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Improved Methods for Cultivation of the Extremely Thermophilic Bacterium *Thermotoga neapolitana*

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Growth medium components and cultivation conditions for the extremely thermophilic bacterium *Thermotoga neapolitana* were optimized. A defined marine salts medium was formulated. Trace amounts of iron stimulated growth of *T. neapolitana*, while zinc inhibited growth at concentrations exceeding 11.1 μM. Other trace metals had no effect on its growth. Of the vitamins tested, only biotin was required for optimal growth. A defined mineral medium containing 5 g of carbohydrates per liter as the carbon source and 0.5 g of cysteine per liter as the sulfur source and reductant supported growth. Growth was stimulated by inclusion of vitamin-free Casamino Acids. Elemental sulfur, cystine, and dimethyl disulfide in the growth medium enhanced growth. Elemental sulfur and cystine relieved growth inhibition by hydrogen. *T. neapolitana* formed colonies in 2 days on plates of complex medium solidified with gellan gum and in 4 days on defined medium. The efficiency of plating was determined when growing cultures were sampled both aerobically and anaerobically and plated under aerobic and anaerobic conditions. Mean plating efficiencies were improved by sampling the growing cultures under strictly anaerobic conditions. Little or no improvement was obtained by inoculating plates inside an anaerobic chamber. Plating efficiencies of approximately 80% were obtained. Polycarbonate jars with aluminum lids withstood repeated incubation at 77°C without significant deterioration of the anaerobic seal and provided the most consistent results.

Phylogenetic analyses have demonstrated that extreme thermophiles occupy the deepest branches of both the archaeal and bacterial lineages (13, 14). This led to a hypothesis that their common ancestor was also an extreme thermophile (1). Comprehensive genetic and physiological characterization of these thermophiles may shed light on the nature of the common ancestor. In addition, these investigations will lend perspective to our understanding of more distally branching organisms by providing benchmarks for comparative analyses. With these goals in mind, this study was undertaken to establish a technical basis for the development of genetic tools to study *Thermotoga neapolitana*, a member of the deepest branch of the bacterial lineage.

T. neapolitana was isolated from a shallow marine sediment in a volcanic region near Lucrino, Bay of Naples, Italy (3). It is an obligate anaerobe with an optimum growth temperature of 77°C. *T. neapolitana* is closely related to the type species *T. maritima* (9). It catabolizes mono- and polysaccharides, while organic acids, alcohols, and amino acids do not support growth. Like many archaeal extreme thermophiles, it reduces elemental sulfur to hydrogen sulfide. Unlike those archaea, it is not dependent upon sulfur reduction for growth. It has been reported that final cell yields are doubled by inclusion of sulfur in the growth medium but that their growth rate remains unaffected (3).

In this report, we present the results of attempts to optimize the conditions necessary for cultivation of *T. neapolitana*. We determined the required medium components and their optimal concentrations to formulate a defined growth medium. To facilitate genetic manipulations, we devised methods to grow *T. neapolitana* in a reproducible manner on solid media. Strict anaerobes are usually grown on solid media contained in bottles (7) or plates which are incubated inside metal cylinders (2). The former are relatively expensive and not readily adapted to standard genetic techniques, while the latter require specially manufactured cylinders. We attempted to develop methods of cultivation on solid media by using less expensive, commercially available supplies.

This report demonstrates that *T. neapolitana* is suitable for studies of the basic molecular biology of extreme thermophiles. It can be grown on both solid and liquid media with minimal anoxic precautions and with readily available materials. Although it is a strict anaerobe at elevated temperatures, at room temperature it can be plated on the bench and then incubated under a nitrogen atmosphere. These features will allow investigators the opportunity to explore many aspects of this evolutionarily important bacterium.

MATERIALS AND METHODS

Organisms and culture conditions. *T. neapolitana* NS-E was kindly provided by H. Jannasch (Woods Hole Oceanographic Institute) via P. Rouviére (University of Wisconsin). It was routinely grown on a common marine medium that is based on one-half-strength Turks Island salts (2, 5). This medium contained the following components (amounts are in grams per liter): NaCl, 20; KCl, 0.335; $MgCl_2 \cdot 2H_2O$, 2.75; $MgSO_4 \cdot 7H_2O$, 3.45; NH_4Cl, 0.25; $CaCl_2 \cdot 2H_2O$, 0.14; K_2HPO_4, 0.14; glucose, 5.0; yeast extract, 0.5; 1,4-piperazine-*N*,*N*′-bis(2-ethanesulfonate) (PIPES) · 1.5 Na, 6.0; cysteine hydrochloride, 0.5; resazurin, 0.001. This was supplemented with 1 ml each of a trace mineral solution and a trace vitamin solution (2). The trace mineral solution contained the following (in grams per liter): nitrilotriacetic acid, 1.5; $MgSO_4 \cdot 7H_2O$, 3; NaCl, 1; $MnCl_2 \cdot 4H_2O$, 0.56; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.37; $CoCl_2 \cdot 6H_2O$, 0.34; $ZnSO_4 \cdot 7H_2O$, 0.32; $NiSO_4 \cdot 6H_2O$, 0.21; $NaSeO_4$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $AlK(SO_4)_2 \cdot 12H_2O$, 0.033; H_3BO_3, 0.01; $CuSO_4$, 0.004. The vitamin solution contained the following (in milligrams per liter): pyridoxine hydrochloride, 10; cal-

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cium pantothenate, 5; nicotinic acid, 5; *p*-aminobenzoic acid, 5; riboflavin, 5; thiamine hydrochloride, 5; lipoic acid, 5; biotin, 2; folic acid, 2; cyanocobalamin, 0.1. Modifications of this basal medium formulation are given elsewhere in the text. For defined medium, yeast extract was omitted. The medium was adjusted to pH 7.5 with NaOH. It was dispensed into serum bottles or tubes, degassed by heating in a steam box until the solution was colorless, stoppered with butyl rubber stoppers, and sealed with aluminum crimp seals, and the atmosphere above the medium was exchanged with oxygen-free nitrogen by five cycles of evacuation and pressurization while the medium was still hot. The atmosphere in the tubes was pressurized to 100 kPa, and the medium was sterilized by autoclaving. Inocula were typically 1% (vol/vol).

For experiments involving sulfur substrates, trace metals were omitted to prevent precipitation of metal sulfides which interfered with optical density measurements. Elemental sulfur and solid cystine were added to 10 ml of *Thermotoga* basal (TB) medium containing starch and yeast extract (TBSY; see Table 1), and the media were tyndallized by being heated twice to 100°C for approximately 1 h each time. Dimethyl disulfide was added to sterile TBSY medium from a stock solution in 100% ethanol. Cysteine was added as a reductant from a sterile solution after the media were sterilized. Since dimethyl disulfide was added from an ethanol solution, the effect of ethanol on growth was assessed and it was found to have little inhibitory effect (data not shown).

Plating of *T. neapolitana*. Solid media were prepared by including 0.7% (wt/vol) GELRITE (Kelco Div. of Merck & Co., San Diego, Calif.). GELRITE was added at a concentration of 14 g/liter to vigorously stirred water to prevent clumping. A solution of 2× medium was prepared, and each solution was sterilized promptly by autoclaving. The two solutions were combined while still hot, and the plates were poured while the medium was still hot. The medium gelled within minutes of being poured to form a clear, firm gel. The plates were immediately placed inside an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich.) for long-term storage. Although the resazurin indicator was pink while the plates were poured, it soon became colorless inside the chamber.

Samples were removed from growing cultures by using disposable tuberculin syringes. When sampling was performed outside the anaerobic chamber, the syringe was flushed twice with sterile nitrogen before a 0.2-ml sample was removed. When cystine was present in the medium, the resazurin in the sample did not change color. After the sample was taken, the syringe needle was inserted into a rubber stopper. When the sample cooled to room temperature, it was diluted and plated. Plates were inoculated by using standard streaking and spreading techniques outside the anaerobic chamber. For plating of dilutions of cultures, a diluent consisting of sterile *Thermotoga* mineral base was used (see Table 1 for the composition of *Thermotoga* mineral base). When dilutions were performed inside the anaerobic chamber, the diluent was degassed by passage through the airlock and left inside the chamber for at least 24 h before use. Aliquots of these dilutions were spread on plates by using a bent Pasteur pipette. When inoculation was performed under aerobic conditions, the plates were returned to the chamber. After both aerobic and anaerobic inoculations, the plates remained inside the chamber overnight to ensure complete reduction of the medium. The plates were sealed inside one of two types of anaerobic jar: either a Vacu-Quik jar (Almore International, Inc., Portland, Oreg.) or a vented BBL GasPak 100 anaerobic jar (Becton Dickinson Labware, Inc., Lincoln Park, N.J.). Pellets of a palladium catalyst (4 g) were placed inside each type of jar. The vent in the lid of the GasPak jar was sealed with a no. 1 black rubber stopper with a hole drilled partially through, and the stopper was wired in place. The 96:4 N_2-H_2 atmosphere inside the chamber provided sufficient reductant to remove traces of oxygen in the jar without inhibiting growth of the organism by excess hydrogen. The sealed jar was removed from the chamber and placed in an incubator at 77°C. Colonies were typically visible after 48 h. To examine the colonies, the jars were allowed to cool to room temperature before being opened. The palladium catalyst was regenerated after each use.

TABLE 1. Composition of TB medium[a]

Component	Final concn (liter^{-1})
Thermotoga mineral base	
PIPES · 1.5 Na salt	6 g
NaCl	20 g
KCl	2 g
$MgSO_4 \cdot 7H_2O$	500 mg
NH_4Cl	250 mg
$CaCl_2 \cdot 2H_2O$	50 mg
K_2HPO_4	50 mg
Trace metals	
$FeSO_4 \cdot 7H_2O$	7 mg
$Na_2WO_4 \cdot 2H_2O$	0.3 mg
Biotin	20 μg
Reducing agent-redox indicator	
Cysteine hydrochloride	500 mg
Resazurin	1 mg

[a] The pH of the medium was adjusted to 7.5. Supplements were added to produce TBS medium (TB medium containing 5 g of soluble starch per liter), TBG medium (TB medium containing 5 g of glucose per liter), or TBSY and TBGY media (0.5 g of yeast extract per liter added to TBS and TBG media, respectively).

Growth measurements. Growth was measured spectrophotometrically with a Spectronic 20 spectrophotometer equipped to hold 18-mm-diameter tubes. Direct cell counts were performed with a Petroff-Hauser counting chamber viewed under a phase-contrast microscope. Viable counts were performed in triplicate by plating cells as described above. Plating efficiency was determined by dividing the number of CFU by the direct cell count and multiplying the result by 100.

Materials. Chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific (Springfield, N.J.). Falcon polystyrene petri dishes were obtained from Becton Dickinson Labware, Inc. Glass culture tubes and butyl rubber serum stoppers were from Bellco Glass, Inc. (Vineland, N.J.). The palladium catalyst was from Coy Laboratory Products, Inc.

RESULTS

Medium composition. To determine the optimal medium for cultivation of *T. neapolitana*, the marine mineral base of Balch et al. was used as a starting formulation (2). Concentrations of each component that yielded maximal growth after 60 h were determined, and the results are listed in Table 1. Addition of magnesium chloride along with magnesium sulfate was found to be unnecessary, so it was omitted from

the medium. The magnesium sulfate concentration was reduced 70-fold with no effect on either growth yields or rates. The other components were present in optimal concentrations, except potassium chloride, which was present at one-sixth of the optimal concentration. When the trace mineral solution was omitted, growth was slightly enhanced. Individual components of the solution were tested, and zinc chloride was found to inhibit growth at concentrations above 11.1 μM. Ferrous sulfate at concentrations of 25 to 50 μM stimulated growth. The other components of the trace mineral solution did not consistently stimulate or inhibit growth. Tungsten has been shown to stimulate hydrogenase activity in *T. maritima*, although no enzyme has been shown to use it as a cofactor (10). We tested the effect of sodium tungstate on growth and found that it had no effect. If it is a required nutrient, it must be present in sufficient quantities as a trace contaminant of other medium components.

Organic nutrients were also tested for their effects on growth. *Thermotoga* species use carbohydrates as sources of energy and carbon (9). Starch gave better growth than lactose or glucose when added in equivalent concentrations (wt/vol). Supplements to starch media stimulated growth in the following decreasing order of stimulation: yeast extract > Bacto-Peptone > vitamin-free Casamino Acids. Maximal growth on defined medium was achieved in 24 h.

Growth often ceased after several transfers in a vitamin-free medium. Growth dependence on individual vitamins was determined by omitting a vitamin from the medium and observing the number of successful transfers that could be made in that medium. Upon the sixth transfer on vitamin-depleted medium, growth in medium lacking biotin gave a final optical density that was only 28% of that of the control tube containing all of the vitamins in the mixture. A tube containing no vitamins gave a final optical density that was only 2% of that of this control. Media lacking other vitamins gave optical densities that were 75 to 93% of that of this control.

Sulfur substrates. The growth yield of *T. neapolitana* in liquid medium was enhanced by addition of elemental sulfur to the medium (Fig. 1). It has been postulated that the sulfur prevents accumulation of inhibitory concentrations of hydrogen, a product of its sugar fermentation (8). Cystine and dimethyl disulfide stimulated growth better than did elemental sulfur (Fig. 1).

Like elemental sulfur, cystine allowed *T. neapolitana* to grow in the presence of an otherwise inhibitory concentration of hydrogen (Fig. 2). When cells grown either with or without elemental sulfur were inoculated into medium containing sulfur under a hydrogen atmosphere, both cultures began growing at the same time (data not shown). A significant lag was observed when cells were grown on cystine but not when they were grown on elemental sulfur (Fig. 2). The inoculum used in the experiment whose results are illustrated in Fig. 2 had been grown with elemental sulfur present, but similar results were obtained with an inoculum grown on cystine (data not shown). This suggests that the mechanism of hydrogen detoxification using cystine may be different from that which uses elemental sulfur.

Cultivation on solid media. Gellan gum (GELRITE) has proven to be superior to agar as a solidifying agent for cultivation of thermophiles (6, 12). We also found significantly better plating efficiencies on 0.7% GELRITE than on 3% agar for *T. neapolitana* (data not shown). The lower concentration of divalent cations in *Thermotoga* mineral base compared with the starting mineral base caused the gel to solidify at a lower temperature, which simplified plate preparation.

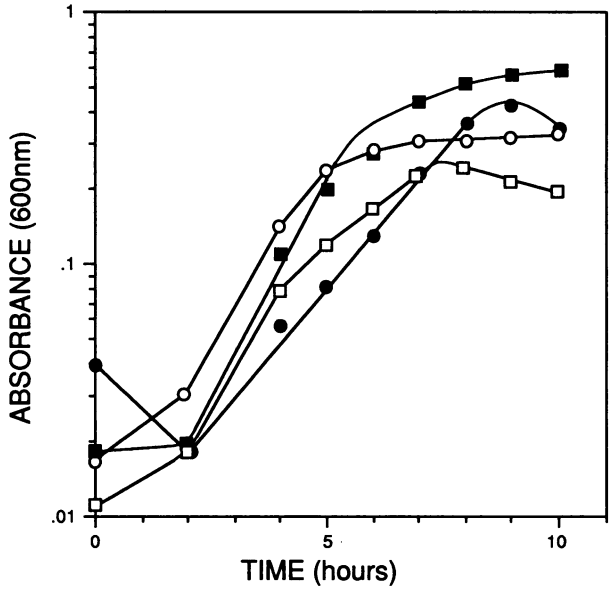

FIG. 1. Growth stimulation by sources of sulfane sulfur. Media (10 ml) contained either cystine (10 mg of sulfur) (■), dimethyl disulfide (6.8 mg of sulfur) (●), elemental sulfur (10 mg of sulfur) (○), or no sulfur (□). Cultures were incubated under a nitrogen atmosphere. Each datum point represents an average of three tubes.

We attempted to use polycarbonate jars that are used to cultivate some anaerobes. Our first attempts made use of vented BBL GasPak 100 anaerobic jars. After several months of use, however, we found that some of the jars allowed the plates to oxidize during incubation. Upon inspection of the jars, significant deterioration of the polycar-

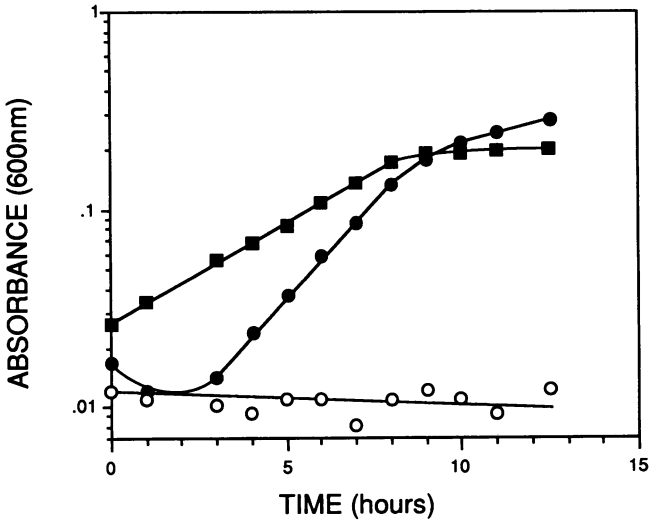

FIG. 2. Relief of hydrogen-induced growth inhibition by elemental sulfur and cystine. Cultures were incubated under an atmosphere of 100% hydrogen. Media (10 ml) contained either cystine (20.4 mg of sulfur) (●), elemental sulfur (20.4 mg of sulfur) (■), or no sulfur (○). The inoculum had been grown in a medium containing elemental sulfur. Each datum point represents an average of three tubes.

TABLE 2. Mean plating efficiencies obtained under different sampling and plating conditions

Time (h) after inoculation	Mean plating efficiency (±1 SD)[a] (%)		
	Aerobic sampling, aerobic plating	Anaerobic sampling, aerobic plating	Anaerobic sampling, anaerobic plating
0	30 ± 2.3	26 ± 8.6	21 ± 0.7
3	52 ± 6.2	84 ± 18.8	81 ± 19.0
5	24 ± 8.2	62 ± 4.2	80 ± 0.07
7	70 ± 4.8	77 ± 7.1	75 ± 14.1
9	44 ± 13.3	81 ± 15.3	81 ± 13.2

[a] Means of two to four determinations, each performed in triplicate, are shown. Cells were grown on TBSY medium (containing 0.05% cystine but lacking trace metals) which had been solidified with 0.7% GELRITE.

bonate rims of the jars was found. The deterioration corresponded to the point of contact between the jar rim and the O-ring contained in the polycarbonate lid. The pressure exerted by the O-ring deformed the polycarbonate at the high incubation temperature. To alleviate the degradation of the polycarbonate, we tried a number of methods to seal the jar, including use of a butyl rubber gasket in combination with the O-ring, fingertip caulk, paraffin, vacuum grease, and a weighted glass lid. None of these gave consistent results.

We have consistently obtained anaerobiosis for several months of continuous use with the Vacu-Quik gas jar. It is fitted with two sealing screws, a pressure gauge, two valved gassing ports, and a Quad-Seal O-ring fitted in an anodized aluminum lid. We did not observe the kind of deterioration of the clear polycarbonate jars that we observed with the GasPak jars.

Using these methods, we were able to obtain mean plating efficiencies of up to 84%, depending upon the growth stage of the inoculum (Table 2). The decrease in plating efficiency observed at 5 h of growth was reproducible and corresponded to the middle of the log phase. To determine the cause of the low plating efficiencies, we tested both the sampling and the plating methods. When samples were withdrawn inside the anaerobic chamber, the mean efficiencies increased at each stage of growth, except for the initial inoculation. At the 5-h time point, a 2.6-fold increase was observed. Although the samples withdrawn under aerobic conditions were not exposed to air sufficient to oxidize the resazurin indicator, oxidation sufficient to kill rapidly dividing cells may have occurred. Plating of these cells inside the anaerobic chamber resulted in a further 1.3-fold improvement in plating efficiency at the 5-h time point and no improvement at the other sampling times. Thus, the most dramatic improvements were obtained when strict anaerobic techniques were used during the sampling phase of plate inoculation.

We also observed profound changes in the morphology of cells during growth, which may play a role in determining plating efficiencies. The inoculum generally contained large spherical cells which fragmented into smaller spheres during the lag phase, resulting in an increase in cell numbers with little increase in optical density (Fig. 3). Upon further incubation, these spheres increased in size, became more oblong, and were frequently present in pairs. At that point, the rod-shaped cells attained the characteristic cell shape with cell envelope material ballooning over the ends of the cells (the "toga") (8). In the late-log phase and the stationary phase, each cell began to condense into a large refractile sphere with less dense cell material protruding from either end. These spheres have been observed in stationary phase

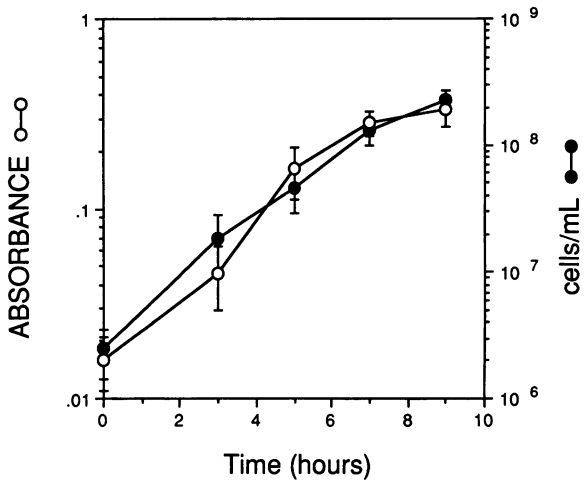

FIG. 3. Kinetics of growth of *T. neapolitana* on TBSY medium containing 0.05% cystine but lacking trace metals. Growth was monitored spectrophotometrically (660 or 600 nm) and by direct cell counting. Each datum point represents the mean of five experiments with ± 1 standard deviation of each point shown.

cultures of *T. maritima* (8) and in *T. neapolitana* grown on complex media (3). All of the cells in the cultures did not progress through these stages in synchrony. The large spheres that formed in the stationary phase were as culturable as the rod-shaped cells. They apparently become less culturable, as evidenced by the fact that the plating efficiency of freshly inoculated cells was substantially lower. We did not measure the loss of culturability of these cells during prolonged storage.

DISCUSSION

We began with a common marine medium that is based on one-half-strength Turks Island salts (2, 5). We found that *T. neapolitana* gave the best cell yields in media with a 26-fold lower Mg^{2+} concentration and a 2.8-fold lower Ca^{2+} concentration than the starting medium. This lower concentration of divalent cations significantly improved the preparation of GELRITE plates, since divalent cations promote its gelling. This was somewhat offset by the fact that the optimal K^+ concentration is 5.2-fold greater than in the starting medium.

In accord with previous findings on *T. maritima* (10), we found that ferrous sulfate stimulated both the growth rate and the final cell yield. Components of the trace mineral solution of Balch et al. were tested for their effects on growth, and zinc was found to inhibit growth at concentrations above 11.1 μM. Tungsten was reported to increase the specific activity of hydrogenase isolated from *T. maritima*, although it had no effect on its growth (10). We did not observe any effect of tungsten on *T. neapolitana* growth, but we included it in the media since it may play a role in cell metabolism.

Growth of *T. neapolitana* declined after several transfers in a medium lacking vitamins. Biotin was necessary for maximal growth. The other vitamins in the original mixture do not stimulate or inhibit growth.

T. neapolitana uses cystine and dimethyl disulfide, in addition to elemental sulfur, as electron sinks. These compounds allow it to grow in the presence of otherwise inhibitory concentrations of hydrogen. The archaeon *Pyrococcus*

furiosus also reduces elemental sulfur to circumvent hydrogen intoxication. *P. furiosus* also uses cystine and dimethyl trisulfide as electron sinks, but dimethyl disulfide cannot be used (4). Elemental sulfur, cystine, and dimethyl trisulfide (but not dimethyl disulfide) form polysulfides in culture media incubated at 98°C for 12 h (4). Since *P. furiosus* uses polysulfides as an electron sink, those researchers suggested that the polysulfides produced from these compounds are the actual electron acceptors. *T. neapolitana* may use other sulfanes, as suggested by the following findings. (i) The results of Blumentals et al. suggest that polysulfides would not be generated from dimethyl disulfide at 77°C during growth of *T. neapolitana* (4). (ii) Small amounts of polysulfide may form from elemental sulfur during tyndallization or incubation, but there is no evidence that suggests that they would form from cystine. (iii) That growth stimulation by cystine may proceed by a mechanism different from that of elemental sulfur is also indicated by growth kinetics. We found that growth with elemental sulfur did not require a period of adaptation, regardless of the source of the inoculum (data not shown), while growth on cystine did appear to elicit a growth lag. This lag was also independent of the growth medium used to grow the inoculum (data not shown). Accumulation of a sulfane from cystine may limit the growth rate. The rate of accumulation of the sulfane may increase as the culture grows. These data suggest that *T. neapolitana* may use sulfane sulfur as an electron acceptor in a manner unlike that found in *P. furiosus*, in which these compounds may induce production of specific proteins (11).

Our efforts to develop methods to grow *T. neapolitana* on solid media have resulted in the finding that polycarbonate jars can be used for cultivation. We found that the Almore Vacu-Quik jar maintained anaerobiosis after prolonged use, while the GasPak jar deteriorated with use. Unlike autotrophic anaerobes, *T. neapolitana* does not require a pressurized atmosphere for growth, so these light-weight jars are adequate.

We obtained reasonable plating efficiencies, even when cells were spread on plates on the laboratory bench. Efficiencies improved and became less dependent upon the growth phase when samples of growing cultures were removed inside an anaerobic chamber. Little improvement in efficiency was noted at most phases of growth when the plates were inoculated inside the chamber as well. Log-phase cells (5 h after inoculation) plated 1.3 times better under anaerobic conditions than under aerobic conditions. If their apparent oxygen sensitivity is due to their rapid metabolism, one should obtain efficiencies greater than 62% by slowing their metabolism before sampling. This can be accomplished by cooling the culture before sampling. This should not affect most routine genetic procedures.

We have found that plates stored under anaerobic conditions immediately after preparation permit faster colony growth than plates stored in air. We are attempting to devise methods which would allow storage of plates under aerobic conditions, so that an anaerobic chamber would not be necessary.

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