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Macromolecular Synthesis and Thymineless Death in *Mycoplasma laidlawii* B[1]

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The relationships between macromolecular synthesis and viability have been studied in the pleuropneumonia-like organism *Mycoplasma laidlawii* B adapted to a semidefined growth medium. This organism exhibited an absolute growth requirement for the nucleosides uridine and thymidine, a partial requirement for guanosine and deoxyguanosine, but no requirement for adenosine, deoxyadenosine, cytosine, and deoxycytosine. Cytosine and deoxycytosine partially satisfied the requirement for uridine. Loss in viability resulted from thymidine deprivation, but not from a deficiency in other growth requirements. This phenomenon of thymineless death in a mycoplasma is similar in many respects to that reported in other bacterial systems. Chloramphenicol specifically inhibited protein synthesis and allowed deoxyribonucleic acid synthesis to proceed to only about 40% of that normally produced per generation period, while causing less inhibition of ribonucleic acid synthesis. Protein synthesis inhibition permitted thymineless death to a survival level of less than 0.5%, but ribonucleic acid synthesis inhibition resulted in a higher (10%) survival level. These results are consistent with previously noted aspects of thymineless death in *Escherichia coli* strains, which suggest that thymineless death is coupled to ribonucleic acid synthesis.

Thymine-requiring bacterial auxotrophs lose viability when they are deprived of thymine. This phenomenon, discovered by Cohen and Barner (7) in *Escherichia coli* 15 T^- and termed "thymineless death" (TLD), has not been adequately explained in spite of an impressive list of proposed hypotheses. These include unbalanced growth (7), nuclear damage (12), episome and prophage induction (9, 21, 30, 44), single-strand breaks in the deoxyribonucleic acid (DNA; 31, 36), and abnormal methylation of the DNA (10; B. H. Rosenberg and D. E. Parker, Biophys. Soc. Abst., p. 39, 1967). It is possible that TLD may result from either of two processes: (i) episome induction, or (ii) an event independent of episome induction. *E. coli* 15 T^-, when cured of its colicine, dies TLD more slowly than does the original strain (21), and the residual TLD may be due to some alteration of the DNA. Although DNA from thymine-starved cultures is reportedly unaltered (46), such DNA has a decreased template activity for ribonucleic acid (RNA) polymerase (25), DNA polymerase (43), and DNA methylase (13), and an increased resistance to nucleases (43). Further, the DNA loses its capacity for conjugational chromosome transfer (20) and transformation (31), and it is more difficult to extract from cells (28).

Protein and RNA synthesis affect TLD. Under some conditions of protein synthesis inhibition, TLD is nearly completely inhibited (35, 44), whereas, under other conditions, limited TLD still occurs (2, 26, 49). RNA synthesis rather than protein synthesis may be essential (12) and, in particular, mRNA synthesis has been implicated (15, 25, 28, 36, 42). However, the base composition of pulse-labeled RNA shows no change in thymine-starved *E. coli* K-12 cells (45). Conditions of protein and RNA synthesis inhibition prior to thymine starvation affect the extent of TLD, and such conditions have been used to study the normal DNA replication cycle in *E. coli* strain TAU (26). Only cells actively replicating DNA are susceptible to TLD (17,

[1] This report was part of a dissertation presented by one of the authors (D. W. S.) to the Graduate School of Stanford University in partial fulfillment of the requirements for the Ph.D. degree. Some of these results were presented at the Annual Meeting of the Biophysical Society, Houston, Tex., 1967.

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26). Inhibition of protein synthesis allows completion of cycles of DNA replication and the acquisition of immunity to TLD (17).

The mycoplasmas are the smallest known self-reproducing organisms (L. Hayflick, ed., *in preparation*); because of their small size and limited genetic content, they may be functionally more primitive than other self-reproducing organisms (32). As part of an examination of DNA replication in mycoplasmas (D. Smith and P. Hanawalt, *in preparation*), it was of interest to look for TLD and to use this in a preliminary study of the regulation of DNA synthesis in such organisms. A strain of *Mycoplasma laidlawii* B that was able to grow in a semidefined broth medium was used in these studies. The nucleosides and tryptophan were specifically added to this medium; they could thus be purposely omitted from the medium. To minimize damage to the mycoplasmas, two techniques for rapid and efficient medium transfer were developed.

MATERIALS AND METHODS

Organism. A strain of *M. laidlawii* B adapted to a casein hydrolysate medium was obtained from M. Tourtellotte. This strain had been derived from the original Laidlaw and Elford isolate (24).

Growth conditions. The organism was maintained in continuous broth culture and on agar plates. The broth culture was maintained by a daily 100-fold dilution from a late exponential-phase culture into fresh medium and thus provided a ready supply of exponentially growing cells. Stock cultures were kept frozen at −20 C; such cultures retained viability for more than 6 months. The semidefined broth medium contained: 10 g of salt-free, vitamin-free casein hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio); 8 g of NaCl; 400 mg of KCl; 200 mg of $MgSO_4$; 60 mg of Na_2HPO_4; 1 mg of thymidine (TdR); 5 mg each of uridine (UR), adenosine (AR), deoxyadenosine (AdR), guanosine (GR), deoxyguanosine (GdR), cytosine (CR), and deoxycytosine (CdR); 10 mg of cystine; 10 mg of tryptophan; 1 mg of coenzyme A; 0.010 mg each of biotin, thiamine, and riboflavine; 0.025 mg each of pyridoxine, pyridoxal, and niacin; 1,000 mg of glutamine; 5 g of glucose; 0.2 mg of phenol red; and 100,000 units of penicillin G, in 1,000 ml of twice-distilled water. The final *p*H was adjusted to 8.0 to 8.2 with HCl; phenol red was added as a *p*H indicator. All vitamins, nucleosides, and penicillin G were grade A from Calbiochem, Los Angeles, Calif. Culture contamination was prevented by using penicillin G and by performing biological operations in a hood equipped with an ultraviolet Sterilamp.

Broth cultures were grown without aeration at 35 to 37 C. Vigorous aeration produced only slightly faster growth. The organism grew as rapidly at 33 C as at 37 C. Growth was assayed by optical density readings at 450 nm (OD_{450}) using a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

The *E. coli* strain TAU-bar[15], auxotrophic for arginine, tryptophan, proline, methionine, uracil, and thymine, was used to determine that uracil and thymine were absent from the casein hydrolysate, but that tryptophan was present at about 5 μg/g of casein hydrolysate. This residual tryptophan was removed by exhaustive growth of the tryptophan-requiring *E. coli* strain A46 (obtained from C. Yanofsky) in the casein hydrolysate medium.

Viability assay. OD_{450} was correlated with the number of cells per milliliter by (i) plating [colony-forming units (CFU) per milliliter], and by (ii) counting cells in a Petroff-Hauser counter. The agar medium for plating contained: 10 g of Tryptose (Difco); 5 g of NaCl; 5 g of tris(hydroxymethyl)aminomethane (Tris; Sigma 121); 8 g of Ionagar (Oxoid); 10 g of glucose; 1.0 g of glutamine; 2 mg of coenzyme A; 0.020 mg each of biotin, thiamine, and riboflavine; 0.050 mg each of pyridoxine, pyridoxal, and niacin; 200,000 units penicillin G; and 2.0 ml of PPLO Serum Fraction (Difco), in 1,000 ml of twice-distilled water, at *p*H 8.0 to 8.2. CFU per milliliter were assayed by plating 0.01-ml drops from dilution tubes onto the surface of the agar plates. Cells were diluted through "1.25 × salts" buffer (0.01 M Tris, *p*H 8, plus the salts of the growth medium at 1.25 times the normal concentrations) or through 0.2 M NaCl, 0.02 M Tris, *p*H 8; no loss of viability occurred after more than 10 hr of suspension in either buffer. Minute colonies of a characteristic "fried egg" morphology (40) formed after 3 to 5 days of growth at 35 to 37 C; these were counted in a Leitz phase-contrast microscope at 50-fold magnification with no staining. Drops were usually plated in duplicate, and drops containing between 5 and 1,000 colonies were counted to provide a plating precision of ±10%.

The efficiency of plating was estimated by counting clusters of cells in a Petroff-Hauser counter at 500-fold magnification. Comparison with CFU per milliliter gave an average number of 3 to 5 CFU per cluster. Since there are probably 3 to 5 cells per cluster, the CFU obtained by plating yields a close estimate of the number of viable cells in broth culture.

An OD_{450} of 0.1 corresponded to 10^8 CFU/ml, and the OD_{450} varied linearly with CFU per milliliter within the OD range 0.01 to 0.1. In the OD range 0.1 to 0.4, a nonlinear relation between OD_{450} and CFU per milliliter was observed, an OD_{450} of 0.45 corresponding to 10^9 CFU/ml. However, formation of CFU per milliliter was exponential in time up to about 2×10^8 CFU/ml, with a doubling time of 3 to 4 hr. The time for mass doubling (OD_{450}) in the OD range 0.01 to 0.1 also was 3 to 4 hr, whereas, in the range 0.1 to 0.4, mass doubling times were typically from 4 to 5 hr. The decrease in the rate of mass doubling in late exponential phase is typical of bacterial cultures (27). Exponentially growing cells (density of 3×10^8 CFU/ml or lower) were used in all experiments.

Medium transfer. Neither centrifugation nor rapid filtration is suitable for harvesting *M. laidlawii* B. During normal centrifugation, the cells clump and lose viability during resuspension. In filtration, the pressure needed to effect filtration through the small-

pore-size filters (0.2 μm or less), and to recover the cells from the filter, lyses or severely injures the cells.

Two methods of medium transfer were developed. The first, with low-speed centrifugation in glass tubes designed to minimize cell clumping, is described elsewhere (D. Smith and P. Hanawalt, *in preparation*). In the second method, sterile Sephadex G50, coarse (Pharmacia Inc., New Market, N.J.) molecular sieve columns were used. The columns were eluted with "1.25 × salts" buffer. A supplement (SUP) containing all ingredients except the salts of the growth medium at appropriate concentrations was added to fractions from the G50 columns to yield the complete growth medium. Of viable cells, 30 to 80% were recovered and growth resumed immediately after transfer to fresh medium; thus, the cells were apparently not injured during the transfer.

The cells eluted in a sharp band just after the void volume. Low-molecular-weight compounds eluted later in broad bands. Control experiments with radioactively labeled compounds demonstrated that less than 0.1% of the total TdR or tryptophan in the medium was present in the Sephadex fractions containing the cells. Time of transfer was about 15 min. The major disadvantage of the method was that the cells were diluted about fivefold during transfer.

Incorporation and assay of radioactivity. M. laidlawii B DNA was labeled with ^{3}H- or ^{14}C-TdR, ^{3}H-BUdR, (bromodeoxyuridine; Schwarz Bio Research Inc., Orangeburg, N.Y.) or with ^{32}P-orthophosphate (ICN). ^{14}C-tryptophan (Calbiochem) was used to label protein, and ^{3}H-UR (Schwarz Bio Research Inc.) was used to label RNA. Incorporated radioactivity was assayed by precipitation with 5 ml of cold 5% trichloroacetic acid (acid-insoluble radioactivity). ^{32}P and ^{3}H-UR incorporated into DNA were assayed as alkaline-resistant (digestion with 1 to 2 N KOH for 2 to 3 hr at 37 C), acid-insoluble radioactivity. The precipitates were collected on HA filters (Millipore Corp., Bedford, Mass.), washed twice with 5 ml of distilled water, dried, and counted in a Packard Tri-Carb liquid scintillation spectrometer as previously described (18). Total radioactivity was assayed by sampling directly onto HA filters, followed by assay as described above.

During one growth cycle in medium containing 1 μg of TdR per ml, approximately 60% of either ^{3}H-TdR or ^{14}C-TdR is incorporated into acid-insoluble material. About 85% of this material remains insoluble after alkaline digestion, whereas about 90% is rendered acid-soluble by pancreatic deoxyribonuclease digestion. Further, 80 to 95% of the acid-insoluble material is recovered as a single band of buoyant density 1.693 g/cc by CsCl equilibrium sedimentation; less than 0.2% of this material appears at the bottom of such gradients. Thus, at least 80 to 95% of the TdR is incorporated into DNA, and less than 0.2% into RNA.

By using the same methods, the ratio of incorporated ^{32}P in RNA to that in DNA was about 3.5; a ratio of 1.9 was obtained for the avian mycoplasma *M. gallisepticum* A 5969 (33). Similarly, about 7% of incorporated ^{3}H-UR was in DNA and the rest in RNA, showing that such incorporation is a good assay for RNA synthesis after correction is made for incorporation into DNA. More than 80% of incorporated ^{14}C-tryptophan was made acid-soluble by digestion (30 min, 60 C) with 200 μg of Pronase (Calbiochem, grade B) per ml. Thus, such incorporation is considered an adequate assay for protein synthesis.

Techniques for CsCl equilibrium sedimentation were as previously described (47).

RESULTS

Growth requirements. Figure 1 presents the growth characteristics of *M. laidlawii* B when selected metabolites were omitted from the growth medium. Viability (CFU per milliliter) is correlated with mass increase (OD_{450}). Typical bacterial growth characteristics were observed in the fully supplemented control, except that an abrupt, total loss of cell viability, without cell lysis, occurred in late stationary phase.

Both mass increase and viability increased parallel to that of the control when either CR and CdR or AR and AdR were removed. However, removal of GR and GdR reduced the

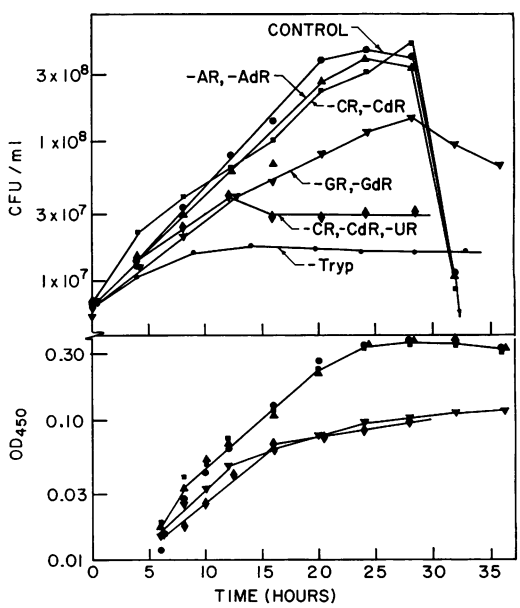

FIG. 1. *Growth characteristics of M. laidlawii B. M. laidlawii B cells from a 1-ml (OD_{450} = 0.25) culture were transferred to 1.25 × salts via a small Sephadex column and supplemented with SUP lacking the appropriate nucleosides. SUP rendered tryptophan-free by using E. coli A46 was used in the minus-tryptophan culture. The cultures were grown anaerobically at 37 C; CFU/ml and OD_{450} were assayed at the times indicated. Symbols: ●, fully supplemented control; ▲, minus AR and AdR; ▼, minus GR and GdR; ■, minus CR and CdR; ◆, minus CR, CdR, and UR; ●, minus tryptophan.*

CFU per milliliter doubling time to about 7 hr and the maximum titer to about 10^8 CFU/ml. OD_{450} behaved similarly. An identical growth curve was obtained when all four purine nucleosides were omitted from the culture. Thus, a conversion of adenine derivatives to guanine derivatives does not explain the residual growth. When CR, CdR, and UR were all removed from the medium, CFU per milliliter increased about fivefold and remained constant, whereas mass increase continued to an OD_{450} of about 0.1. When only UR was omitted (*not shown*), CFU per milliliter increased slowly to a final low titer (10^8 to 2×10^8 CFU/ml), but the OD_{450} increased to about 0.2, suggesting that cell size abnormally increases and showing that CR or CdR, or both, will partially satisfy the pyrimidine nucleoside requirement. Substitution of the free bases for the nucleosides was not systematically studied. When thymine, or 5-bromouracil, was substituted for TdR, very little incorporation occurred (4% of the control containing TdR). The cells lost viability but increased in mass. These are characteristics typical of TLD. Thus, thymine or 5-bromouracil will not substitute adequately for TdR.

With growth medium depleted of tryptophan by exhaustive growth of *E. coli* A46, *M. laidlawii* B grew normally (*not shown*) when the medium was replenished with tryptophan. However, very little increase in viability occurred in the absence of tryptophan (Fig. 1). The OD_{450} remained less than 0.02. Thus, tryptophan is an absolute growth requirement. 5-Methyltryptophan, at 50 μg/ml, a false feedback inhibitor of the tryptophan operon in *E. coli* (34) which prevents growth of wild-type *E. coli* when present at 0.5 to 1 μg/ml (1), had no observable effect on the growth properties of *M. laidlawii* B. Thus, *M. laidlawii* B does not seem to contain a functional tryptophan operon.

Requirement for TdR and TLD. Table 1 shows the effect of TdR removal on RNA and DNA synthesis. For the first one to two generations, considerable RNA was synthesized. After growth for five generations, less than 3% of the normal amount of DNA synthesis per generation had occurred, and this residual synthesis was distributed roughly uniformly over the five-generation growth period. Thus, TdR is an absolute requirement for DNA synthesis. The residual 3% synthesis is possibly due to both the DNA "turnover," which occurs in *M. laidlawii* B (D. Smith and P. Hanawalt, *in preparation*), and to nonconservative "repair" replication. Such repair replication occurs in *M. laidlawii* B after ultraviolet irradiation (D. Smith and P. Hanawalt, *in preparation*), and repair replication has been demonstrated in *E. coli* TAU-bar as a result of thymine deprivation (36).

TABLE 1. *RNA and DNA synthesis in the absence of TdR*[a]

Time ^{32}P was added after removal of thymidine	^{32}P incorporated (counts/min)[b]	
	RNA synthesis	DNA synthesis
hr		
0 (control)	258,000 (100%)	71,000 (100%)
0 (0.0 generation)	54,000 (20.9%)	2,120 (3.0%)
2 (0.5 generation)	15,500 (6.0%)	2,035 (2.9%)
9 (2.2 generation)	7,500 (2.9%)	1,160 (1.6%)

[a] *M. laidlawii* B cells were transferred to four fresh cultures via a Sephadex column. TdR (5 μg/ml) was added to the control, and 15 μc of ^{32}P was added to each of the four cultures at the times shown. ^{32}P incorporation into RNA and DNA was periodically assayed, and the distribution of ^{32}P in nucleic acids after 23 hr of growth was further determined by CsCl equilibrium sedimentation.

[b] Figures in parentheses indicate percentages of control with TdR.

Of the eight nucleosides present in the growth medium, only withdrawal of thymidine resulted in a loss of viability (Fig. 2). Death occurred exponentially after an initial lag of 10 to 15 hr. However, in a few experiments, the lag was only 2.5 to 5 hr (Fig. 7 and 8). This "short" lag is comparable, in generation times, to that observed in *E. coli* strains resistant to TLD (7, 8, 26). The final rate of exponential killing was the same in all experiments. During this killing, the 1/e time was about 2.5 hr as compared with about 20 min for *E. coli*, about 0.5 generation times for both organisms. Loss of viability proceeded to a fraction of less than 10^{-4}, whereas OD_{450} (*not shown*) increased about 10-fold.

A slow loss of viability extending over many hours was observed in thymine-requiring *E. coli* when thymine was present at a low concentration, e.g., 0.05 μg/ml (27). Thus, it was necessary to show that small amounts of TdR were not present in these experiments. Medium transfer via Sephadex reduced the TdR content to about 0.1% (0.005 μg of TdR per ml). Passage of the cells through a second column further reduced the TdR content to 5×10^{-7} μg/ml. When this was done, there was no detectable difference in the kinetics of TLD. Thus, the observed long lag and slow kinetics of death are not due to the presence of a residual amount of TdR.

In the presence of BUdR (Fig. 2), growth

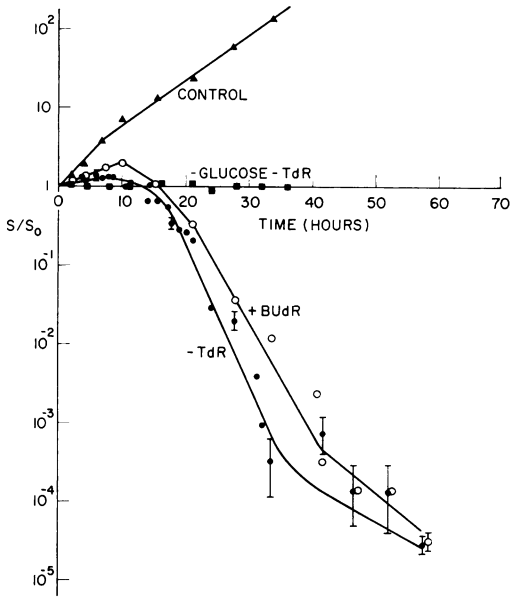

FIG. 2. *TLD in M. laidlawii B. Cultures containing all supplements, lacking TdR and glucose, lacking TdR, and containing BUdR (5 μg/ml) but no TdR were inoculated with cells transferred to 1.25 × salts via a small Sephadex column. The TLD curve shows data from many similar experiments. Growth was anaerobic at 37 C. Symbols: ▲, fully supplemented control; ●, minus TdR; ■, minus TdR and glucose; ○, minus TdR but plus BUdR.*

proceeded exponentially at a slower rate (10-hr CFU-per-milliliter doubling time) for about one doubling; then the cells lost viability similarly to that of TLD. Thus, BUdR is pathogenic for *M. laidlawii* B, as it is for *E. coli* (7, 14).

In *E. coli*, TLD requires active metabolism (7, 11). This is also true for *M. laidlawii* B; in the absence of glucose and TdR, viability remained constant for more than 35 hr (Fig. 2).

Effects of chloramphenicol (CAP) on M. laidlawii B. CAP, via a ribosome interaction specifically blocking peptide bond formation (22, 50), preferentially inhibits protein synthesis in *E. coli*. The effects of CAP on growth and macromolecular synthesis in *M. laidlawii* B were examined preparatory to its use for protein synthesis inhibition.

Figure 3 shows the effects of several concentrations of CAP upon cell growth. At 3 μg/ml, CAP increased the generation time, but a normal maximum titer was obtained. At 15 μg/ml, CAP stopped cell division after perhaps one doubling, whereas 200 μg of CAP per ml caused a slow loss of viability. Essentially no mass increase occurred in the presence of 15 μg of CAP per ml (Fig. 4).

At 2 μg/ml and 5 μg/ml, CAP inhibited but did not stop DNA synthesis (Fig. 4). However, with 15 to 25 μg of CAP per ml, a nearly constant amount of DNA synthesis occurred, with no further synthesis or degradation. At higher CAP concentrations, some DNA degradation occurred. Thus, 15 μg of CAP per ml appears to be an optimal concentration for studies on the regulation of DNA synthesis in this system.

CAP inhibited RNA synthesis, but to a lesser extent than DNA synthesis. This also is true for *E. coli* (12, 23). Also, as with *E. coli* (23), there was no increase in the rate of RNA synthesis at low CAP concentrations in this casein hydrolysate medium. Figure 4 further shows that 2 μg of CAP per ml markedly reduced subsequent protein synthesis, and 15 μg of CAP per ml nearly totally inhibited protein synthesis. Thus, CAP appears to have the same general effects in *M. laidlawii* B as in other organisms.

DNA synthesis in the absence of protein synthesis was examined both by removing tryptophan from the growth medium and by adding 15 μg of CAP per ml to the medium (Fig. 5). In both cases, DNA synthesis proceeded for about one generation time (3.5 hr), yielding an amount of DNA equivalent to 40% of that normally synthesized per generation time (*see also* Fig. 4). This is the value expected if nearly all the cells

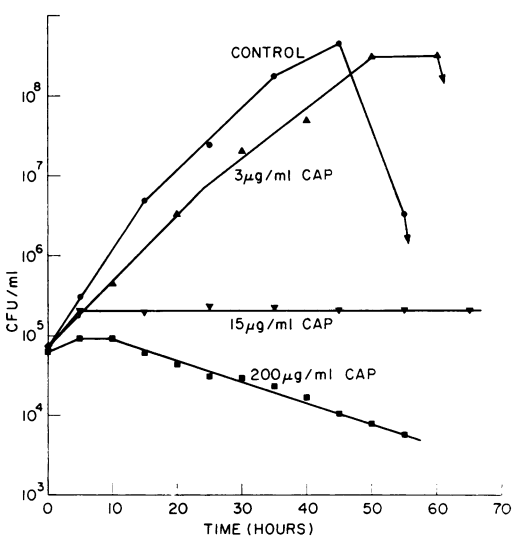

FIG. 3. *Survival of M. laidlawii B in the presence of CAP. After medium transfer via a large Sephadex G50 column, 5-ml M. laidlawii B cultures containing TdR and 0, 3, 15, and 200 μg of CAP per ml were grown anaerobically at 37 C. Samples were plated at the times shown. Symbols: ●, no CAP (control); ▲, 3 μg of CAP per ml; ▼, 15 μg of CAP per ml; ■, 200 μg of CAP per ml.*

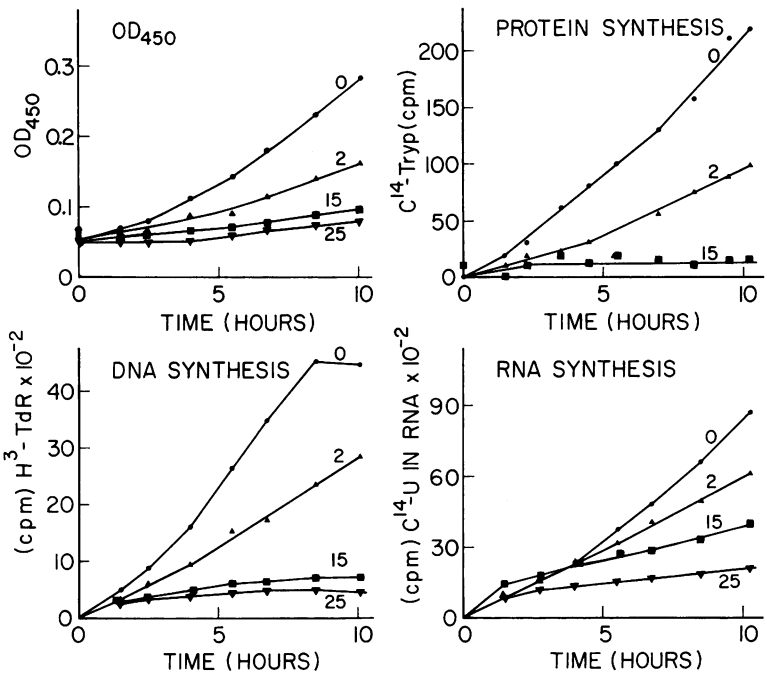

FIG. 4. *Macromolecular synthesis in the presence of CAP. Cultures (5 ml) containing 0, 2, 15, and 25 μg of CAP per ml were inoculated with exponentially growing M. laidlawii B cells to a final* OD_{450} *= 0.05 and were grown anaerobically at 37 C. Acid-insoluble* ^{3}*H-TdR (0.33 μc/ml) assayed DNA synthesis, and acid-insoluble minus alkaline-resistant acid-insoluble* ^{14}*C-uracil (0.33 μc/ml) assayed RNA synthesis. Protein synthesis was measured by acid-insoluble* ^{14}*C-tryptophan (0.08 μc/ml) in a separate but similar experiment. Symbols: ●, no CAP (control); ▲, 2 μg of CAP per ml; ■, 15 μg of CAP per ml; ▼, 25 μg of CAP per ml.*

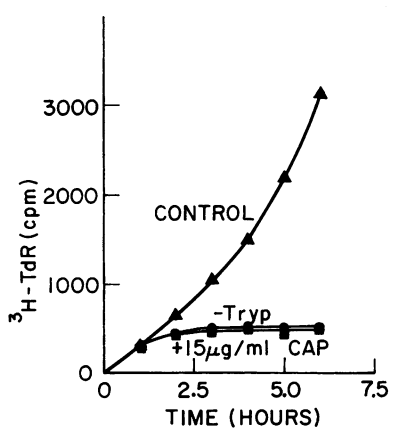

FIG. 5. *DNA synthesis in the absence of protein synthesis. Tryptophan-free culture medium (13.5 ml) containing 25 μc of* ^{3}*H-TdR was inoculated with cells transferred to 1.25 × salts via Sephadex G50 and split into three equal parts. Tryptophan (5 μg/ml) was added to one (control), tryptophan (5 μg/ml) and CAP (15 μg/ml) to another, and the third received no further supplements. DNA synthesis was assayed via acid-insoluble radioactivity. Symbols: ▲, control; ■, plus CAP; ●, minus tryptophan.*

were synthesizing DNA at the time protein synthesis was stopped, and if already begun rounds of DNA replication were completed, but no new replication rounds were initiated (26). There is no difference in the amount of DNA synthesis obtained in the presence of 15 μg of CAP per ml or in the absence of tryptophan.

TLD in the absence of protein and RNA synthesis. When both uracil and thymine are removed from cultures of *E. coli* TAU-bar, almost no TLD occurs (15). This possible dependence of TLD upon RNA synthesis in *M. laidlawii* B was tested by removal of UR and TdR, and by removal of all pyrimidines (Fig. 6). In the absence of UR and TdR, normal kinetics of TLD were observed, with some deviations at low survival levels. However, when all four pyrimidines were omitted from the growth medium, about 10% of the cells appeared to be resistant to TLD. Thus, conditions that strongly inhibit, or prevent, RNA synthesis in *M. laidlawii* B also inhibit TLD.

The dependence of TLD on protein synthesis when inhibited by CAP is shown in Fig. 7. At 3 μg/ml, CAP permitted TLD to proceed with kinetics nearly identical to that of the control

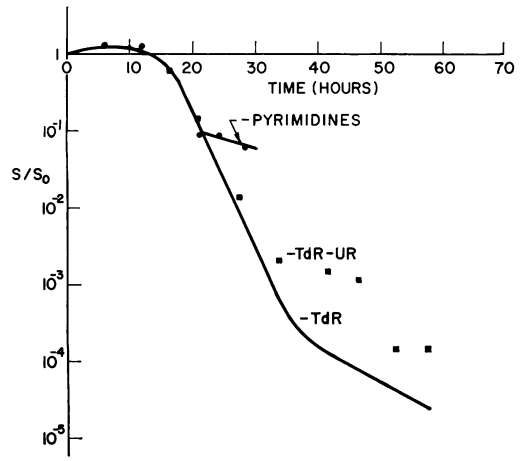

FIG. 6. *Effects of removal of pyrimidines on TLD. Growth medium lacking TdR, growth medium lacking TdR and UR, and growth medium lacking TdR, UR, CR, and CdR (all pyrimidines) were inoculated with cells transferred to 1.25 × salts via a Sephadex column. Growth was anaerobic at 37 C. The TLD curve from Fig. 2 is reproduced for reference. Symbols: ●, minus all pyrimidines; ■, minus TdR and UR.*

(no CAP), a control illustrating the "short lag" TLD. At higher CAP concentrations, killing proceeded exponentially, but at a greatly reduced rate. A concentration of 15 μg of CAP per ml resulted in killing to a survival of no more than 0.5%, and 200 μg of CAP per ml resulted in a net survival of less than 10%. Correction was made for the slow loss of survival in the presence of both 200 μg of CAP per ml and TdR (Fig. 3); the total survival in medium containing 200 μg of CAP per ml, but no TdR, was less than 0.5%.

When protein synthesis was inhibited by the removal of the required amino acid tryptophan (Fig. 8), viability initially followed that of the control containing tryptophan, a control also illustrating the "short lag" TLD. However, at about 30% survival, loss of viability continued, but at a greatly reduced rate. This final rate of killing was nearly the same as that observed for TLD in the presence of 15 μg of CAP per ml (Fig. 7). Killing again continued to a survival of less than 0.5%.

DISCUSSION

Growth studies with *M. laidlawii* B are consistent with the known nucleoside pathways in *E. coli* (4, 29). In *E. coli*, CdR is first converted to uracil, and CR to UR, before phosphorylation of uracil and UR to uridine monophosphate (UMP). UMP is the direct precursor of all four pyrimidine triphosphates. Thus, in the presence of UR, neither CR nor CdR would be required, as is observed in *M. laidlawii* B. In the absence of UR, CR and CdR serve as the UMP precursor; this is partially true in *M. laidlawii* B, although growth is inhibited. TdR is not a UMP precursor. Further, ^{3}H-uracil can be readily incorporated into RNA by *M. laidlawii* B in the presence of excess UR. This suggests that *M. laidlawii* B can convert uracil directly into UMP without first forming the nucleoside, as is also true for *E. coli*. The requirement for at least one of these three nucleosides shows that *M. laidlawii* B does not contain a functional orotic acid pathway for pyrimidine biosynthesis. Since the same enzyme fraction catalyzes the conversion of UDP to dUDP and the conversion of CDP to dCDP (3), *M. laidlawii* B probably can synthesize dUMP from UR, CR, or CdR, and thus apparently lacks a functional thymidylate synthetase. Unlike *E. coli*, *M. laidlawii* B apparently cannot convert thymine to TMP.

Some slow growth occurs in the absence of all purine nucleosides, suggesting that *M. laidlawii* B can slowly synthesize purines. Since AR and AdR do not stimulate this growth, *M. laidlawii* B apparently cannot convert AR to GMP, either via inosinate and inosinic monophosphate (IMP) or by opening the six-membered purine ring with subsequent resynthesis of IMP, the

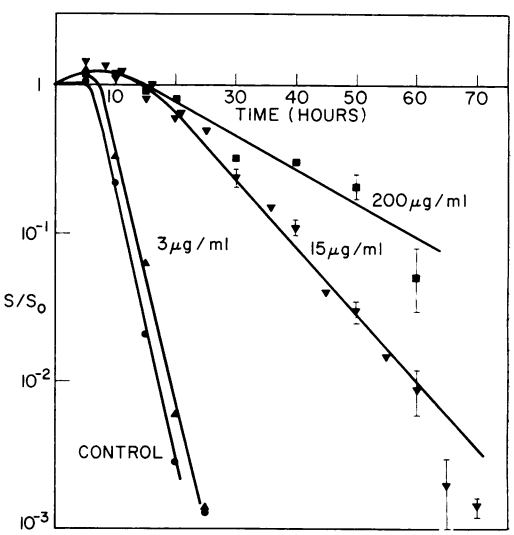

FIG. 7. *TLD in the presence of CAP. M. laidlawii B cells from the same medium transfer described in Fig. 3 were used to inoculate 5-ml cultures containing 0, 3, 15, and 200 μg of CAP per ml, but no TdR. Growth was anaerobic at 37 C. Samples were plated at the times shown. Symbols: ●, no CAP (control); ▲, 3 μg of CAP per ml; ▼, 15 μg of CAP per ml; ■, 200 μg of CAP per ml.*

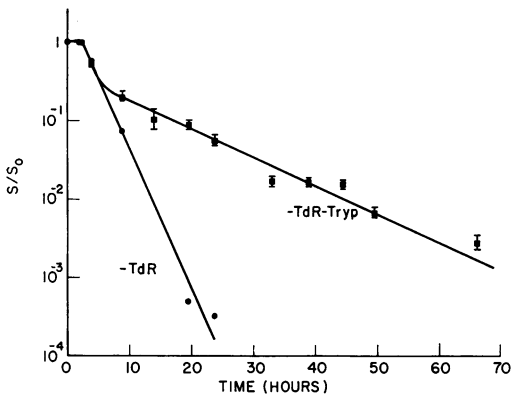

FIG. 8. *TLD in the absence of tryptophan. Tryptophan-free culture medium lacking TdR was inoculated with cells transferred to 1.25 × salts via Sephadex and split into three equal portions. One-third was supplemented with 5 μg of tryptophan per ml and 1 μg of TdR per ml (fully supplemented control), tryptophan (5 μg/ml) was added to another third (minus TdR control), and the final third remained minus TdR and minus tryptophan. The fully supplemented control grew normally (not shown). Samples were plated at the times shown. Symbols: ●, minus TdR; ■, minus TdR and minus tryptophan.*

two pathways used by *E. coli*. However, since the casein hydrolysate medium contains about 300 μg of histidine per ml, this may be preventing the opening of the ring by feedback inhibition of a pyridine-adenosine triphosphate pyrophosphorylase. Also, as an alternative to de novo purine biosynthesis, it is possible that histidine partially spares the purine requirement, as is true for *Lactobacillus casei* (6).

Razin (39) found that *M. laidlawii* strain A requires only thymidine, adenine, guanosine, and cytosine. This is consistent with our results, except that adenine should not be required in the presence of GR. Razin also noted that the TdR requirement could be replaced by folinic acid, and similar results have been observed in lactic acid bacteria (48). This could mean that *M. laidlawii* B contains a functional thymidilate synthetase but cannot convert dUMP into TMP because of an inability to synthesize the needed reduced folic acids, although such deficiency would possibly be lethal. Substitution of folinic acid for TdR, or its effect on TLD, were not studied here.

Tryptophan is an absolute growth requirement, but *M. laidlawii* B does not suffer the "tryptophanless death" reported in *Bacillus subtilis* (38). Studies with the analogue 5-methyl-tryptophan showed that *M. laidlawii* B does not possess a tryptophan operon sensitive to this analogue. Little is known about the regulation of protein biosynthesis in *M. laidlawii* B, and it is possible that tryptophan synthase would be of interest in biochemical evolution studies (5).

TLD in *M. laidlawii* B is characterized by a lag period (2 to 5 hr, "short" lag, or 10 to 15 hr, "long" lag), followed by a slow exponential loss of viability. The rate of killing, in generation periods, is comparable to that of many *E. coli* strains, although these strains exhibit lag times varying from zero to about two generation periods (8). The lethal event, or events, in TLD may be the same in the two organisms if the probability of the event occurring were proportional to the generation period. Such an event might be associated with a growth-limiting function, for example, with DNA replication, or with a function that normally occurs a fixed number of times per generation period, for example, with basal level messenger RNA synthesis (19).

The relatively long lag time of 0.5 to about 3 generations renders *M. laidlawii* B "resistant" to TLD (8). It is not understood why two different lag times appear to be exhibited. Although no TdR is present after medium transfer, another thymine precursor, e.g., thymidine triphosphate, may be inadequately removed during medium transfer. This possibility, however, is unlikely (Fig. 7). Only one medium transfer was performed, yet two of the subsequent four cultures exhibited the "short" lag (0- and 2-μg/ml CAP curves), whereas the other two exhibited the "long" lag.

Because of the slow kinetics of TLD in *M. laidlawii* B, it is considered unlikely that the lethal event is induction (or diversion) of a prophage or other episome. Lysates of ultraviolet-irradiated cells contained no agents which could infect and lyse viable *M. laidlawii* B cells. Ultraviolet (and visible) irradiation, however, does effect cell lysis (D. Smith and P. Hanawalt, *in preparation*), but thymine-starved cells exhibit no such lysis, ruling out induction of a *nondefective* episome. The possibility of induction of a defective episome cannot be eliminated, although no bacteriophage has yet been reported that is infective for any mycoplasma.

The possible role of growth of an induced prophage in TLD remains unresolved. Although such induction results in cell death, *E. coli* 15 T^-, when cured of its known defective prophage, still suffers TLD, although at a reduced rate (21). Other genetic markers besides that for the prophage also affect the rate of death (21). Thus, TLD is not totally explainable by prophage induction. Further, *E. coli* K-12, lysogenic for bacteriophage λ, suffers TLD with the same

kinetics as *E. coli* K-12 nonlysogenic for λ (8), demonstrating that thymineless induction of a prophage need not increase the rate of TLD.

During the generation following protein synthesis inhibition, further DNA synthesis occurs, equivalent to 40% of that normally synthesized per generation period (Fig. 5). This strongly suggests that nearly all of the cells are actively synthesizing DNA in exponentially growing cultures, and that protein inhibition permits completion of already begun rounds of replication with no initiation of new rounds (17, 26). Further evidence that most of the exponentially growing cells are replicating DNA is provided by analysis of the buoyant density distribution of intact *M. laidlawii* B chromosomes isolated in CsCl density gradients after growth in BUdR-containing medium (D. Smith, *in preparation*). However, more direct methods should be used to determine the fraction of cells synthesizing DNA in exponentially growing cultures of *M. laidlawii* B. When protein synthesis is inhibited by removal of tryptophan, about 30% of the cells are "immune" to TLD, losing viability at a greatly reduced rate. This suggests that about 30% of the cells have completed rounds of replication, cannot reinitiate a new round in the absence of protein synthesis, and are thus immune to TLD (17, 26). However, when protein synthesis is inhibited by addition of CAP (Fig. 7), TLD proceeds only at a greatly reduced rate after a long lag. A similar difference in the extent of TLD, when protein synthesis is inhibited by using these two methods, has been observed in *E. coli* 15 T^- (2, 35).

A 2-μg/ml concentration of CAP caused nearly a twofold reduction in the rate of protein synthesis, but only a slight reduction in the rate of both RNA synthesis (Fig. 4) and TLD (Fig. 7). Similarly, 15 μg of CAP per ml inhibited nearly all protein synthesis, but reduced the rate of both RNA synthesis and TLD only about twofold. Thus, the lethal event associated with TLD is apparently associated with RNA synthesis rather than with protein synthesis, as is also apparently true for *E. coli* (12, 15, 25) and *B. subtilis* (40). In the absence of all pyrimidine nucleosides, about 10% of the cell population remains "immune" to TLD (Fig. 6), again suggesting an association of at least some RNA synthesis with the lethal event in TLD.

Pauling and Hanawalt (36) showed that a nonconservative mode of DNA replication similar to that found in ultraviolet-irradiated *E. coli* (37) occurs after thymine starvation of *E. coli* strain TAU-bar. A model was proposed in which single-strand breaks are introduced into the bacterial DNA as a result of transcription during normal growth, and that, in the absence of thymine, these breaks cannot be repaired and are possibly enlarged by an excision enzyme of the dark repair system. This hypothesis can also account for most of the observed properties of TLD in *M. laidlawii* B. TLD would necessarily require RNA synthesis, and the rate of TLD would be correlated with the generation period, as is observed. A mode of nonconservative DNA replication similar to that of *E. coli* TAU-bar has been found in ultraviolet-irradiated *M. laidlawii* B (D. Smith and P. Hanawalt, *in preparation*), strongly suggesting the presence of a dark repair system in this organism. Also, a small amount of nonconservative DNA replication has been found in normally growing *M. laidlawii* B, as well as in *E. coli* (16), and could be the result of repair of such breaks. Further, during the course of TLD in *M. laidlawii* B, a small residual amount of DNA synthesis is observed (Table 1). Such synthesis could arise from reutilization of thymine derivatives released during enlargement of the single-strand breaks. Single-strand breaks have been found in the DNA of thymine-starved *B. subtilis* (31) and might account for the decreased template activity for RNA polymerase, DNA polymerase, and DNA methylase. Regions of the DNA containing excised gaps would be particularly sensitive to shear during isolation and, hence, such single-strand breaks might be missed when usual DNA isolation methods are used.

These studies have shown that *M. laidlawii* B probably contains the same, or similar, pathways for nucleic acid synthesis as do other bacteria. Further studies, however, are needed to demonstrate the actual presence of the requisite enzymes of these pathways. The response of *M. laidlawii* B to addition of CAP is apparently identical to that of *E. coli*, and the aspects of TLD presented here are similar to those of *E. coli*. One must conclude that the organisms are more similar than different, and that the mycoplasmas are, with the exception of some functions such as possibly a slower DNA replication system, nearly as highly developed biochemically as are other bacterial systems.

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