JOURNAL OF BACTERIOLOGY, May 1967, p. 1509-1512
Vol. 93, No. 5

Influence of Urea on the Growth of T-Strain Mycoplasmas

DENYS K. FORD AND JOYCE MACDONALD
Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

Received for publication 15 November 1966

T-strain mycoplasmas require urea for propagation, but urea metabolism also occurs in nonpropagating viable cultures. Ammonia results from this metabolism and alkalinizes the medium. Ammonium ions and an alkaline *p*H both inhibit the multiplication of T strains and reduce the viability of T strains in broth. These toxic effects of urea metabolism currently limit the growth of T strains in broth. Stock T-strain cultures are optimally maintained in continuous culture if the routine medium at *p*H 6.0 is supplemented with 0.05% urea and 0.002% phenol red, but an incubation temperature of 30 C is preferable to 37 C for subculture at 24-hr intervals.

T-strain mycoplasmas, first described by Shepard (2), were noted to multiply logarithmically for 16 to 18 hr in broth media, after which time the number of colony-forming units (CFU) decreased progressively so that cultures incubated for 48 hr usually contained no viable organisms (1). Subsequent studies showed that medium in which the organisms had grown to maximal concentration, about 5×10^6 to 10×10^6 CFU per ml, would support the further growth of T-strain mycoplasmas if it was supplemented with a dialysate of horse serum. The growth-promoting factor in the dialysate was heat-stable and was not removed by ether; it was a small molecule, as indicated by Sephadex G-25 separation, and was positively charged, as shown by its removal by Dowex-50 resin. At this point in the study, information was obtained (M. C. Shepard, *personal communication*) that T-strain mycoplasmas metabolized urea with the formation of ammonia. It became immediately apparent that the growth factor in the dialyzed horse serum could be replaced by urea and was destroyed by urease. The following observations amplify the effect of urea on the growth of T-strain mycoplasmas.

MATERIALS AND METHODS

T-strain 354, isolated in 1962 from the urethral exudate of a patient with nongonococcal urethritis, was used for the study.

The routine broth and agar employed for the culture of the T strain consisted of Difco PPLO broth (80%), horse serum (10%) obtained from Microbiological Associates, Inc., Bethesda, Md., and Oxoid yeast extract (10% of a 10% solution).

Colony counts were performed by placing duplicate 0.01-ml drops of 10-fold serially diluted culture medium on agar with a 10-μ liter micropipette, the counts being performed at 100 times magnification.

Urea was measured by the conversion to ammonia by the action of urease. Ammonia was estimated by the Seligson-Nessler procedure. A 2-ml amount of appropriately diluted culture medium was added to 3 g of a mixture of 2 parts $K_2CO_3 \cdot 1.5H_2O$ with 1 part $KHCO_3$ in a 50-ml bottle containing two stainless-steel stirring bars. The bottle was immediately stoppered with a stopper containing a ground-glass receiving rod dipped in 1 N H_2SO_4. The bottle was rotated in a horizontal position at 50 rev/min for 40 min. The receiving rod was then washed off in 10 ml of 1:10 Nessler's solution, and the resulting color was read within 1 min on a Beckman DB spectrophotometer at wavelength 420 mμ. The NH_3-N content of the medium was determined from a standard graph of known $(NH_4)_2SO_4$ solution. The above procedure was done before and after the addition of urease to the culture medium; the urea concentration of the medium was calculated from the difference between the two readings.

The *p*H was measured with a Beckman model G *p*H meter, adjustments of the medium being achieved through the addition of 0.1 N HCl or 0.1 N NaOH.

Variation of the NH_4^+ concentration of broths was accomplished through the addition of appropriate amounts of NH_4Cl.

RESULTS

The NH_3-N and urea-N contents of the constituents of the medium were determined, and it was evident (Table 1) that the urea content of the medium, equivalent to 0.003% urea, was derived from the horse serum component. Urea was absent from dialyzed horse serum, and T-strain mycoplasmas could not be grown in medium sup-

TABLE 1. *Free NH_3-N and urea-N concentrations of broth constituents*

Broth constituent	Free NH_3-N	Free NH_3-N and urea-N	Urea-N
	μg/ml	μg/ml	μg/ml
PPLO Broth Base.....	18	18	0
Yeast solution.....	215	215	0
Horse serum.....	6	146	140
Routine medium[a].....	36	50	14[b]

[a] Routine medium contained 80% broth base, 10% yeast solution, and 10% horse serum.
[b] Equivalent to 0.003% urea.

plemented with dialyzed horse serum unless an additional supplement of urea was also added.

Ammonia formation during log-phase growth of T-strain 354 in routine medium containing 0.003% urea was compared (Table 2) with ammonia formation in medium supplemented with 0.05 and 1% urea. In the unsupplemented medium at 12 hr, 3.5×10^6 CFU resulted from the metabolism of the 0.003% urea, producing 17.0 μg per ml of NH_3-N. In the medium supplemented with 0.05% urea, 8.2×10^6 CFU resulted in the production of 234 μg per ml of NH_3-N, and, in the medium supplemented with 1% urea, 5.9×10^6 organisms resulted in the production of 464 μg per ml of NH_3-N. It was evident, therefore, that urea was metabolized for other purposes in addition to that relating directly to multiplication of the organisms. The amount of urea metabolized was proportional to the amount present in the medium in addition to the number of organisms present. Maximal growth in this experiment was the same in the 0.05% as in the 1% urea broth, but at 24 hr the viability of organisms in the 1% urea broth was reduced by a factor of 80.

T-strain mycoplasmas prefer a *p*H below 7, and the formation of significant amounts of ammonia causes an alkaline shift in the medium. Table 3 shows the maximal CFU counts, the final *p*H, and the final NH_3-N concentration of cultures containing successive 0.01% increments of urea from 0.01 to 0.1%. When T strains were grown in media containing 0.05% urea, the initial *p*H of 6 was changed to approximately 7.4. Concentrations of urea above 0.05% resulted in media of sufficient alkalinity to inhibit growth of the organisms and to cause an increased death rate.

Experiments were performed to determine more precisely the effects of NH_4^+ and *p*H on the growth rate and death rate of T-strain 354. Replicate broths containing 10% horse serum, but no added urea, were adjusted to increasing *p*H and increasing NH_4^+ concentrations, and were inoculated with identical inocula of organisms. Counts were performed at 18 hr (Table 4). Even in the absence of added NH_4^+, inhibition of growth was evident at *p*H 7.5. In the presence of increasing NH_4^+ concentrations, progressively greater inhibition of multiplication resulted. Both an alkaline *p*H and increasing NH_4^+ concentrations were therefore inhibitory, but the inhibition of the two together was additive so that, at *p*H 7.0, 100 μg per ml of NH_4^+ was significantly inhibitory, and this concentration of NH_4^+ would result from the supplementation of routine broth with only 0.01% urea.

Earlier experiments indicated that the death rate of T strains was enhanced by increasing the *p*H or the NH_4^+ concentration, or both. To evaluate this in greater detail, an 18-hr broth culture was divided into several parts. After appropriate adjustment of the *p*H and NH_4^+ concentration, the culture was incubated at 37 C for 6 hr; colony counts were then performed. Table 5 indicates that there was a definite, but not marked, additive lethal effect of both *p*H and NH_4^+ during the

TABLE 2. *Growth of T-strain 354 and production of NH_3-N at different urea concentrations*

Time	Routine broth containing 0.003% urea			Broth + 0.05% urea (potential NH_3-N, 247 μg/ml)			Broth + 1.0% urea (potential NH_3-N, 4,680 μg/ml)		
	CFU/ml (× 10^6)	Free NH_3-N	NH_3-N from urea	CFU/ml (× 10^6)	Free NH_3-N	NH_3-N from urea	CFU/ml (× 10^6)	Free NH_3-N	NH_3-N from urea
hr		μg/ml	μg/ml		μg/ml	μg/ml		μg/ml	μg/ml
0	0.01	36		0.01	36		0.01	36	
2	0.02	36	0	0.02	38	2	0.02	38	2
4	0.02	39	3	0.02	39	3	0.02	41	5
6	0.07	38	2	0.07	40	4	0.09	41	5
8	0.26	40	4	0.35	54	18	0.36	56	20
12	3.5	53	17	8.2	270	234	5.9	500	464
16	4.2	56	20	12.0	275	239	12.0	850	814
24	3.9	56	20	8.7	275	239	0.11	1,200	1,164

TABLE 3. *Maximal CFU counts, pH, and NH_3 production from urea concentrations between 0.01 and 0.1%*

Urea supplementation of routine broth	Potential NH_3-N	Observed NH_3-N	pH	CFU/ml (× 10^6)	
				18 hr	24 hr
%	μg/ml	μg/ml			
Nil	60	62	6.4	7.1	9.7
0.01	106	106	6.7	16.8	16.0
0.02	153	150	6.9	21.7	18.3
0.03	200	195	7.1	23.0	22.0
0.04	247	238	7.3	26.7	24.7
0.05	293	285	7.4	28.0	23.0
0.06	340	330	7.6	27.2	24.3
0.07	386	370	7.7	26.2	22.8
0.08	433	425	7.8	27.0	19.0
0.09	479	487	7.9	22.0	17.0
0.1	526	506	7.9	23.0	17.0

TABLE 4. *Effect of pH and NH_4^+ concentration on the multiplication of T-strain 354*

NH_4^+ concn[a]	Growth at 18 hr (CFU/ml × 10^6)				
	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
μg/ml					
Nil	5.2	3.1	2.0	0.4	0.1
100	4.1	2.4	0.5	0.1	0.1
200	1.8	1.1	0.1	<0.1	<0.1
400	1.2	1.0	<0.01	<0.01	<0.01
800	1.4	0.6	<0.01	<0.01	<0.01
1,000	1.0	0.25	<0.01	<0.01	<0.01

[a] Supplement added to routine broth.

period of observation. Comparison of Tables 4 and 5 demonstrates that inhibition of multiplication is more important than increased death rate in the limitation by *p*H and NH_4^+ of maximal CFU counts of broth cultures.

In view of the inhibitory effect of *p*H above 6.0 and also of the increased production of NH_3-N in media containing excess urea, the multiplication of T-strain 354 was studied when urea was added in small increments and the *p*H was controlled to below 7.0. A broth culture containing 0.05% urea was incubated for 12 hr, after which the *p*H was adjusted to 6.0 and urea was added to a concentration of 0.02% at hourly intervals. In this experiment, the maximal CFU count, 50.5 × 10^6 at 20 hr, was only moderately elevated over levels obtained in 0.05% urea medium without subsequent urea supplementation, 28 × 10^6 (Table 3). At the peak count, the NH_3-N level of the medium was 1,025 μg per ml, equivalent to an NH_4^+ concentration of 1,318 μg per ml; this NH_4^+ concentration was well into the range

TABLE 5. *Effect of pH and NH_4^+ concentration on the death rate of T-strain 354*

NH_4^+ concn[a]	CFU/ml (× 10^6) after 18-hr growth, 6-hr exposure time				
	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
μg/ml					
Nil	4.2	4.0	3.1	2.1	1.3
100	3.5	3.9	3.2	2.2	1.6
200	2.9	2.6	2.2	2.3	1.8
400	2.3	3.0	2.6	2.3	1.4
800	1.4	1.5		2.0	0.7
1,000	1.6	0.5	0.6	0.8	0.6

[a] Supplement added to routine broth.

causing both inhibition of multiplication and increased death rate at *p*H 6.0 to 6.5.

DISCUSSION

The metabolism of urea with the production of NH_4^+ results in an obstacle to the propagation in broth of T-strain mycoplasmas to titers that are easily obtained with other *Mycoplasma* strains. Even if *p*H is controlled to the optimal range for T-strain growth (*p*H 6 to 6.5), there is a direct toxic effect of the NH_4^+. Whereas T strains can multiply in the presence of small amounts of urea so that 5 × 10^6 to 10 × 10^6 organisms per ml can result from the metabolism of 0.003% urea, the presence of higher concentrations of urea results in the rapid metabolism of urea and rapid production of NH_4^+ without corresponding multiplication. Moreover, urea is metabolized in cultures having a stable CFU count.

During the course of the present work, unsuccessful attempts were made to remove the NH_4^+. Unfortunately, at an acid *p*H, ammonia cannot be removed by aeration of the cultures or by precipitation as NH_4MgPO_4. In addition, pilot experiments with Dowex-50 indicated that this was not likely to be a profitable approach. Because the reason for the utilization of urea by T strains could not be determined, it was not possible to present the organisms with an alternative substrate which might not result in the accumulation of toxic substances.

One practical application of the better understanding of the metabolism of urea has been the incorporation of 0.05% urea into routine broth media. This concentration of urea results in more uniform growth of T strains to higher titers (20 × 10^6 to 30 × 10^6 CFU per ml) than unsupplemented broth (5 × 10^6 to 10 × 10^6 CFU per ml). If 0.002% phenol red is incorporated into the medium, a readily observed *p*H change

from 6 to 7.5 results, indicating that a viable culture is present; this is helpful because broth cultures show no opalescence even under optimal conditions. The choice of 0.05% urea also arises from the fact that, if the starting *p*H is 6.0, maximal *p*H and NH_4^+ concentration will not be rapidly toxic, and cultures will usually remain viable for 24 hr. In contrast, broth supplemented with 1% urea results in such marked alkalinity and NH_4^+ concentrations that cultures are almost uniformly dead within 20 hr. It has been found necessary, however, to incubate stock cultures at 30 C instead of 37 C to subculture at 24-hr intervals with the certain assurance of maintaining optimal growth and viability. Until these measures were incorporated into the laboratory routine, stock cultures in continous propagation would intermittently fail to subculture.

ACKNOWLEDGMENTS

This investigation was supported by Federal Health Grant 609-7-137, Medical Research Council Grant MA-2128, and the Canadian Arthritis and Rheumatism Society.

LITERATURE CITED

1. FORD, D. K. 1962. Culture of human genital "T-strain" pleuropneumonia-like organisms. J. Bacteriol. **84**:1028–1034.
2. SHEPARD, M. C. 1956. T-form colonies of pleuropneumonia-like organisms. J. Bacteriol. **71**: 362–369.