JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1982, p. 798–802 Vol. 16, No. 5
0095-1137/82/110798-05$02.00/0
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Growth Requirements of *Haemophilus somnus*

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Received 17 May 1982/Accepted 26 July 1982

The growth factor needs of *Haemophilus somnus*, which have not been defined to date, were found to be provided by 1% IsoVitaleX (IVX; BBL Microbiology Systems) in tryptose broth. Some growth, however, occurred in unsupplemented tryptose broth. Of the ingredients of IVX, cocarboxylase was found to stimulate growth to about the same degree as the total supplement. Cocarboxylase was without direct effect in 2% peptone broth, which supported no growth of 25 *H. somnus* strains until supplemented with IVX, optimally at the 10% level. This could be substituted for by proportional amounts of cysteine or cystine, but by no other IVX ingredient. Cysteine-cystine and IVX but not cocarboxylase supplementation allowed *H. somnus* to grow in Eagle minimal medium, a completely synthetic medium, but attempts at serial passage were unsuccessful.

The bacterium at present most commonly referred to as *Haemophilus somnus* was originally described as a *Haemophilus*-like organism because of its inability to grow satisfactorily in various meat infusion and digest media unless these were supplemented with blood or a contaminating feeder bacterium allowing satellitic growth was present (11). Subsequent studies showed the freshly isolated organism to be capable of propagating on media enriched with other supplements, including yeast extract or hydrolysate and serum (4, 5, 13, 14), whereas other investigators found bloodless media unsatisfactory even with established laboratory cultures (16). Limited growth has been observed occasionally in some unsupplemented media (1, 2).

The minimal needs in terms of chemically defined growth factors supplied by the enrichments have not been identified. There is general agreement, however, that they do not include requirements for factors X (hemin) or V (NAD), which are associated with recognized members of the genus *Haemophilus* (1, 4, 10, 11, 15, 17, 18; W. E. Bailie, Ph.D. thesis, Kansas State University, Manhattan, 1969). In fact, the ability of *H. somnus* to convert delta-aminolevulinic acid to porphyrin, which is correlated with X-factor independence, has been repeatedly demonstrated (2). Asmussen and Baugh reported a significant stimulatory effect of thiamine pyrophosphate (cocarboxylase [COC]) on the growth of *H. somnus* cultures in brain heart infusion broth, as determined turbidimetrically. They described 7 of 10 of their test strains as having an "absolute requirement for thiamine monophosphate or thiamine pyrophosphate" (1).

The object of the present study was the identification of growth factor requirements for the propagation of *H. somnus*. A necessary preliminary step was the identification of a truly basal medium, i.e., one that would not support any growth of *H. somnus* in the absence of chemically defined supplement(s).

The success obtained in the propagation of *Legionella* spp. with the aid of IsoVitaleX (IVX; BBL Microbiology Systems, Cockeysville, Md.; 13) and in the identification of the critical ingredients (8) recommended that mixture of potential growth factors to our use as a supplement. Once its effectiveness had been established (1, 2), its synthetic nature promised to permit a further narrowing down of constituents to those essential for the growth of *H. somnus*.

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MATERIALS AND METHODS

Bacterial cultures. Five cultures were employed in the search for a basal medium and identifiable growth factors. Two originated from bovine meningoencephalitis (2078, 632), and three originated from bovine pneumonia (734, 805, 2106). Two had been isolated in Colorado (632, 705) and were made available to us through the cooperation of A. B. Hoerlein. The remaining three were recovered in California. The validity of the conclusions reached on the basis of these exploratory experiments was then tested with the aid of an additional 20 cultures from a collection of strains previously described (3).

The strains were reconstituted from the freeze-dried state with nutrient broth (Difco Laboratories, Detroit, Mich.) and cultured on chocolate agar. Incubation was

TABLE 1. Comparative growth at 48 h of five *H. somnus* strains through eight passages in tryptose broth supplemented with four different IVX-derived formulas

Supplement	No. of observations	Group mean (10^7 CFU/ml)[a]	SEM
IVX	40	$7.5^{A,B}$	0.85771
IVX less NAD	40	8.7^{A}	0.85771
IVX less COC	40	2.9^{C}	0.85771
COC	40	5.4^{B}	0.85771
None	32	1.8^{C}	0.95895

[a] Means with identical superscripts do not significantly differ from one another statistically (protection level, 96% over all comparisons). CFU, Colony-forming units.

at 37°C in a candle jar. Growth was harvested with a loop and suspended in 1 ml of skim milk in screw-capped tubes (13 by 100 mm). Suspensions were stored at −70°C. Sufficient tubes of each of the five principal strains were prepared to permit the use of a freshly thawed sample in each experiment and, thus, to minimize the subpassaging of test cultures.

The identity of the cultures as *H. somnus* was confirmed by cultural tests (17).

Media. Chocolate agar was prepared from GC Medium Base (Difco) according to directions. The 1% supplement added was enrichment no. 1. (Bakte Bennett Laboratory, Berkeley, Calif.) consisting of (grams/liter): glucose (250), L-cysteine hydrochloride (26), L-glutamine (10), L-cystine (1.1), adenine (1), NAD (0.25), guanine (0.03), thiamine hydrochloride (0.003), ferric nitrate (0.02), *para*-aminobenzoic acid (0.013), vitamin B-12 (0.01), and COC (1.1).

Tryptose, nutrient, and proteose-peptone broths were prepared from dehydrated media (Difco) by the manufacturer's directions. Peptone and tryptone broth were 2% solutions of the respective substances (Difco) in 0.5% sodium chloride solutions. Further processing was as for the dehydrated media. In experiments involving the supplementation of peptone broth, the supplemented media, after the adjustment of the pH to 6.8 with 2 M Na_2HPO_4, were sterilized by passage through a 0.2-μm Nalgene filter (Sybron Corp., Rochester, N.Y.) and dispensed in 5-ml amounts in screw-capped vials (13 by 100 mm).

TC-Eagle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) was handled according to the supplier's instructions.

Supplements. One drop of bovine defibrinated blood (about 0.05 ml) was added to every 5 ml of tryptose or peptone broth or MEM in the preparation of positive control culture media.

IVX was added under sterile precautions to a final concentration of 1 or 10%. The prepared, commercially obtained product was duplicated and modified by various combinations of stock solutions of its several ingredients: vitamin B-12 (Eastman-Kodak [EK]), L-glutamine (EK), adenine (EK), guanine hydrochloride (EK), *p*-aminobenzoic acid (EK), L-cystine (Aldrich Chemical Co., Milwaukee, Wis.), glucose (Nutritional Biochemicals, Cleveland, Ohio), NAD^+ (EK), COC (Sigma Chemical Co., St. Louis, Mo.), ferric nitrate (Mallinckrodt Chemical Co., St. Louis, Mo.), thiamine hydrochloride (EK), cysteine hydrochloride (J. T. Baker Chemical Co., Philipsburg, N.J.). All solutions were stored at −15°C.

Inocula. In early screening tests, tryptose broth media with their respective supplements were inoculated by the transfer of a loopful of colonial growth from 48-h chocolate agar cultures which had been seeded directly with thawed skim-milk suspensions. Serial subcultures were made by the passage of three drops (about 0.15 ml) from the previous culture by Pasteur pipette. Control media of unsupplemented and adequately supplemented tryptose broth were similarly inoculated. All culture media that developed turbidity on incubation were subcultured to chocolate agar as a control of viability and purity.

In the critical experiments, plate counts were used as a measurement of the adequacy of the various media and of the significance of certain supplements. Inocula of cultures intended for plate counts were prepared from suspensions corresponding in optical density to a MacFarland nephelometer tube no. 1. In practice, they were 1:10 dilutions of suspensions adjusted to a light absorbancy of 0.14 at 640 nm in a spectrophotometer (Spectronic 20; Bausch & Lomb, Rochester, N.Y.). A 0.1-ml amount of such a diluted suspension was transferred to 5 ml of broth as an inoculum. In experiments involving serial passages, 0.1-ml amounts were transferred to each 5 ml of broth. In experiments utilizing peptone broth, this amount was reduced to 0.03 ml per 5 ml of broth. Appropriate controls in the form of unsupplemented and adequately supplemented (blood or IVX) media were included.

Statistical procedures. The growth data of one series of passages were analyzed by means of a one-way analysis of covariance (BMDP1V; 6): broth, plain, and with one of four different additives. The covariate was passage. Pairwise comparisons of group means were made by Duncan's multiple-range test (7). The significance of differences among the variously supplemented peptone broth cultures was determined by Student's *t* test.

RESULTS

Preliminary experiments revealed that tryptose broth enriched with 1% IVX was a suitable medium for the propagation of the five *H. somnus* strains tested as judged by the production of visible turbidity within 24 h. The omission of NAD from the IVX formula did not affect these results, but the combined exclusion of NAD and COC resulted in the cessation of discernible growth in three of the five strains on the third subpassage. In a more comprehensive attempt to assess the significance of COC in the propagation of *H. somnus* in tryptose broth, growths in unsupplemented tryptose broth and tryptose broth supplemented with IVX deprived of COC were compared over an eight-passage trial with growth in tryptose broth supplemented with IVX, COC, or IVX from which an ingredient other than COC had been excluded. Growth occurred in all media with all strains. The analysis of covariance, however, determined signifi-

TABLE 2. Growth of *H. somnus* in peptone broth with various supplements[a]

Supplement	IVX component deleted	No. of strains tested (No. of determinations)	Mean plate count at 48 h (CFU/ml × 10^7)	*P*
Blood	NA	4	2.8 ± 1.2	>0.1
IVX	—[b]	5 (9)	2.1 ± 1.1	>0.1
IVX	COC, Thi	4	0.6 ± 0.24	<0.002[c]
IVX	CY,CY	5	0	
IVX	Glt, Gu, Ad	5	2.5 ± 2.0	>0.1
IVX	B-12, P, Fe	5	3.4 ± 2.9	>0.1
COC	NA	4	0	
CY, CY	NA	5	2.4 ± 1.3	>0.1
CY, CY, COC	NA	5	1.9 ± 1.0	>0.1
None	NA	5 (9)	0	

[a] The mean inoculum size was 3.5 ± 1.6 × 10^4 CFU/ml of medium. Supplements were added at a rate corresponding to 10% IVX. Abbreviations: NA, not applicable, Thi, thiamine hydrochloride; CY, CY, cysteine-cystine; Glt, L-glutamine; Gu, guanine hydrochloride; Ad, adenine; B-12, vitamin B-12; P, *p*-aminobenzoic acid; Fe, ferric nitrate

[b] —, None.

[c] Significant deviation from the mean plate count of all cultures in which growth occurred (Student's *t* test).

cantly better growth in the media with, rather than without, COC (Table 1). The statistical analysis further found no significant difference between growth patterns in the two media lacking COC. The experiment also showed a statistically significant improvement of growth ($P < 0.01$) with an increasing number of passages irrespective of the presence or the nature of supplements.

Although an overall stimulatory effect of COC was thereby confirmed, it became clear that tryptose broth, being capable of supporting the continuous growth of *H. somnus* in the absence of any supplementation, was not the appropriate basal medium for the determination of minimal growth factor needs. A search for a medium incapable of such growth support by itself but capable of it when supplemented with IVX identified, among proteose-peptone, peptone, and tryptone broths, only peptone broth as the medium meeting these specifications: one of the two strains used in the screening was found to multiply by one log in proteose-peptone broth, whereas both grew comparably in tryptone broth. In plain peptone broth not only was there no multiplication demonstrable but also no viable remnant of the inoculum within 24 h. When, on the other hand, peptone was supplemented with IVX, multiplication regularly occurred and, with four of five strains, was comparable to that attained in the blood broth controls. It was improved by a boost in the IVX content to 10%. The addition of COC, even at a rate corresponding to 10% IVX, failed to render peptone broth suitable for the propagation or survival of any test strain. Nor did the deletion of COC and thiamine from IVX cancel the growth factor activity of that supplement although the amount of growth was significantly reduced by such a deletion (Table 2).

In an experiment intended to identify the constituent of IVX responsible for the growth factor activity, a number of fractions were prepared and added to peptone broth at a rate corresponding to 10% IVX (Table 2). It became apparent (i) that only fractions containing cysteine-cystine had such activity, (ii) that cysteine-cystine alone was adequate as a growth promoter to the same degree as complete IVX, and (iii) that the addition of COC to the cysteine-cystine fraction resulted in no further enhancement of growth. (Subsequent tests showed either cysteine [0.25%] or cystine [0.04%] to be satisfactory as cofactors.) Of IVX constituents other than cysteine-cystine, the fraction containing glucose-guanine-adenine appeared to be essential to the growth of strain 714 (not included in Table 2). The presence of ferric iron did not result in any noticeable increase in growth.

An attempt was made to substitute a completely synthetic medium, MEM, for peptone broth. When this medium, a mixture mainly of amino acids, purines, pyrimidines, and vitamins but lacking cysteine and COC, was supplemented with large amounts of cysteine (0.25%), appreciable growth occurred, roughly comparable to that seen in peptone blood broth. No such growth was observed with smaller amounts of cysteine or with large additions of COC (Table 3). Subcultures from cysteine-enriched MEM cultures, however, uniformly failed to grow.

A collection of 20 *H. somnus* cultures from a variety of geographical and anatomical or pathological sources (3) was tested for the ability to grow through three passages of peptone broth enriched with 0.25% cysteine hydrochloride.

TABLE 3. Growth of 4 *H. somnus* strains in MEM with various supplements

Supplement	Mean plate count at 48 h (10^7 CFU/ml)
Blood	5.85 ± 2.29
Cysteine (0.25%)	4.0 ± 3.75
Cysteine (0.1%)	0
Cysteine (0.05%)	0
COC (0.05%)	0
None	0

[a] Inoculum size ranged from 3×10^4 to 2×10^5 CFU/ml.

Growth, comparable in quantity to that obtained in 10% IVX-supplemented peptone broth, was observed in all instances. No growth occurred in unsupplemented peptone broth with any of the strains, including three atypical ones that grew well on plain infusion and tryptose media from the time of their primary isolation.

DISCUSSION

The identification of so-called *H. somnus*, like that of legitimate members of the genus *Haemophilus*, rests largely on the demonstration of certain cultural requirements (17). Such a demonstration presupposes the existence of a set of base-line cultural conditions under which the agent cannot propagate until the critical factors are added. Various laboratories have used a number of poorly defined media, e.g., brain heart infusion (1) and tryptose (17), to provide the nutritional dimension of these base-line conditions. Unfortunately, as has been known since the agent was first described, *H. somnus* may achieve variable amounts of growth on some such media, especially after repeated laboratory passage (11), and recently even freshly isolated field strains have been encountered that were capable of normal growth on unenriched tryptose agar (2). Such media would, therefore, appear to be less than ideal for the identification of absolute growth factor requirements. We therefore discarded the use of tryptose broth when quantitative procedures revealed that most of our strains grew consistently, if modestly, in this medium.

In peptone broth, a truly basal medium incapable of supporting any growth of 25 *H. somnus* cultures in the absence of supplement, the growth factor activity of COC observed in other media (1, 2; L. R. Stephens, Ph.D. thesis, University of Guelph, Guelph, Ontario, Canada, 1982) was shown to be at most stimulatory, rather than essential, to the growth of the bacteria. Instead, the component of IVX which rendered the previously inadequate peptone broth suitable for *H. somnus* propagation was shown to be cysteine-cystine. The addition of these amino acids, but not of COC, permitted the growth of *H. somnus* strains for one and only one passage in the completely synthetic MEM. Apparently this medium lacks, in addition to cysteine, some other essential ingredient(s) carried over with the inoculum in the first passage but diluted to subliminal levels on further passage.

The requirement for cysteine or cystine is not unique to *H. somnus*. A need for these sulfur-containing amino acids has long been known for *Francisella tularensis* (9) and has recently been identified in *Legionella pneumophila* (13), in the cultivation of which L-cysteine hydrochloride and ferric pyrophosphate have replaced the IVX used in the original isolation (8). The benefit of ferric ion was not apparent with our *H. somnus* cultures.

Our identification of cysteine-cystine as an apparently indispensable requirement for the growth of *H. somnus*, a trait shared by other, unrelated taxa, does little to aid in resolving the unsettled taxonomic status of this species. The current trend to place these bacteria into the emerging family *Pasteurellaceae* (12) is traceable to their perceived resemblance to *Haemophilus* spp., which goes back to their original description. For nonenteric, gram-negative, facultatively anaerobic fermenters, this appears to be an appropriate provisional placement, especially as it agrees with many other traits prevalent in that group (12). Further studies, particularly on nucleic acid homologies, can be expected to clarify some of the taxonomic aspects of this species sufficiently for a more definitive classification.

ACKNOWLEDGMENTS

We thank D. W. Chladek, R. E. Corstvet, A. B. Hoerlein, and J. Nicolet for supplying us with some of their cultures and T. B. Farver for guidance in the statistical procedures.

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