOquant Quantitative Peroxide Assay Kit (Pierce Chemical Co., Rockford, IL), based on H_2O_2 -mediated oxidation of Fe^{2+} under acidic conditions followed by reaction of the Fe^{3+} with xylenol orange (Jiang et al. 1990).

The effect of cell density on the disappearance of H_2O_2 added to cultures was measured by removing 2.0-mL portions from 60-mL cultures of the wild type, grown in CCM broth without pyruvate, at various times after inoculation and exposing them to 15 µM H_2O_2 for 30 min, at which time the residual H_2O_2 was measured. The ability of the medium itself to destroy H_2O_2 was tested by removing 2.0-mL portions of cultures as described above and pelleting the cells before adding the H_2O_2 to the supernatant medium. In other experiments, instead of testing equivalent volumes of culture the cells were compared by diluting samples from log phase cultures (19- to 21-h-old) in sterile

medium to give the same cell density as that of 3- to 4-h-old cultures (1 x 10^5 CFU/mL) before exposing them to 15 µM H_2O_2.

The mutagenesis procedure was based on the method of Christman et al. (1985). *S. volutans* was grown for 22 h in 6 mL of CCM broth without pyruvate to a density of 2 x 10^6 cells/mL in a slanted 20 x 120-mm tube as described above. Diethyl sulfate (0.01 mL) was added and the tube was agitated vigorously for 20 sec. After incubation at 25°C for 20 min, 1.0 mL of the culture was used to inoculate 5 mL of CCM broth. After incubation for 22 h at 30°C, H_2O_2 was added to a concentration of 35 µM and the culture was incubated for 20 h on a rotary shaker at 100 oscillations per min at 30°C. A 0.02-mL portion was cultured in CCM (with pyruvate, to destroy residual peroxide) for 48 h. Cells were then inoculated in CCM broth without pyruvate and incubated for 22 h. They were exposed to 35 µM peroxide for 3.5 h, plated on CCM overlay plates (Alban and Krieg 1996), and incubated for 4 days under an atmosphere of 6 % O_2 and 94% N_2. Eleven colonies were selected and maintained by serial transfer in semisolid CCM medium without pyruvate. When the 11 strains were tested for peroxide resistance as described below, all showed greater resistance than that of the wild type and the most resistant strain was used for subsequent study. No decrease in its resistance occurred even after several months of daily transfers. The effect of H_2O_2 on viability was tested by growing cultures in 60 mL of CCM broth without pyruvate at 30°C. Samples were removed at various cell densities and transferred into sterile tubes, after which H_2O_2 was added to the desired final concentration. Tubes were incubated at 30°C on a rotary shaker at 100 oscillations per min. Samples taken immediately prior to addition of peroxide and

periodically thereafter were diluted in CCM broth (with pyruvate, to destroy residual peroxide) and plated on CCM medium. Colonies were enumerated after incubation at 30°C for 4 days under an atmosphere of 6 % O_2 and 94% N_2.

Oxygen tolerance could not be tested by the method described by Padgett and Krieg (1986) because the organisms failed to grow reproducibly on the surface of agar media (Alban and Krieg 1996). Consequently, the following method was used. Cells were grown to midlog phase (20 h; 2 x 10^6 CFU/mL) at 30°C in CCM broth without pyruvate. Pyruvate was omitted because it enhances O_2 tolerance in *S. volutans*, presumably by its ability to destroy H_2O_2, which would obscure the peroxide-destroying ability of the cells. The culture was serially diluted in CCM broth and plated in CCM agar overlay medium minus pyruvate. Plates were incubated in Oxoid jars under various O_2 levels at 30°C for 3 to 4 days.

For enzyme assays, cell-free extracts were prepared from cells grown statically in 1-L Erlenmeyer flasks containing 700 mL of CCM broth minus pyruvate. The initial inoculum consisted of 21 mL of a 20-h-old culture grown in CCM broth minus pyruvate; this large inoculum was needed when pyruvate was omitted from the medium (Alban and Krieg 1996). After 20 h the cells were harvested at 14,000 x *g*, washed twice in potassium phosphate buffer (10 mM, pH 6.9) and suspended in 2.5 mL of buffer. Cells were disrupted by sonication at 4°C and centrifuged at 40,000 x *g* for 15 min to remove intact cells and large debris. The soluble and membrane fractions were obtained by centrifuging fresh crude extracts at 100,000 x *g*. The membrane fraction was washed twice with 10 mM phosphate buffer (pH 6.9) before use. The periplasmic protein

fraction was obtained by treatment of whole cells with Tris and EDTA according to the method of Garrard (1971). Alkaline phosphatase but not cytoplasmic aconitase was liberated by this procedure, indicating that no detectable damage had occurred to the cytoplasmic membrane at the time of release of the alkaline phosphatase.

NADH peroxidase (EC 1.11.1.1) activity was assayed at 25°C by the method of Poole and Claiborne (1986). The reaction was initiated by addition of cell extract and the linear decrease in absorbance at 340 nm was followed for 2-3 min. Background NADH oxidase activity was measured similarly and subtracted from the observed rate. Activities were compared at pH 5.0, 5.4, 5.8, 6.4, 6.7, 6.9, 7.2, and 7.8. Catalase (EC 1.11.1.6) was measured by monitoring a decrease in the absorbance of H_2O_2 at 240 nm as described by Beers and Sizer (1952). Donor: H_2O_2 oxidoreductase (EC 1.11.1.7) activity was assayed by monitoring the oxidation of *o*-dianisidine at 450 nm as described by Hoffman et al. (1979). Aconitase was assayed by the method of Racker (1950). The reaction mixture for assaying alkaline phosphatase consisted of 1 mL of 1.0 M Tris buffer (pH 8.0) containing 1 mM $MgCl_2$ and 20 mM disodium *p*-nitrophenyl phosphate. The reaction was initiated by addition of periplasmic protein extract and the increase in absorbance at 405 nm was monitored over a 2-min period. All specific activities were expressed in micromoles of substrate converted per min (international units) per mg protein.

The relationship of the density of growing cultures of the wild type to their resistance to 15 μM H_2O_2 is shown in Fig. 1. Resistance increased with cell density until the end of active growth, when it began to decrease. Wild type cells 3- to 4-h-old which had grown to a density of 1 x 10^5 mL^{-1} could destroy only 11-15% of the added H_2O_2,

with approximately 12 µM H_2O_2 remaining at 30 min. Log phase cells 19- to 21-h-old which had reached a density of 1.5 x 10^6 mL^{-1} were able to destroy the added H_2O_2 completely, with no detectable levels of H_2O_2 remaining at 30 min. The medium *per se* was unable to destroy H_2O_2 regardless of growth phase. Although the log phase cells seemed to be more resistant than the 3-4-h-old cells, when they were diluted in sterile medium to the same density as the younger cells (i.e., 1.5 x 10^6 cells mL^{-1} were diluted to 1 x 10^5 mL^{-1}), they were able to destroy only 11-15% of the added H_2O_2 and thus had the same poor resistance to H_2O_2 as the younger cells.

Levels of peroxide greater than 10 µM were lethal to wild type cells of *S. volutans* (Fig. 2). The H_2O_2-resistant mutant had the same doubling time as that of the wild type (2.8 h) but exhibited 100% survival when exposed to 40 µM H_2O_2, whereas the wild type was rapidly killed (Fig. 2). Furthermore, the mutant exhibited 1% survival after exposure to 80 µM H_2O_2 for 60 minutes whereas all of the wild type cells were killed. For comparison, H_2O_2 levels of approximately 1 mM are needed to kill *E. coli* (Imlay & Linn, 1987).

The ability to destroy H_2O_2 in the culture medium correlated well with peroxide tolerance (Fig. 3). When exposed to 40 µM H_2O_2, the mutant was able to destroy >90% of the peroxide within 30 min. With the wild type 30% of the peroxide remained in the medium even at 90 min (Fig 3, inset). Both the wild type and the mutant lacked any detectable catalase activity (<0.69 I.U./mg protein). Both had barely detectable amounts of donor: H_2O_2 oxidoreductase activity (0.0003 I.U. mg^{-1} protein). The mutant, however, had relatively high NADH peroxidase activity (0.072 I.U. mg^{-1}) in the soluble fraction

whereas the wild type had no detectable activity (<0.0002 I.U. mg^{-1} protein). The pH optimum was 6.9. No detectable NADH peroxidase activity occurred in the periplasmic and membrane fractions. NADPH could also be used as the electron donor but showed approximately 30% less activity than NADH. Comparison of the O_2 tolerance of the mutant and wild type indicated that the mutant was not able to grow at higher O_2 levels than the wild type and in fact was slightly more O_2 sensitive (Table 1). Neither could grow under an air atmosphere. Despite the widespread distribution of *S. volutans* in nature, genetic characterization studies of this organism have not yet been done. Studies of increased production of enzymes that scavenge toxic forms of oxygen, and the regulatory mechanisms such as OxyR for such enzymes, have been done mainly with facultatively anaerobic *Enterobacteriaceae*, which are resistant to much higher levels of H_2O_2 (approximately 1 mM) and have little phylogenetic relatedness to microaerophiles such as *S. volutans*, *Campylobacter* species, or *Helicobacter* species. Although the genetic basis of the mutation in *S. volutans* is not yet known, it is clear that the increased ability of the mutant to destroy H_2O_2 did not result in the expected increase in tolerance to O_2.

The finding that dense cultures 19- to 21-h-old were much more resistant to H_2O_2 than a sparse population of 3- to 4-h-old cells indicates a relationship of H_2O_2 resistance to cell density in *S. volutans*. When the dense suspensions were diluted, however, the cells

showed the same susceptibility as the 3- to 4-h-old cells, indicating that the phase of the growth curve was not involved but merely the fact that the cells were at a higher

concentration. Thus it was important to use equivalent cell densities instead of an equivalent volumes of culture when estimating peroxide resistance at various stages of growth (Fig. 2). This precaution may seem obvious but it has not always been followed (e.g., see Dowds et al. 1987 and Jamieson et al. 1994). Cell density is also an important consideration when comparing the peroxide resistance of a mutant with a wild type, as emphasized by the classic work of Ma and Eaton (1992) in their comparison of catalase-positive *E. coli* with a catalase-negative mutant.

Population density may not only affect H_2O_2 resistance but also O_2 tolerance in microaerophiles (for examples see Krieg & Hoffman 1986.) In the present study colonies of *S. volutans* were obtained under O_2 levels as high as 40% when dense inocula (2×10^6 cells mL^{-1}) were spread onto the surface of CCM agar plates, even without the protective effect of pyruvate (data not shown). This is in accord with Krieg & Hoffman (1986) who recommended that dilute cultures should be used when comparing the O_2 tolerance of microaerophiles.

NADH peroxidase has been reported only in a few gram positive fermentative bacteria such as *Enterococcus faecalis* and *Lactobacillus casei* (Condon 1987) and *Clostridium perfringens* (Zavadora et al. 1974). Its occurrence in the H_2O_2-resistant mutant of *S. volutans* is the first report of it in a gram negative bacterium and, moreover, in a bacterium having a strictly respiratory type of metabolism. Its occurrence suggested that the mutant might be more O_2 tolerant; however, it was not, despite other studies that have linked peroxidases to an increased tolerance to O_2. For instance, in a study of a wide range of bacteria Rolfe et al. (1978) concluded that the occurrence of *o*-dianisidine

peroxidase activity, but not superoxide dismutase or catalase activity, correlated well with aerotolerance. Two aerotolerant variants of *S. volutans* were reported to exhibit increased levels of *o*-dianisidine peroxidase (Padgett and Krieg 1986); no correlation of the H_2O_2 tolerance of the variants with the wild type could be made, however, because no method of obtaining reliable colony counts for survival experiments was then available. Unlike NADH peroxidase, the biological electron donor for *o*-dianisidine peroxidase is not known. There are few reports linking NADH peroxidase with O_2 tolerance. Oxygen-sensitive mutants of *S. faecalis* have been reported to have lost NADH peroxidase activity (Britton 1979) and an aerotolerant mutant of *Clostridium perfringens* was reported to have gained NADH peroxidase activity (Zavadora et al. 1974). Several possible explanations can be suggested to explain the findings with *S. volutans*. (i) The finding that a culture of low density cannot cope with a given level of H_2O_2 as well as a more dense culture suggests that individual cells growing on solid media may have so few neighbors that it makes little difference whether they have a higher ability to destroy H_2O_2. (ii) Although resistance to H_2O_2 was higher in the mutant than in the wild type, this resistance was approximately an order of magnitude less than that of aerotolerant organisms such as *E. coli* and thus it may simply not be enough to protect individual cells on an agar medium. (iii) The metabolic requirements for synthesis of cell materials during growth may require so much NADH that little may be available for peroxidase activity. In the study by Padgett et al.(1986), in which aerotolerant mutants possessed increased activities of *o*-dianisidine peroxidase, it is possible that biological electron donors (whose identity is unknown) were sufficiently abundant so as to allow greater

removal of H_2O_2 by this enzyme than is possible with NADH peroxidase. (iv) It is possible that the addition of H_2O_2 to the medium causes damage to the cell's membranes before intracellular NADH peroxidase can destroy it.

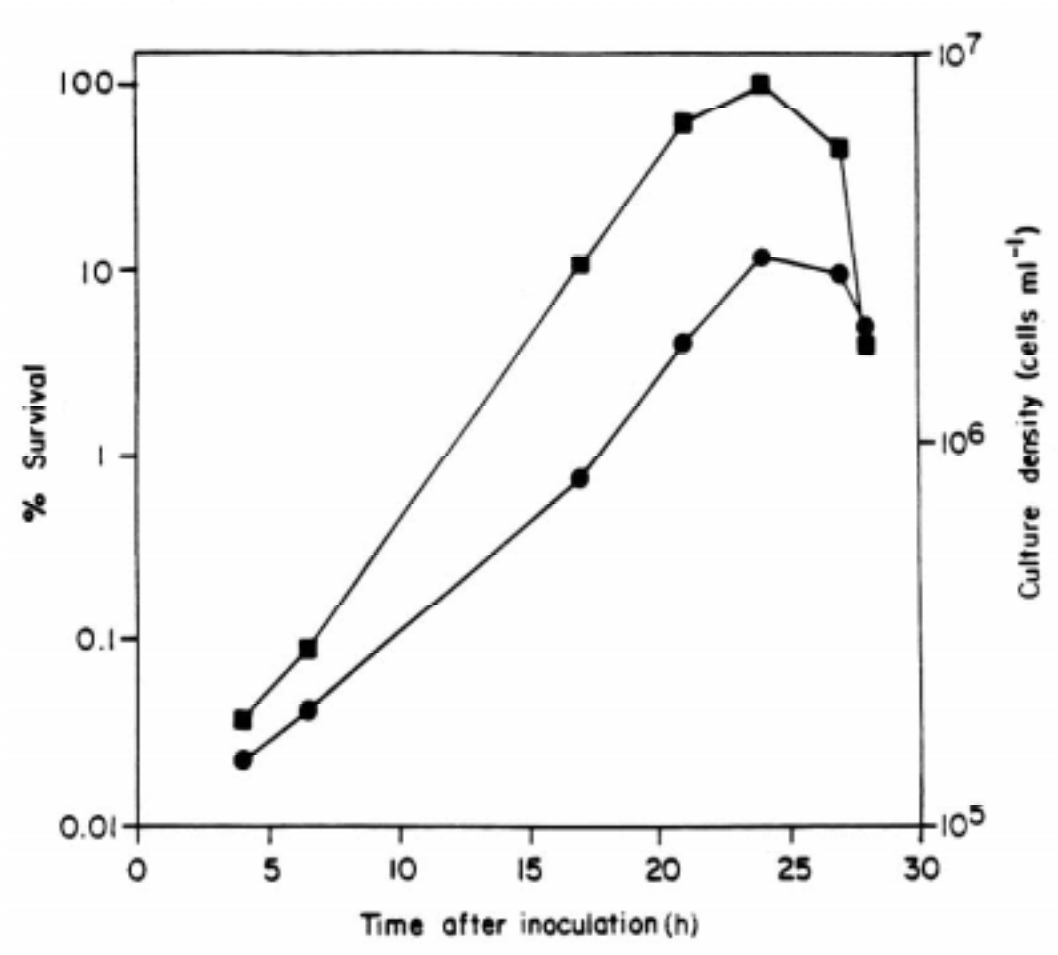

Fig. 1. Effect of cell density on the resistance of *S. volutans* ATCC 19554 to H_2O_2. Cells were inoculated into CCM broth minus pyruvate to an initial density of 6 x 10^4 CFU/mL. Samples were removed periodically, H_2O_2 was added to a final concentration of 15 µM H_2O_2, and the percent survivors was measured at 30 min relative to a sample taken before H_2O_2 addition. (●), culture density as estimated by plate counts in a CCM agar overlay medium. (■), percent survival following H_2O_2 challenge. Data points represent the mean values from triplicate plates from a representative experiment.

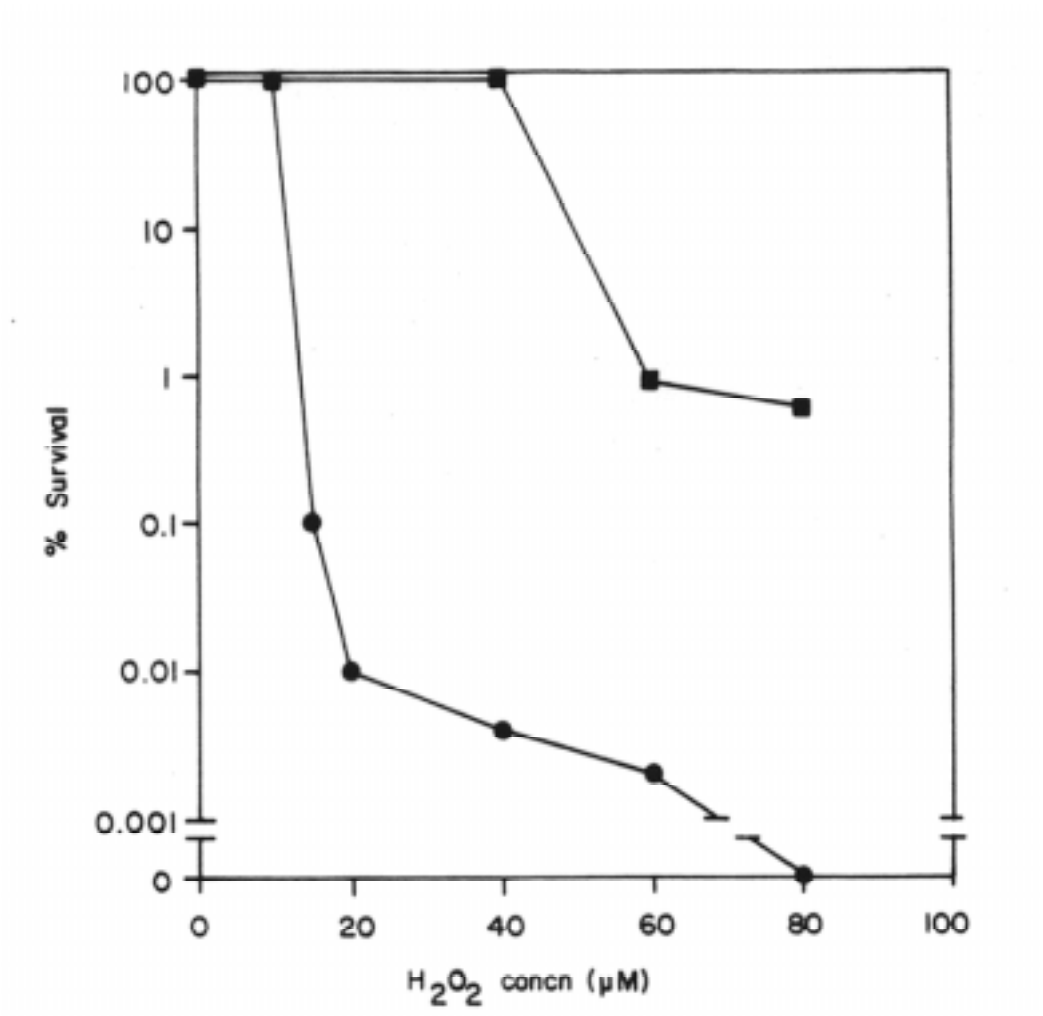

Fig. 2. Resistance of wild type (●) and mutant (■) cells of *S. volutans* to H_2O_2. Cells were grown exponentially in CCM minus pyruvate to a density of 2 x 10^6 CFU/mL (which represents 100% survival). Cells were challenged with various concentrations of H_2O_2 for 60 min as described in Methods, diluted, and plated in a CCM agar overlay medium to determine cell survival. Data points are the means of triplicates from a representative experiment.

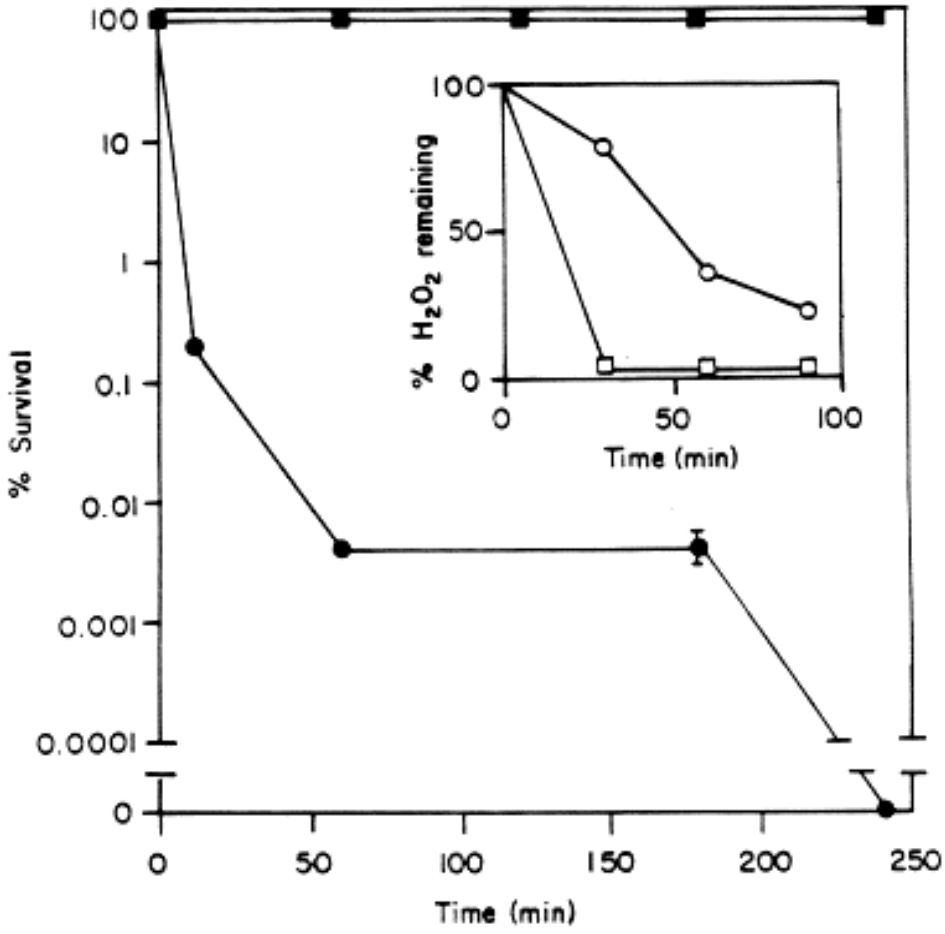

Fig. 3. Resistance of wild type (●) and mutant (■) cells of *S. volutans* to H_2O_2 compared with ability of the cells to destroy H_2O_2. Cells were at a density of 2 x 10^6 CFU/mL. Percent survival after exposure to 40 µM H_2O_2 is shown for various times of exposure. Inset: Destruction of H_2O_2 by wild type (○) and mutant (□) vs. time of exposure. Data points are the means of triplicates from a representative experiment. Standard deviations are shown when they exceed the size of the data symbol.

Table 1. Growth response of the wild type and mutant *Spirillum volutans* at various O_2 concentrations*

O_2 concn (%)	Percentage of cells forming colonies	
	Wild type	Mutant
1	29 ±19 †	10 ± 4
6	10 ± 6	4 ± 3
12	7 ± 7	5 ± 7
21	0 ± 0	0 ± 0

* Cells from mid-log phase cultures were enumerated with a hemacytometer and, based on subsequent dilutions in CCM broth minus pyruvate, 150-250 cells were inoculated into CCM agar overlay medium minus pyruvate.

† Values are the means of triplicate plates from three separate experiments.

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Chapter 3

Identification of a gene for a rubrerythrin/ nigerythrin-like protein in *Spirillum volutans* by using amino acid sequence data from mass spectrometry and NH_2-terminal sequencing

SUMMARY

A hydrogen peroxide-resistant mutant of the catalase-negative microaerophile *Spirillum volutans*, constitutively expresses a 21.5 kDa protein that is undetectable and noninducible in the wild type cells. We cloned part of the gene that encodes the protein by using amino acid sequence data obtained by both mass spectrometry and NH_2-terminal sequencing. The deduced 158 amino acid polypeptide shows high relatedness to rubrerythrin and nigerythrin previously described in the anaerobes *Clostridium perfringens* and *Desulfovibrio vulgaris*. The protein also shows high similarity to putative rubrerythrin proteins found in the anaerobic archeons *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, and *Methanobacterium thermoautotrophicum*. This is the first report of this type of protein in an organism that must respire with oxygen. The methodology may be useful for rapid cloning of genes in other bacteria.

INTRODUCTION

Spirillum volutans is a large microaerophilic spirillum found in stagnant pond water. Although it has a strictly respiratory type of metabolism with O_2 as the only known terminal electron acceptor it does not grow on solid media at O_2 levels above 12% (v/v). The fundamental physiological and biochemical basis for its microaerophily is not known but might be related to the organism's lack of catalase that presumably allows susceptibility to very low levels of H_2O_2 formed spontaneously in aerobic culture media, especially under illumination (Padgett and Krieg 1986). H_2O_2 levels greater than 10 μmol l^{-1} are lethal to the wild type cells (Alban and Krieg 1998). For comparison, H_2O_2 levels of approximately 1 mmol l^{-1} are needed to kill *E. coli* (Imlay and Linn 1987). *S. volutans* becomes more oxygen tolerant when catalase is added to the culture medium (Padgett *et al.* 1982). Moreover, variants have been isolated having increased *o*-dianisidine peroxidase activity whereas the wild type has barely detectable activity (Padgett and Krieg 1986). An inability to destroy exogenous H_2O_2 , however, cannot be the only reason for the microaerophilic behavior because neither exogenous catalase nor peroxidase can permit aerobic growth of cells on solid media (Padgett and Krieg 1986) and because a recently isolated H_2O_2-resistant variant of *Sprillum volutans* is no more tolerant to oxygen than the wild type (Alban and Krieg 1998). In other bacteria exposure to H_2O_2 induces a number of proteins (Christman *et al.* 1985; Morgan *et al.* 1986; Murphy *et al.* 1987; Dowds and Hoch 1991). In many instances H_2O_2-resistant mutants overexpress proteins involved in peroxide protection such as catalase and glutathione

reductase, which has led to discovery of regulatory elements such as the OxyR protein (Christman *et al.* 1985; Christman *et al.*1989). Little is known, however, about the response of microaerophiles to peroxide stress. The purpose of the present study was to identify proteins that are overexpressed by the H_2O_2-resistant mutant of *S. volutans* (Alban and Krieg 1998) in an attempt to elucidate how this mutant copes with levels of H_2O_2 that are lethal to the wild type.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions.

Spirillum volutans ATCC 19554 and an H_2O_2-resistant mutant derived from it (Alban and Krieg, 1998) were maintained at 30^{o} C with daily transfer in 5.0-ml portions of colony count medium (CCM; Alban and Krieg 1996) lacking agar and pyruvate. The broth was contained in slanted 20 × 120-mm tubes with loose screw caps. The inoculum for each culture consisted of 0.2 ml of the previous day's culture.

Preparation of cell-free extracts and DNA isolations

Cells were grown statically in 4-l flasks containing 800 ml of CCM broth minus pyruvate. The initial inoculum consisted of 21 ml of a 20-h-old culture grown in CCM broth minus pyruvate; this large starter inoculum was needed when pyruvate was omitted from the medium (Alban and Krieg 1996). At 20 h the cells were harvested at 14 000 *g*, washed twice in potassium phosphate buffer (10 mmol l^{-1}, pH 6.9) and suspended in 2.0 ml of

buffer. In some cases, cells were exposed to various levels of H_2O_2 prior to the harvesting step. Cells were disrupted by sonic oscillation at 4°C and centrifuged at 40 000 *g* for 15 min to remove intact cells and large debris. Protein concentrations in cell extracts were estimated by the method of Bradford (1976) with a Bio-Rad (Hercules, CA) protein assay kit. DNA isolations from cell pellets were performed using the method of Marmur (1961) as modified by Yousten and Rippere (1997).

Electrophoresis

SDS-PAGE was performed according to the methods of Laemmli (1970) in a 12 % polyacrylamide separating gel and a 4 % polyacrylamide stacking gel. Following electrophoresis gels were stained with 0.025 % Coomassie blue. Molecular weight standards were phosphorylase B (97 400), bovine serum albumin (66 200), L-glutamic dehydrogenase (55 000), ovalbumin (42 700), aldolase (40 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400) (Promega, Madison, WI).

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed according to the method of O'Farrell (1975) using a Model 250 SE system (Hoefer Scientific Instruments, San Francisco, CA). Isoelectric focusing (IEF) was run to equilibrium using a 4 % polyacrylamide tube gel (7.5 cm in length and 1.5 mm internal diameter). The IEF gel also contained 9.2 mol l^{-1} urea, 2.0 % Triton X-100 , 1.6 % Bio-Rad ampholytes (pH 5-7) and 0.4 % Bio-Rad ampholytes (pH 3-10). Protein samples to be loaded on isoelectric focusing gels were mixed 1:1 with sample loading buffer

containing 9.5 mol l^{-1} urea, 2% Triton X-100, 5 % β-mercaptoethanol, 1.6 % Bio-Rad ampholytes (pH 5-7), and 0.4 % Bio-Rad ampholytes (pH 3-10) and incubated at 25^{o}C for 30 min. Eighty–100 μg protein was loaded on each isoelectric focusing gel. Subsequent SDS-PAGE was performed as described above. 2-D PAGE gels were silver stained as described by Wray *et al.* (1981).

Nondenaturing gel electrophoresis was carried out in 7.5 % polyacrylamide gels at 4^{o}C using the buffer system described by Laemmli (1970) with the omission of SDS.

NH_2-terminal sequencing

Following SDS-PAGE, proteins were transblotted onto a polyvinylidene difluoride membrane (Bio-Rad) using a Transphor Elecrophoresis Unit (Hoefer Scientific Instruments). The membrane was stained with 0.025 % Coomassie brilliant blue R 250 and the protein of interest was sequenced using a model 477A Sequenator (Applied Biosystems, Foster City, CA).

Liquid chromatography-mass spectrometry (LC-MS)

The 21.5 kDa protein spot from a Coomassie blue-stained 2-D PAGE gel was cut from the gel, reduced and alkylated, and subsequently digested by trypsin as described by Schevchenko *et al*. (1996). The peptides formed in this digest were extracted from the gel matrix and the resulting extract evaporated to less than 20 μl for LC-MS analysis. The LC-MS system consisted of a Finnigan-MAT TSQ7000 system with an electrospray ion source interfaced to a 10 cm × 75 mm id (internal diameter) POROS 10 RC reversed-

phase capillary column (Finnegan, San Jose, CA). One-μl volumes of the extract were injected and the peptides eluted from the column by an acetonitrile/0.1 mol l^{-1} acetic acid gradient at a flow rate of 0.6 μl min^{-1}. The digest was analyzed by capillary LC-electrospray mass spectrometry to measure the molecular weight of the peptides present in the digest. Amino acid sequences for the peptides detected were determined by interpreting collisionally activated dissociation (CAD) spectra obtained using LC-electrospray-tandem mass spectrometry with argon as the collision gas. Following completion of the mass spectrometry experiments, the digest was fractionated by capillary LC using a self-packed 10 cm × 75 mm id POROS 10 RC reversed phase capillary column with a LC Packings capillary flow cell in an ABI UV/vis detector. Approximately half of the digest was fractionated and the collected fractions re-analyzed by LC-MS to determine peptides in that fraction. Selected fractions were then analyzed by Edman degradation using a Procise protein sequencer (Applied Biosystems) to confirm the amino acid assignments from the CAD spectrum interpretations.

Primers and PCR amplification

Degenerate oligonucleotide primers were constructed by GIBCO BRL custom primer facility (Gaithersburg, MD). PrimerIT and primerNT were constructed against nucleotide sequences which could code for internal peptide NELGQSK and the NH_2-terminal region of the protein, respectively. The sequences of the degenerate PCR primers were as follows: primerIT, 5'- YTTNSWYTGNCCNADYTCRTT-3'; primerNT, 5'-GTNACNTTYCARAAYYTNGAR-3'. PCR amplification mixtures (25 μl volumes)

contained 5 % glycerol, 200 μmol l^{-1} of each deoxynucleoside triphosphate, 3 mmol l^{-1} $MgCl_2$, 6 μmol l^{-1} of both primerIT and primerNT, 1U Taq polymerase (Promega, Madison, WI), and 12.5 ng of genomic DNA isolated from the H_2O_2-resistant mutant. A PTC-100 thermocycler (MJ Research, Inc., Watertown, MA) was used for amplification. Amplification temperatures were as follows: 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min.

Cloning and sequencing of DNA fragment

PCR products were separated on a 1 % agarose gel. A 459 bp fragment was purified from a 25-μl PCR reaction using a Wizard PCR Preps DNA purification kit (Promega). The purified product was ligated into a pGEM-T vector (Promega) and transformed into *E. coli* strain JM109 according to the manufacturer's protocol. Cells were spread onto agar plates containing 16 g l^{-1} peptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 10 μg ml^{-1} IPTG**,** 40 μg ml^{-1} ampicillin, and 40 μg ml^{-1} X-Gal. Transformants were selected and plasmid DNA was isolated by an alkaline-lysis/ polyethylene glycol (PEG) precipitation procedure (Applied Biosystems). To confirm the presence of the correct insert, aliquots of plasmid DNA were digested with 1 U each of PstII and SacII in buffer H (Promega). Nucleotide sequences were determined using dye terminator chemistry and Taq polymerase (dideoxynucleotide chain-termination method) using a model 377 Prism DNA Sequencer (Applied Biosystems). Synthesis was primed using T7 and Sp6 primers.

Analysis of similarity

The TBLASTN program was used as described by Altshul *et al.* (1990) to compare the DNA and deduced protein sequence to all potential translation products in the available database. The deduced protein sequence was aligned with other rubrerythrin, nigerythrin, and putative rubrerythin sequences using MegAlign in the DNAstar program (DNASTAR, Inc., Madison, WI).

Enzyme assays

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by two methods. The first was the quantitative hematoxylin method of Martin *et al.* (1987). Specific activities were expressed as units mg^{-1} protein. In the second method, SOD activity was determined qualitatively by the staining method of Beauchamp and Fridovich (1971) following nondenaturing polyacrylamide gel electrophoresis of crude cell extracts. NADH peroxidase activity stains were performed according to the method of Brown *et al.* (1995) following nondenaturing polyacrylamide gel electrophoresis of crude cell extracts.

RESULTS

One- and two-dimensional protein electrophoresis

Cell-free extracts of the wild type and variant were separated on a SDS-PAGE gel and stained with Coomassie blue (Fig. 1). The protein banding patterns were compared and found to be identical except for a unique protein band 21.5 kDa in size possessed by the

variant. The protein band unique to the variant consisted of only one protein as determined by two-dimensional PAGE and was undetectable in the wild type (Figs. 2a and 2b). Exposure of the wild type cells to sublethal peroxide levels (10 μmol l^{-1}) for 1.5 h did not induce this protein. The protein was present in the variant regardless of whether or not cells were exposed to H_2O_2.

NH_2-terminal sequencing, mass spectrometry, PCR, cloning and sequencing

NH_2-terminal sequence analysis was performed on the 21.5 kDa protein and 30 amino acid residues were assigned. Mass spectrometry performed on the trypsin-digested protein allowed the identification of five internal polypeptide fragments (Fig. 3). Successful cloning of the gene fragment relied upon the use of two synthetic, degenerate 21-mer PCR primers. "PrimerNT" was deduced from the N-terminal residues VTFQNLE and "primerIT" was deduced from one of the internal sequence fragments (NEI/LGQSK) obtained by mass spectrometry (Fig. 3). A 459-bp PCR product was obtained using the 21-mer degenerate PCR primers under relatively stringent conditions (hybridization temperature > 51^{o}C). The 459 bp product was subsequently purified and cloned into a pGEM-T vector. Highly competent *E. coli* cells strain JM109 were transformed and positive clones (white colonies) were selected for plasmid preps. The plasmid DNA of two positive clones was sequenced by the Biomolecular Sequencing Facility at the University of Virginia, Charlottesville, VA, using primers T7 and Sp6 (Fig. 3).

Sequence analysis

The 459 bp PCR product (Fig. 3) encoded a 153 codon open reading frame. Five codons could not be definitively identified due to DNA sequence ambiguities. Two of these amino acids were assigned based upon mass spectrometry data (residues 138 and 157, Fig. 3) and another amino acid was assigned based upon NH_2-terminal sequence information (residue 11, Fig. 3). The final two DNA sequence ambiguities indicated two possible amino acids at residues 143 and 148 (Fig. 3). In addition the mass spectrometry data allows the assignment of residue 154 as leucine or isoleucine not phenylalanine as the respective codon indicates. This false phenylalanine codon occurred because the degenerate primer was constructed against this region. The NH_2-terminal sequence data identified another 5 amino acids that were not encoded within the PCR product. When all of the sequence information is included together the predicted molecular weight of the 158-residue peptide is 17.8 kDa which is 83 % of the 21.5 kDa protein estimated by SDS-PAGE. The deduced 158 amino acid sequence was compared to all potential translation products in the available DNA sequence database using the TBLASTN program (Altshul *et al.* 1990). The TBLASTN search identified the similar proteins and MegAlign was used to align these sequences with the protein sequence of *S. volutans*. When the rubrerythrin, putative rubrerythrin, and nigerythrin sequences are aligned by MegAlign (Fig 4), the rubrerythrin/nigerythrin-like sequence in *S. volutans* shows high similarity to other sequences in the database. For example, the rubrerythrin/nigerythrin- like sequence in *S. volutans* shows 32 % identity and 43% similarity to the putative rubrerythrin sequence # 1 of *Archaeoglobus fulgidus* (Klenk *et al.* 1997), 30 % identity and 45 % similarity to putative rubrerythrin sequence #2 of *M. thermoautotrophicum* (Smith *et al.*

1997), and 30 % identity and 31 % similarity to the nigerythrin sequence of *D. vulgaris* (Lumppio *et al.*1997). Kurtz and Prickril (1991) observed the prescence of two E-X-X-H sequences in internally homologous stretches of rubrerythrin in *D. vulgaris*. Two E-X-X-H sequences provide Glu and His ligands to the diiron clusters in ribonucleotide reductase R2 protein (Norland and Eklund 1993) and methane monooxygenase hydroxylase (Rosenzweig *et al.* 1993) and it has been suggested that these two E-X-X-H sequences have a similar function in rubrerythrin (Kurtz and Prickril 1991). We find that this sequence is conserved among these proteins in other organisms and is contained within a much larger conserved tandem repeat amino acid sequence within the protein (Fig. 4). Kurtz and Prickril (1991) observed small (33 residues in length) intrapeptide sequence homology in rubrerythrin from *D. vulgaris*. We observed what appears to be large (> 45 residues in length) tandemly repeated intrapeptide sequence homology in the rubrerythrin-like protein of *S. volutans*. Additionally, these intrapeptide sequences can also be observed in rubrerythrin from other organisms (Fig. 4).

Enzyme activity

There was little quantitative difference between the SOD activity in the wild type (23 U mg^{-1}) and the variant (26 U mg^{-1}). In addition, no qualitative difference in SOD activity was observed in nondenaturing gels. Nondenaturing gels failed to exhibit any bands having NADH peroxidase activity.

DISCUSSION

During an attempt to identify proteins involved in protection of an H_2O_2 resistant mutant of *S. volutans* we identified a protein that was only detected in the peroxide-resistant mutant. By using amino acid sequence data obtained from both mass spectrometry and NH_2-terminal sequencing, we successfully cloned and sequenced most of the gene which encodes this novel protein. The sequence indicated a close relatedness of the protein to rubrerythrin and nigerythrin. We have no direct evidence that the rubrerythrin/ nigerythrin-like protein has a role in the H_2O_2 resistance of the mutant. However, no other proteins could be detected that were uniquely expressed in the mutant and thus it seemed likely that the novel protein plays a key role in protection of the cells from peroxide.

This is the first report of a rubrerythrin/nigerythrin-like protein occurring in an organism that must respire with oxygen. Previous reports of rubrerythrin or nigerythrin have been limited to the anaerobic bacteria *C. perfringens* (Lehmann *et al.* 1996) and *D. vulgaris* (Pickril *et al.* 1991; Lumppio *et al.* 1997). As a result of recent genome sequencing projects, putative rubrerythin genes have also been identified in the anaerobic archeons *Methanococcus jannaschii* (Bult *et al.* 1996), *Methanobacterium thermoautrophicum* (Smith *et al*. 1997), and *Archaeoglobus fulgitidus* (Klenk *et al.* 1997), and the anaerobe *Thermatoga maritima* (personal communication: The Institute for Genomic Research (TIGR)), a member of the domain *Bacteria*. In some instances more than one copy of putative rubrerythrin genes exists in these archeons.

When the amino acid sequence of the *S. volutans* protein is compared to the previously reported sequences of rubrerythrin, nigerythrin, and to the putative rubrerythrin sequences many conserved regions are observed (Fig. 4). In particular there are two sequences that are tandemly repeated and are common to all rubrerythrin sequences from the other organisms. The conserved nature of this tandem repeat suggests that it is directly involved in the physiological function of the protein.

The repeated sequences may represent two distinct domains responsible for binding a pair of substrate molecules or a substrate with bipartite symmetry. Within the tandemly repeated regions of rubrerythrin and nigerythrin of *D. vulgaris* there are a pair of E-X-X-H sequences which serve as diiron-binding sites (Kurtz and Prickril 1991; Lumppio *et al.* 1997). The protein of *S. volutans* also has two E-X-X-H sequences that could serve an identical function.

A physiological function for rubrerythrin and nigerythrin has not yet been defined although different biological functions have been proposed. Recently, Lehmann *et al.* (1996) identified rubrerythrin in *C. perfringens* and proposed a superoxide dismutase function for it. This was consistent with the fact that expression of rubrerythrin in *E. coli sod* mutants afforded the cells protection against oxidative stress (Lehmann *et al.* 1996). In *S. volutans*, however, we found no qualitative difference and little quantitative difference between the SOD activities of the wild type and the mutant. The complete amino sequence of nigerythrin was recently reported by Lumppio *et al.* (1997), who suggested that nigerythrin and rubrerythrin may protect cells against oxidative stress by both scavenging iron so as to avoid oxidative damage caused by the Fenton reaction and

donating iron to oxidatively damaged [4Fe-4S] proteins. We suggest a somewhat different possibility. This is based on (i) the failure to find other proteins unique to the mutant on 2-D gels and (ii) the occurrence of detectable NADH peroxidase-like activity in the mutant. In *S. volutans* the protein may serve as a reductant with an oxidation-reduction potential low enough to reduce intracellular H_2O_2 to H_2O. The two iron atoms bound to the E-X-X-H sites might be reduced by NADH or another reductant and could subsequently reduce H_2O_2. The NADH peroxidase activity found in the mutant but not in the wild type (Alban and Krieg 1998) might be due to this mechanism rather than to an NADH peroxidase *per se*.

Another possibility is that this rubrerythrin-like protein is one member of an H_2O_2 regulon but does not have a direct role in the H_2O_2 resistance of the variant. Other investigators have identified stress proteins that were later found to be encoded by genes that are members of complex regulons (for examples see Christman *et al.* 1985; Morgan *et al*., 1986; Murphy *et al.* 1987). It is also possible that H_2O_2 could be destroyed in *S. volutans* by nonenzymatic reaction with the novel protein. However, the latter explanation does not account for the high NADH peroxidase-like activity in the mutant (Alban and Krieg, 1998).

The discovery of a ruberythrin/ nigerythrin-like protein in an oxygen-respiring microaerophile rather than only in anaerobes may not be surprising. Microaerophiles seem to have some features that are characteristic of anaerobes and others characteristic of aerobes. For example, all microaerophiles so far tested oxidize pyruvate via pyruvate ferredoxin/flavodoxin oxidoreductase which was once thought to occur only in anaerobes,

yet they possess a respiratory chain that uses O_2 as a terminal electron acceptor. In addition, it has recently been reported that *D. vulgaris* (Hildenborough) which contains rubrerythrin is capable of oxygen-dependent growth at very low oxygen concentrations (< 0.1 % v/v O_2) (Johnson *et al.* 1997) and thus could be considered a microaerophile rather than an obligate anaerobe. It could be interesting to see whether any strictly aerobic organisms possess this protein.

The present report shows that proteins separated by one and two-dimensional electrophoresis can be rapidly identified and the genes encoding these proteins can be cloned and sequenced by using a combination of mass spectrometry and NH_2-terminal amino acid sequencing. This method could be a valuable tool in the study of stress proteins.

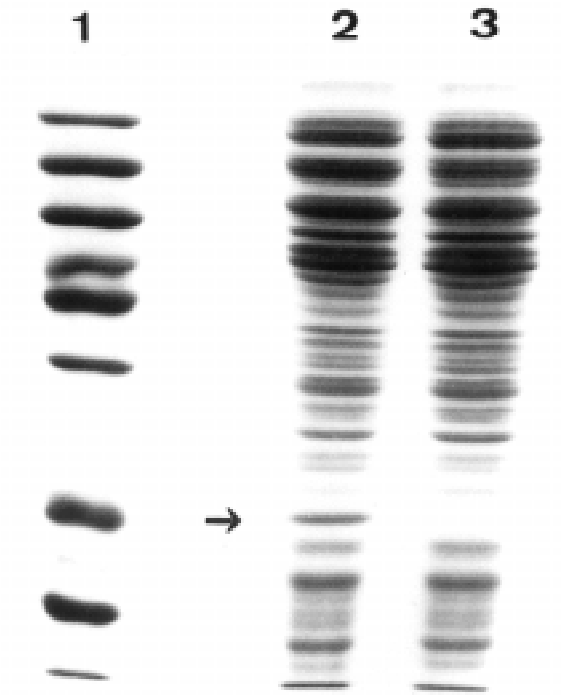

Fig. 1 Coomassie-stained SDS-PAGE gel of the whole-cell proteins of the peroxide-resistant mutant (Lane 2) and wild type *S. volutans* (Lane 3). Lane 1: protein molecular weight markers (top to bottom: phosphorylase B (97 400), bovine serum albumin (66 200), L-glutamic dehydrogenase (55 000), ovalbumin (42 700), aldolase (40 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400)). The arrow indicates the 21.5 kDa protein band only observed in the mutant.

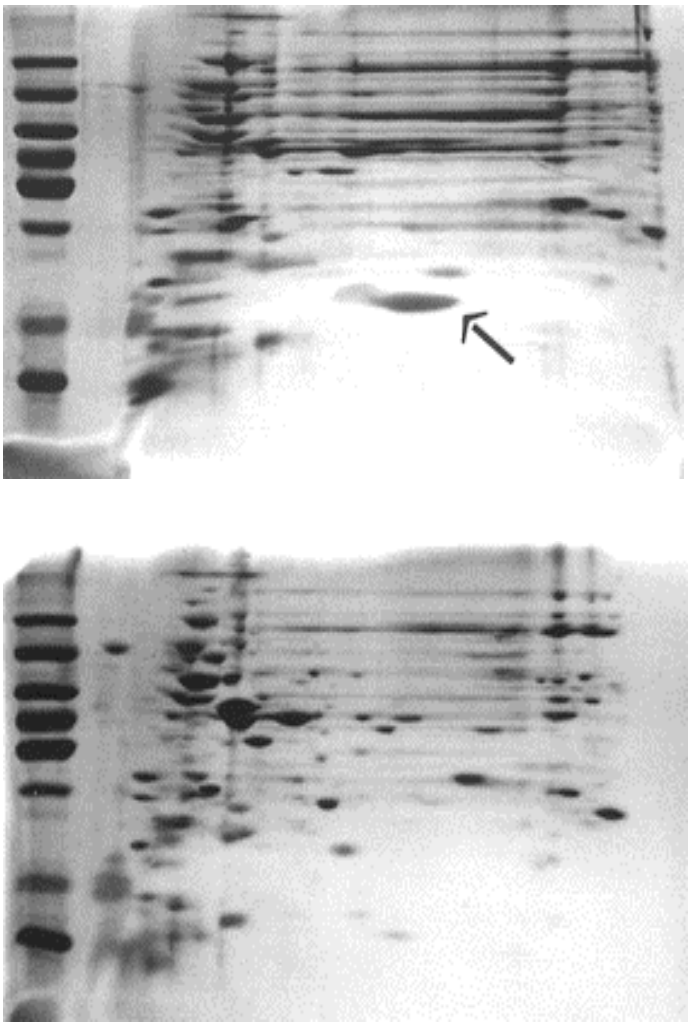

Fig. 2 Silver-stained two-dimensional polyacylamide gels of whole-cell proteins of the peroxide-resistant mutant (top) and the wild type (bottom). Arrow points to 21.5 kDa rubrerythrin/nigerythrin-like protein detected only in the mutant. Protein molecular weight markers are in the far left lane of both gels and are the same molecular mass as Fig.1,

lane 1.

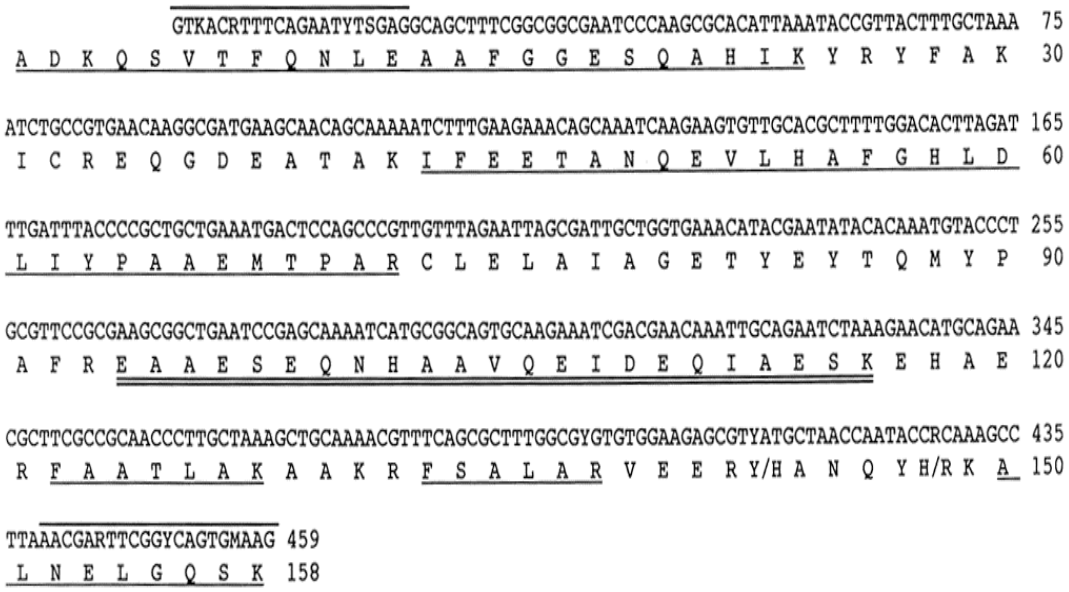

Fig. 3 Nucleotide sequences of the PCR product containing most of the rubrerythrin/nigerythrin-like gene and the predicted amino acid sequence for the protein.The first 30 amino acids were assigned by NH_2-terminal sequencing and the DNA sequence encoding the first five amino acids was not in the PCR product. Underlined amino acids were determined by mass spectrometry. Double underlined sequences were determined by Edman sequencing of tryptic peptide. Amino acid positions 143 and 148 are uncertain due to DNA sequence ambiguity. Lines above the ends of the DNA sequence indicate the PCR primer sequence. Ambiguous codons at amino acid positions 138 and 157 were assigned based on mass spectrometry data.

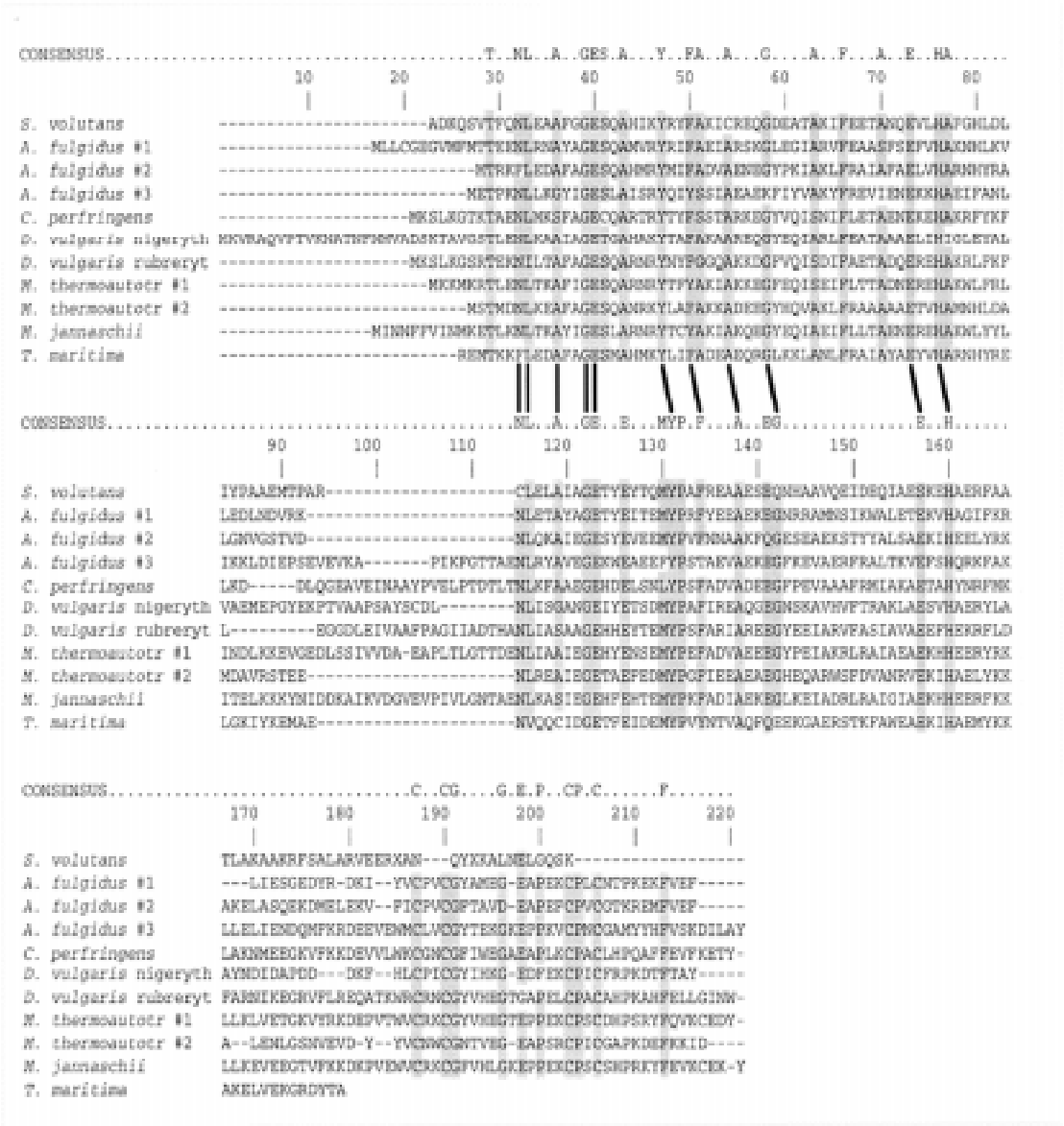

Fig. 4 Alignment of the amino acid sequences of rubrerythrin and nigerythrin occurring in other organisms and the novel rubrerythrin/nigerythrin-like protein of *S. volutans*. Shaded regions indicate conserved regions and the consensus amino acids are indicated above the sequence alignments. Black lines indicate the most highly conserved residues within the tandemly repeated sequences.

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Summary and Conclusions

On the basis of experiments conducted in this study, the following conclusions were reached:

(i) Studies of the adaptive responses of *S. volutans* to various stresses such as heat shock and peroxide are now possible by using the newly developed colony count medium (CCM).

(ii) *Spirillum volutans* is capable of a heat shock response. Cells exposed to 40^{o}C for 100 min allows greater survival at 45^{o}C compared with cells having no prior exposure.

(iii) Cells of *Spirillum volutans* are extremely sensitive to exogenous peroxide. Cells exposed to levels greater than 10 μM are rapidly killed.

(iv) A mutant was isolated which can survive and grow at lethal levels of peroxide. The mutant was not more aerotolerant therefore a lack of catalase and the inability of cells to destroy peroxide is not the sole reason for the microaerophilic behavior of the cells.

(v) The peroxide-resistant mutant showed NADH peroxidase-like activity whereas the wild type cells had low, barely detectable levels. The peroxide-resistant mutant also constitutively expressed a 21.5 kDa protein as determined by one and two-dimensional PAGE. This protein was undetectable and noninducible in the wild type cells. Greater than 80 % of the gene was sequenced and the deduced 158 amino acid polypeptide showed high similarity to rubrerythrin and nigerythrin previously described only in anaerobic *Bacteria* and anaerobic *Archaea*. This is the first report of this type of protein in an organism that must respire with oxygen. The rubrerythrin/nigerythrin-like protein may play a role in the peroxide resistance of the mutant.

(vi) The methodology used to clone the gene may be useful for rapid cloning of genes in other bacteria.

Future Areas of Investigation

The following areas related to my work deserve further investigation:

(i) Now that rubrerythrin has been discovered in the oxygen-respiring microaerophile, *S. volutans* it would be interesting to investigate its occurrence in other microaerophilic bacteria such as *Gallionella ferruginea* which is closely related to *S. volutans*, and *Campylobacter* species. Based on 16*S* rRNA sequencing, *S. volutans* is most closely related to *Thiobacillus thioparus* (Maidak et al. 1997) and the presence of rubrerythrin in this organism may also be worth investigating. Perhaps rubrerythrin is a highly conserved oxidative stress protein that has played a significant role for thousands of years. A DNA probe could be constructed using the DNA sequence of rubrerythrin of *S. volutans*. Colony hybridization could be done under low-moderate stringency. The use of an organism whose complete sequence is known and does not contain a rubrerythrin-like open reading frame (such as *Helicobacter pylori*) could be used as a negative control.

(ii) A putative rubrerythrin gene occurs in the archeon, *Methanococcus jannashii* which is positioned in the neighborhood of several putative electron-transfer proteins like rubredoxin, alkyl hydroperoxide reductase, heterodisulfide reductase, and desulfoferredoxin. Since the physiological function of

rubrerythrin has not yet been determined it would be interesting to investigate whether it is a member of an important operon for electron transfer proteins in *S. volutans*. Perhaps it is transcribed with other stress proteins or important electron carriers. DNA fragments approaching 2 kb could be sequenced which contain the rubrerythrin and neighboring genes. A large fragment of DNA could be cloned and recovered for sequencing if a DNA library of *S. volutans* is screened for by a DNA probe created against the rubrerythrin gene sequence described in this study.

(iii) Other mutants can be isolated by the mutagenesis procedure described in this study which are resistant to paraquat and/or higher levels of peroxide to investigate the role of other important proteins in the survival of *S. volutans* to oxidative stress.

(iv) Little is known about DNA repair mechanisms of microaerophilic bacteria. It would be interesting to study the DNA repair capabilities of *S. volutans* and the mutant described in this study compared to aerotolerant bacteria.

Appendix A

Enzyme assays

NADH peroxidase assays

$$NADH + H_2O_2 \rightarrow NAD + 2H_2O$$

For the spectrophotometric measurements the method of Poole and Claiborne (1986) was used and the reaction mixture was as follows:

Potassium acetate 0.1 M (pH 5.8) ……2 ml
6 mM EDTA ……..……...150 µL
7.8 mM H_2O_2……..……500 µL
4.8 mM NADH ……..……100 µL

cell extract initiates the reaction……..……..250 µL or less (add H_2O accordingly)

Monitor the decrease in ABS at 340 nm and follow Δ ABS for 2-3 minutes. Negative controls are no extract and no H_2O_2. The background NADH oxidase activity must be subtracted from the observed NADH peroxidase activity.

***o*-dianisidine peroxidase**

$$H_2O_2 + o\text{-dianisidine (reduced)} \rightarrow o\text{-dianisdine (oxidized)} + 2\ H_2O$$

o-dianisidine peroxidase activity was determined by monitoring the oxidation of *o*-dianisidine at 450 nm. This assay was described by Hoffman (Ph.D dissertation, VPI and SU, 1977) and was a modification of the method of Guidotti *et al.* (1961). The reaction mixture consisted of the following:

o-dianisidine (5 mg/ml)…….....0.025 ml
0.1 M potassium phosphate buffer (pH 6.4)…..2.0 ml
H_2O_2 (5.6 x 10^{-2} M)…….....0.5 ml
Distilled H_2O……...0.375 ml
Cell extract……...0.1 ml

Hoffman (Ph.D dissertation, VPI and SU, 1977) experimentally determined the molar extinction coefficient of *o*-dianisidine to be $9{,}450^{-1}cm^{-1}$.

Quantitative SOD assay

The hematoxylin assay is based upon studies by Martin et al. (1985) which showed that the autooxidation of reduced hematoxylin to the oxidized form (hematein) is dependent upon superoxide radicals. Hematein absorbs light at 550 nm. The superoxide radicals required for the reaction are generated as intermediates in the course of autooxidation. If SOD is present, it will compete for the superoxide radicals and thus will inhibit the autooxidation and decrease the rate of increase in absorbance at 550 nm. The reaction mixture consist of:

Potassium phosphate buffer, 50 mM containing
1 mM EDTA (pH 7.5) ……..2.7 ml
Hematoxylin 10 mM in 10 mM potassium
phosphate buffer (pH 6.0)*……0.065 ml
Cell extract, dialyzed for 48 h against 2 volumes of
50 mM potassium phosphate buffer (pH 7.4)
containing 1 mM EDTA…… variable
H_2O……q.s to 3.0 ml

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

Under these conditions (Δ ABS = 0.02 min^{-1}), the hematoxylin assay is one-half as sensitive as the xanthine oxidase-cytochrome c method (Martin et al. 1987). Therefore, 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 absorbance is 0.02 min^{-1} the relation between SOD units and percent inhibition may be determined from the graph in Fig. 6 of Martin et al. (1987). The advantage of this method is that it does not require purified proteins such as xanthine oxidase, horseradish peroxidase, catalase, or cytochrome *c*.

Qualitative SOD assay

Staining of polyacrylamide gels for SOD activity was based on the method of Beauchamp and Fridovich (1971) and described by Hoffman (Ph.D dissertation, VPI and SU, 1977). Gels were soaked for 40 min in the dark in a solution containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.25 mM nitro blue tetrazolium, 30 µM riboflavin, and 20 mM TMED. The gels were developed by exposing them to light for approximately 20 min. The photochemical generation of superoxide radicals proceeds according to the following reactions:

$$\text{TMED-}H_2 + \text{Riboflavin} \Rightarrow \text{TMED} + \text{Riboflavin-}H_2$$

$$\text{Riboflavin-}H_2 + O_2 \Rightarrow \text{Riboflavin} + O_2\cdot^-$$

The generated superoxide radicals reduce nitro blue tetrazolium to the water-insoluble formazan form which is dark blue in the polyacrylamide gel. Superoxide dismutase activity in the gel appears as an achromatic band.

Various enzyme activities from Ch.3

Aconitase activity

Periplasm < 0.00001 IU mg^{-1} protein
Cytosol 0.01 IU mg^{-1} protein

Alkaline phosphatase

Periplasm 2.34×10^{-3} IU mg^{-1}

NADH peroxidase

Cytosol 0.12 IU mg^{-1}
Membrane < 0.00001 IU mg^{-1}
Periplasm 0.001 IU mg^{-1}

Pyruvate destroys H_2O_2 as follows:

$$H_2O_2 + CH_3COCOOH \rightarrow CH_3COOH + CO_2 + H_2O$$

Appendix B

DNA isolations, ligations, transformations, and plasmid preps

DNA isolations

DNA isolations were based on the method of Yousten and Rippere (1997). 2.5 L cell cultures grown in CCM minus pyruvate were harvested by centrifugation at 14,000 x g, washed twice in potassium phosphate buffer (10 mM, pH 6.9), and suspended in 20 ml of cell suspension buffer (10 mM Tris, 1.0 mM EDTA, 0.35 M sucrose, 0.1 mg/ml lysozyme) and incubated at 37^{o}C for 30 min. 20 ml lysing solution (100 mM Tris, 20 mM EDTA, 0.3 M NaCl, 2 % (w/v) SDS, 2 % β-mercaptoethanol, 100 µg/ ml proteinase K was added and the mixture was incubated at 55^{o}C for 1 h. The protein was removed by multiple phenol-chloroform extractions and DNA was precipitated with 0.6 vol isopropanol. DNA was dried, suspended in 20 ml TE, 250 µL RNase (1 mg/ml RNase A, 4000 U/ml T1) was added and incubated 1 h at 37^{o}C. The DNA was chloroform extracted and precipitated with ethanol.

Purification of PCR product by Wizard PCR Preps DNA purification system (Promega)

The Wizard PCR Preps System provides a reliable way to purify double-stranded PCR amplified DNA. PCR products are purified effectively away from contaminants, including primers and dNTPs. In a 1.7 ml sterile microcentrifuge tube 25 µL of a PCR reaction and 50 µL of purification buffer (50 mM KCl, 10 mM Tris pH 8.8, 1.5 mM $MgCl_2$, 0.1 % Triton X-100) was added and vortexed. 1 ml PCR prep resin was then added and briefly vortex 3 times over one min period. Using a sterile 3 ml syringe the

mixture was pushed through a Wizard Minicolumn into a new sterile microcentrifuge tube. Column was washed by pushing 2 ml of 80 % isopropanol through column with syringe. Column was dislodged from syringe and attached to the microcentrifuge tube. Tube was centrifuged for 45 sec. To elute DNA 50 µL TE (10 mM Tris pH 7.5, 1 mM EDTA) was added to the top of the column and attached to new sterile microcentifuge tube. The tube was then centrifuged for 30 sec.

Ligation of PCR product into a pGEM-T Vector (Promega)

The ligation procedure was performed according to the manufacturer's protocol. The ligation reaction consisted of :

T4 DNA ligase 10 X buffer ……1 µL
PGEM-T Vector ……1 µL
Purified PCR product …….7 µL
T4 DNA ligase (3 U/µL)…….1 µL

Reaction was incubated overnight at 4^{o}C.

DNA Transformations

DNA transformation reactions were performed according to the manufacturer's protocol. 2 µL of the ligation reaction was added to a 1.5 ml sterile microcentrifuge tube on ice. 50 µL of freshly thawed competent *E. coli* JM109 cells (supplied in Promega pGEM-T kit) were then added to the tube. The contents of the tube were gently mixed and placed on ice

for 20 min. The tube was then placed in a 42^{o} C water bath for 45 sec to heat shock the cells. The tube was placed in ice. 950 µL of SOC medium was added to the tubes and incubated for 1.5 h at 37^{o}C with shaking (150 rpm). Cells were concentrated by centrifugation and 100 µL of transformed cells were spread-plated on duplicate plates containing 16 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl, 10 µg/ml IPTG, 40 µg/ml ampicillin, and 40 µg/ml X-Gal.

SOC medium (100 ml)

Bacto-Tryptone…….. 2.0 g
Bacto-Yeast Extract……..... 0.5 g
1 M NaCl……. 1 ml
1 M KCl ……. 0.25 ml
1M Mg^{2+} stock,
(1M $MgCl_2 \bullet 6\ H_2O$ filter sterilized)…….... 1 ml
2 M glucose, filter sterilized…… 1 ml

Add Bacto-Tryptone, Bacto-YE, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool. Add 2 M Mg^{2+} stock and 2 M glucose, each to the final concentration of 20 mM. Increase volume to 100 ml with sterile H_2O. Filter the complete medium. The final pH should be 7.0.

Preparation of Plasmid DNA: A Modified Mini Alkaline-Lysis/ PEG Precipitation Procedure

The following procedure was developed by Applied Biosystems, Inc. and allows the preparation of quality template DNA for DNA sequencing. This protocol was supplied by the UVA Biomolecular DNA sequencing facility. White colonies were picked from the antibiotic containing plates described above and used to inoculate 5 ml of Terrific Broth containing 100 µg/ml ampicillin. Cultures were grown at 37^{o}C for 24 h and 1.5 ml of culture was pelleted by centrifugation in a sterile microcentrifuge tube. The supernatant was aspirated and the pellet was suspended in GTE buffer (50 mM glucose, 25 mM Tris, 10 mM EDTA (pH 8.0)) . 300 µL of freshly prepared 0.2 N NaOH / 1% SDS was added and mixed until the solution clears. The tube was placed on ice for 5 min. The solution was neutralized by adding 300 µL of 3.0 M potassium acetate (pH 4.8) and placed on ice for 5 min. Cellular debris was removed by centrifugation for 10 min at 25^{o} C and the supernatant was transferred to a new sterile microcentrifuge tube. RNase A (DNase-free) was added to a final concentration of 20 µg/mL and the tube was incubated at 37^{o}C for 20 min. After the RNase treatment, the supernatant was extracted twice by 400 µL chloroform. Each chloroform extraction was done by mixing layers by hand and centrifuging mixture 1 min to separate phases. The extracted supernatant was transferred to a new microcentrifuge tube each time. DNA was precipitated by adding an equal volume of 100 % isopropanol and immediately centrifuging the tube for 10 min at 25^{o} C. The DNA pellet was washed with 500 µL of 70 % ethanol and dried under a vacuum. The resulting pellet was dissolved in 32 µL of deionized H_2O and the plasmid DNA was

precipitated by first adding 8 µL of 4 M NaCl and then adding 40 µL of autoclaved 13 % polyethylene glycol (PEG). After thorough mixing, samples were incubated for 15 min at 4^{o} C. The supernatant was carefully removed and the pellet was rinsed with 500 µL of 70 % ethanol. Finally, the pellet was dried under vacuum and then resuspended in 20 µL of deionized H_2O and stored at $^{-}20^{o}$C.

Terrific Broth

To prepare Terrific Broth, add 100 mL of a sterile solution of 0.17 M KH_2PO_4 to 900 ml of base broth (base broth = 12 g Bacto-Tryptone, 24 g Bacto-yeast extract, 4.0 ml glycerol, q.s. to 900 mL with deionized water and then autoclave).

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