Some Properties of Two Erythromycin-dependent Strains of *Escherichia coli*

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Strains of *Escherichia coli* can be isolated that require erythromycin for growth. With one strain, AM, a range of antibiotics, including chloramphenicol, tetracycline, spectinomycin, kasugamycin and rifampicin, will substitute for erythromycin on solid and in liquid media; nalidixic acid supports growth in liquid but not on solid media. With a second strain, 103, chloramphenicol, tetracycline and spectinomycin support growth in liquid media but on solid medium only chloramphenicol substitutes for erythromycin. In media of higher than normal ionic strength, strain AM, but not strain 103, can grow in the absence of antibiotics. Possible reasons for these complex phenotypes are discussed.

INTRODUCTION

In *Escherichia coli*, mutated ribosomal proteins can cause resistance to antibiotics such as spectinomycin, streptomycin, gentamicin and erythromycin (Reynolds, 1984). Mutation can also generate organisms that require an antibiotic for growth. If the drug is one that acts on ribosomes, a potential mechanism is that antibiotic binding restores function to altered ribosomes. In *E. coli*, streptomycin resistance or dependence both result from single amino acid changes in ribosomal protein S12 (Birge & Kurland, 1969); streptomycin may allow growth of dependent strains by decreasing proof-reading by their over-accurate ribosomes (Ruusala et al., 1984). Dependence on other antibiotics has been less studied. In spectinomycin- and kasugamycin-dependent mutants of *Bacillus subtilis* there are separate mutations for resistance and dependence; some kasugamycin-dependent mutations create spectinomycin dependence when introduced into a spectinomycin-resistant strain (Henkin et al., 1975; Pai et al., 1981). In *E. coli*, kasugamycin dependence and resistance can be caused by separate mutations or result simultaneously from an altered ribosomal protein S9 (Dabbs, 1983). Some rifampicin- and spectinomycin-dependent strains are double mutants; in one rifampicin-requiring strain, this drug could be replaced by kasugamycin but not by other ribosomally-targeted antibiotics (Dabbs & Looman, 1981).

The present work describes the phenotypes of two erythromycin-dependent strains of *E. coli*, AM (Dabbs, 1979) and 103 (Wild, 1988). Revertants of strain AM to independence often lack a ribosomal protein; such organisms have been used to help locate that protein on non-mutant ribosomes (Dabbs et al., 1981; Stoffler et al., 1984) and to explore the functions in which the protein is involved (Subramanian & Dabbs, 1980). Some erythromycin-independent revertants from strain 103 show defects in ribosome assembly and in the regulation of rRNA synthesis (Wild, 1988). However, the causes of the erythromycin dependence of strains AM and 103 are unclear. The present work reports observations on their phenotypes that relate to their antibiotic dependence.

Abbreviation: m.g.t., mean generation time.
Bacteria and media. Experiments used E. coli strain A19 (Gesteland, 1966) and two erythromycin-dependent derivatives, strains AM (Dabbs, 1979) and 103 (Wild, 1988). Unless otherwise stated, organisms were grown at 37 °C in DP-broth, which contained, per litre, 10 g tryptone, 10 g yeast extract, 5 g glucose, 3 g KH₂PO₄, 7 g K₂HPO₄, 500 mg trisodium citrate, 3H₂O and 100 mg MgSO₄. 7H₂O. DP-agar was the same medium solidified with 2% (w/v) agar. DT-medium contained tryptone, yeast extract and glucose as for DP-medium but with 0·1 M-Tris/HCl, pH 7·4, and other inorganic constituents (Blundell & Wild, 1969) replacing the phosphates and other salts of DP-medium. D2T-medium was the same as DT-medium but with the concentrations of Tris and other salts doubled. CT-medium contained the 0·1 M-Tris buffer and inorganic constituents of DT-medium plus, per litre, 2 g glucose, 1 g Casamino acids and 8 mg uracil.

Growth. Dependent strains were maintained on DP-agar containing 200 µg erythromycin ml⁻¹. Growth in liquid media was measured as the optical density of cultures at 450 nm (OD₄₅₀). For some experiments, protein, RNA, or DNA synthesis was followed by addition, per ml of DP-medium, of 23 pCi (92·5 kBq) ¹⁴C-leucine, or 0·025 pCi (0·925 kBq) [2-¹⁴C]uracil, or 0·33 pCi (12·2 kBq) [6'-³H]thymidine plus 7·5 pg unlabelled thymidine. Samples (0·5 ml) from cultures, taken in quadruplicate, were added to 0·5 ml of 10% (w/v) trichloroacetic acid (TCA) at 0°C filtered through Whatman GF/B glass fibre circles, washed on the filter with 5% TCA, then ethanol, and dried at 80 °C in a vacuum oven. Radioactivities were measured in scintillant containing 5 g butyl-PBD per litre of toluene.

Preparation of extracts and density-gradient centrifugation. The techniques were as previously described (Wild, 1988).

RESULTS

Growth with erythromycin

The parent strain, A19, had a mean generation time (m.g.t.) in DP-medium of about 26 min. Growth was slowed substantially by 25 µg erythromycin ml⁻¹ (m.g.t. 50 min) and prevented by 100 µg ml⁻¹ and above. Both dependent strains were at least partially resistant. Strain 103 grew exponentially (m.g.t. about 70 min) with 100 µg erythromycin ml⁻¹. At this concentration the growth of strain AM, although exponential, was slow (m.g.t. 120 min); the 25 µg erythromycin ml⁻¹ routinely used for growth experiments gave an m.g.t. of about 60 min. In medium without erythromycin, both dependent strains grew well for 3–4 generations; growth then stopped rather abruptly and there was some subsequent lysis (Fig. 1a).

An irreversible change occurred soon after erythromycin deprivation. When strain 103 was inoculated to OD₄₅₀ 0·05 in DP-medium without erythromycin, the number of viable organisms ml⁻¹ (measured from the colonies formed from dilutions spread on DP-agar containing 100 µg erythromycin ml⁻¹) halved (from 4 × 10⁷ ml⁻¹ to 2 × 10⁷ ml⁻¹) during the first doubling of the OD₄₅₀. In a similar experiment with strain AM, the OD₄₅₀ increased about tenfold in the first 3 h in erythromycin-free medium but the number of viable organisms ml⁻¹ remained almost constant. No gross change in the morphology of either strain was observed to account for these disparities.

Macromolecular synthesis by dependent strains

Erythromycin binds to ribosomes. Changes in the distribution of ribosomes and ribosomal sub-units were therefore sought in dependent organisms deprived of antibiotic. Strain AM was inoculated to OD₄₅₀ 0·05 into DP-medium without and with 25 µg erythromycin ml⁻¹. At OD₄₅₀ 0·5, when the culture deprived of erythromycin had just ceased to grow (cf. Fig. 1a), both cultures were harvested, and extracts were made and centrifuged. The sedimentation profiles were very similar to each other and to that of their parent, strain A19 (Wild, 1988); most ribonucleoprotein was in 70S ribosomes, with a small proportion in 'native' 30S and 50S ribosomal sub-units.

The effect of erythromycin on RNA and protein synthesis was examined. The parent strain, A19, was grown with 25 µg erythromycin ml⁻¹. Growth, and both RNA and protein synthesis were inhibited. However, whereas the protein synthesized per OD₄₅₀ unit was very similar to that in the control, more RNA was synthesized per OD₄₅₀ unit during erythromycin inhibition (Fig. 2a, b). Growth of strain A19 with erythromycin thus caused an imbalance in which protein synthesis was inhibited more than RNA synthesis.
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Fig. 1. Growth of, and RNA and protein synthesis by, dependent strains. Strains AM (○, ●) and 103 (□, ■) were grown overnight with 25 and 100 µg erythromycin ml⁻¹ respectively and then inoculated into medium containing [¹⁴C]uracil and [³H]leucine without (open symbols) and with (filled symbols) the same concentration of erythromycin. (a) Growth of the cultures; (b), (c), radioactivity incorporated by strain AM into RNA and protein respectively.

Similar experiments were carried out with the dependent strains. Strain AM grew exponentially with 25 µg erythromycin ml⁻¹ but more slowly than during the first 2–3 doublings without antibiotic (Fig. 1a). During this period, RNA synthesis per OD₄₅₀ unit was virtually the same in both cultures (Fig. 1b) but protein synthesis per OD₄₅₀ unit was less in the culture that received erythromycin (Fig. 1c). Results with strain 103 were similar and are not shown. Thus erythromycin inhibits protein synthesis more than RNA synthesis in dependent as well as sensitive organisms. With strain AM in the absence of erythromycin, both RNA and protein continued to be made during the onset of cell lysis and after the absorbance had begun to decrease (Fig. 1b, c). An inability to make RNA or protein is thus not responsible for the cessation of growth in deprived cultures.

DNA synthesis by strains A19 and AM was also compared (Fig. 3). The rates of incorporation of [³H]thymidine by strain A19, per OD₄₅₀ unit, were linear and about 20% greater during growth inhibition by 25 µg erythromycin ml⁻¹. Incorporation by strain AM, growing with 25 µg erythromycin ml⁻¹, was very similar to that of strain A19. In the absence of erythromycin, rather more [³H]thymidine was incorporated than with the antibiotic present; DNA continued to be made after the OD₄₅₀ of the culture reached its maximum. Thus neither the halting of growth
nor the large difference between the viable number and mass of cells ml\(^{-1}\) can be ascribed to a failure of DNA synthesis in the absence of the antibiotic.

**Replacement of erythromycin by other antibiotics**

Chloramphenicol would substitute for erythromycin in supporting exponential growth of strain AM from OD\(_{450}\) 0.05 to a high optical density (\(> 2\)). Chloramphenicol was most effective at a low concentration (0.25–0.5 \(\mu\)g ml\(^{-1}\)) that increased the m.g.t. of the parent strain A19 only from 26 to 28 min. Higher concentrations (\(> 1 \mu\)g ml\(^{-1}\)) inhibited the growth of both parent and dependent strains. Lower concentrations allowed more extensive growth of strain AM than in the absence of antibiotic but growth was not maintained. Spectinomycin (5 or 10 \(\mu\)g ml\(^{-1}\)) or tetracycline (0.05 or 0.10 \(\mu\)g ml\(^{-1}\)) also gave exponential growth of strain AM to high OD\(_{450}\). These concentrations did not inhibit growth of strain A19, and 5 \(\mu\)g spectinomycin ml\(^{-1}\) had no effect on RNA or protein synthesis by this organism (result not shown). Another protein synthesis inhibitor, kasugamycin, also supported growth of strain AM to high OD\(_{450}\), with an optimum concentration (75 \(\mu\)g ml\(^{-1}\)) that had no effect on growth of the parent strain, A19. Surprisingly, rifampicin would also substitute for erythromycin; the optimum concentration (1 \(\mu\)g ml\(^{-1}\)) caused a short (about 15 min) lag in the growth of strain A19 but had no effect on the subsequent rate of growth. Nalidixic acid also had some effect; at 2 \(\mu\)g antibiotic ml\(^{-1}\), rapid growth for 3–4 doublings was succeeded by slower but exponential growth to high OD\(_{450}\).

Because of the large discrepancy between OD\(_{450}\) measurements and cell viabilities noted above, various antibiotics were also tested for their ability to replace erythromycin as regards effects on bacterial colony formation. Strain AM (grown with 25 \(\mu\)g erythromycin ml\(^{-1}\)) was washed and about \(10^4\) organisms spread on plates of DP-agar. Antibiotic solution (50 \(\mu\)l) was added to a centre well and plates were stored at 4 °C overnight for diffusion to occur. Subsequent incubation was at 37 °C. With erythromycin a dense halo of colonies grew in 2–3 d around a clear central zone. Presumably the halo formed within a range of erythromycin concentrations adequate to support growth but not high enough to be inhibitory. Similar haloes formed with chloramphenicol, spectinomycin, tetracycline, rifampicin and kasugamycin in the centre well. There was no halo with nalidixic acid.

With strain 103 in liquid DP-medium, low concentrations of chloramphenicol (0.5 \(\mu\)g ml\(^{-1}\)), tetracycline (0.1 \(\mu\)g ml\(^{-1}\)) and spectinomycin (30 \(\mu\)g ml\(^{-1}\)) would substitute for erythromycin. However, kasugamycin, nalidixic acid and rifampicin (each tested over a range of
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Fig. 3. DNA synthesis by strains A19 and AM. (a) Strain A19 (□, ■) grown without erythromycin, and (b) strain AM (○, ●) grown with 25 μg erythromycin ml⁻¹, were inoculated into DP-medium with added [³H]thymidine (0.33 μCi, plus 7.5 μg unlabelled thymidine ml⁻¹) without (open symbols) and with (filled symbols) 25 μg erythromycin ml⁻¹.

Fig. 4. Growth of strain AM in Tris-buffered media. Strain AM, grown in DP-medium with 25 μg erythromycin ml⁻¹, was inoculated into DP-medium (○), DP-medium plus 2% NaCl (■), DT-medium (●) and D2T-medium (■), all without erythromycin. For clarity, the curve for DP-medium + NaCl is displaced by 1 h along the time axis.

concentrations) gave little more of an increase in OD₄₅₀ than that after erythromycin withdrawal. On DP-agar, only chloramphenicol would replace erythromycin. A range of concentrations of either tetracycline or spectinomycin consistently failed to give growth haloes, even though these antibiotics supported growth in liquid medium.

**Growth of dependent strains without antibiotics**

Strain AM was grown in DP-medium containing 25 μg erythromycin ml⁻¹ and then inoculated into DT-medium without erythromycin. [DT-medium has the inorganic constituents of DP-medium (mainly 0.06 M-phosphate buffer) replaced by 0.1 M-Tris plus other salts.] The increase in OD₄₅₀ before growth ceased was about five times that in DP-medium (Fig. 4). In D2T-medium (with the concentration of Tris buffer and other salts doubled), strain AM grew exponentially without erythromycin and could be subcultured. With strain 103, substitution of DT- or D2T-medium for DP-medium had no effect on the extent of growth in the absence of erythromycin.

Strain AM (but not strain 103) would also grow without erythromycin in CT-medium, which contains the Tris buffer and salts of DT-medium plus glucose, Casamino acids and uracil. A further difference between the strains was that AM (but not 103) grew erratically without erythromycin in DP-medium containing 2% NaCl (Fig. 4) and could be subcultured.

**DISCUSSION**

Strains AM and 103 were both isolated as erythromycin-dependent mutants. However, erythromycin is only one of a range of antibiotics that supports their growth. With strain AM,
the twin criteria of growth in liquid and on solid media showed that spectinomycin, tetracycline, kasugamycin and rifampicin can substitute for erythromycin. Nalidixic acid supported growth in liquid but not solid medium. These antibiotics were most effective within a narrow concentration range that had little or no effect on growth of the parent strain. Presumably too little antibiotic cannot maintain the dependency; too much is inhibitory. Strain 103 differed from strain AM; in liquid cultures, chloramphenicol, tetracycline and spectinomycin supported growth, but on solid media only chloramphenicol would substitute for erythromycin. This also suggests that conditions for growth are delicately balanced and makes it difficult to assess the significance of the failure to find concentrations of kasugamycin, rifampicin or nalidixic acid to allow growth of this strain.

An erythromycin-dependent mutant (strain FS141) of *E. coli*, described by Sparling & Blackman (1973), had an erythromycin-resistance mutation at about 73 min on the chromosome and a separate dependence mutation, *mac*, at about 26 min. Some properties of strains AM and 103 are similar to those of strain FS141. With the latter, the effects of erythromycin deprivation on the $OD_{450}$ increase were only evident after 1-2 mass doublings; addition of erythromycin 1.5 h after its withdrawal failed to restore growth, suggesting (as with strains AM and 103) that an irreversible process is triggered by drug deprivation. However, with strain FS141, erythromycin could be replaced only by the related macrolide oleandomycin. Chloramphenicol gave some mass increase but only at very high concentration ($400\,\mu g\,ml^{-1}$); tetracycline and spectinomycin were without effect. Strain FS141 more closely resembles strain 103 than strain AM but the phenotypes do not allow conclusions to be drawn about the variety of genotypes involved. The same problem underlies the findings that the antibiotic requirements of strain AM (but not strain 103) can be satisfied by an increase in the ionic strength of media.

There is at present no satisfactory explanation of the phenotypes of strains AM and 103. One possibility is that their dependence is ribosomal. Strain 103 has an altered ribosomal protein L25 but it is not clear whether this is related to the dependency (Wild, 1988); strain AM has a more acidic ribosomal protein L4 but the dependence mutation has not been located (Stoffler et al., 1984). On this basis, erythromycin interacts with dependent ribosomes to restore function. So does chloramphenicol, which like erythromycin binds to 50S ribosomal sub-units close to the peptidyl-transferase reaction centre (Reynolds, 1984). Interactions of tetracycline, spectinomycin and kasugamycin with the smaller ribosomal sub-unit can be transmitted to the larger (Saltzman & Apirion, 1976). Ribosome conformation might change in media of higher ionic strength. Although rifampicin acts primarily on initiation of RNA synthesis, drug-treated organisms show marked changes in their ribosomes (Blundell & Wild, 1971). Secondary effects of nalidixic acid on ribosomes are also possible. A second possibility is that synthesis of RNA and protein by dependent strains is 'unbalanced' (Dabbs, 1983). Balance can be restored either genetically by mutations to independence that alter ribosomes so as to slow protein synthesis, or phenotypically by antibiotics that preferentially inhibit protein synthesis, as does erythromycin and at least some of the other antibiotics that can substitute for it (Holmes & Wild, 1966). However, the antibiotics that replace erythromycin are most effective at concentrations that scarcely inhibit growth or, with spectinomycin at least, RNA and protein synthesis. If rebalanced synthesis does enable dependent strains to grow, very subtle changes must be involved. A third possibility is that strain AM has a membrane defect that is minimized by antibiotics or altered by conditions of growth as, for example, is porin synthesis (Ramakrishnan et al., 1987). Ribosomally targeted antibiotics can interact with cell membranes. For example, loss of viability by gentamicin-resistant *E. coli* was prevented by NaCl or sucrose in the medium (Bosl & Bock, 1981). A third of spectinomycin-resistant mutants isolated by Miyoshi & Yamagata (1976) were simultaneously sucrose-dependent. In these mutants, a protein missing from cytoplasmic membranes was released from the membranes of sensitive organisms by spectinomycin (Mizuno et al., 1977). The antibiotic dependence of strain AM could be an example of similar interactions.

In distinguishing these hypotheses, knowledge of the location of the dependent mutation may be particularly helpful. Dependent ribosomes may need an antibiotic to function properly in
protein synthesis. Membrane proteins may be missing or altered in dependent organisms. These suggestions are being tested.

REFERENCES


