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R-Factor elimination by thymine starvation

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SUMMARY

R-factor 1818, which is refractory to curing by acridines or ethidium bromide from *Escherichia coli* K12 and *Klebsiella aerogenes* 418 hosts, was eliminated from thymineless mutants of these organisms under conditions of thymine starvation. Neither R-7268 nor R-TEM could be eliminated by this method. The concurrence of R-TEM with R-1818 inhibited the curing of R-1818, whereas R-7268 had no effect on R-1818 elimination.

R-factors and other episomes have been eliminated from Gram-negative bacteria by treatment of the cells with acridine dyes (Hirota & Iijima, 1957; Watanabe & Fukasawa, 1961), sodium dodecyl sulphate (Tomoeda *et al.* 1968) or ethidium bromide (Bouanchaud, Scavizzi & Chabbert, 1969). However, these treatments do not eliminate some elements which have been deduced by other evidence to be extrachromosomal. In this category are included the colicin factors *Col I* and *Col E1*, which are refractory to acridine orange treatment. These were, however, eliminated from thymineless mutants of *Escherichia coli* K12 grown under conditions of thymine deprivation (Clowes, Moody & Pritchard, 1965).

R-factor 1818 (Table 1) in *E. coli* K12 *met*$^-$F$^-$58.161/*sp* (Meynell & Datta, 1966) or in *Klebsiella aerogenes* 418 (Smith & Hamilton-Miller, 1963) was found to be refractory to curing by growth in acridines or ethidium bromide; this led us to attempt its elimination by growing thymineless R-factor containing strains under conditions of thymine deprivation.

High level (> 30 μg/ml) thymine-requiring mutants (*thy*$^-$) of K12 and 418 were isolated (Smith, 1967) and R-factor 1818 was transferred into them from *E. coli* J6–2(1818) (Smith, 1969). When these *thy*$^-$ mutants harbouring R-1818 were deprived of thymine in suitably supplemented Davis Mingioli medium (DM) (Davis & Mingioli, 1950) containing 2 μg/ml thymine, as used by Clowes *et al.* (1965), the *thy*$^-$ cells underwent thymineless death (Barner & Cohen, 1954). However, the small proportion of secondary mutants to thymine low requirement (*tlr*) (Alikhanian *et al.* 1966) present in the original inoculum were able to multiply. Thus, clones from *thy*$^-$ inocula which had undergone 24 h incubation in 2 μg/ml thymine were found to be almost exclusively of the *tlr* type and none had lost their R-factor.

Since the method of thymine deprivation was found to suffer from this technical limitation, thymine starvation was then tested, i.e. the experiments were repeated using no thymine in the starvation medium. The *thy*$^-$ strains harbouring R-1818

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were grown overnight in suitably supplemented DM at 37 °C and 0·1 ml of washed overnight suspension was added to DM supplemented with respect to growth requirements, but without any added thymine and incubation continued. Samples were plated on nutrient agar supplemented with 60 μg/ml thymine and greater than 100 clones from each time of sampling were tested for R-1818 elimination by replica plating. It was found (Fig. 1; Table 2, lines 1 and 2) that during thymine starvation the incidence of R^- cells increased up to 100 times over control rates; greatest R-factor curing occurred during the period of most rapid thymineless death at 2–3 h after the onset of thymine starvation.

Table 1. *R-factors used* (*Datta & Kontomichalou, 1965*)

R-factor*	Resistances†
1818	Am Sm Su Tc
7268	Am Cm Sm Su
TEM	Am^H Sm

* R-1818 and R-7268 have been renamed R-46 and R-1 respectively by Meynell & Datta (1966). R-factors 1818 and 7268 were originally isolated in Dr E. S. Anderson's laboratory.

† Am, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Am^H, resistance to high levels of ampicillin which was sufficient to differentiate R-TEM from R-7268 and R-1818.

Table 2. *R-factor elimination by thymine starvation*

Washed cultures were suspended in complete DM medium lacking thymine and samples plated on fully supplemented medium. The resulting clones were tested for R-factor content by replica plating on to fully supplemented DM plates containing appropriate antibiotics. Antibiotics used: for R-1818, tetracycline, 10 μg/ml; for R-7268, chloramphenicol, 10 μg/ml; for R-TEM, ampicillin, 400 μg/ml. Antibiotic sensitive clones were checked to ensure loss of all R-factor resistances by streaking on media containing suitable antibiotics. Samples from K12(TEM/1818) *thy*$^-$ and K12(1818/TEM) *thy*$^-$ cultures taken at times other than 2 and 3 h of thymine starvation also showed no R-factor elimination greater than in control, unstarved cultures.

Strain and R-factor	Spontaneous occurrence of R^- cells (%)	Survivors after 2 h (%)	R^- cells after 2 h (%)	Survivors after 3 h (%)	R^- cells after 3 h (%)
1. K12(1818) *thy*$^-$	< 0·19	2·1	10·0	0·26	2·5
2. 418(1818) *thy*$^-$	< 0·08	46·1	5·5	19·2	8·0
3. K12(7268) *thy*$^-$	< 0·43	1·2	< 0·24	0·44	< 0·41
4. K12(TEM) *thy*$^-$	< 0·77	3·2	< 0·48	0·71	< 1·0
5. K12(7268/1818) *thy*$^-$	< 0·34	2·9	5·3*	0·31	< 0·76*
6. K12(1818/7268) *thy*$^-$	< 0·28	8·9	5·7*	2·2	2·8*
7. K12(TEM/1818) *thy*$^-$	< 0·97	2·7	< 0·36†	0·31	< 0·48†
8. K12(1818/TEM) *thy*$^-$	< 0·48	0·92	< 0·53†	0·27	< 0·79†

* Refers to R-1818 loss only: no elimination of R-7268 was observed.

† Refers to loss of either R-factor.

Attempts to eliminate R-factors 7268 and TEM (Table 1) by this method were unsuccessful (Table 2, lines 3 and 4). Strains containing two R-factors were constructed and examined to test the effect of the presence of refractory R-factors

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on the curing of R-1818 by thymine starvation. For example, K12(1818/7268)*thy*$^-$ was prepared by mating K12(1818)*thy*$^-$ with J6–2(7268) and plating on K12-supplemented DM+Cm, 10 μg/ml; clones which grew were streaked on DM+Tc, 10 μg/ml, and DM+Cm, 10 μg/ml, to check for the retention of R-1818 and introduction of R-7268 respectively. Other strains containing two R-factors were prepared with analogous techniques.

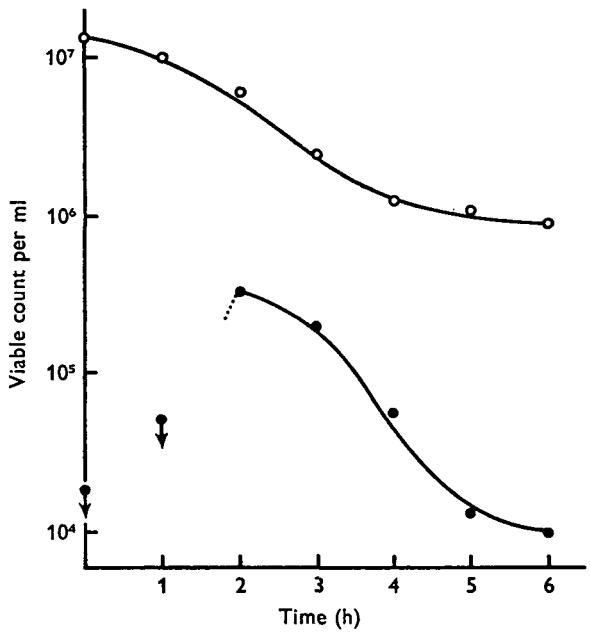

Fig. 1. Thymineless death and increase in R$^-$ population produced by thymine starvation of *Kleb. aerogenes* 418(1818)*thy*$^-$. ○, Overall viable count; ●, viable count of R$^-$ cells. Number of R$^-$ cells in initial population = $< 1{\cdot}7 \times 10^4$/ml, after 1 h = $< 5{\cdot}0 \times 10^4$/ml.

To test if the double R$^+$ strains contained each R-factor as a separate replicon, they were mated with J6–2 as recipient. It was found that each R-factor transferred independently, and examination of the drug-resistance patterns of recipients confirmed that no recombination had occurred. When the double-R$^+$ strains were subjected to thymine starvation it was found that the presence of R-7268 did not inhibit the curing of R-1818 from cells undergoing thymineless death (Table 2, lines 5 and 6), whereas the presence of R-TEM did inhibit R-1818 elimination (Table 2, lines 7 and 8). The order in which the pairs of R-factors were transferred into K12*thy*$^-$ did not affect these results.

The mechanism of R-1818 curing by thymine starvation is apparently R-factor specific, since R-1818 but not R-7268 or R-TEM is eliminated, and not host-strain specific, since curing occurs in thymineless strains of both *E. coli* and *Kleb. aerogenes*. Single-strand breaks, or nicks, occur in episomal (Freifelder, 1969) and chromosomal (Walker, 1970) DNA during thymine starvation. Although no direct correlation can

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yet be made between strand breakage and the biological effects of thymine starvation, the facts that chloramphenicol inhibits thymineless death (Cummings & Kusy, 1969), nicking (Weissbach & Shuster, 1969) and R-factor 1818 elimination (R.J.P. in preparation) and that readdition of thymine neither seals nicks (Freifelder, 1969; Weissbach & Shuster, 1969), nor decreases the proportion of R^- cells (R.J.P. in preparation) suggest that nicking may be at least partly responsible for R-1818 elimination.

Freifelder (1969) has suggested three possible causes of nicking: an endonuclease is either synthesized or activated, or a repair enzyme (ligase) is inhibited; he favours the ligase inhibition hypothesis. However, Weissbach & Shuster (1969) demonstrated production of single-strand breaks in bacteriophage lambda during thymine starvation of the host cell, which they ascribed to a λ endonuclease. If nicking is the cause of R-factor elimination, it would either have to occur more readily in the R-factor genome than in the chromosome, or the R-factor would have to be more susceptible to the effects of nicking. The former could be due to production of an endonuclease, more specific for R-factor DNA; the latter to a relative lack of ligase repair activity on the R-factor genome. The protective effect of R-TEM on R-1818 curing could possibly be explained by an R-TEM specific ligase acting *trans* on R-1818 DNA.

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