GROWTH ENHANCEMENT AND SELECTION ATTEMPTS FOR *SPIRILLUM VOLUTANS*

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Thesis submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Microbiology

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August, 1987

Blacksburg, Virginia

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(ABSTRACT)

Project objectives were to develop an improved plating medium to achieve reproducible colony counts and accurate quantification for growth or survival of *Spirillum volutans*; and to develop an improved method for selection of *S. volutans* from nature.

Growth enhancement studies began with casein hydrolysate-succinate-salts medium (CHSS). Casein hydrolysate concentration, type of casein hydrolysate, pH, and phosphate concentration optima were determined. Growth of *S. volutans* doubled after 24 h with the addition of potassium phosphate buffer (1 mM final concentration) to CHSS medium (CHSS-P). Addition of supplements to CHSS-P medium, and modified Bordetella and Brucella media failed to increase the cell yields of *S. volutans*. Punctiform colonies did form in CHSS-P medium (0.7% agar) pour-plates, yet at a recovery rate much lower than reported by Padgett et at. (1982) for surface growth on spread plates. The role of amino compounds as possible growth factors was investigated. Thin-layer chromatography, gas chromatography, and amino acid analysis were employed to identify those amino compounds (if any) that decreased or disappeared in the medium after 48 h of growth. None could be found. Numbers of viable cells were not greatly increased, however, viability

was prolonged by growing S. volutans in dialysis sacs suspended in CHSS-P medium.

The tolerances of S. volutans to several antimicrobial compounds were determined. Attempts at using hay infusion, Pringsheim, and CHSS-P media supplemented with various compounds as selective agents did not result in predominance of S. volutans.

IN DEDICATION

This thesis is dedicated to the following people who have touched my life dearly. Their essence will always enable me to smell sweetness where sour abounds.

Mrs. Minnie Shoobs Friedman

Mr. Howard Simmons

ACKNOWLEDGMENTS

Exuberance, wonder, classical friction upon cold fact revealing the sassy spirit of the man. Dr. Noel Roger Krieg has taught me that science is jubilant fascination anchored in quantitative examination. No other person on earth can embellish his students with a greater love for microbiology, and for this I thank him! I am also grateful for the summer stipend he awarded me.

I extend my appreciation to my committee members, Drs. Robert M. Smibert and Eugene M. Gregory, for their direction and helpful suggestions during the course of my project. Special thanks go to Drs. Ernest R. Stout and F. M. Anne McNabb for supplying me with GTA funding, and giving me their cooperation and support with my adjunct teaching positions.

To my parents, Paul and Bernice, I give my thanks for their emotional and financial support.

Finally, I give my most heart-felt appreciation to my wife Mary. Her devoted friendship and love have given me the courage and self esteem to complete this degree.

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INTRODUCTION

"It's no longer enough for me to fight dwarfs -- I need giants!"

--- Cyrano de Bergerac, Act I, Scene VII

The first description of S. volutans is credited to C. G. Ehrenberg in 1838. His measurements indicated that the spirillum was of enormous size compared to most other bacteria. In the ensuing years many attempts were made to isolate the giant spirillum from mixed cultures, but it was not until 1962 that the first pure cultures were obtained. The major difficulties were that (1) no enrichment system was available that allowed the spirilla to predominate in mixed cultures, and (2) the organism did not form colonies on solid media. Rittenberg and Rittenberg (1962) found that S. volutans swam faster than most other bacteria in the mixed cultures, and they were able to isolate the organism by taking advantage of this high degree of motility. They inoculated the mixed culture at the end of a long capillary tube filled with a sterile medium and found that the spirilla were able to outdistance the contaminating organisms as they traveled along the length of the tube. The tube was then broken at a point behind the spirilla and the contents of the tube were expelled into a sterile liquid medium. Although this procedure was ingenious, it was not an easy one, and the Rittenbergs made many attempts before finally obtaining a pure culture. Unfortunately, the capillary tube method still remains the only way to successfully isolate S. volutans.

Other isolation methods, such as streaking plates of solidified media, have not proven satisfactory because colonies of _S. volutans_ are very difficult to obtain. Indeed, colonies were not obtained at all until the work of Padgett et al. in 1982. One factor contributing to the difficulty is that _S. volutans_ is a microaerophile, as first shown by Wells and Krieg in 1965. Using nutrient broth, these investigators showed that, although the organisms required oxygen for respiration, they could grow only at oxygen concentrations from 1 to 12%. Other liquid media have since been developed that allow better growth of _S. volutans_ and one medium, CHSS broth, even allows growth to occur under aerobic conditions. However, even CHSS broth, when solidified with agar, fails to allow the growth of colonies of _S. volutans_ under aerobic conditions. Colonies have been obtained, with difficulty, by incubating cultures on solidified CHSS under 6 to 12% oxygen (Padgett et al., 1982). However, other conditions are required besides a low oxygen level. It is necessary to protect the solidified medium from light, to add chemical agents that destroy toxic forms of oxygen such as hydrogen peroxide and superoxide radicals, and to incubate the cultures in a highly humid atmosphere. Even with these conditions, comparisons of the numbers of cells inoculated onto plating media versus the number of colonies that developed indicate that only 22 to 72% of the cells are able to form colonies. It is not known what factors are responsible for this high degree of variability and for the failure of all the inoculated cells to form colonies.

One factor related to the variability of colony counts is that even liquid media such as CHSS broth are not entirely satisfactory for

the growth of S. volutans. This is indicated by the fact that only low cell densities are achieved in the liquid media that are available. If higher cell densities could be achieved by modifying the liquid media, the solidified versions of such improved media would probably yield much more reliable and reproducible colony counts.

This background information provided the basis for the present research project. The goals of the project were two-fold. One goal was to develop a selection system in which S. volutans would either predominate in mixed cultures or would at least comprise a reasonably large proportion of the total population, so that its isolation could be accomplished by plating on solidified media rather than by using the capillary tube method. The second goal was to improve CHSS broth for S. volutans so that higher cell densities could be obtained.

LITERATURE REVIEW

Isolation and Cultivation of Spirillum volutans

Initially, S. volutans could be grown only in mixed cultures. For example, in 1951 this fastidious bacterium was observed in the waters of a beet-sugar refinery cooling tower by E. G. Pringsheim (Rittenberg et al., 1962). Pringsheim maintained it in mixed culture at a concentration of about 1.5 x 10^6 cells per ml. The medium consisted of wheat or barley grains, soil, and water. S. volutans was successfully enriched for using Pringsheim soil medium only when the enrichment broth was allowed to incubate at ca 25°C for four months (Williams et al., 1957).

Mixed cultures were not satisfactory for physiological and biochemical characterization of the organism, and in 1962 Rittenberg and Rittenberg tried to obtain pure cultures. They eventually achieved their goal by taking advantage of the spirillum's rapid motility. By the use of a long capillary tube filled with medium, they attained separation by allowing S. volutans to outswim the other bacteria. Once the spirilla had migrated far enough away from the other organisms, the capillary tube was broken at a point up stream from the mixed culture. The isolated spirilla were then inoculated into various sterile media.

Although S. volutans had been separated from the other bacteria, the Rittenbergs found that it could not grow by itself. They tried many different media with no success. Growth of pure cultures occurred only when S. volutans was placed in a sterile medium contained in a dialysis sac that was in turn suspended in a mixed culture. However, the Rittenbergs subsequently found that filter-sterilized extracts from

sonicated cultures of Escherichia coli could support S. volutans growth when added to an asparagine- mineral salts medium. Extracts from statically grown cultures of E. coli were more stimulatory for S. volutans growth than from aerated cultures (Rittenberg et al., 1962).

In determining the optimal growth conditions for S. volutans, Rittenberg and Rittenberg found that pH was a critical factor. The highest cell yields were seen between pH 6.5 and 7.5. Values higher than pH 7.5 proved to be lethal. A solution of asparagine (0.05% w/v) was shown to be stimulatory (Rittenberg et al., 1962).

Wells and Krieg (1965) were the first to grow S. volutans in the absence of other bacteria or bacterial extracts. They were able to achieve this after discovering that the spirillum was a microaerophile: in wet mounts the organisms formed a band close to, but not quite at the edges of the coverslip. This behavior suggested that the bacteria were migrating to a position that was intermediate between anaerobiosis and aerobiosis. Subsequently, growth could be obtained in nutrient broth if cultures of S. volutans were incubated in a microaerophilic environment of 9% O_2 or less. Thus, the symbiotic growth of S. volutans with other bacteria as observed by Rittenberg and Rittenberg was most likely based on the ability of the other bacteria to lower the surrounding O_2 tension to a level suitable for growth of the spirilla (Wells et al., 1965).

Aerotaxis studies by Caraway and Krieg (1974) revealed that S. volutans exhibited positive chemotaxis toward self-created oxygen gradients, and that the migration was related to oxygen consumption and not substrate consumption. Thus, the cells have the capability of

seeking out a suitable oxygen level, as suggested originally by Wells and Krieg (1965).

McElroy et al. (1967) improved the culture medium for S. volutans. They used a peptone-succinate-salts (PSS) medium (peptone, 10 g/l; succinic acid, 2 g/l; $(NH_4)_2SO_4$, 1.0 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; $FeCl_3 \cdot 6H_2O$, 0.002 g/l) in which the pH was adjusted to 6.8 with KOH. Since freshwater spirilla have a low sodium ion tolerance, NaOH was not used in pH adjustment (Hylemon et al., 1973a). This medium was later modified by Caraway and Krieg (1972) who decreased the levels of peptone and succinic acid to 5.0 g/l and 1.0 g/l respectively. The modified medium was termed MPSS broth and gave higher cell yields.

A casein hydrolysate-succinate-salts (CHSS) medium devised by Bowdre and Krieg (1974) allowed the microaerophilic spirillum to grow in the air under 21% O_2 in a broth culture. This medium was a modification of MPSS, in that the 5 g/l of peptone was replaced with 2.5 g/l of "vitamin-free salt-free" casein hydrolysate (Bowdre et al., 1974). Shortly after this development, Bowdre et al. (1976) devised a defined medium for S. volutans. Here, Bowdre and coworkers (1976) discovered the importance of dihydroxyphenyl compounds, such as norepinephrine, on growth. Addition of such compounds to the defined medium allowed an increase in the tolerance of S. volutans to oxygen.

Bowdre et al. (1976) also discovered that the addition of a low concentration of NaCl (0.01% final volume) was stimulatory for aerobic growth of S. volutans. Padgett et al. (1982) found that aerobic growth of S. volutans was not supported in CHSS medium containing a different lot of casein hydrolysate. Using flame photometry Padgett and coworkers

(1982) found that the lot of casein hydrolysate not yielding growth contained 2 mM Na^+ in contrast to the 65 mM Na^+ content of the lot that did permit growth. When between 0.01% and 0.02% NaCl was added to CHSS containing the Na^+ deficient lot of casein hydrolysate, aerobic growth of S. volutans was supported (Padgett et al., 1982).

Growth studies of both CHSS broth and the defined growth medium in tubes used a 24-h log phase culture of S. volutans as an inoculum. However, growth studies using CHSS broth employed a final concentration for inocula of 2 x 10^3 cells/ml, where as those with defined growth medium used a final concentration for inocula of 2 x 10^4 cells/ml (Bowdre et al., 1976; Padgett et al., 1981).

Role of Oxygen Toxicity on the Growth of S. volutans

Various possible explanations for the microaerophilic nature S. volutans are given below.

Respiratory rates of S. volutans may be too low to maintain a reduced cell interior. Cole and Rittenberg (1971) assayed for five tricarboxylic acid cycle enzymes (fumarase, malate dehydrogenase, aconitase, isocitrate dehydrogenase, and succinate dehydrogenase) and also NADH oxidase. All these enzyme activities were present at lower levels in S. volutans than in two other aerobic spirilla assayed (Cole et al., 1971). Cole and Rittenberg showed that S. volutans possessed cytochromes b and c and a cytochrome oxidase. No cytochrome a was found. In comparing the respiration rates of S. volutans with two aerobic spirilla, Cole and Rittenberg found that the aerobic organisms had respiratory rates 2 to 40 times that of S. volutans depending upon the

substrate used. It should be noted that when no compounds were added to the suspending buffer (50 mM phosphate buffer), the endogenous respiration rates were approximately 16 times higher for A. itersonii than for S. volutans (Cole et al., 1971). However, Caraway and Krieg (1974) questioned the validity of Cole and Rittenberg's results. They found that 0.05 M phosphate buffer inhibits the respiration of S. volutans. When 0.01 M phosphate buffer, or a synthetic buffer (BES) buffer was used instead, much higher rates of respiration were obtained.

Despite discrepancies in respiration values, these two research groups both agreed that succinate was an excellent oxidizable substrate for S. volutans (Caraway et al., 1974; Cole et al., 1971). According to Cole and Rittenberg (1971), the main supply of electrons for oxidative phosphorylation came from succinate via succinate dehydrogenase (Cole et al., 1971). Caraway and Krieg (1974) noted that malate and fumarate also supported high respiration rates in S. volutans.

Sterile growth media for S. volutans may undergo autooxidation or photooxidation resulting in the generation of toxic derivatives of oxygen. Padgett et al. (1982) found that addition of potassium metabisulfite, norepinephrine, catalase, or superoxide dismutase (SOD) to MPSS broth permitted S. volutans to grow aerobically. A combination of catalase plus SOD had a synergistic effect. Moreover the organism was found to be extraordinarily sensitive to low levels of exogenous hydrogen peroxide (H_2O_2). Illumination of MPSS broth generated enough H_2O_2 to render the medium inhibitory to growth. A combination of catalase plus SOD prevented this inhibition. Thus, it seems that both

H_2O_2 and superoxide radicals were generated in the media (Padgett et al., 1982).

S. volutans might possess insufficient types or levels of certain enzymes that could protect it from toxic forms of oxygen. In searching for an explanation of the microaerophilic nature of S. volutans Padgett et al. (1982) thought that superoxide radicals (O_2^-) might be involved. SOD is the enzyme responsible for eliminating O_2^-. Padgett et al. (1982) found that S. volutans possesses cytoplasmic SOD activity at a level of 12-14 U/mg protein. They found similar values for SOD activities in cells grown under 6% and 21% O_2. Thus, it appears that the SOD activity in S. volutans is not stimulated by O_2 (Padgett et al., 1982).

The aerotolerance of S. volutans is increased (Padgett et al., 1982) when exogenous bovine SOD is added to MPSS broth, despite the occurrence of SOD activity in the cells. Thus, the cellular SOD seems incapable of protecting the cells against exogenous O_2^-.

The true culprit may not be O_2^- but rather H_2O_2, formed by dismutation of O_2^-, or hydroxyl radicals (OH·), formed by the reaction between O_2^- and H_2O_2. Hydroxyl radicals have the shortest life of all the toxic forms of oxygen and are the most reactive with biological compounds.

In the case of S. volutans, H_2O_2 seems to be more toxic than O_2^- (Padgett et al., 1982). The organism possesses no catalase activity, and catalase added to MPSS broth can increase the aerotolerance of S. volutans. However, a combination of SOD and catalase accomplishes a much greater enhancement of aerotolerance than either enzyme alone (Padgett et al., 1982); thus O_2^- may still play a role. The role seems less

important than that of H_2O_2, however, because Padgett (Ph. D. dissertation, 1981) obtained preliminary evidence that the action of SOD on superoxide radicals enhanced the killing rate of S. volutans exposed to a flux of superoxide radicals. Hence, the dismutation of O_2^- to H_2O_2 and O_2 led to higher toxicity than with O_2^- per se. When catalase was added to this system some protection was offered (P. J. Padgett, unpublished data). These results indicate H_2O_2 and OH·, formed by reaction with O_2^- and H_2O_2, might have been the major toxic agent.

In comparing various microorganisms, aerobic or facultative organisms are more tolerant to O_2 and its derivatives than microaerophiles. S. volutans can only tolerate a very low level of H_2O_2, viz., 0.029 μM (Padgett et al., 1982).

Generation of H_2O_2 in S. volutans might occur through the action of oxidases. This might contribute to the sensitivity of the organism to oxygen: the higher the oxygen level during growth, the more H_2O_2 would be metabolically generated.

S. volutans might possess key oxygen-sensitive cell constituents. Single-stranded breaks might occur in its DNA in the presence of low levels of H_2O_2, as happens in Treponema pallidum (Steiner et al., 1984). Other primary sites of attack within S. volutans besides DNA might account for O_2 toxicity. Two of these might be sulfhydryl-containing enzymes, which are subject to oxidation, and membrane lipids, which are subject to peroxidation. Enzymes can become inactivated if their -SH groups become oxidized. If long-chain fatty acids become oxidized, subsequent inactivation of certain enzymes and production of singlet oxygen could occur (Halliwell, 1979). With regard to -SH groups on

enzymes, Padgett et al. (1986) has shown S. volutans to be highly sensitive to the drug metronidazole, a drug to which only anaerobes have hitherto been reported to be sensitive. Metronidazole owes its inhibitory activity to its reduction by ferredoxin, an oxygen-labile iron-sulfur protein. Thus the possibility exists that S. volutans may contain ferredoxin, and that it might be inactivated by oxygen or hydrogen peroxide.

S. volutans might lack sufficient reducing power to maintain a low intracellular oxygen level. In anaerobes oxygen toxicity can also occur if O_2 can divert reducing equivalents normally used for anabolism (Morris, 1979). In some organisms, especially eucaryotes, sufficient levels of glutathione are maintained to offer protection for oxygen-labile enzymes against oxygen. Studies of various bacteria have shown that there is little correlation between glutathione levels and oxygen tolerance; nevertheless, the possibility that S. volutans may have only low levels of this reducing agent has not been investigated.

Enrichment and Selection of Spirilla

Aquatic spirilla are divided into three distinct genera: Aquaspirillum, Oceanospirillum, and Spirillum. The majority of species contained in all three genera possess poly-β-hydroxybutyrate granules. A brief description of the genera Aquaspirillum and Oceanospirillum will be given below. The genus Spirillum has only one species (S. volutans) which was described earlier.

Members of the genus Aquaspirillum are Gram-negative, usually bipolarly flagellated, mostly helical bacteria ranging in diameter from

0.2 to 1.4 µm. These microorganisms thrive in freshwater, eutrophic, lentic habitats with oxygen demands ranging from aerobic to microaerobic. Aquaspirilla are usually catalase- and phosphatase-positive. They use Krebs cycle acids as carbon sources but usually do not catabolize carbohydrates. They do not grow in media supplemented with 3% NaCl. The optimal temperature for growth is 30°C (Krieg, 1976; Krieg, 1984a).

Members of the genus *Oceanospirillum* are gram-negative, bipolarly flagellated, helical spirilla having a diameter between 0.3 µm and 1.4 µm. They are strict aerobes that use organic acids or amino acids as sole carbon sources. Oceanospirilla typically form coccoid bodies during stationary phase of growth. They can be found in coastal sea water or associated with seaweed. In either case, sea water or Na^+ is a requisite for growth. The optimum temperature is between 25°C and 32°C (Krieg, 1984b).

Spirilla have been found in a variety of environments, but never as the predominant organisms. The most frequent isolations have occurred from fresh water (*Spirillum* sp., *Aquaspirillum* sp.), soil (*Aquaspirillum* sp.), and salt water (*Oceanospirillum* sp.) (Krieg, 1981).

Preliminary enrichment of spirilla is often accomplished through natural infusions. Botanical infusions proved successful in cultivating both *S. volutans* and *Aquaspirillum* sp. Hay infusions have been used for *S. volutans*, and infusions of freshwater algae have been used for aquaspirilla (Wells et al., 1965; Williams et al., 1957). Jannasch (1965) expanded upon Williams and Rittenberg's work by creating an infusion composed of aquatic plant debris, source water, calcium malate

or calcium lactate, potassium phosphate, and magnesium sulfate. After incubation for a week, this infusion yielded spirilla just beneath the surface (Jannasch, 1965).

Aquaspirillum and *Oceanospirillum* species have been enriched for from freshwater and seawater infusions of *Corbicula japonica*, a mussel indigenous to Japan's fresh waters (Terasaki, 1980). The mussel was boiled to generate a broth. Portions of mussel and aquatic soil samples were added to the cooled broth. The broth was incubated at ambient temperature until an abundance of spirilla appeared (Terasaki, 1980).

Jannasch (1967) demonstrated that a marine spirillum grew more rapidly than a species of *Pseudomonas* under low nutrient conditions in a chemostat. At low dilution rates (0.4 h^{-1}) the spirillum outnumbered the *Pseudomonas* 7:1. At higher dilution rates (0.7h^{-1}) the *Pseudomonas* sp. outgrew the spirillum. Jannasch (1967) concluded that organisms such as spirilla that possess a low K_s, and thus a high affinity for the nutrient substrate, will predominate in environments with low nutrient availability.

Aquaspirillum serpens showed a sharp and unexpected washout from an aerated chemostat culture when the substrate concentration fell to 10 mg/l (Jannasch, 1977). Although the amount of lactate in the culture vessel was adequate for growth, the cell density was too sparse in relation to the oxygen content of the medium. Thus, high cell densities of *Aquaspirillum serpens* grow microaerophilically " ...to overcome the unfavorable effect of a high redox potential..." (Jannasch, 1977). The early washout could be prevented by decreasing the O_2 content of the gas atmosphere.

An extreme example of using low levels of organic carbon compounds in enriching for spirilla can be seen with Aquaspirillum autotrophicum. Schweizer and Aragno (1975) were able to enrich for this organism by placing membrane filters seeded with eutrophic lake samples on mineral agar. Due to the organism's membrane-bound H_2-inducible hydrogenase, and its autotrophic growth requiring microaerobic conditions, the mineral agar plates were incubated under 60% H_2, 30% air, and 10% CO_2. Under these conditions A. autotrophicum could outgrow most of the heterotrophs that were present (Schweizer et al., 1975; Aragno and Schlegel, 1978). Eventual isolation could be achieved through subsequent transfers onto mineral agar plates incubated under similar gaseous conditions (Aragno et at., 1978).

Another extreme example of the enriching effect of low nutrient concentrations is represented by Aquaspirillum delicatum. This organism was isolated from samples of stored distilled water. The samples were spread onto agar plates containing low amounts of casitone (0.03 g/l) and yeast extract (0.01 g/l) (Leifson, 1962; Krieg, 1981).

Williams and Rittenberg (1957) successfully isolated spirilla from primary enrichment cultures prepared by inoculating samples into autoclaved eutrophic pond water. Under these low nutrient conditions the spirilla predominated and could be isolated after transfer to agar media (Williams et al., 1957).

Like S. volutans, enrichment of Aquaspirillum fasciculus was achieved by the use of hay infusions, with subsequent inoculation into Pringsheim soil medium (Strength et al., 1976). Pringsheim soil medium

is a good preliminary enrichment step for spirilla, because of its low nutrient content.

Pond water supplemented with the calcium salts of malate or lactate proved to be a good selective substrate for freshwater spirilla (Williams et al., 1957; Jannasch, 1965). Addition of nitrogenous compounds such as ammonium salts to this enrichment will enable other bacteria to outgrow the spirilla (Williams et al., 1957).

Many spirilla appear larger than accompanying procaryotes when infusions are viewed under a microscope. This large size has been used in selection methods. For instance, secondary enrichment of Aquaspirillum bengal was accomplished by Kumar et al. (1974) by centrifuging enrichment broths at 1,000 x g. The resulting pellet contained predominantly the large spirilla (Kumar et al., 1974).

A different procedure was used to separate Oceanospirillum maris subsp. williamsae from a contaminating vibrioid organism. The spirillum was enriched for by agglutinating the vibrioid cells by means of specific antibodies. After removal of the flocs of vibrioid cells by low-speed centrifugation, the resulting supernate contained predominantly O. maris subsp. williamsae (Linn et al., 1978).

The isolation of Aquaspirillum gracile was based on the small size of the cells (Canale-Parola et al., 1966). A cellulose acetate filter (pore size = 0.45 μm) was placed on the surface of a medium consisting of peptone (0.5%), yeast extract (0.05%), Tween 80 (0.002%), K_2HPO_4 (0.01%), and agar. The surface of the filter was inoculated with fresh water samples containing A. gracile. The spirilla penetrated the filter

and grew in the underlying agar, and were subsequently isolated from pure colonies on streak plates (Canale-Parola et al., 1966).

Jannasch (1965) employed a modification of the hanging drop method to isolate freshwater spirilla. Individual drops of diluted enrichment + 0.005% ascorbic acid (each drop containing one spirillum) were incubated to allow proliferation of the organisms. The growth was subsequently transferred to solid media (Jannasch, 1965).

Jannasch (1965) isolated spirilla by taking advantage of their aerotactic behavior and motility. In a manner very similar to that of Rittenberg and Rittenberg (1962) the capillary tube method was used for isolating *Aquaspirillum* sp. The aquatic spirilla successfully migrated away from contaminants along a gradient of increasing O_2 tension (Jannasch, 1965).

An unusual tactic response ever exhibited by a bacterium was observed by Blakemore (1975). In 1979 he and his colleagues separated *Aquaspirillum magnetotacticum* from other microbes in a New England bog sample. The initial isolation method involved placing bar magnets adjacent to the upper portion of an enrichment culture. After the magnetotactic spirilla migrated from their microaerobic zone of growth to the area of the culture vessel adjacent to the magnet, samples were withdrawn and placed in a pre-reduced selective medium for subsequent isolation (Blakemore et al., 1979). The only source of O_2 in the selective medium came from the inoculum.

Another unusual spirillum was isolated by Yayanos et al. (1979). These investigators grew and subsequently isolated colonies of a barophilic spirillum by culturing the organism on a salt water-silica

gel medium containing tryptone (0.5%), yeast extract (0.25%), and glucose (0.1%) (Dietz et al., 1978). The plates were incubated at 3°C under 500 bars of pressure (Yayanos et al., 1979).

Inoue (1976) isolated an obligately psychrophilic spirillum, with a temperature preference of 9°C, from Syowa Station in Antarctica (Inoue et al., 1976). The organism, "Spirillum pleomorphum," was selectively enriched by taking advantage of its temperature optimum (Inoue, 1976; Inoue et al., 1976)

Successful enrichment and isolation of spirilla from natural sources is for the most part a tedious procedure, and depends on taking advantage of any unusual properties of the organisms.

MATERIALS AND METHODS

Glassware Preparation

Glassware, plastic graduated cylinders, and plastic beakers were washed in a Heinicke glassware washer using an 8-min. wash cycle, 8-min. rinse cycle with tap water, and a 30 sec. rinse using water purified by reverse osmosis. Items to large to be cleaned in the washer were washed by hand and rinsed copiously with tap water. Powdered phosphate base detergent was used in both machine and hand washing procedures. Immediately before using plastic and glassware, the items were rinsed 3 to 5 times with glass-distilled water (Sybron/Barnstead glass still). The only glassware not treated with distilled water prior to use were culture tubes. Rinsing culture tubes with distilled water seemed impractical, since many experiments required 400 or more tubes.

During the early stages of this project, glassware was soaked in dilute sulfuric acid for at least 2 hours, rinsed 10 times in tap water, and 5 times in distilled water after having been washed as stated above. This practice was eventually dismissed since it had no noticeable effect on bacterial growth.

In many cases the caps for culture tubes were boiled three consecutive times in purified H_2O. The purified water was changed between boiling.

Complex Media

Casein hydrolysate-succinate-salts (CHSS) medium was prepared as described by Bowdre et al. (1974). Vitamin-free, salt-free, acid-

hydrolyzed casein (ICN Biochemicals catalog # 104778 [lot # 11523]) was used throughout the study. Below is the protocol followed in making this medium.

The following procedures were performed under ordinary room illumination.

1. All dry chemicals (both defined and complex) were added to a glass or plastic beaker filled with distilled water. The level of H_2O added was approximately 98% of the final volume of the medium.
2. Stock solutions of $FeCl_3 \cdot 6H_2O$ and $MnSO_4 \cdot H_2O$ (0.002 g/l each) were added to the broth.

The following procedures were performed under dim red illumination.

3. The pH of the medium was adjusted to 7.0 using KOH. If pH was accidentally brought higher than 7.0, HCl was used to readjust the pH to 7.0.
4. The volume was adjusted to 100% with distilled water.
5. After dispensing the medium into appropriate receptacles and autoclaving, the medium was then stored in a dark cupboard for at least 12 h before use.

The following variations of CHSS medium were also used.

CHSS-P Medium = CHSS medium supplemented with 1 M potassium phosphate buffer in order to achieve a final concentration of 1 mM.

CHSS-Bis Medium = CHSS medium supplemented with potassium metabisulfite (0.02 g/1). The potassium metabisulfite was added to the medium after the pH was adjusted.

CHSS-Bis-BES Medium = CHSS-Bis medium supplemented with BES [n,n bis-(2 hydroxyethyl 2 aminoethane sulfonic acid)] buffer. The final concentration of BES buffer was 1.0665 g/1 (Bowdre et al., 1976).

CHSS-P-Bis Medium = CHSS-Bis medium supplemented with 1 mM final concentration of potassium phosphate buffer.

CHSS-P-BTB Medium = CHSS-P medium supplemented with bromothymol blue. The indicator was initially dissolved in dilute KOH and added to CHSS-P broth prior to adjusting the pH of the medium. The final concentration of bromothymol blue was 0.0025%.

Modified brucella broth was made by adding 1.5% peptone 20 (Gibco), 0.5% peptone 100 (Gibco), 0.2% yeast extract (Gibco), 0.1% glucose, 0.01% NaCl, and 0.1% citric acid to distilled water (90% of the final medium volume). The aforementioned procedures were done under ordinary room illumination. The remainder of the protocol was as described for CHSS medium, under dim red illumination.

Pringsheim soil medium was prepared by adding 1 barley seed and ca. 3 cm of potting soil to an 18 mm x 145 mm test tube (Rittenberg et

al., 1962). Each tube was filled to half volume with tap water and sealed with a sleeve cap. The medium was sterilized by autoclaving.

Defined Media

Defined medium (DM) for growing S. volutans was prepared as previously stated by Bowdre et al. (1975). Many of the autoclavable reagents were added as separate stock solutions. Three separate stock solutions (100 x) were made in distilled water. Two of the stock solutions were $MnSO_4 \cdot H_2O$ and K_2HPO_4. The third stock solution, composed of NaCl, $CaCO_3$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 7H_2O$, H_3BO_3, and $Na_2MoO_4 \cdot 2H_2O$, was acidified with 1% of 12 N HCl. These stock solutions were stored at 4°C. Solutions of $FeCl_3 \cdot 6H_2O$ and DL-nor-epinephrine·HCl that were sterilized by filtration were added to the autoclaved portion of DM within 24 h of their preparation.

The constituents for a defined medium based on the defined medium for Bordetella pertussis developed by Stainer and Scholte (1971) were as follows: L-proline, 0.24 g/l; L-glutamic acid, 0.67 g/l; NaCl, 0.10 g/l; KH_2PO_4, 0.50 g/l; KCl, 0.20 g/l; $MgCl_2 \cdot 6H_2O$, 0.10 g/l; $CaCl_2$, 0.02 g/l; Trizma buffer, 6.08 g/l. S. volutans is sensitive to sodium ions, yet growth is enhanced with minute quantities of NaCl (Bowdre et al., 1976). For this reason the NaCl concentration was reduced, and glutamic acid was used in place of sodium glutamate. All of the above constituents were added to distilled H_2O. The pH was adjusted to 7, and the medium was dispensed in 80-ml amounts into cotton-stoppered 250-ml Erlenmeyer flasks and sterilized by autoclaving. This medium was termed glutamate + proline basal medium (G+PBM).

Semisolid, "Soft," and Solid Media

Semisolid media were obtained by adding 1.5 g of agar (Gibco, bacteriological grade) to 1 l of liquid medium. "Soft agar" and solid agar media contained 7.0 g and 15.0 g of agar per 1 l of broth.

Culture Maintenance

S. volutans ATCC 19554 was transferred daily in cotton-stoppered 250-ml Erlenmeyer flasks filled with 80 ml of CHSS-P broth, using 1 ml of the previous culture as inoculum for the next.

Standard Inoculum

For broth and semisolid media a 1.0% - 1.2% (v/v) inoculum of *S. volutans* was used. The inoculum was taken from a 24-h culture that had been transferred for at least 3 days at 24-h intervals.

Culture Supernates

Cultures of *S. volutans* grown for 24 h, 48 h, and 72 h were centrifuged (12,100 x g) and the supernates collected. The culture supernates were sterilized by passage through a Gelman acrodisc filter (pore size = 0.2 μm). In some instances they were sterilized after first being concentrated 20-fold with a flash evaporator (Buchler). All concentrates were stored at -20°C.

Sterilization of Liquid Supplements for Media

Heat-labile solutions were sterilized by filtration (2.0 µm pore size; Gelman acrodisc or a Millipore filter apparatus).

Supplements for CHSS-P Medium

KCl. This was added to CHSS-P broth in various amounts prior to adjusting the pH.

Catalase. The stock solution was prepared by dissolving 6 mg of bovine liver catalase (Sigma lot # 53F-7070) in 10 ml of distilled water. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

Fructose. The stock solution was prepared by dissolving 8 g of fructose in 15 ml distilled water and adjusting the pH to 7. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

β-Hydroxybutyrate. The stock solution was prepared by dissolving 2 g of β-hydroxybutyrate in 10 ml distilled water, and adjusting the pH to 7. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

Cycloheximide. The stock solution was prepared by dissolving 25 mg of cycloheximide in 5 ml of distilled water. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

5-Fluorouracil. The stock solution was prepared by dissolving 100 mg of 5-fluorouracil in 2 ml of distilled water. This solution was added to the medium prior to autoclaving.

5-Fluorocytosine. The stock solution was prepared by dissolving 100 mg of 5-fluorocytosine in 2 ml of distilled water. This solution was added to the medium prior to autoclaving.

Iodoacetic acid. The stock solution was prepared by dissolving 100 mg of iodoacetic acid in 2 ml of distilled water. This solution was added to the medium prior to autoclaving.

Potassium fluoride. The stock solution was prepared either by dissolving 1 g of potassium fluoride in 10 ml of distilled water, or by dissolving 300 mg of potassium fluoride in 20 ml of distilled water. This solution was added to the medium prior to autoclaving, or filter sterilized and added after the medium was autoclaved.

Tannic acid. The stock solution was prepared by dissolving 10 mg of tannic acid in 2 ml of distilled water. This solution was added to the medium prior to autoclaving.

Basic fuchsin. The stock solution was prepared by dissolving 4 mg of basic fuchsin in 1 ml of ethanol followed by 9 ml of distilled water. This solution was added to the medium prior to autoclaving.

A stock solution containing lincomycin (60 mg) and 5-fluorocytosine (45 mg) was prepared by dissolving these compounds in 21 ml of a solution containing 50% 2 N KOH and 50% distilled water. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

A stock solution containing basic fuchsin and other supplements was prepared by dissolving the following compounds in 10 ml of ethanol: basic fuchsin, 12 mg; tannic acid, 75 mg; and cycloheximide, 75 mg. The

solution was brought up to 100 ml total volume with distilled water. The solution was added to the medium after the medium was autoclaved.

Supplement for DM

L-Glutamic acid. The stock solution was prepared by dissolving 1 g of L-glutamic acid in distilled water. The solution was adjusted to pH 6.95. Distilled water was then added to bring the volume up to 100 ml. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

Supplements for Pringsheim Soil Medium

Nystatin. The stock solution was prepared by dissolving 24.2 mg of nystatin in 58 ml of distilled water. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

Cycloheximide. The stock solution was prepared by dissolving 2.5 mg of cycloheximide in 5 ml of distilled water. This solution was sterilized by filtration and added to the medium after the medium was autoclaved.

Supplements for Modified Bordetella Medium

Ferrous sulfate. The stock solution was prepared by dissolving 8 mg of $FeSO_4 \cdot 7H_2O$ in distilled water (Stainer et al., 1971).

A stock solution containing cystine and other supplements was prepared by dissolving the following compounds in 10 ml of distilled water: cystine, 32 mg; ascorbic acid, 16 mg; niacin, 3.2 mg; and glutathione, 80 mg. The solution was adjusted to pH 7 (Stainer et al., 1971).

The above solutions were sterilized by filtration, and added separately to the G+PBM. Two groups of assay flasks were established; those that contained G+PBM + ferrous sulfate, and those that contained G+PBM + ferrous sulfate and the cystine + supplement solution. Final concentrations of the constituents sterilized by filtration corresponded to those found in the medium of Stainer and Scholte (1971).

Antibiotic Solutions

Stock solutions of oxacillin-sodium salt (Sigma Chemical Co.), clindamycin-HCl (Sigma Chemical Co.), lincomycin-HCl (Sigma Chemical Co.), and cloxacillin-sodium salt (Sigma Chemical Co.) were prepared by dissolving the antibiotics in distilled water. The solutions were sterilized by filtration. Various concentrations of these antibiotics were added to CHSS-P broth.

"Soft Agar" Pour Plates

Various dilutions of a 24-h-old culture of *S. volutans* were made in sterile CHSS-P broth and inoculated into molten (45°C) CHSS-P-Bis medium containing 0.7% agar. The entire procedure was performed under dim red illumination to avoid photochemical generation of hydrogen peroxide in the medium. After the media had gelled the plates were incubated under an atmosphere of 6% O_2: 94% N_2, using aluminum pressure cookers whose gaskets and been sealed in place with silicone rubber. Growth response was measured by counting colonies after 4 days of incubation.

Semisolid Spread Plates

Media containing 0.7% agar were prepared with DM. Prior to inoculation the plates were inverted and stored in a dark cupboard for 24 h to eliminate drops of water of syneresis.. Then they were transferred to a humidified chamber for an additional 24 h to allow the media to absorb moisture that may have been lost during drying. A 0.1-ml inoculum from a 24-h-old culture of S. volutans grown in CHSS-Bis-BES broth was used. After the plates were spread with inoculum they were incubated under an atmosphere of 6% O_2:94% N_2, using aluminum pressure cookers whose gaskets had been sealed in place with silicone rubber. The entire procedure was done under dim red illumination to avoid generation of toxic forms of oxygen.

Auxanographic Pour Plates Using Antibiotic Discs

One milliliter from a 24-h-old culture of S. volutans was inoculated into molten (45°C) CHSS-P-Bis medium containing 0.7% agar. This seeded agar was poured in 20-ml amounts into petri plates. After the agar had solidified, antibiotic impregnated discs were placed equidistant from one another on the plates. The plates were inverted and incubated under an atmosphere of 6% O_2:94% N_2, using aluminum pressure cookers whose gaskets had been sealed in place with silicone rubber. The entire procedure was done under dim red illumination. Responses to antibiotics were determined by observing the level of growth surrounding the antibiotic impregnated discs on a plate.

Auxanographic Pour Plates Using Solid and Liquid Compounds

Minute quantities of various test compounds were placed equidistant from each other on the surface of CHSS-P-Bis pour plates (4 compounds/plate). Inocula used for these plates was either 2 ml from a 48-h-old S. volutans culture grown in CHSS or 2 ml from an 18-h-old S. volutans culture grown in CHSS + 5 mM phosphate buffer. Crystals of solid test compounds were added to plates by means of a sterile platinum spatula, while drops of liquid test compounds were added with a sterile pasteur pipet. The plates were inverted and were incubated for 2 days under an atmosphere of 6% O_2:94% N_2, using aluminum pressure cookers whose gaskets had been sealed in place with silicone rubber. The entire procedure was done under dim red illumination. Responses to test compounds were determined by observing the level of growth surrounding the test compound on a plate.

Growth Enhancement of S. volutans

Growth Medium Used in Microscopic Observations of S. volutans

Screw-cap tubes containing DM that had been supplemented with various concentrations of the L-glutamic acid solution were inoculated with one drop from a 2-day-old culture of S. volutans grown in DM. The tubes were incubated at 30°C. Motility patterns were observed microscopically.

Effect of Potassium Phosphate Buffer on Growth

CHSS broth was prepared entirely under ordinary room illumination. Various amounts of potassium phosphate buffer (pH 7) were added to

flasks of this medium (Costilow, 1981). The flasks were inoculated with a 22-h-old culture of *S. volutans* grown in CHSS broth. Growth responses were estimated by visual inspection after 25 h of incubation at 30°C.

Effect of Substitution of Peptones for Casein Hydrolysate on Growth

The following peptones were substituted for the vitamin-free, salt-free, acid hydrolyzed casein (ICN) used in CHSS medium at 2.5 g/l and 5.0 g/l: tryptose (Difco), yeast autolysate (Pfizer), trypticase (BBL), Bacto-tryptone (Difco), peptonized milk (Difco), proteose peptone (Difco), meat peptone no. 100 (Gibco), casein hydrolysate no. 20 (Gibco), and gelatin hydrolysate no. 190 (Gibco). The media was dispensed in 80-ml amounts into cotton-stoppered 250-ml Erlenmeyer flasks. Each flask was inoculated with the standard inoculum and incubated at 30°C. Growth responses were measured turbidimetrically using a Klett-Summerson colorimeter.

Comparison of Various Brands and Lots of Casein Hydrolysate

The following brands and lots of casein hydrolysate were substituted for the lot normally used: ICN lot # 1444, ICN lot # 10421, NBC (no lot # given), and Gibco peptone no. 40 (an enzymatic digest of casein) lot # 11335. Note: both ICN and NBC stand for Nutritional Biochemical Corporation. The NBC refers to older lots prior to the name change to ICN. The media were dispensed in 80-ml amounts into cotton-stoppered 250-ml Erlenmeyer flasks. Each flask was inoculated with the standard inoculum and incubated at 30°C. Growth responses were measured turbidimetrically using a Klett-Summerson colorimeter.

Growth of S. volutans in Various Concentrations of Casein Hydrolysate

Flasks of CHSS-P medium were prepared (100 ml/flask) using different concentrations of casein hydrolysate (ICN lot # 11523) in each flask. The medium was initially made without casein hydrolysate and the pH adjusted to 7. Each medium was again adjusted to pH 7 after the casein hydrolysate was added. The inoculum consisted of 1 ml from a 37-h-old culture of *S. volutans* grown in CHSS-P broth. The flasks were incubated for 27 h at 30°C. Growth was measured turbidimetrically on a nephelometer constructed from a Hitachi-Perkin Elmer spectrophotometer.

Growth of S. volutans in CHSS-P Broth Supplemented with Yeast Autolysate

Filter sterilized yeast autolysate (a gift from Dr. R. M. Smibert) was serially diluted in sterile distilled water. Various amounts of the yeast autolysate dilutions were added to flasks filled with CHSS-P broth. These flasks then received a 1% inoculum (v/v) from a 24-h-old culture of *S. volutans* grown in CHSS-P broth, and were incubated at 30°C. Growth was measured turbidimetrically with a Klett-Summerson colorimeter.

Growth of S. volutans in a 24-h Culture Filtrate

S. volutans was grown for 24 h in CHSS-Bis-BES broth. A small portion of the culture was removed and diluted in sterile CHSS-Bis-BES broth to give a cell density of 24,250 cells/ml, as determined with a hemocytometer. The remaining culture was sterilized by filtration and

inoculated with 0.1 ml of the diluted culture. The medium was then reincubated at 30°C. Growth response was measured by visual inspection.

Growth of S. volutans in CHSS-P Broth with Different pH Values

CHSS-P broth was initially made without casein hydrolysate and the pH adjusted to 7. After addition of casein hydrolysate the pH of the medium was again adjusted to 7. The broth was then added to flasks (100 ml/flask) and each flask adjusted to a different pH. HCl (1 N) or 2 N KOH were used in adjusting the pH. The flasks were inoculated with 0.1 ml from a 37-h-old culture of *S. volutans* grown in CHSS-P broth and were incubated for 27 h at 30°C. Growth was measured turbidimetrically on a nephelometer constructed from a Hitachi-Perkin Elmer spectrophotometer.

Effect of Maintenance of a Constant pH on the Growth of S. volutans

Flasks filled either with CHSS-P or CHSS-P-BTB were inoculated with the standard inoculum of *S. volutans*. Flasks were incubated at 30°C.

Bromothymol blue was used as a pH indicator (yellow is acid, green is neutral, and blue is basic). At various times throughout the incubation period, sterile 0.5 N HCl was added to each CHSS-P-BTB flask until neutrality was reached. Growth was measured turbidimetrically using a Klett-Summerson colorimeter.

Growth of S. volutans in the presence of H_2

A 0.5 ml portion of a 48-h-old culture of *S. volutans* grown in CHSS-Bis-BES broth was inoculated into each of two 50-ml flasks

containing 21 ml of DM. One flask was incubated aerobically and the other under an atmosphere of 80% air:20% H_2 (17% of this atmosphere was O_2). After incubation for 2 days at 30°C, the cultures were centrifuged, suspended in 7 ml each of 50 mM phosphate buffer, and their turbidity determined using a Klett-Summerson colorimeter.

Dialysis Sac Cultures

S. volutans was grown in CHSS-P-filled dialysis sacs submerged in a much greater volume of CHSS-P broth. Dialysis tubing (Spectrapor No. 2, 45-mm diameter membrane tubing) moistened with distilled water was tied off at one end, and reinforced at the same end with a knot tied with fishing line. The sac was slipped over a straight length of polypropylene tubing having perforations near the bottom (Fig. 1). The open end of the sac was secured to the polypropylene tubing by a piece of fishing line. This sac was suspended in a 500 ml Erlenmeyer flask filled with 400 ml of CHSS-P broth so that the holes in the polypropylene tubing were submerged. The tube was secured in an upright position by nonabsorbent cotton. After the assembly was autoclaved, sterile liquefied paraffin was used to seal the exposed upper portions of the dialysis tubing in order to ensure sterility. Ten ml of sterile CHSS-P broth were then added aseptically to each dialysis sac after the flasks had cooled to room temperature. Each dialysis sac and control flask (a cotton-stoppered 250-ml Erlenmeyer flask filled with 80 ml of CHSS-P broth) were inoculated with the standard inoculum of *S. volutans*, and incubated at 30°C. After incubating for various periods, growth responses were measured turbidimetrically (using a Klett-Summerson

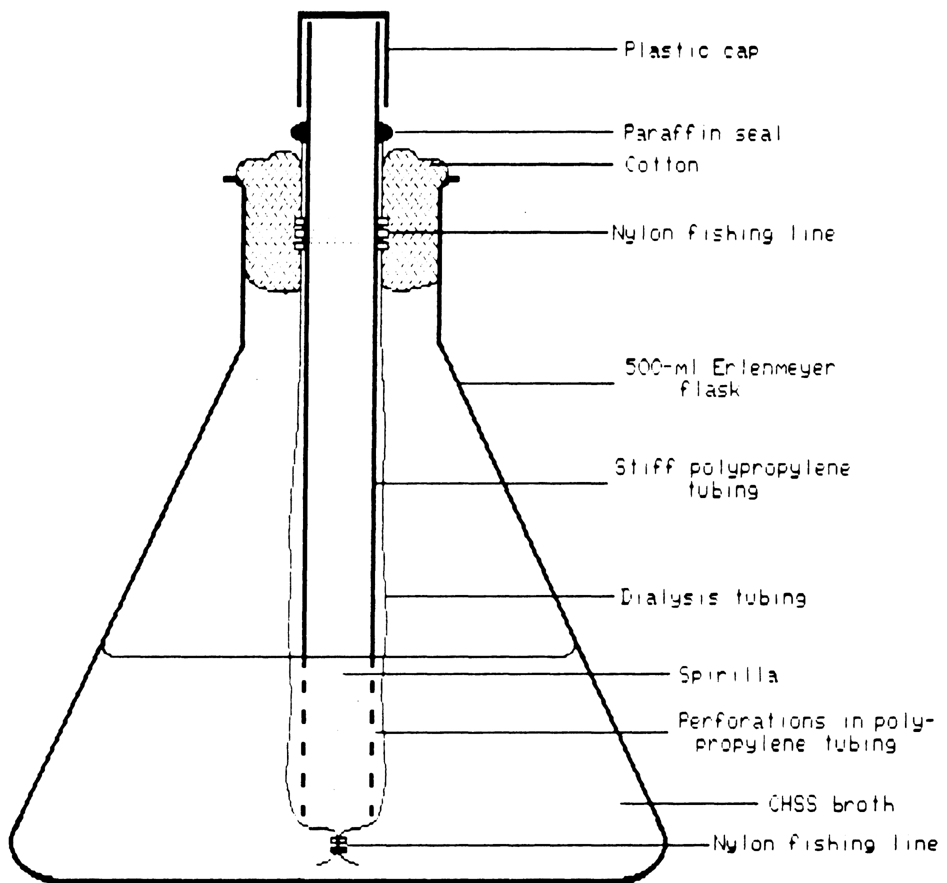

Fig. 1. Dialysis flask apparatus.

colorimeter), and culture viability was determined by using the Reed and Muench (1938) method of 50% end point dilution.

Aeration of S. volutans Cultures in CHSS-P Broth

Freshly inoculated and 24-h-old CHSS-P broth cultures (in flasks) of *S. volutans* were incubated at 30°C in a reciprocating shaker water bath (Blue M Electric Co.) at 54 cycles per min. protected from light by an aluminum shield and a black cloth. Growth responses were measured turbidimetrically using a Klett-Summerson colorimeter.

Thin Layer and Paper Chromatography

Uninoculated CHSS-P concentrate and concentrated supernates from CHSS-P-grown cultures were spotted on either thin layer chromatography (TLC) plates (Eastman chromagram 6061, silica gel) or Whatman # 1 chromatography paper. Ascending chromatography was used for the TLC plates: descending chromatography was used for paper chromatograms. Development was done with a solvent consisting of n-propanol and 30% ammonium hydroxide (7:3) (Sherma, 1972). Compounds were detected by spraying with ninhydrin followed by heating.

Fatty Acid Analysis

Volatile and nonvolatile fatty acid determinations of uninoculated CHSS-P concentrate and a 48-h-supernate concentrate were performed by gas-liquid chromatography as described by Holdeman et al. (1975). A Beckman GC-4 gas chromatograph was used.

Amino Acid Analysis

Attempts at analyzing amino acids found in the CHSS-P medium concentrate, 24-h supernate concentrate, and the 48-h supernate concentrate by gas-liquid chromatography were done using the Amino Acid Kit of Alltech Associates and a Beckman GC-4 gas chromatograph equipped with linear temperature programming.

Analysis of Amino Acids with an Amino Acid Analyzer

Amino acid analyses (courtesy of Dr. D. Peterson, MCV, Richmond, VA) were performed on concentrated CHSS-P medium supernates obtained from cultures grown for various periods. Samples were passed through a cation exchange column (Dowex 50) on a Durrum MBF analyzer with fluorescent detection system. This amino acid analyzer used a Pickering three- buffer system.

Attempts at Developing a Selective Medium for *S. volutans*

Selection of S. volutans in Hay Infusion Semisolid Medium Supplemented with β-D-Thioglucose

β-D-Thioglucose was used as a possible selective supplement because it might be inhibitory to glucose-utilizing organisms without being inhibitory to *S. volutans*, since this spirillum does not catabolize carbohydrates (Krieg, 1976). For a more detailed rational see Results.

The liquid portion of a hay infusion was mixed with agar in order to make a semisolid medium. The medium was added to two screw cap tubes and then autoclaved. β-D-Thioglucose (1 g/l final concentration) was

added to one of the tubes. Both media were stored in a dark cupboard overnight.

The next day each tube received a 0.5% (v/v) inoculum of hay infusion liquid and a 0.5% (v/v) inoculum from a 24-h-old S. volutans culture grown in CHSS-P broth. The tubes were incubated at 30°C for 2 days. Growth in each tube was examined microscopically.

Selection of S. volutans in Pringsheim Medium Supplemented with Nystatin and Cycloheximide

Autoclaved tubes of Pringsheim medium and Pringsheim medium supplemented with the nystatin solution and the cycloheximide solution were filled with sterile tap water to within 1 cm from the top of the tubes. A 1 ml inoculum from a 3 to 4 day-old hay infusion (hay - VPI & SU dairy barns; pond water - Foxridge reservoir, Blacksburg, VA) was added to one tube of each medium. After inoculation the tubes were placed in a vented anaerobe jar, and the atmosphere inside the jar was adjusted to 2% O_2:94 N_2. The jar containing the tubes was incubated at 30°C. After 48 h samples from each tube were observed microscopically.

Selection of S. volutans in CHSS-P Medium Supplemented with Lincomycin and Cycloheximide

Solutions of lincomycin and cycloheximide were added to flasks containing CHSS-P broth. Flasks containing CHSS-P broth and CHSS-P broth supplemented with lincomycin (100 mg/l final concentration) and cycloheximide (5 mg/l final concentration) received either the standard inoculum of S. volutans or both a 1% inoculum of pond water (VPI & SU

duck pond) plus the standard inoculum of S. volutans. The flasks were incubated at 30°C for 24 h. Growth was examined by visual inspection. Samples were removed from one flask from each triplicate set and observed microscopically.

Selection of S. volutans in CHSS-P Semisolid Medium Supplemented with Lincomycin, 5-Fluorocytosine, Potassium Fluoride, Basic Fuchsin, Tannic Acid, Triton X-100, and Cycloheximide

All of the antimicrobial compounds that S. volutans was found tolerant to were incorporated into CHSS-P semisolid medium (0.2% agar) (Tables 18 and 19) in order to make a selective medium.

Under dim red illumination sterile screw cap tubes (15 x 150 mm) were filled with either 15 ml or 12.5 ml of tempered CHSS-P semisolid (0.2% agar) medium. The semisolid medium was tempered 21 min. at 37°C. To the tubes containing 12.5 ml of CHSS-P semisolid (0.2% agar) medium the lincomycin-5-fluorocytosine stock solution, potassium fluoride stock solution, basic fuchsin-tannic acid-cycloheximide stock solution, and Triton X-100 were added. The final volume of these tubes was ca. 15 ml. Since liquid reagents were being added to several of the tubes of medium after the medium was autoclaved, 0.2% agar was used instead of 0.15%. All of the CHSS-P semisolid medium tubes were stored in a dark cupboard for 14.5 h.

Several of the CHSS-P semisolid medium tubes and CHSS-P semisolid medium tubes supplemented with antimicrobial compounds received either the standard inoculum of S. volutans, a 1% inoculum from an 11-day-old hay infusion (hay - VPI & SU dairy barns; pond water - VPI & SU duck

pond) or a 1% inoculum from the 11-day-old hay infusion plus the standard inoculum of S. volutans. The flasks were incubated at 30°C.

All of the tubes were viewed microscopically after 24 h and 48 h of incubation. A 1% inoculum from one of the CHSS-P medium tubes supplemented with antimicrobial compounds (seeded 26 h earlier with S. volutans and hay infusion) was added to a tube of CHSS-P semisolid (0.2% agar) medium. A similar inoculation was made after 51 h of incubation. Also, a 1% inoculum from one of the CHSS-P medium tubes supplemented with antimicrobial compounds (seeded 51 h earlier with S. volutans) was added to a tube of CHSS-P semisolid (0.2% agar) medium. All tubes were incubated at 30°C and observed visually or microscopically after 22 h and 48 h of incubation.

RESULTS

Growth Enhancement of S. volutans

Observation of the Microaerophilic Nature of S. volutans on Wet Mounts

The microaerophilic nature of S. volutans was demonstrated by Wells et al. (1965). While observing a covered wet-mount of S. volutans, they noticed cells migrating into dense bands about 2 mm from the edge of the coverslip (Wells et al., 1965). Since gas exchange of the culture medium with the atmosphere occurs only at the edge of the coverslip, the level of O_2 present where the band formation occurred was less than 21%.

In the present study when a large drop from one of the DM + 0.005% L-glutamic acid culture tubes was placed on a microscope slide and viewed microscopically (100x total magnification, darkfield), a large number of cells were seen to migrate towards the center of the drop forming a massive clump of motile cells. The same phenomenon was observed with a sample from a DM + 0.16% L-glutamic acid culture, though to a lesser degree.

A large drop from the same DM + 0.005% L-glutamic acid tube was placed on a microscope slide with a coverslip. When viewed microscopically as stated above, intense migration was seen in the center of the wet mount approximately 60 to 120 μm from an air bubble. This intense migration was seen as dense clumps of motile cells. Migration to a lesser degree occurred approximately 2 mm inward from one of the edges of the coverslip.

Peptone Substitution for Casein Hydrolysate in CHSS Medium

Different peptones were substituted for casein hydrolysate in CHSS medium in hope of stimulating the growth of *S. volutans*. None of the peptones tested yielded turbidity readings above 31 klett units (Table 1). No growth improvement was seen when any of the peptone concentrations were increased two-fold. Moreover, growth inhibition occurred when the concentration of proteose peptone was increased.

Effect of Potassium Phosphate Levels on Growth in CHSS Medium

As indicated in Tables 1 and 2, potassium phosphate at a concentration of 1 mM markedly enhanced growth (by a factor of 1.7) compared to that in CHSS broth lacking any phosphate supplement. Turbidity was lower at 48 h for the cultures grown in CHSS supplemented with 1 mM potassium phosphate (Table 1). The turbidity decrease was possibly due to cell lysis. Levels of phosphate higher than 1 mM were less effective than 1 mM.

Based on these results, CHSS-P medium was used in many subsequent experiments as the basal medium.

Comparison of Various Brands and Lots of Casein Hydrolysate in CHSS Medium

Five different lots of casein hydrolysate were tested in CHSS broth for their effectiveness in supporting growth of *S. volutans*. When either the ICN lot # 1444 or the NBC casein hydrolysate was used in CHSS broth inoculated with *S. volutans*, a maximum turbidity of ca. 25 klett

TABLE 1

Growth of S. volutans in Response to Various Peptones Substituted for Casein Hydrolysate in CHSS Broth[1]

Peptone	Growth response[2]			
	24 h		48 h	
	2.5 g/l	5.0 g/l	2.5 g/l	5.0 g/l
Vitamin-free, salt free, acid hydrolyzed casein (ICN)	15	NT[3]	23	23
Vitamin-free, salt free, acid hydrolyzed casein (ICN) + 1 mM phosphate[4]	25	NT	16	NT
Bacto-tryptone (Difco)	NT	NT	20	11
Trypticase (BBL)	NT	NT	19	16
Tryptose (Difco)	NT	NT	13	0
Yeast autolysate (Pfizer)	NT	NT	0	0
Proteose peptone (Difco)	19	10	23	12
Meat peptone no. 100 (Gibco)	7	NT	10	NT
Casein hydrolysate no. 20 (Gibco)	24[4]	24[4]	26[4]	31[4]
Gelatin hydrolysate (Gibco)	NT	14[4]	22	25

[1]The standard inoculum of S. volutans was used.

[2]Growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized screw-cap tubes were used as cuvettes. Values reported as the mean of two flasks.

[3]NT = not tested.

[4]Only one flask was tested.

TABLE 2

Effect of Potassium Phosphate Buffer on the Growth of *S. volutans* in CHSS Broth

Final concentration of potassium phosphate buffer added to CHSS (mM)	Turbidity[1]
0	++
1.0	++++
3.5	+++
5.0	+++
7.5	++
15.0	++

[1]Turbidity was estimated by visual inspection. Growth was ranked from "0" (no growth) to "++++" (highest turbidity seen).

units was obtained after 24 h (Table 3). A subsequent 36% decrease in turbidity was seen in these cultures after 48 h, possibly due to cell lysis. In contrast, cultures grown in CHSS prepared with ICN lot # 10421 or Gibco lot # 11335 had essentially the opposite effect, viz., the higher turbidity occurred at 48 h (Table 3).

Effect of Various Concentrations of Casein Hydrolysate on Growth in CHSS Broth

An experiment was done to determine the optimal concentration of casein hydrolysate (ICN lot # 11523) to use in CHSS-P broth.

As indicated in Table 4, the quantity of casein hydrolysate could be decreased by half or doubled with little effect on growth. However, by increasing the concentration of casein hydrolysate to 0.75%, or by decreasing the concentration of casein hydrolysate 5-fold, growth was inhibited.

Effect of Yeast Autolysate on Growth in CHSS-P Broth

During the production of yeast extracts the product is often heated (R. M. Smibert, personal communication). Because yeast autolysates are not heated, but filter sterilized, it was felt that yeast autolysate might provide some heat-labile growth factor that casein hydrolysate might lack.

As shown in Table 5, none of the concentrations of yeast autolysate tried seemed to affect the growth of S. volutans. The influence yeast autolysate had on the final pH of the medium was negligible.

TABLE 3

Effect of Different Brands and Lots of Casein Hydrolysate on Growth of *S. volutans* in CHSS Broth[1]

Casein hydrolysate (0.25 g/l)[2]	Growth response[3]	
	24 h	48 h
ICN lot # 11523	17	25
ICN lot # 1444	25	15
ICN lot # 10421	17	19
NBC (no lot # given)	26	16
Gibco lot # 11335	16	26

[1]The standard inoculum of *S. volutans* was used.

[2]Both ICN and NBC refer to Nutritional Biochemical Corporation. NBC refers to older batches prior to the change of name to ICN. Gibco lot # 11335 is peptone no. 140 (an enzymatic digest of casein). ICN lot # 11523 is the current lot used in CHSS medium.

[3]Growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized screw-cap tubes were used as cuvettes. Values were reported as the mean of two flasks.

TABLE 4

Effect of Various Concentrations of Casein Hydrolysate on Growth of S. volutans in CHSS-P Broth

Final concentration of casein hydrolysate (ICN lot # 11523) (%)	Growth response[1]
0.05	8
0.10	54
0.25	60
0.50	55
0.75	32
1.00	6

[1]Turbidity was measured on a nephelometer constructed from a Hitachi-Perkin Elmer spectrophotometer. Values were reported as the result of one flask.

TABLE 5

Effect of Yeast Autolysate on the Growth of *S. volutans* in CHSS-P Broth

Final concentration of yeast autolysate (%)	Growth response[1]	pH of medium[2]
0	23	7.76[3]
10^{-1}	19	7.65
10^{-2}	19	ND[4]
10^{-3}	18	7.69
10^{-4}	17	7.66
10^{-5}	18	7.68

[1]Growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized tubes were used as cuvettes. Values were reported as the mean of two flasks.

[2]After 26 h of incubation one flask out of each set of duplicate flasks was analyzed for pH.

[3]Value was reported as the mean of duplicate flasks.

[4]ND = not done.

Effect of β-Hydroxybutyrate, D-Fructose, and Catalase on Growth

CHSS-P broth was supplemented with β-hydroxybutyrate, D-fructose, or catalase in an attempt to improve growth of S. volutans. It was thought that addition of β-hydroxybutyrate to CHSS medium might enhance the growth, because poly-β-hydroxybutyrate (PHB) granules are produced by S. volutans as a reserve energy source (Krieg, 1976). Fructose was tested, because although most spirillum species do not utilize sugars, utilization of fructose has been demonstrated in Aquaspirillum itersonii and Aquaspirillum peregrinum (Hylemon, et al., 1973b). Catalase was tested, because it has been shown to extend the oxygen tolerance of S. volutans in MPSS medium (Padgett et al., 1982).

However, none of the three supplements enhanced the growth of S. volutans. In fact, β-Hydroxybutyrate at a level of 0.004 g/ml inhibited growth (Table 6).

Effect of KCl on Growth in CHSS-P Broth

Since S. volutans is sensitive to sodium ions, yet requires small amounts of NaCl to grow (Bowdre et al., 1976), it was thought that chloride ions might be stimulatory for S. volutans growth. For this reason various levels of KCl were added to CHSS-P broth.

As indicated in Table 7, all concentrations of KCl tried appeared inhibitory for S. volutans.

TABLE 6

Effect of β-Hydroxybutyrate, Fructose, and Catalase on Growth of *S. volutans* in CHSS-P Broth

Supplement	Concentration (g/l)	Growth response[1]
None	0	25
β-Hydroxybutyrate	0.04	26
	0.4	28
	4.0	3
Fructose	0.1	26
	1.0	25
	10.0	25
Catalase	0.075	25
	0.15	26[2]

[1]Growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized screw-cap tubes were used as cuvettes. Values were reported as the mean of three flasks.

[2]Value was reported as the mean of two flasks.

TABLE 7

Effect of KCl on Growth of *S. volutans* in CHSS-P Broth[1]

Concentration of KCl (g/l)	Growth response[2]
0	0.446[3]
5	0
10	0
100	0

[1]The standard inoculum of *S. volutans* was used.

[2]Turbidity (expressed as absorbance units) was measured on a spectrophotometer (Bausch and Lomb Spectronic 2000). Values were reported as the mean of triplicate flasks.

[3]Value was reported as the mean of quadruplicate flasks.

Effect of Increased Levels of Succinic Acid on Growth

Since succinic acid is the major carbon and energy source in CHSS-P medium, increasing the levels of succinic acid in CHSS-P medium might enhance the growth of *S. volutans*. However, as shown in Table 8, a two-fold increase in succinic acid appeared to be slightly inhibitory, and a four-fold increase was even more inhibitory.

Growth of S. volutans in a CHSS-Bis-BES Broth-Filtrate

This experiment was done in order to see if some compound secreted by living cells or liberated by lysed cells could afford protection for a small inoculum to grow aerobically in broth.

No growth was ever observed.

Optimum pH for Growth of S. volutans

An experiment was performed to determine whether or not neutrality was the optimal pH for the growth of *S. volutans*.

As shown in Table 9, a pH of 7 seemed to be optimal for the growth of *S. volutans*.

Effect of Maintaining a Constant pH on the Growth of S. volutans

As previously mentioned, a pH value of 7.0 seemed to be the optimal pH for supporting the growth of *S. volutans* (Table 9). Also, it was noted that after only one day after inoculation with *S. volutans*, CHSS-P medium attained a pH of ca. 7.9. If increasing alkalinity in the CHSS-P medium is limiting the growth of *S. volutans*, then maintaining

TABLE 8

Growth of S. volutans in CHSS-P Broth with Various Concentrations of Succinic Acid[1]

Concentration of succinic acid (g/l)	Growth response[2]
1	18
2	14
4	5

[1]The standard inoculum of S. volutans was used.

[2]After 24 h of incubation growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized screw-cap tubes were used as cuvettes. Values were reported as the mean of two flasks.

TABLE 9

Growth of S. volutans in CHSS-P Medium Adjusted to Various pH Levels

Initial pH of CHSS-P broth	Growth response[1]
6.0	9
6.5	37
7.0	60
7.5	28
8.0	20

[1]Relative turbidity was measured on a nephelometer constructed from a Hitachi-Perkin Elmer spectrophotometer.

the medium at neutrality might improve cell yields. The results of this experiment are shown in Table 10.

The incremental addition of HCl to CHSS-P-BTB medium may have induced a longer lag phase resulting in reduced turbidity at 25 h (Table 10). However, by maintaining the CHSS-P-BTB medium at neutrality, nearly twice the turbidity was obtained after 49 h compared to cultures not periodically readjusted to pH 7.

Growth of S. volutans in Modified Bordetella Broth

Both glutamic acid and proline are the most abundant amino acids found in casein (Tristram, 1953). Consequently glutamate + proline medium of Stainer et al. (1971) might be a suitable growth medium for *S. volutans*.

Duplicate flasks containing G+PBM with supplements were inoculated in duplicate, with one flask of CHSS-P broth serving as a control. All flasks were inoculated with the standard inoculum of *S. volutans*.

Visual inspection of culture flasks after 24 h and 72 h of incubation revealed that the flasks containing glutamate + proline had lower turbidities than the control flask (data not shown).

Modified Brucella Semisolid Medium

Since the microaerophile *Campylobacter jejuni* grows well in a 3% Brucella broth (Albimi) semisolid medium under normal atmospheric conditions, it was thought that *S. volutans* might also be able to do so. To avoid the potentially inhibitory effects of Na^+ on *S. volutans*, the concentration of NaCl was decreased to 0.1 g/l; potassium metabisulfite

TABLE 10

Effect of pH Maintenance on the Growth of *S. volutans*

Medium	Growth response[1]	
	25 h	49 h
CHSS-P	27	19
CHSS-P + 0.0025% bromothymol blue[2]	19	37

[1]Growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized screw-cap tubes were used as cuvettes. Values were reported as the mean of two flasks.

[2]Sterile 0.5 N HCl was added to each flask at 18 h, 21 h, 24 h, 27 h, 35.5 h, 39.5 h, 42.5 h, and 45 h post inoculation to readjust the pH to neutrality (medium turned green).

was substituted for sodium bisulfite; and citric acid was substituted for sodium citrate.

As indicated in Table 11, S. volutans grew much better from a small inoculum in semisolid CHSS-P medium than in the modified, semisolid Brucella medium.

Influence of Hydrogen Gas on the Growth of S. volutans

The microaerophile Campylobacter sputorum was found to grow better in the presence of H_2 gas (Smibert, 1984). For this reason, the possibility that S. volutans might grow better in the presence of H_2 was investigated. However, as shown in Table 12, an atmosphere containing 80% air:20% H_2 appeared to be inhibitory for S. volutans.

DM Spread Plates

Successful growth of S. volutans on the surface of solid medium was achieved by Padgett et al. (1986) when inoculated CHSS-Bis plates were incubated in a humidified chamber. This procedure had never been tried with DM. In order to increase the moisture level that the spirilla would be exposed to, DM "soft agar" was used.

Growth did occur after 4 to 6 days of incubation. However, even though care was taken to spread the agar plates evenly with an ethanol-flamed glass spreader, colonies tended to occur into nebulae-like formations on the agar surface. Well isolated colonies such as those obtained by Padgett et al. (1982) were never obtained on this medium.

TABLE 11

Ability of *S. volutans* to Grow in Modified Brucella Semisolid Medium

Medium	Inoculum[1] (ml)	Number of Tubes Showing Growth[2]
CHSS-P	1.0	3
	0.1	3
Modified Brucella	1.0	3[3]
	0.1	1[4]

[1]Inocula were from a 24-h culture of *S. volutans* that had been serially transferred for 3 consecutive days.

[2]Growth was estimated by visual inspection after 24 h of incubation. Cultures were prepared in triplicate.

[3]One tube showed growth after 24 h. The other two tubes exhibited slight growth after ca. 84 h.

[4]Slight growth was observed after 48 h.

TABLE 12

Growth of *S. volutans* Under an Atmosphere of 80% Air:20% H_2

Atmospheric conditions	Growth response[1]
Air	83
80% air:20% H_2	15

[1]Growth (expressed as klett units) was measured with a Klett-Summerson colorimeter using the red (660 nm) filter. Optically standardized sleeve-cap tubes were used as cuvettes. Values were reported as the result of one flask. Cultures grown in DM for 2 days were concentrated 3-fold in 50 mM potassium phosphate buffer before measuring turbidity.

CHSS-P-Bis Agar Pour Plates

Padgett and coworkers (1982) previously determined the need for humid conditions in culturing S. volutans on the surface of solid medium. Since humidity would be at a maximum for subsurface colonies, S. volutans was used to seed molten (45°C) CHSS-P-Bis agar (0.7%) plates.

A 10^{-4} dilution yielded pour plates with average plate count of 232 colonies This corresponded to an inoculum of 12,600 cells as determined with a hemocytometer. Thus, the recovery rate was only 1.8%, much lower than the 22 to 72% reported by Padgett et al. (1982) for surface growth on spread plates of CHSS-Bis medium containing 1.5% agar and unsupplemented with phosphate.

Growth of S. volutans on CHSS-P-Bis Auxanographic Pour-Plates with Compounds

Small quantities of various compounds were added to the surface of CHSS-P-Bis pour-plates seeded with S. volutans in order to determine which compounds were stimulatory for growth.

Plates having areas of dense growth surrounding a compound were double checked by passing the plate through a scanning densitometer (Quick Scan Jr., Helena Labs). Peaks appearing above the baseline were regarded as areas of more intense growth, and troughs below the baseline were regarded as zones of inhibition. The baseline was set at colony densities formed away from any added surface compounds (Fig. 2).

Acetyl choline chloride, ammonium molybdate, DL-epinephrine and EDTA all were surrounded by a zone of inhibition circumscribed by a zone of stimulation (Table 13, Fig. 2). Consequently, experiments were done

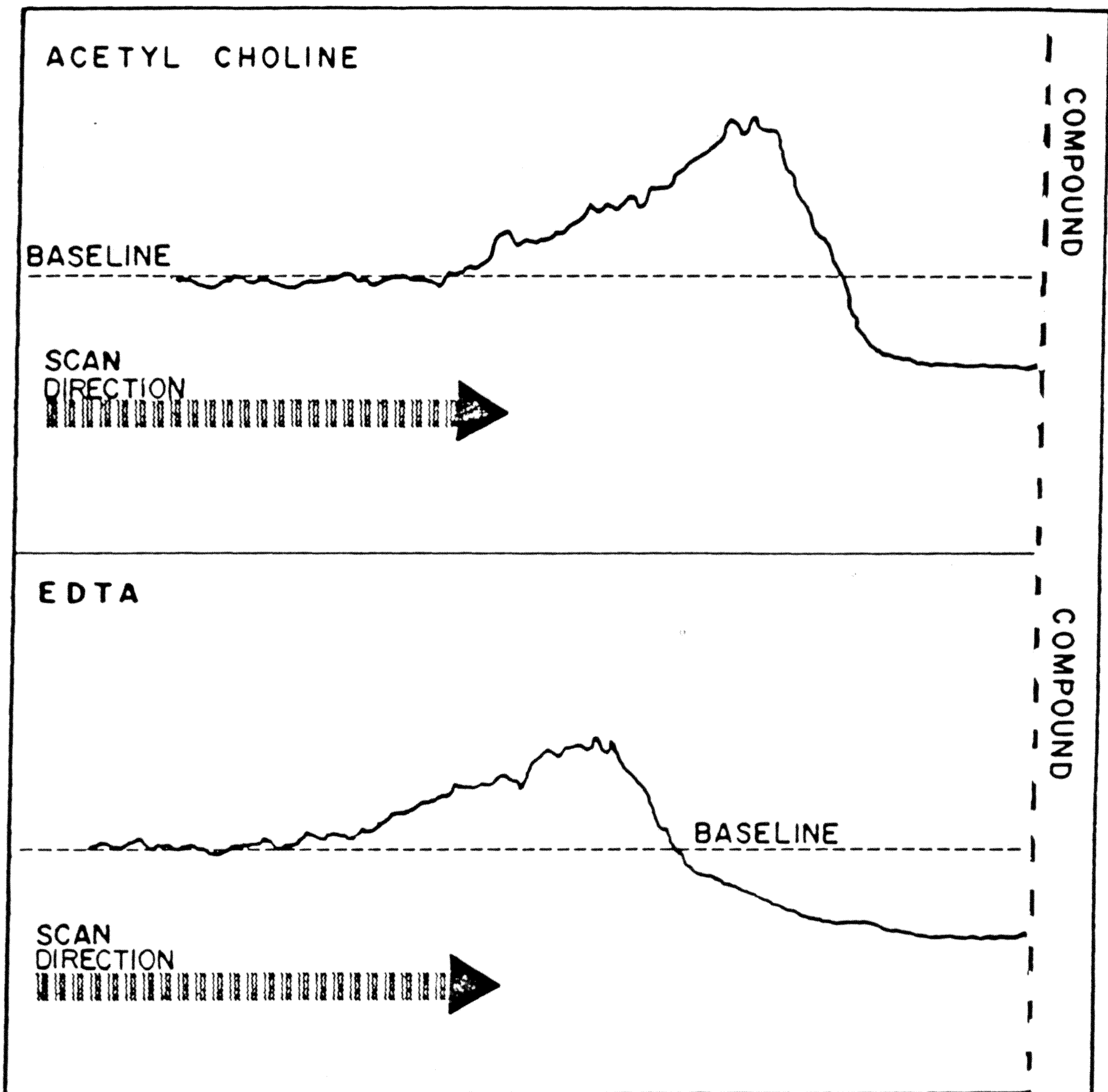

Fig. 2. Scanning densitometry of CHSS-P-Bis auxanographic pour-plates containing acetyl choline and EDTA. Peaks depict areas of growth stimulation, while troughs depict areas of growth inhibition.

TABLE 13

Growth of S. volutans on CHSS-P-Bis Auxanographic Pour Plates Supplemented with Various Compounds

Compound	Effect of compound on growth[2]		
	S	I	N
Acetylcholine	X	X	
Adenine		X	
Adonitol			X
Aesculin			X
Alanine, L			X
Alanyl glycine, DL			X
Aluminum ammonium sulfate			X
Aluminum chloride			X
Aluminum potassium sulfate	X		
Aminoacetic acid (glycine)			X
Aminobenzoic acid, p			X
Aminobenzoyl glutamic acid, p			X
Aminodimethylaniline oxalate, p		X	
Aminolevulinic acid, δ			X
Aminonaphthol sulfonic acid		X	
Aminopterin			X
Ammonium acetate		X	
Ammonium chloride			X
Ammonium molybdate	X	X	
Ammonium nitrate			X
Ammonium persulfate		X	
Aniline oxalate			X
Anthrone			X
Arabinose, L			X
Arginine monohydrochloride, L, (+)			X
Arsenic trioxide			X
Ascarite			X
Ascorbic acid		X	
Asparagine, L			X
Aspartic acid		X	
Aspartic acid, DL			X
Barium carbonate			X
Barium chloride			X
Barium hydroxide			X
Benzoic acid			X
BES buffer		X	
Biotin			X
Bismuth subnitrate		X	

TABLE 13, continued . . .

Compound	Effect of compound on growth[2]		
	S	I	N
Boric acid			X
Brom cresol green			X
Butanediamine, 1,4			X
Butanediol, 2,3			X
Butyric acid		X	
Cadaverine			X
Calcium carbonate			X
Calcium chloride		X	
Calcium gluconate			X
Calcium nitrate			X
Calcium pantothenate			X
Calcium phosphate			X
Canada balsam		X	
Capric acid		X	
Caproic acid		X	
Carmine			X
Catechol		X	
Cedarwood oil			X
Cellobiose, β, D(+)			X
Cetyl pyridinium chloride		X	
Charcoal			X
Chloral hydrate			X
Chloramphenicol		X	
Chloromercuribenzoate, p, (sodium)		X	
Choline		X	
Citric acid		X	
Citrulline, L			X
Cobaltous chloride		X	
Cobaltous sulfate		X	
Cupric acetate		X	
Cupric chloride			X
Cupric nitrate		X	
Cupric oxide		X	
Cupric sulfate		X	
Cysteine		X	
Cystine			X
Desferal		X	
Dextrin			X
Diaminopimelic acid, DL, α,ε			X
Diastase of malt		X	

TABLE 13, continued . . .

Compound	Effect of compound on growth[2]		
	S	I	N
Dichloroindophenol, 2,6, sodium			X
Dicumarol			X
Dimethyl sulfoxide (DMSO)			X
Dimethylaminobenzaldehyde, p			X
Dithiobis-2-nitrobenzoic acid, 5,5'		X	
Dulcitol			X
Epinephrine, DL	X	X	
Erythritol			X
Ethylenediaminetetraacetic acid (free acid)	X	X	
Ferric citrate		X	
Ferric sulfate			X
Ferrichrome			X
Ferrous sulfide			X
Folinic acid			X
Fructose, D		X	
Fumaric acid			X
Gluconic acid			X
Glucosamine			X
Glucose, D		X	
Glutamic acid, L			X
Glutamine			X
Glutathione		X	
Glycerol		X	
Glycerophosphate, β		X	
Guanine		X	
Guanosine			X
Histidine			X
Hydroxyproline		X	
Hypoxanthine			X
Indoleacetic acid			X
Inosine			X
Inositol, i			X
Isocitric acid, DL		X	
Isoleucine, L		X	
Kynurenic acid			X
Leucine, L			X
Lysine, L			X
Magnesium sulfate			X
Malic acid, L		X	
Manganous sulfate		X	

TABLE 13, continued . . .

Compound	Effect of compound on growth[2]		
	S	I	N
Mannose			X
Quinolinol, 8		X	
Vitamin B12			X
Vitamin D3			X
Xanthine			X

[1]S = stimulatory. More intense growth was seen in the area surrounding the compound than on the rest of the plate. I = inhibitory. Less intense growth was seen in the area surrounding the compound than on the rest of the plate. N = no effect. Growth was the same intensity in the area surrounding the compound as on the rest of the plate. S + I = zone of inhibition circumscribed by a zone of stimulation occurred around the compound.

with adding various concentrations of acetyl choline chloride and EDTA to CHSS-P broth (data not shown) to determine if these compounds were truly stimulatory. None of the concentrations of either compound proved stimulatory for broth cultures.

Growth in CHSS-P Broth Dialysis Sacs

Diffusion of nutrients into and diffusion of waste products out of a dialysis sac should replenish nutrients needed for growth of *S. volutans*, eliminate toxic catabolic products from the immediate vicinity of the spirilla, and maintain a stable pH. With the above requirements being met, *S. volutans* might grow to high turbidities.

The turbidity of the control flasks was indeed higher than that in the dialysis sac cultures at 24 h (Fig. 3). The longer lag phase seen in the dialysis sac cultures might possibly be attributed to a need of the organisms to pre-condition the medium prior to exponential growth. Preconditioning would take longer within the dialysis sacs, due to passive diffusion occurring across the dialysis membrane.

Turbidity in the dialysis sacs continued to increase markedly after 48 h, yet the viable cell numbers as determined by the end point dilution method of Reed and Muench (1938) remained fairly constant (compare Figs. 3 and 4). Therefore, the large turbidity seen at 233 h for the dialysis sac cultures was largely due to the accumulation of dead cells and cell debris. Although living cells were present, many lysed cells and cell debris were observed microscopically in the cultures.

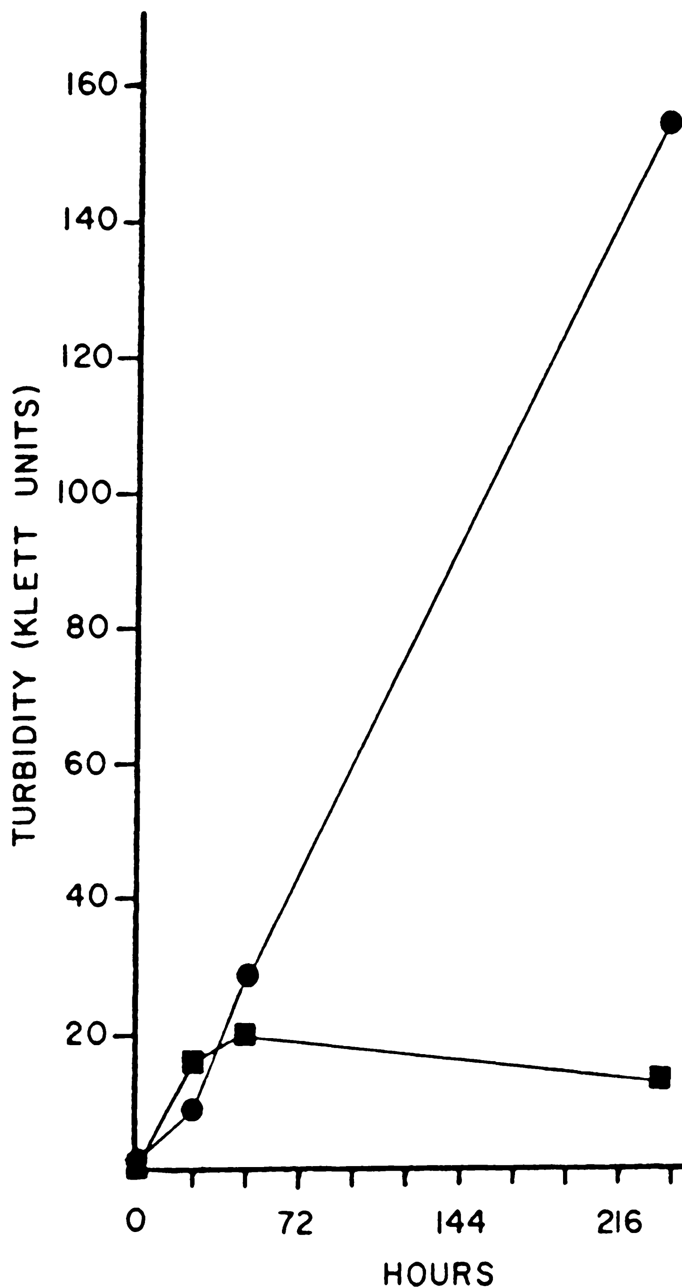

Fig. 3. The growth responses of S. volutans in dialysis sacs and batch cultures. All cultures were grown in CHSS-P broth. ■-■ = control (flask cultures grown statically). ●-● = dialysis sac cultures. Values reported as the mean of three flasks or three dialysis sacs. The standard inoculum of S. volutans was used.

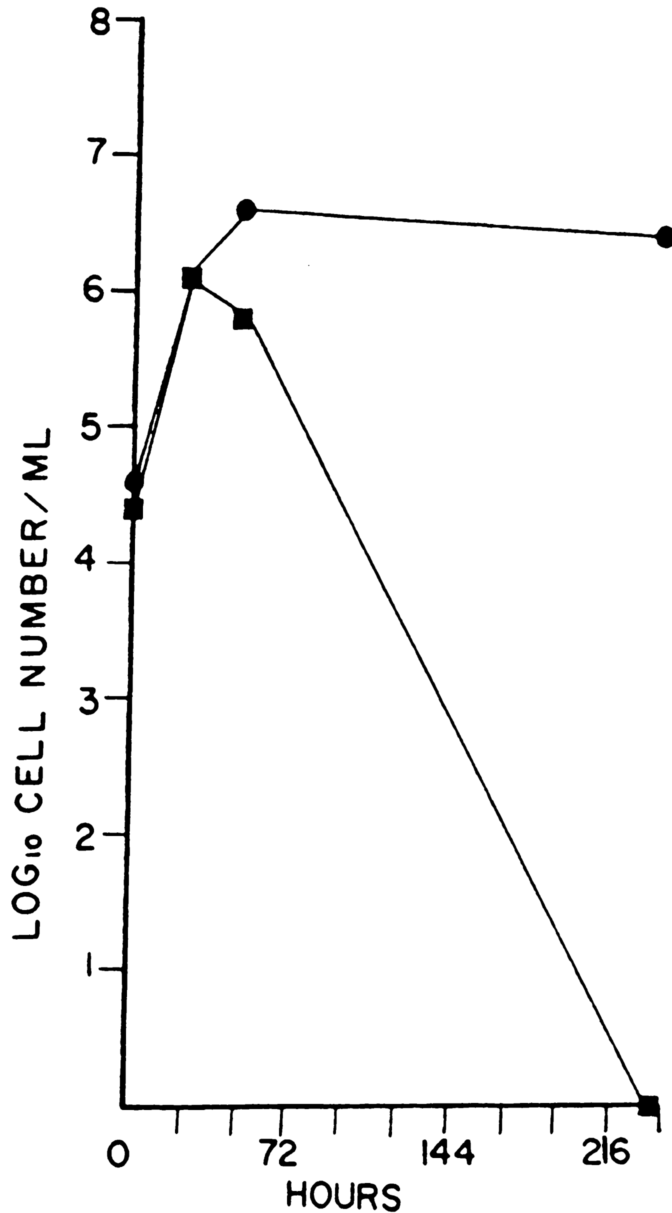

Fig. 4. Viability of S. volutans in dialysis sac cultures and batch cultures. All cultures were grown in CHSS-P broth. Viablilty is expressed as the 50% end point dilution as obtained by the Reed and Muench method (1938). ■■ = control (flask cultures grown statically). ●● = dialysis sac cultures. Values were reported as the combined results from dilution tubes (CHSS-P semisolid) of three flasks or three dialysis sacs. The standard inoculum of S. volutans was used initially for each flask or dialysis sac.

Aeration of S. volutans Cultures in CHSS-P Broth

Caraway and Krieg (1972) found that if S. volutans were cultured initially under 6% O_2 in MPSS broth, and then incubated under 21% O_2, a higher turbidity resulted compared to that of cultures incubated continuously under 6% O_2. Consequently, if S. volutans were to be grown statically in CHSS-P broth for 24 h and then aerated by agitating the cultures for an additional 24 h, the aeration might stimulate growth. Cultures initially aerated by agitation should yield little or no growth, because of the microaerophilic nature of the organism.

Surprisingly, turbidities of between 50 and 60 Klett units were obtained for S. volutans flask cultures regardless of whether they were aerated initially or after the first 24 h (Fig. 5). Cultures grown initially under static conditions reached peak growth (22 Klett units) after 24 h and growth declined after 48 h (Fig. 5).

Thin Layer and Paper Chromatography

By determining which compounds disappear from the medium during growth of S. volutans, the nature of nutrients that limit growth might be identified. Amino compounds were analyzed first, because growth responses were found to vary between brands and batches of casein hydrolysate (Table 3). Thin layer and paper chromatography were performed on concentrated CHSS-P medium supernates obtained from cultures grown for various periods. Solvent systems, and sprays were selected that separated and stained amino compounds.

The farthest migrating amino compound (FMAC) found in the CHSS-P concentrate and the 24-h supernate concentrate was absent from the 48-h

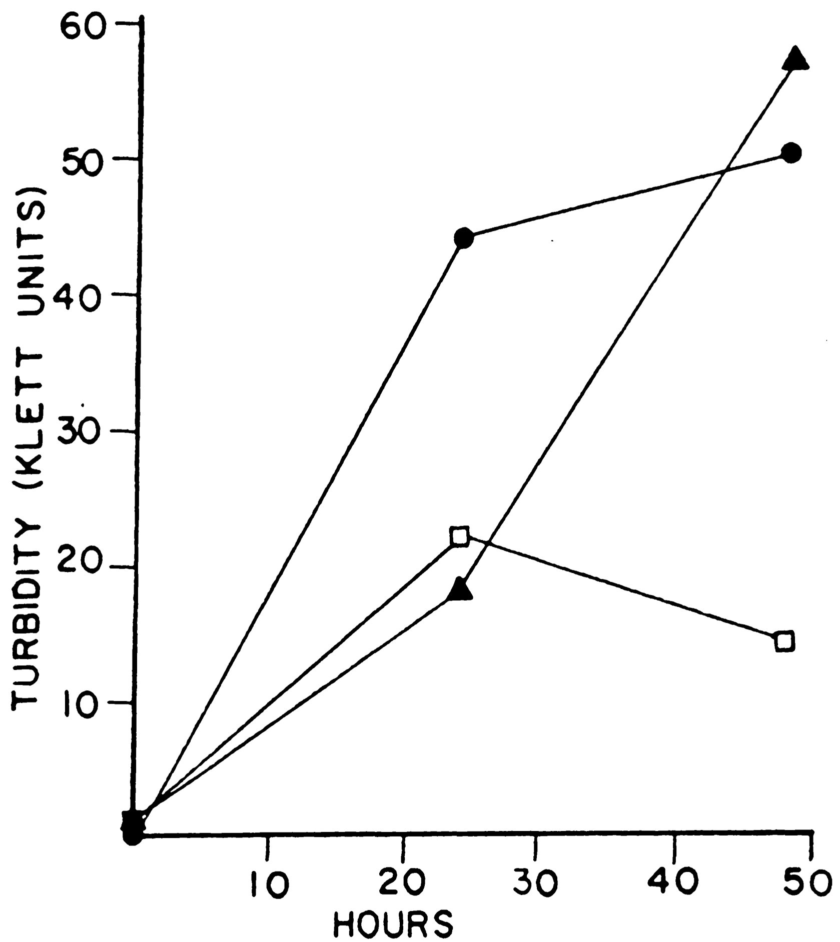

Fig. 5. Growth responses of S. volutans grown statically and on a shaker water bath in CHSS-P broth. □-□ = control (flask cultures incubated statically). ●-● = flask cultures incubated on a shaker water bath. ▲-▲ = flask cultures incubated statically for first 24 h and then incubated on a shaker water bath for the remaining 24 h. Values reported as the mean of three flasks. The standard inoculum of S. volutans was used.

supernate concentrate as determined by TLC (Fig. 6). Paper chromatography yielded similar results. In order to elucidate what the identity of the FMAC might be, the following experiments were performed.

1] Different amino compounds were spotted separately or on top of spots of CHSS concentrates on TLC plates (Table 14). All these compounds migrated more slowly than the FMAC of the CHSS concentrate.

2] The FMAC was eluted from an unstained silica matrix on a TLC plate by pulverizing the corresponding area of silica. This powdered material was made into a slurry and passed through a non-absorbent cotton column to remove particulate matter. The filtrate produced was later re-chromatographed (data not shown). None of the FMAC could be detected in the silica filtrate.

3] Unstained portions of a paper chromatogram corresponding to the approximate location of the FMAC were excised and placed face down onto CHSS agar pour plates seeded with S. volutans. The plates were incubated at 30°C in the dark under a humidified atmosphere of 6% O_2:94% N_2. All of the bioautography plates showed decreased growth under the entire area where the paper chromatogram had been placed.

4] After confirming that casein hydrolysate alone, when chromatographed, yielded the same FMAC as CHSS broth concentrate (data not shown), attempts were made to extract this compound from the rest of the amino compounds. Three different biphasic solvent systems were shaken and separated

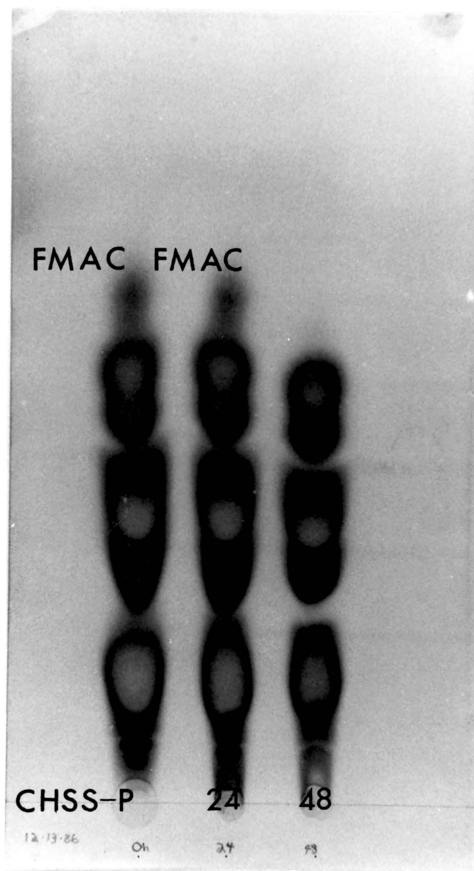

Fig. 6. Thin layer chromatography of concentrates prepared from sterile CHSS-P broth and from cultures grown in CHSS-P broth for 24 and 48 h.

TABLE 14

Amino Compounds Chromatographed in Order to Identify FMAC

Amino compound	Adjacent[1]	On top
Alanine	X	X
Arginine	X	X
Asparagine	X	
Cadaverine	X	
Cystine	X	X
Glucosamine		X
Glutamic acid	X	X
Glycine	X	X
Histidine	X	X
Hydroxyproline	X	X
Indole	X	
Isoleucine		X
Leucine		X
Lysine	X	X
Methionine	X	X
Norleucine	X	
Phenylalanine		X
Proline		X
Putrescine	X	
Serine		X
Threonine	X	X
Tryptophan	X	
Tyrosine		X
Valine		X
D-(+)Galactosamine		X
DL-α-Aminocaprylic acid		X
DL-β-Aminobutyric acid		X
DL-β-Aminoisobutyric acid		X
L-DOPA	X	
L-α-Aminoadipic acid		X
o-Phospho-L-serine		X
p-Aminohippuric acid		X
α-Aminoisobutyric acid		X
τ-Amino-n-butyric acid		X
δ-Aminolevulinic acid		X
ε-Amino-n-caproic acid		X

[1]The amino compounds listed were either spotted directly on top of, or adjacent to a 48-h CHSS-P concentrate spot on a silica gel TLC plate.

by a separatory funnel. The first system was a 1:1 mixture of aqueous casein hydrolysate solution and n-butanol. This system was used to separate polar from non-polar compounds. The second and third systems were chloroform:30% NH_3OH (1:1 v/v), and chloroform:6N HCl (1:1 v/v). The casein hydrolysate was dissolved in the HCl and the 30% NH_3OH prior to mixing with the chloroform. It was hoped that acidic and basic amino compounds might be extracted into the HCl and NH_3OH phases respectively. Only the n-butanol:H_2O extraction separated the FMAC from many of the other amino compounds; it appeared in the butanol phase. Although this separation occurred it was incomplete because the majority of the FMAC remained in the aqueous phase, as determined by TLC.

The data presented in Table 14 indicate that the FMAC is not any of the amino acids, amino sugars, nor polyamines tested. Its identity remains unknown.

Fatty Acid Analysis by Gas-Liquid Chromatography

To determine whether any fatty acids present in CHSS-P broth were used up by S. volutans during growth for 48 h, volatile and nonvolatile fatty acids were analyzed using a Beckman GC-4 gas chromatograph (Holdeman et al., 1975).

No volatile fatty acids were found in either the CHSS concentrate or the 48-h supernate-concentrate (data not shown). Only small amounts of pyruvic, lactic, fumaric, and oxaloacetic acids were detected in concentrates of 48-h-old cultures, and were presumably formed by the

spirilla. Forty-seven percent of the succinate disappeared after 48 h, as calculated from the areas under the succinate peaks (Figs. 7 and 8). Since at least half of the original succinate remained, it is unlikely that lack of succinate is a factor limiting growth of S. volutans in CHSS-P broth.

Analysis of Amino Acids with an Amino Acid Analyzer

The system of acetylation and propylation of amino acids for gas chromatographic analysis failed to yield reproducible chromatograms, and for this reason use of the system was abandoned.

Amino acid analysis was performed on the CHSS-P medium concentrate, the 24-h supernate concentrate, and the 48-h supernate concentrate in hopes of finding which amino acid(s), if any, were being depleted after 48 h of growth.

None of the amino acids seemed to be significantly depleted after 48 h, in fact, some amino acids appeared to increase slightly (Table 15). This slight increase may have been due to cell lysis.

Attempts to Devise a Selection System for S. volutans

Many of the conventional selective procedures for spirilla cannot be used for S. volutans. One of the greatest obstacles in developing isolation procedures for S. volutans is the inability of small inocula to yield colonies on solid media. Also, only scant growth of this orga-

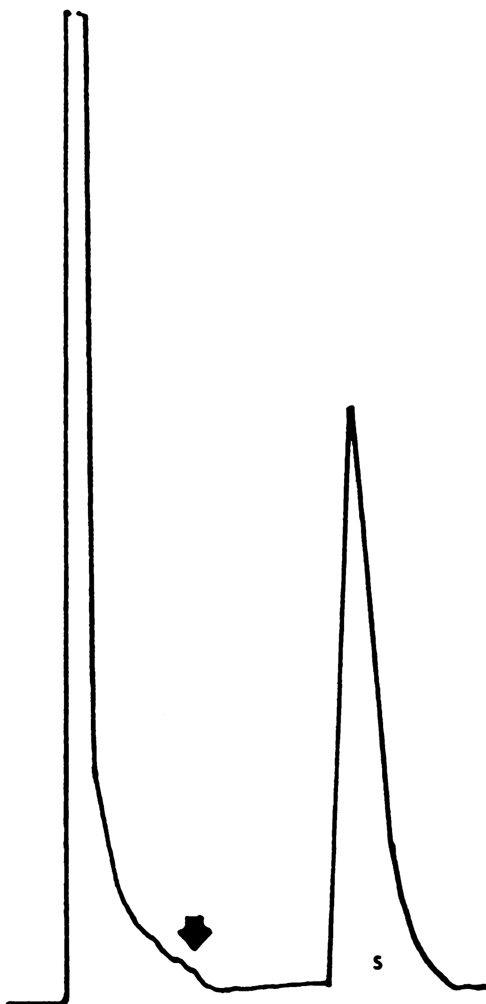

Fig. 7. Gas chromatogram of nonvolatile fatty acids found in the CHSS concentrate. Attenuation = 6,400 until the arrow, and then the attenuation = 12,800. s = succinic acid.

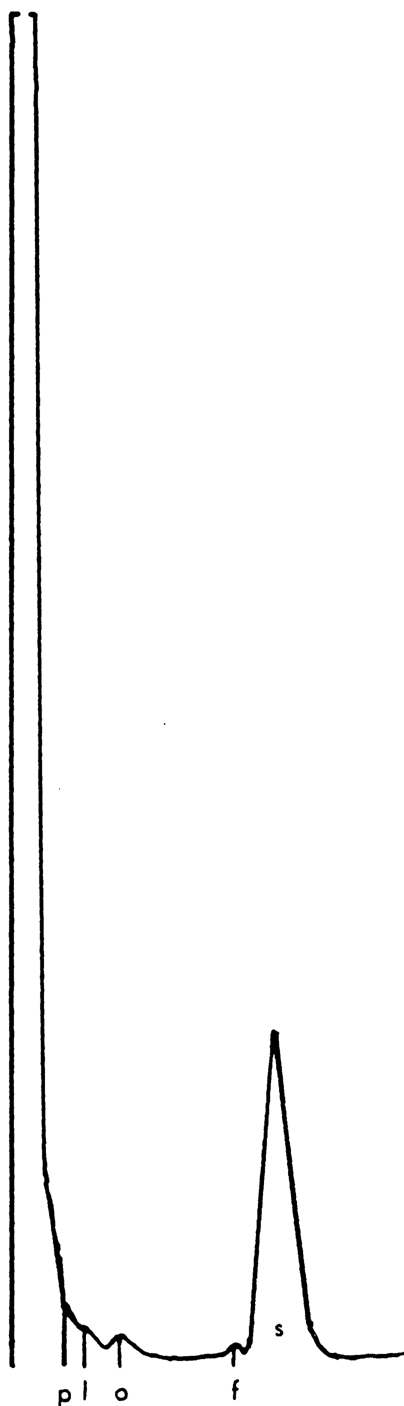

Fig. 8. Gas chromatogram of nonvolatile fatty acids found in the 48-h supernate-concentrate. p = pyruvic acid; l = lactic acid; o = oxaloacetic acid; f = fumaric acid; s = succinic acid. Attenuation = 12,800.

TABLE 15

Concentration of Amino Acids Found in Concentrated CHSS-P Medium-Supernates[1]

Amino acid	Percent of total amino acids analyzed in		
	CHSS-P[2]	24-h[3]	48-h[4]
Aspartic acid[5]	9.4	9.5	7.7
Threonine	5.6	5.7	5.5
Serine	9.5	9.3	8.8
Glutamic acid	27	27	24
Glycine	5.2	5.2	4.6
Alanine	7.1	7.0	6.1
Valine	5.7	5.5	8.5
Methionine	2.1	2.3	2.1
Isoleucine	3.1	2.9	5.4
Leucine	7.0	7.2	8.1
Tyrosine	2.0	2.0	2.3
Phenylalanine	2.4	2.4	2.5
Histidine	2.5	2.4	2.7
Lysine	8.4	8.7	8.6
Arginine	3.2	3.2	3.4

[1]Samples were analyzed on a Durrum MBF amino acid analyzer.

[2]CHSS-P = CHSS-P broth concentrate.

[3]24-h = 24-h supernate concentrate.

[4]48-h = 48-h supernate concentrate.

[5]Asparagine and glutamine were converted to their respective acids by the preparative method used.

nism occurs on all of the liquid media used to date. The large size of S. volutans precludes use of a filtration method, such as that used in isolating Aquaspirillum gracile (Canale-Parola et al., 1966). The low salt tolerance and the neutral pH requirement of S. volutans preclude increased salt concentrations or acidic or alkaline pH values from being used in selection of S. volutans.

The only method known for enriching and isolating S. volutans is the cumbersome capillary tube method (Rittenberg et al., 1962). Though it has been successful, this method is limited by the problem of contamination, and the low probability that the few mechanically separated cells will grow. For these reasons alternate methods of selection and isolation were sought.

By obtaining a selective medium that enables S. volutans to be the predominant organism, subsequent isolation procedures would undoubtedly be more effective.

Antibiotic Susceptibility Tests

In order to develop a selective medium for isolating S. volutans from nature, knowledge of which antibiotics and other antimicrobial compounds S. volutans is resistant to could prove useful. To determine antibiotic susceptibilities and resistance, auxanographic plates were prepared as described in materials and methods.

As indicated in Table 16, the only antibiotics to which S. volutans was resistant were oxacillin, clindamycin, lincomycin, and cloxacillin.

TABLE 16

Growth Response of S. volutans to Discs Impregnated with Antibiotics and Chemotherapeutic Agents on CHSS-P-Bis Pour Plates

Impregnated disc	Amount on disc	Zone of inhibition produced
Amikacin	30 μg	+
Ampicillin	10 μg	+
Bacitracin	10 μg	+
Carbenicillin	100 μg	+
Cefamandole	30 μg	+
Cefoxitin	30 μg	+
Cefuroxime	30 μg	+
Cephaloridine	5 μg	+
Cephalothin	30 μg	+
Cephazolin	30 μg	+
Cephradine	30 μg	+
Chloramphenicol	30 μg	+
Chlortetracycline	30 μg	+
Clindamycin	10 μg	-
Cloxacillin	1 μg	-
Colistin	10 μg	+
Doxycycline	30 μg	+
Erythromycin	15 μg	+
Furazolidone	100 μg	+
Fusidic acid	10 μg	+
Gentamicin	30 μg	+
Kanamycin	5 μg	+
Lincomycin	2 μg	-
Mecillinam	25 μg	+
Methicillin	5 μg	+
Metronidazole	5 μg	+
Mezlocillin	75 μg	+
Minocycline	30 μg	+
Nalidixic acid	30 μg	+
Neomycin	30 μg	+
Nitrofurantoin	300 μg	+
Nitrofurazone	100 μg	+
Novobiocin	5 μg	+
Oxacillin	1 μg	-
Oxytetracycline	30 μg	+
Penicillin	10 U	+
Polymyxin B	300 U	+
Rifampicin	RD2[1]	+
Rifampin	5 μg	+

TABLE 16, continued . . .

Impregnated disc	Amount on disc	Zone of inhibition produced
Sisomicin	10 μg	+
Spectinomycin	10 μg	+
Streptomycin	10 μg	+
Sulfamethoxazole + trimethoprim	23.75 + 1.25 μg	+
Sulfisoxazole	2 μg	+
Tetracycline	30 μg	+
Tobramicin	10 μg	+
Trimethoprim	5 μg	+
Vancomycin	30 μg	+

[1]RD2 = reactive dose 2.

Tolerance of S. volutans to Oxacillin, Lincomycin, and Cloxacillin

Various concentrations of those antibiotics toward which S. volutans exhibited resistance (Table 16) were added to CHSS-P broth (Washington, 1985). The maximum concentrations of these compounds which caused no visible decrease in the turbidity of a 24-h CHSS-P broth culture of S. volutans are indicated in Table 17. The highest tolerance was exhibited toward lincomycin.

Tolerance of S. volutans Towards Cycloheximide and Antimicrobial Compounds Other than Antibiotics and Chemotherapeutic agents

In order to develop a selective medium for S. volutans, the tolerance of this organism toward cycloheximide and various antimicrobial agents other than antibiotics was determined. Selection of which agents to test was based on the following.

1] Flagg and Wilson (1977) demonstrated that, with regard to uptake, Escherichia coli could not discriminate between thiogalactosides (which are nonmetabolizable) and galactosides (which can be metabolized for energy production). Since S. volutans, unlike many other bacteria, does not use carbohydrates as carbon and energy sources, it was thought that addition of thiogalactosides to mixed cultures containing S. volutans might inhibit many of the contaminants without inhibiting S. volutans: only the contaminants would take up the nonmetabolizable carbohydrate analog. However, thiogalactosides are difficult to obtain, and for use at substrate concentrations they are very ex-

TABLE 17

Tolerance of *S. volutans* to Oxacillin, Lincomycin, and Cloxacillin

Antibiotic	Tolerance[1] (μg/ml)
Oxacillin	2
Lincomycin	400[2]
Cloxacillin	2

[1]The maximum concentration of antibiotic that caused no visible decline in turbidity of *S. volutans* in CHSS-P broth. The standard inoculum of *S. volutans* was used. Values were reported as the result of duplicate flasks.

[2]Value was reported as the result of 5 replicate tubes. Assay was performed with CHSS-P semisolid medium.

pensive. On the other hand, thioglucose -- a nonmetabolizable analog of glucose -- can be readily obtained and is reasonably inexpensive. Consequently, it was of interest to determine if S. volutans might be tolerant to this glucose analog.

2] Aquaspirillum serpens has been reported to have been successfully selected by the addition of 300 µg/ml of 5-fluorouracil to mixed cultures (Cody, 1968). Consequently, it was of interest to determine whether S. volutans might also be resistant to this pyrimidine analog and also to a similar analog, 5-fluorocytosine.

3] Iodoacetic acid and potassium fluoride are inhibitors of the Embden-Meyerhof-Parnas pathway of carbohydrate dissimilation. Since S. volutans does not use sugars, and therefore does not use the Embden-Meyerhof-Parnas pathway as an energy-yielding pathway, it seemed likely that this organism might be tolerant to these inhibitors.

4] Certain dyes at low concentrations have been used as selective agents in various bacteriological media, such as those used for enteric bacilli, Brucella species, mycobacteria, and others (Freeman, 1979). Consequently, the tolerance of S. volutans to two such dyes, brilliant green and basic fuchsin, was tested.

5] Tannins from plant debris are probably associated with the natural environment of S. volutans (i.e., stagnant water rich in organic matter). Tannins are complex phenolic

compounds known to inhibit the growth of many bacteria, and they probably play an important role in the resistance of various plants to bacterial infection. Therefore, the tolerance of S. volutans to tannic acid was determined.

6] S. volutans has been observed to retain its motility (although it cannot grow) in media containing 40% Triton X-100. Since detergents affect cell membranes and are toxic to many bacteria, it was of interest to see whether S. volutans might be able to grow in presence of a lower level of Triton X-100.

7] Cycloheximide is an antibiotic that inhibits eucaryotic cells but not procaryotic cells, and it is a known fungicide (Windholz, 1976). Since hay infusions containing S. volutans also contain many fungi (and protozoa), it was desirable to determine if S. volutans, which is a procaryote, would indeed tolerate the presence of this compound.

As shown in Table 18, S. volutans was resistant to the levels employed of all of the above compounds except brilliant green dye and iodoacetic acid. In the case of thioglucose, although S. volutans did exhibit growth in the presence of 0.1% of this glucose analog, the growth was very scant, indicating that some inhibition had occurred.

TABLE 18

Tolerance of *S. volutans* to Cycloheximide and Various Antimicrobial Compounds Other than Antiobiotics and Chemotherapeutic Agents

Compound	Concentration	Growth response[1]
Brilliant green	12.5 mg/l	-[2]
β-D-Thioglucose	100.0 mg/l	FT[2]
Cycloheximide	5.0 mg/l	+[3]
5-Fluorouracil	150.0 mg/l	-[3]
5-Fluorocytosine	300.0 mg/l	+[3]
Iodoacetic acid	150.0 mg/l	-[3]
Potassium fluoride	2,000.0 mg/l	+[4]
Triton X-100	2.0 % (v/v)	+[3]
Tannic acid	10.0 mg/l	+[3]
Basic fuchsin	1.6 mg/l	+[3]

[1]"+" = growth occurred in all tubes or flasks tested. "-" = no growth occurred in any of the tubes or flasks tested. FT = faint growth occurred in all tubes or flasks tested. The standard inoculum of *S. volutans* was used to inoculate tubes of CHSS-P semisolid medium or flasks of CHSS-P broth. All flasks and tubes were incubated for 24 h at 30 °C.

[2]Two replicates tested.

[3]Three replicates tested.

[4]Five replicates tested.

Selection of S. volutans in Hay-Infusion-Semisolid Medium Supplemented with β-D-Thioglucose

Semisolid medium was used in order to take advantage of the microaerophilic nature of S. volutans. It was hoped that S. volutans would be the predominant organism in the region of the tubes just below the surface. Also, if carbohydrate metabolizing microbes indiscriminately took up β-D-thioglucose, the carbohydrate present in excess, it was felt that they might starve (Flagg et al., 1977). This in turn would help S. volutans, which does not metabolize sugars, in predominating.

A sample was removed from just below the surface of each tube, and examined microscopically. Both cultures contained a wide variety of procaryotes and eucaryotes. Unfortunately, only a few cells of S. volutans were seen.

Selection of S. volutans in Pringsheim Medium Supplemented with Nystatin and Cycloheximide

The addition of nystatin and cycloheximide to Pringsheim medium might reduce the numbers of eucaryotic organisms in a mixed culture containing S. volutans.

After 48 h of incubation both the Pringsheim medium and the Pringsheim medium supplemented with nystatin (12 mg/l final concentration) and cycloheximide (25 mg/l final concentration) were observed microscopically. The Pringsheim medium with supplements had far fewer protozoa than the other tube, yet the tube with supplements still possessed many other motile organisms including some large motile

spirilla. Whether or not these large spirilla were S. volutans was unknown.

Selection of S. volutans in CHSS-P Medium Supplemented with Lincomycin and Cycloheximide

It was thought that the combination of lincomycin (effective against gram + bacteria) and cycloheximide might be good selective supplements when used in concert. Because the number of S. volutans naturally occurring in pond water is too low to be assessed microscopically, tubes were inoculated with both pond water and S. volutans.

As shown in Table 19, lincomycin and cycloheximide did not appear to be effective selective agents for S. volutans.

Selection of S. volutans in CHSS-P Semisolid Medium Supplemented with Lincomycin, 5-Fluorocytosine, Potassium Fluoride, Basic Fuchsin, Tannic Acid, Cycloheximide, and Triton X-100

The antimicrobial compounds S. volutans was resistant to (Tables 17 and 18) were incorporated into CHSS-P medium in hopes of creating an effective selective medium. Semisolid medium was used in order to take advantage of the microaerophilic nature of S. volutans. It was hoped that S. volutans would be the predominant organism in the region of the tubes just below the surface. No cells of S. volutans were ever seen in the 11-day-old hay infusion. For this reason tubes inoculated with hay infusion were also inoculated with S. volutans.

A sample was removed from just below the surface of each tube, and examined microscopically. The results are shown in Table 20. The CHSS-P medium supplemented with antimicrobial compounds did appear to inhibit the growth of most of the microorganisms from the hay infusion. Unfortunately, the growth of S. volutans was also inhibited (Table 20). Protozoa were present in the hay infusion, yet none were ever witnessed in any of the control or selective medium tubes (Table 20). The rapid growth rate of aerobic bacteria with subsequent nutrient depletion probably precluded any protozoa from proliferating in the CHSS-P medium.

The tubes of CHSS-P semisolid (0.2% agar) medium inoculated with the 26-h-old S. volutans + hay infusion culture (grown in CHSS-P medium supplemented with antimicrobial compounds) was observed microscopically after 22 h. The culture contained many motile and nonmotile rod-shaped bacteria and a few small motile spirilla. No cells of S. volutans were seen. The tube of CHSS-P semisolid (0.2% agar) medium inoculated with 51-h-old S. volutans + hay infusion culture (growth in CHSS-P medium supplemented with antimicrobial compounds) was observed microscopically after 48 h. The majority of the bacteria seen were vibrioid cells. Very few of these were motile. No eucaryotes or S. volutans were present. Growth never appeared in the tube of CHSS-P semisolid (0.2% agar) medium inoculated with the 51-h-old S. volutans culture (grown in CHSS-P medium supplemented with antimicrobial compounds) after 48 h of incubation. This indicates that S. volutans could not tolerate all of the antimicrobial compounds added together.

TABLE 19

Selection of *S. volutans* in CHSS-P Medium Supplemented with Lincomycin and Cycloheximide

Inoculum[1]	Addition of cycloheximide and lincomycin[2]	Microscopic observation[3]
S. volutans	-	Culture appeared pure. Cells appeared normal in size and shape (20 to 30 cells/field). Ca. 80% of the cells were motile.
S. volutans	+	Culture appeared pure. Cells appeared normal in size and shape (20 to 30 cells/field). Ca. 80% of the cells were motile.
S. volutans + pond water	-	Culture contained predominantly motile and nonmotile rods, a few motile spirilla, and very few *S. volutans* (<1/field).
S. volutans + pond water	+	Culture contained predominantly motile and nonmotile rods, a few motile spirilla, and very few *S. volutans* (<1/field).

[1]The standard inoculum of *S. volutans* was used. A 1% (v/v) inoculum of pond water (VPI & SU duck pond) was used.

[2]Cycloheximide = 5 mg/l final concentration. Lincomycin = 100 mg/l final concentration.

[3]Each observation was made on one flask out of a set of three incubated for 24 h at 30 °C. All of the assay flasks were turbid. Microscopic observations were made using 100x darkfield total magnification and 900x phase contrast total magnification (Leitz-Wetzlar).

TABLE 20

Selection of S. volutans in CHSS-P Semisolid Medium Supplemented with Lincomycin, 5-Fluorocytosine, Potassium Fluoride, Basic Fuchsin, Tannic Acid, Cycloheximide, and Triton X-100

Inoculum[1]	Addition of lincomycin, 5-fluorocytosine, potassium fluoride, basic fuchsin, tannic acid, cycloheximide, and Triton X-100[2]	Microscopic observation[3]
S. volutans	-	After 24 h many motile and and nonmotile cells of S. volutans were present. Cells appeared normal in size and shape. After 48 h many motile and nonmotile cells of S. volutans were present. Locomotion was slower than after 24 h. Some unusually long nonmotile cells of S. volutans were present.
S. volutans	+	After 24 and 48 h only nonmotile cells of S. volutans were seen (20/field at 24 h). Some debris was seen (possibly cell material). Cells appeared normal in size and shape.
S. volutans + hay infusion	-	After 24 h S. volutans cells were seen (20/field) amid a plethora of motile and nonmotile rods. After 48 h many motile and nonmotile rods and a few small motile spirilla were seen. No S. volutans was seen.

TABLE 20, continued . . .

Inoculum[1]	Addition of lincomycin, 5-fluorocytosine, potassium fluoride, basic fuchsin, tannic acid, cycloheximide, and Triton X-100[2]	Microscopic observation[3]
S. volutans + hay infusion	+	After 24 h a few cells of of S. volutans (20/ field) were seen amid a few nonmotile rods. After 48 h only nonmotile cells of S. volutans were seen.
Hay infusion	-	After 24 h many motile and nonmotile rods were seen. After 48 h a mixture of motile and nonmotile rods and small motile spirilla were seen.
Hay infusion	+	After 24 h only very few rod-shaped bacteria were seen. After 48 h two of the tubes contained no observable growth. One tube contained vibrioid and helical bacteria (none resembled S. volutans).

[1]The standard inoculum of S. volutans was used. A 1% inoculum of hay infusion (hay - VPI & SU dairy barns; pond water - VPI & SU duck pond) was used.

[2]Final concentrations of antimicrobial agents: lincomycin, 190 mg/l; 5-fluorocytosine, 143 mg/l; potassium fluoride, 1 g/l; basic fuchsin, 0.8 mg/l; tannic acid, 5 mg/l; cycloheximide, 5 mg/l; Triton X-100, 1% (v/v).

[3]Microscopic observations were made using 100x darkfield total magnification and 900x phase contrast total magnification (Leitz-Wetzlar). Observations using 900x phase contrast total magnification were made on one, two, or three tubes out of a set of three replicate tubes incubated at 30 °C. Observations using 100x darkfield total magnification were made on two or three tubes out of a set of three replicate tubes incubated at 30 °C.

DISCUSSION

Growth Enhancement of *S. volutans*

In trying to improve the growth response of a bacterium the parameters of optimum pH, temperature, nutrient concentrations, osmotic potential, atmosphere, and in some cases even pressure must be considered. The optimum growth temperature and lighting conditions for medium preparation and growth of *S. volutans* have previously been determined (Hylemon et al., 1973b; Padgett et al., 1981). Satisfactory gaseous conditions and medium pH have also been established for this spirillum (Wells et al., 1965; Bowdre et al., 1976; Padgett et al., 1981). For these reasons the present study concentrated on enhancing growth through nutritional avenues. During one such experiment *S. volutans* was confirmed to be still microaerophilic by virtue of its migration patterns in a drop of culture (see Results). However, it was surprising that *S. volutans* was able to grow in CHSS-P broth on a shaker, i.e., under conditions of high aeration; in fact, better growth occurred under the aerated conditions than under static conditions. The addition of phosphate to CHSS broth, which supports growth to a higher cell density than occurs in broth without the added phosphate, provides the best explanation for this phenomenon. Since no phosphate is provided in the formulation of CHSS broth, the only phosphate that is ordinarily present must come from contaminating quantities in the other constituents of the medium. Caraway et al. (1974) found that too much phosphate (50 mM) was detrimental to the respiration of *S. volutans*.

However, in the present report, only 1 mM phosphate was added to the medium. This low level of phosphate might have allowed S. volutans to produce more high energy phosphate compounds, which in turn could lead to higher respiration rates. With a higher rate of respiration, S. volutans might be better able to maintain a reduced cell interior and proliferate more readily under aerated conditions (Kuenen et al., 1979). Alternatively, the additional phosphate might allow the cells to synthesize more nucleic acids or phospholipids, thereby allowing a greater cell density to develop. It is also possible that during the 22 years since the strain was isolated, selection of a more oxygen-tolerant variant strain may have occurred. This may be attributed to the many serial passages in both semisolid media (in which a continuous gradient of oxygenation occurs) and, in the present study, in broth cultures incubated statically under an air atmosphere. That aerotolerant mutants can be derived from S. volutans has recently been reported by Padgett et al. (1986).

By itself, DMSO did little for stimulating the growth response of S. volutans (Table 14). However, due to its penetrability and OH· quenching properties (Fox et al., 1983) DMSO might possibly work well in concert with SOD and catalase in protecting S. volutans from toxic oxygen radicals. Having catalase and SOD quench extracellular O_2^- and H_2O_2, and DMSO remove intracellular ·OH, S. volutans might grow better on solid media.

Growth of S. volutans on DM spread plates most likely appeared only where high proportions of viable cells were located. This would

account for the nebulae formations seen on DM plates incubated for 4 to 6 days.

The fact that KCl was inhibitory for S. volutans (Table 7) raises the question as to whether the Cl^- of the Na^+ of NaCl is the toxic culprit. It had previously been assumed that S. volutans was sensitive to Na^+ (Hylemon et al., 1973b). If Cl^- is damaging to S. volutans, then the pH maintenance experiment might have been successful had HCl not been used to adjust the pH. Perhaps the use of succinic acid (free acid form) might have proven more satisfactory.

Future research should be done in order to determine which ions, Na^+ or Cl^-, S. volutans is sensitive to. Such experimentation might possibly reveal that (1) S. volutans was not getting enough Na^+ in its diet, in which case an increase in Na^+ would prove stimulatory; and (2) by avoiding the addition of HCl-salts of compounds to media, the growth of S. volutans might increase.

According to Barry (1976) the area of "stimulated" growth circumscribing a zone of inhibition surrounding an antibiotic impregnated disc on a petri plate can be artifactual. The more intense growth can result from nutrients being made available from the adjacent zone of no growth (Barry, 1976). This is probably the best explanation for the zones of "stimulation" that surrounded EDTA, acetyl choline, ammonium molybdate, and DL-epinephrine on the CHSS-P-Bis auxanographic plates (Table 14 and Fig. 2).

With the discovery that very high turbidities of S. volutans could be achieved in dialysis sac cultures, it was thought that this technique would be a suitable way to obtain a high density of viable cells.

However, the viable cell count was approximately the same as that in ordinary broth cultures; thus, most of the cells contributing to the high turbidity were dead cells. The dialysis technique was found to have one useful aspect, however: viable cells were still present in the dialysis sac cultures at the end of 10 days of incubation, whereas in ordinary broth cultures no viable cells can usually be found after 3 days. This indicates that dialysis sac cultures can be useful for maintenance of S. volutans, and can eliminate the need for daily transfer.

The reason why a high viable-cell density could not be achieved in the dialysis sac cultures is presently elusive. According to Bail (1938), with any fluid culture the total yield of bacteria per unit volume of medium tends to be constant for a given species. This population of bacteria has been labeled the M concentration by Bail. As noted by Lamanna et al. (1973), frequently one may remove the organisms at an M concentration, reinoculate the medium, and find that growth occurs (although never to the same level as the original population). In these cases the cessation of growth by the original culture cannot have been due to the exhaustion of food, or accumulation of toxic metabolic products. Such observations have suggested that a certain amount of physical space which has been thought of as "biological space" is required to support the growth of individual bacteria.

Unfortunately, the concept of biological space is merely descriptive and not explanatory. Considering that the average length of a S. volutans cell is 60 μm, and that the amplitude of its helix is 6.5 μm (Hylemon et al., 1973a), the volume occupied by one cell of S.

volutans would be 2 x 10^3 μm^3. Therefore, 1 ml of broth could contain a maximum of 5.02 x 10^8 cells of S. volutans. Consequently, S. volutans, at a concentration of 5 x 10^6 cells/ml, might be approaching the maximum viable cell density permissible in broth. Each viable spirillum in the dialysis sac might need a certain volume of surrounding medium in order for toxic waste products to diffuse away from the cell. Another possible explanation is that the dead cells in the dialysis sac might release a soluble growth factor upon lysing. If the quantity of growth factor was limiting, this would explain why the viable count never exceeded approximately 5 x 10^6 cell/ml. The diffusion of oxygen into dialysis flasks also might be a limiting factor. Thus, agitating dialysis flasks might permit higher cell yields.

That one or more limiting nutrients are indeed restricting the growth of S. volutans in CHSS broth is indicated by the decrease in growth response that occurs when the level of casein hydrolysate is decreased five-fold. On the other hand, it appears that casein hydrolysate also contains toxic factors (probably certain amino acids), since an increase in concentration to 0.75%, a level which is used in many bacteriological media, results in inhibition, while an increase to 1.0% (again, a reasonable level in bacteriological media) results in severe inhibition. Thus, the viable cell density of the organism seems to be the result of not having a sufficient level of certain nutrients in casein hydrolysate; yet these nutrients cannot be increased without also increasing the level of one or more toxic constituents of casein hydrolysate. If the level of the limiting nutrient(s) could be increased without simultaneously increasing the toxic factors, we would predict

without simultaneously increasing the toxic factors, we would predict that a much higher viable-cell density would occur. Unfortunately, all attempts at discovering the identity of the limiting nutrients have failed. The disappearance of the FMAC from CHSS broth during growth (Fig. 6) suggests that this compound might be an important limiting nutrient; however, the FMAC could not be identified as any of a wide range of amino acids or other nitrogenous compounds. It might possibly be a decarboxylated amino acid due to its migration pattern on TLC plates (R. H. White and E. M. Gregory, personal communication); however it is not putrescine or cadaverine (Table 15). Whether or not the FMAC seen on TLC plates and paper chromatograms is even stimulatory for S. volutans has yet to be determined; the main problem lies in obtaining a sufficient amount of the material to test.

Tyrrell et al. (1958) developed the biphasic dialysis system for growing large quantities of bacteria in a small volume of liquid. They accomplished this by culturing bacteria in broth medium that was placed over top of solid medium of the same composition. In such a biphasic system nutrients are slowly released from the agar underlay, while waste products diffuse into the agar from the broth (Krieg and Gerhardt, 1981). A biphasic culture system using a "soft agar" overlay on top of a solid agar underlay might prove useful in enhancing the growth of S. volutans on solid media.

Attempts at Developing a Selective Medium for S. volutans

Of the three antibiotics tested (Table 18), S. volutans showed the highest tolerance towards lincomycin (400 μg/ml). Gram positive organisms have been shown to be sensitive from as little as 0.21 μg/ml to as high as 23.7 μg/ml (Lewis et al., 1963). Though preliminary work with lincomycin did not prove fruitful, lincomycin in combination with other antimicrobial compounds might serve as useful media supplements in selecting for S. volutans.

Combining all of the antimicrobial compounds that S. volutans was found tolerant to (Tables 18 and 19) into CHSS-P medium resulted in an unsuccessful selective medium. Since this medium did inhibit the growth of many procaryotes, different combinations and concentrations of the antimicrobial agents used might prove useful. All single additions and double combinations of antimicrobial agents in media tried thus far have not proven satisfactory. Low speed centrifugation of a selective culture might prove successful in separating protozoa, fungi, and S. volutans from smaller bacteria. Subsequent transfer of the protozoa-fungi-S. volutans fraction into a selective medium containing nystatin and cycloheximide might permit S. volutans to predominate.

SUMMARY

Project objectives were to develop an improved plating medium in order to achieve reproducible colony counts and accurate quantification for growth or survival of Spirillum volutans; and to develop an improved method for selection of S. volutans from nature.

Growth enhancement studies began with the casein hydrolysate-succinate-salts medium (CHSS). Optima of which pH, casein hydrolysate concentration, type of casein hydrolysate, and phosphate concentration to use were determined. Growth of S. volutans doubled after 24 h with the addition of potassium phosphate buffer (1 mM final concentration) to CHSS medium (CHSS-P). Addition of supplements to CHSS-P medium, and modified Bordetella and Brucella media failed to increase the cell yields of S. volutans. Punctiform colonies did form in CHSS-P medium (0.7% agar) pour-plates, yet at a recovery rate much lower than reported by Padgett et at. (1982) for surface growth on spread plates. Investigations into the role of amino compounds as possible growth factors was done. Thin-layer chromatography, gas chromatography, and an amino acid analyzer were employed to identify those amino compounds (if any) that decreased or disappeared in the medium after 48 h of growth. None could be found. Growth, though not greatly increased, was prolonged by growing S. volutans in dialysis sacs suspended in CHSS-P medium.

The tolerances of S. volutans to several antimicrobial compounds were determined. Attempts at using hay infusion, Pringsheim, and CHSS-P media supplemented with various compounds as selective media did not result in predominance of S. volutans.

LITERATURE CITED

Aragno, M. and H. G. Schlegel. 1978. Aquaspirillum autotrophicum, a new species of hydrogen-oxidizing, facultatively autotrophic bacteria. Int. J. Syst. Bacteriol. **28**:112-116.

Bail, O. 1929. Ergebnisse experimenteller populationsforschung. Zitätsf. Immun. **60**:1-22.

Barry, A. L. 1976. The Antimicrobic Susceptibility Test: Principles and Practices. Lea & Febiger. Philadelphia.

Blakemore, R. P. 1975. Magnetotactic bacteria. Science **190**:377-379.

Blakemore, R. P., D. Maratea, and R. S. Wolfe. 1979. Isolation and pure culture of a freshwater magnetic spirillum in chemically defined medium. J. Bacteriol. **140**:720-729.

Bowdre, J. H. and N. R. Krieg. 1974. Water quality monitoring: bacteria as indicators. Virginia Polytechnic Inst. & State University Water Resources Research Center. Bull. **69**:1-20.

Bowdre, J. H., N. R. Krieg, P. S. Hoffman, and R. M. Smibert. 1976. Stimulatory effect of dihydroxyphenyl compounds on the aerotolerance of Spirillum volutans and Campylobacter fetus subspecies jejuni. Appl. Environ. Microbiol. **31**:127-132.

Canale-Parola, E., S. L. Rosenthal, and D. G. Kupfer. 1966. Morphological and physiological characteristics of Spirillum gracile sp.n. Antonie van Leeuwenhoek J. Microbiol. Serol. **32**:113-124.

Caraway, B. H. and N. R. Krieg. 1972. Uncoordination and recoordination in Spirillum volutans. Can. J. Microbiol. **18**:1749-1759.

Caraway, B. H. and N. R. Krieg. 1974. Aerotaxis in Spirillum volutans. Can. J. Microbiol. **20**:1367-1377.

Cody, R. M. 1968. Selective isolation of Spirillum species. Appl. Microbiol. **16**:1947-1948.

Cole, J. A. and S. C. Rittenberg. 1971. A comparison of respiratory processes in Spirillum volutans, Spirillum itersonii, and Spirillum serpens. J. Gen. Microbiol. **69**:375-383.

Costilow, R. N. 1981. Biophysical factors in growth. pp. 66-78. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, G. B. Phillips (eds.). Manual of Methods for General Bacteriology. American Society for Microbiology. Washington, DC.

Dietz, A. S. and A. A. Yayanos. 1978. Silica gel media for isolating and studying bacteria under hydrostatic pressure. Appl. Environ. Microbiol. **36**:966-968.

Flagg, J. L. and T. H. Wilson. 1977. A protonmotive force as the source of energy for galactoside transport in energy-depleted Escherichia coli. J. Membr. Biol. **31**:233-255.

Fox, R. B. and W. K. Fox. 1983. Dimethyl sulfoxide prevents hydroxyl radical-mediated depolymerization of hyaluronic acid. In J. C. de la Torre (ed.). Biological Actions and Medical Applications of Dimethyl Sulfoxide. New York Academy of Sciences. New York.

Freeman, B. A. 1979. Burrows Textbook of Microbiology. 21st ed. W. B. Saunders Co. Philadelphia.

Goodman, T. G. and P. S. Hoffman. 1983. Hydrogenase activity in catalase-positive strains of Campylobacter spp. J. Clin. Microbiol. **18**:825-829.

Halliwell, B. 1979. Oxygen-free-radicals in living systems: dangerous but useful? pp. 195-221. In M. Shilo (ed.). Strategies of Microbial Life in Extreme Environments. Verlag-Chemie, New York.

Holdeman, L. V. and W. E. C. Moore. (ed). 1975. Anaerobe Laboratory Manual. V. P. I. Anaerobe Laboratory. Blacksburg, VA.

Hylemon, P. B., J. S. Wells, Jr., J. H. Bowdre, T. O. Macadoo, and N. R. Krieg. 1973a. Designation of Spirillum volutans Ehrenberg 1832 as type species of the genus Spirillum Ehrenberg 1832 and designation of the neotype strain of S. volutans. Int. J. Syst. Bacteriol. **23**: 20-27.

Hylemon, P. B., J. S. Wells, Jr., N. R. Krieg, and H. W. Jannasch. 1973b. The genus Spirillum: a taxonomic study. Int. J. Syst. Bacteriol. **23**:340-380.

Inoue, K. 1976. quantitative ecology of microorganisms of Syowa Station in Antarctica and isolation of psychrophiles. J. Gen. Appl. Microbiol. **22**:143-150.

Inoue, K. and K. Komagata. 1976. Taxonomic study on obligately psychrophilic bacteria isolated from Antarctica. J. Gen. Appl. Microbiol. **22**:165-176.

Jannasch, H. W. 1965. Die Isolierung heterotropher aquatischer Spirillen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig., Suppl. **1**:198-203.

Jannasch, H. W. 1967. Enrichments of aquatic bacteria in continuous culture. Arch. Mikrobiol. **59**:165-173.

Jannasch, H. W. 1977. Growth kinetics of aquatic bacteria. In F. A. Skinner and J. M. Shewan (eds.). Aquatic Microbiology. Academic Press. New York.

Krieg, N. R. 1976. Biology of chemoheterotrophic spirilla. Bacteriol. Rev. **40**:55-115.

Krieg, N. R. 1981. The genera Spirillum, Aquaspirillum, and Oceanospirillum, pp. 595-608. In M. P. Starr, H. Stolp, H. G. TrÜper, A. Balows, and H. G. Schlegel (eds.). The Prokaryotes. vol. 2. Springer-Verlag. New York.

Krieg, N. R. 1984a. Genus Aquaspirillum, pp. 72-90. In J. G. Holt and N. R. Krieg (eds.). Bergey's Manual of Systematic Bacteriology. vol. 1. Williams and Wilkins. Baltimore, MD.

Krieg, N. R. 1984b. Genus Oceanospirillum, pp. 104-110. In J. G. Holt and N. R. Krieg (eds.). Bergey's Manual of Systematic Bacteriology. vol. 1. Williams and Wilkins. Baltimore, MD.

Krieg, N. R. and P. Gerhardt. 1981. Solid culture. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, G. B. Phillips (eds.). Manual of Methods for General Bacteriology. American Society for Microbiology. Washington, DC.

Kuenen, J. G., H. M. Hassan, N. I. Krinsky, J. G. Morris, N. Pfennig, H. Schlegel, M. Shilo, G. D. Vogels, U. Weser, and R. Wolfe. 1979. Oxygen toxicity group report. pp.223-241. In M. Shilo (ed.). Strategies of Microbial Life in Extreme Environments. Verlag-Chemie, New York.

Kumar, R., A. K. Banerjee, J. H. Bowdre, L. J. McElroy, and N. R. Krieg. 1974. Isolation, characterization, and taxonomy of Aquaspirillum bengal sp.nov. Int. J. Syst. Bacteriol. **24**:453-548.

Lamanna, C., M. F. Mallette, and L. N. Zimmerman. 1973. Basic Bacteriology. Williams & Wilkins Co. Baltimore, MD.

Leifson, E. 1962. The bacterial flora of distilled and stored water. III. New species of the genera Corynebacterium, Flavobacterium, Spirillum and Pseudomonas. Int. Bull. Bacteriol. Nomen. Taxon. **12**:161-170.

Lewis, C., H. W. Clapp, and J. E. Grady. 1963. In vitro and in vivo evaluation of lincomycin, a new antibiotic. pp. 570-582. Antimicrobial Agents and Chemotherapy - 1962.

Linn, D. M. and N. R. Krieg. 1978. Occurrence of two organisms in cultures of the type strain of Spirillum lunatum: proposal for rejection of the name Spirillum lunatum and characterization of

Oceanospirillum maris subsp. williamsae and an unclassified vibrioid bacterium. Int. J. Syst. Bacteriol. **28**:132-138.

Luria, S. E., and J. E. Darnell, Jr. 1967. General Virology. John Wiley & Sons, Inc. New York.

McElroy, L. J., J. S. Wells, Jr. and N. R. Krieg. 1967. Mode of extension of cell surface during growth of Spirillum volutans. J. Bacteriol. **93**:499-501.

Morris, J. G. 1979. Nature of oxygen toxicity in anaerobic microorganisms, pp.149-162. In M. Shilo (ed.). Strategies of Microbial Life in Extreme Environments. Verlag-Chemie, New York.

Padgett, P. J., W. H. Cover, and N. R. Krieg. 1982. The microaerophile Spirillum volutans: cultivation on complex liquid and solid media. Appl. Environ. Microbiol. **43**:469-477.

Padgett, P. J. and N. R. Krieg. 1986. Factors relation to the aerotolerance of Spirillum volutans. Can. J. Microbiol. **32**:548-552.

Reed, L. J. and H. Meunch. 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. **27**:493-497.

Rittenberg, B. T. and S. C. Rittenberg. 1962. The growth of Spirillum volutans Ehrenberg in mixed and pure cultures. Arch. Mikrobiol. **42**:138-153.

Schweizer, C., and M. Aragno. 1975. Etude des hydrogénobactéries dans un petit lac (le Loclat, ou lac de Saint-Blaise). Bull. Soc. Neuchatel. Sci. Nat. **98**:79-87.

Sherma, J. 1972. Principles and techniques. In G. Zweig and J. Sherma (eds.). Handbook of Chromatography. vol. 1. CRC Press. Cleveland, OH.

Smibert, R. M. 1984. Genus Campylobacter. pp. 111-118. In J. G. Holt and N. R. Krieg (eds.). Bergey's Manual of Systematic Bacteriology. vol. 1. Williams and Wilkins. Baltimore, MD.

Stainer, W. D. and M. J. Scholte. 1970. A simple chemically defined medium for the production of phase I Bordetella pertussis. J. Gen. Microbiol. **63**:211-220.

Steiner, B. M., G. H. W. Wong, P. Sutrave, and S. Graves. 1984. Oxygen toxicity in Treponema pallidum: deoxyribonucleic acid single-stranded breakage induced by low doses of hydrogen peroxide. Can. J. Microbiol. **30**:1467-1476.

Strength, W. J., B. Isani, D. M. Linn, F. D. Williams, G. E. Vandermolen, B. E. Laughon, and N. R. Krieg. 1976. Isolation and characterization of Aquaspirillum fasciculus sp. nov., a rod-shaped, nitrogen-fixing bacterium having unusual flagella. Int. J. Syst. Bacteriol. **26**:253-268.

Tarrand, J. J., N. R. Krieg, and J. Döbereiner. 1978. A taxonomic study of the Spirillum lipoferum group, with descriptions of a new genus, Azospirillum gen.nov. and two species. Azospirillum lipoferum (Beijerinck) comb.nov. and Azospirillum brasilense sp.nov. Can. J. Microbiol. **24**:967-980.

Terasaki, Y. 1980. Enrichment and isolation of aerobic chemoheterotrophic spirilla from mud and sand samples. J. Gen. Appl. Microbiol., **26**:395-402.

Tristram, G. R. 1953. The amino acid composition of proteins. In H. Neurath and K. Bailey (eds.). The Proteins. vol. 1. part A. Academic Press Inc. New York.

Tyrrell, E. A., R. E. MacDonald, and P. Gerhardt. 1958. Biphasic system for growing bacteria in concentrated culture. J. Bacteriol. **75**:1-4.

Washington II, J. A. 1985. Susceptibility tests: agar dilution. pp. 967-971. In E. H. Lennette (ed.). Manual of Clinical Microbiology. American Society for Microbiology. Washington, D.C.

Wells, J. S. Jr., and N. R. Krieg. 1965. Cultivation of Spirillum volutans in a bacteria-free environment. J. Bacteriol. **90**:817-818.

Williams, M. A. and S. C. Rittenberg. 1957. A taxonomic study of the genus Spirillum Ehrenberg. Int. Bull. Bacteriol. Nomen. Taxon. **7**:49-111.

Windholz, M., S. Budavari, L. Y. Stroumtsos, and M. N. Fertig. (eds.) 1976. The Merck Index. Merck and Co. Inc. Rahway, NJ.

Yayanos, A. A., Dietz, A. S., and R. V. Boxtel. Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. Sci. **205**:808-810.

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