Lifetime of Messenger Ribonucleic Acid for Penicillinase Synthesis in Several Strains of Bacilli

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1. Previous studies of penicillinase synthesis in *Bacillus licheniformis* showed that enzyme synthesis after the addition of actinomycin continues for far longer in the constitutive mutant 749/C than in the parental inducible strain (Yudkin, 1966). This result was interpreted as indicating a difference in the lifetime of specific messenger RNA in the two strains. Other bacilli have now been examined in an attempt to see whether this difference is general. 2. There was no difference in the lifetime of messenger RNA for penicillinase synthesis between an inducible and a constitutive strain of *Bacillus cereus*. 3. Three freshly isolated constitutive mutants of *B. licheniformis* also had short-lived messenger RNA, like their inducible parent. 4. A reinvestigation of mutant 749/C confirmed the original finding that, on treatment with actinomycin, it continued to synthesize penicillinase far longer than did its parent. 5. An inducible revertant of mutant 749/C was indistinguishable from the original inducible strain, and appeared to have lost both constitutivity and long-lived messenger RNA in the back mutation.

Though most m-RNA* in bacteria is known to be unstable, there have been several reports of species of m-RNA that are comparatively long-lived (see, e.g., Pollock, 1963; Yudkin & Davis, 1965; Forchhammer & Kjeldgaard, 1967). Moses & Calvin (1965) and Yudkin (1965) suggested independently that a difference in lifetime of m-RNA might correspond to a difference in function: in particular, that short-lived m-RNA might code for inducible proteins and long-lived m-RNA for constitutive proteins. This idea gained some support from observations of two penicillinase-producing strains of *Bacillus licheniformis* (Yudkin, 1966). In the wild-type strain (749) that synthesized penicillinase inducibly the m-RNA specific for penicillinase appeared to be much more unstable than in a mutant (749/C) that synthesized the enzyme constitutively.

I have now extended these observations by examining the stability of the m-RNA coding for penicillinase in a pair of strains of *Bacillus cereus*, of which one synthesizes the enzyme inducibly and the other constitutively. In addition, I have studied other constitutive mutants of *B. licheniformis*, and also a strain that has reverted from constitutivity to inducibility. These experiments, like the earlier studies of penicillinase synthesis (Yudkin, 1966), involved the use of the antibiotic actinomycin D. This drug inhibits the synthesis of

RNA, and in its presence the rate of protein synthesis gradually declines (Kirk, 1960). Since this decline is believed to reflect the degradation of m-RNA, the time at which protein synthesis ceases is taken as a measure of the lifetime of the corresponding m-RNA (for discussion see Yudkin, 1966).

The present results suggest that unusually long-lived m-RNA for penicillinase synthesis is characteristic only of the constitutive mutant 749/C of *B. licheniformis* originally isolated by Pollock (1963).

MATERIALS AND METHODS

Parent organisms. *Bacillus cereus* strain 569 (wild-type, inducible for penicillinase synthesis) and 569/H (constitutive for penicillinase synthesis) were obtained through the kindness of Professor M. R. Pollock, as were the analogous strains of *Bacillus licheniformis*, 749 (inducible) and 749/C (constitutive). They were all grown as described previously (Yudkin, 1966).

Mutants. Constitutive strains of *B. licheniformis* were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Cells from a stationary-phase culture of strain 749 were washed once with 0.1 M-sodium acetate buffer, pH 5.0, resuspended in the same buffer at 10 mg. dry wt./ml., and incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.8 mg./ml.) at 37° for 30 min. The cells were washed in buffer and inoculated on to starch-agar plates (Dyke, Jevons & Parker, 1966). Constitutive colonies were picked after flooding the plates with a solution containing benzylpenicillin and iodine (Dyke *et al.* 1966).

* Abbreviation: m-RNA, messenger RNA.

Of the three mutants chosen for study, two (NG A and NG C) were fully constitutive, and the other (NG 1) semi-constitutive (Table 1).

A spontaneous revertant (SP 4) of the constitutive strain 749/C was found by picking a colony that gave no penicillinase reaction on the plate. Its properties with respect to penicillinase synthesis were indistinguishable from the wild-type strain 749.

All the strains of *B. licheniformis* used in this work are listed in Table 1.

Penicillinase. Induction and estimation of the enzyme were as previously described (Yudkin, 1966), save for the following modifications. Samples were taken into a tube containing chloramphenicol (final concn. 100 µg./ml.) and plunged into ice. A-grade pipettes were used throughout, to improve the accuracy of the assays.

Antibiotics. Actinomycin D was generously supplied by Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A., and cephalosporin C was given by Professor E. P. Abraham.

RESULTS

In *B. licheniformis*, penicillinase synthesis after the addition of actinomycin continued for far longer in the constitutive mutant 749/C than in the inducible strain 749 (Yudkin, 1966). In an attempt to see whether this difference was general, I com-

pared the constitutive mutant 569/H of *B. cereus* with its inducible parent 569. However, in both strains penicillinase synthesis ceased about 10 min. after actinomycin had been added to cultures growing at 25°.

I next sought to show whether other constitutive mutants of *B. licheniformis* possessed the long-lived m-RNA that is characteristic of mutant 749/C. In fact, two newly isolated constitutive mutants turned out to have short-lived m-RNA, like the wild-type strain 749. Both ceased to make penicillinase about 6 min. after the addition of actinomycin at 30°. The same was true of a semi-constitutive mutant, whether or not it was induced to make penicillinase at maximum rate. The amount of penicillinase synthesized after the addition of actinomycin was 10–14% of the quantity that the culture contained at the time when actinomycin was added; this observation was true for all the strains tested.

These discouraging results cast doubt on the original conclusion (Yudkin, 1966) that strain 749/C possessed an unusually long-lived m-RNA coding for penicillinase. I have therefore reinvestigated the response of strains 749 and 749/C to actinomycin. Five separate experiments were carried out with each strain at 25°. For strain 749 the times at which the synthesis of penicillinase ceased after the addition of actinomycin were 9, 9, 10, 12 and 12 min., with a mean of 10 min. (s.d. 1.4 min.). For strain 749/C the values were 20, 35, 35, 35 and 40 min., with a mean of 33 min. (s.d. 6.8 min.). Student's *t* test shows that the difference between the mean values is highly significant ($P < 0.01$).

Fig. 1 shows typical curves of the production of enzyme. The total percentage increase in penicillinase after the addition of actinomycin is about the same in the two strains. In five experiments the inducible strain synthesized 11% (s.d. 2.8%) of its

Table 1. *Penicillinase synthesis by various strains of B. licheniformis*

Strain	Penicillinase (units/mg. dry wt.)	
	Not induced	Fully induced
749	15	1200
749/C	2400	2400
NG A	2000	1950
NG C	1500	1600
NG 1	300	1200
SP 4	15	1000

Fig. 1. Effect of actinomycin on penicillinase synthesis by (a) strain 749 and (b) strain 749/C. Actinomycin (1 µg./ml.) was added at time 0 to cultures growing at 25°. The content of penicillinase/ml. of culture was measured by an iodometric method. Results are expressed as percentage increases in the content of penicillinase at time 0.

Fig. 2. Effect of actinomycin on penicillinase synthesis by (a) strain 749 and (b) strain SP4. Actinomycin ($1\mu\text{g./ml.}$) was added at time 0 to cultures growing at 30° . The content of penicillinase/ml. of culture was measured by an iodometric method. Results are expressed as percentage increases in the content of penicillinase at time 0.

content of penicillinase at time 0; and the constitutive strain synthesized 12% (S.D. 4.3%).

There remained the possibility that the apparent difference in lifetime of m-RNA was not connected with the constitutivity of mutant 749/C. This strain could conceivably have been the product of two separate mutations, one of which led to constitutivity and the other to enhanced stability of the m-RNA. One would then expect that when mutant 749/C reverted to inducibility the revertant would retain the long-lived m-RNA characteristic of mutant 749/C. In fact, however, a revertant (SP4) derived from mutant 749/C behaved, on treatment with actinomycin, exactly like the wild-type strain (Fig. 2).

One can summarize these results by saying that, of the eight strains tested, only one (749/C) continued to synthesize penicillinase for an unusually long time after treatment with actinomycin.

DISCUSSION

All of the constitutive strains used in this work synthesized more penicillinase than their fully induced wild-type parents. This fact can be explained in several ways; but the simplest explanation is that the m-RNA responsible for penicillinase synthesis is present in higher concentration in the constitutive than in the inducible organisms. If one assumes in addition that the m-RNA is translated, and decays, at the same rate in the constitutive strains as in the inducible strains, it follows that in the presence of actinomycin the quantity of penicillinase in the culture should

show the same percentage increase, in the same time, in all the strains.

This is, in fact, what happens in all the strains except mutant 749/C. In that organism penicillinase synthesis continues for far longer after the addition of actinomycin than it does in the other strains. However, a revertant of the mutant 749/C behaves in the same way, when treated with actinomycin, as the wild-type organism. It is therefore plain that a single mutation is responsible for the various ways in which the mutant 749/C differs from its parent. The characteristics of this strain are that it (a) produces twice as much penicillinase during normal growth as the wild-type, (b) synthesizes penicillinase constitutively, (c) after treatment with actinomycin produces no more enzyme (proportionately) than the wild-type, and (d) continues to synthesize enzyme at a low rate for an exceptionally long time after the addition of actinomycin. The first three of these characteristics are common to the other constitutive strains tested in this work, but the explanation that served for the others, namely that there is a higher concentration of specific m-RNA that is translated and destroyed at the normal rate, is incompatible with characteristic (d). A trivial explanation in terms of some special reaction of mutant 749/C to actinomycin appears to be excluded, since both this and the wild-type strain show precisely the same pattern of amino acid incorporation after treatment with the antibiotic (Yudkin, 1966).

Mutant 749/C was isolated after treatment of wild-type cultures with ethyl methanesulphonate (Pollock, 1963), whereas the other mutants used in this work were induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It is conceivable that this difference might have a bearing on the results; nevertheless there appears to be no simple way of accounting for the anomalous behaviour of mutant 749/C.

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