Respiration Rate, Growth Rate and the Accumulation of Streptomycin in 
Escherichia coli

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Using chemostat cultures of Escherichia coli it was possible to vary respiration rates while maintaining a constant growth rate. This allowed the effect of variations in respiration rates on the accumulation of streptomycin to be studied in cultures at constant growth rates. At a particular dilution rate cultures exhibited higher respiration rates when phosphate limited growth than when carbon limited growth. A ubiquinone-deficient strain had a lower rate of respiration at a particular dilution rate than a related ubiquinone-sufficient strain. In spite of these differences in respiratory activity, the accumulation of streptomycin was identical in carbon- and in phosphate-limited chemostat cultures of ubiquinone-deficient and ubiquinone-sufficient strains. Moreover, accumulation of streptomycin in an anaerobic chemostat culture occurred at the same rate as that in an aerobic chemostat. There was however a lag of 1.5 h before accumulation commenced in the anaerobic culture, a feature that was not apparent in the aerobic culture. These results indicate that the lower rates of respiration in slow-growing bacteria are not responsible for the decreased accumulation of streptomycin in slow-growing compared to fast-growing cultures. Moreover, it seems unlikely that quinones are involved directly (e.g. as carriers) in streptomycin accumulation, since removal of 90% of cellular ubiquinone, or replacement of ubiquinone with a structural analogue, did not affect accumulation as long as mutant and parent cultures grew at the same rate.

INTRODUCTION

In a previous study using chemostat cultures of Escherichia coli and Bacillus megaterium, the rate of accumulation of streptomycin was proportional to the growth rate of the culture before addition of the antibiotic (Muir et al., 1984). That is, streptomycin was accumulated more rapidly by fast-growing than by slow-growing bacteria. Respiration rates of bacteria also vary with dilution rates. Thus for chemostat cultures of Klebsiella aerogenes, where growth was limited by available carbon source, respiration rates were greater for cultures at higher than at lower dilution rates (Neijssel & Tempest, 1975). In view of recent studies (see Discussion), which point to the importance of respiratory energy in streptomycin accumulation, we considered it was possible that the rapid respiration rates of fast-growing cultures were responsible for the greater accumulation of streptomycin observed at higher dilution rates. We therefore investigated the relationship between growth rates, respiration rates and the rates of accumulation of streptomycin in chemostat cultures of E. coli.

Abbreviations: AUM, aminoglycoside uptake medium; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
Methods

Bacterial strains. All strains used were derivatives of E. coli K12. Strain JP2140 [ile-1 his-29(am) trpA9605(am)] was obtained from A. J. Pittard, University of Melbourne, Australia. The streptomycin-sensitive strains NSW60 (ubiA420 menA401) and NSW62 (menA401) were constructed in the following way. Strain AN144 (Hfr ubiA420 metB rpsL ref), obtained from I. G. Young, Australian National University, Canberra, Australia, was mated overnight in L-broth with NSW33, a spontaneous nalidixic acid-resistant derivative of JP2140. A recombinant clone, NSW56 (metB ubiA420), unable to grow on succinate as sole source of carbon, was isolated. Strain NSW60 (ubiA420 menA401) is a met+ transductant derived from a cross using NSW56 and phage P1kc propagated on a menA01 strain (AN386). Strain NSW62 (menA401) was derived by selecting a clone capable of growth on succinate following a transduction cross with NSW60 and phage P1kc from a ubi+ strain. Genetic nomenclature conforms to that recommended by Demerec et al. (1968), and the symbols for gene loci are those of Bachmann & Low (1980).

Media and growth of cells. Cultures were maintained on plates consisting of nutrient broth (Oxoid) enriched with 0.5% (w/v) brain heart infusion (Oxoid) and supplemented with 2% (w/v) agar (Difco). Cultures from transduction and conjugation experiments were grown on plates consisting of medium A (Davis & Mingioli, 1950) with 2% agar, supplemented with glucose or succinate (each 30 mM), thiamin. HCl (3 μM), L-isoleucine (0.38 mM), L-valine (0.43 mM), L-histidine (0.52 mM), L-tryptophan (0.2 mM), L-methionine (0.17 mM), p-hydroxybenzoate (0.1 or 1.0 mM), uracil (0.2 mM) and nalidixic acid (50 μg ml⁻¹), as required. All batch culture experiments, strains were grown in nutritive broth supplemented with 0.5% (w/v) brain heart infusion. Generation times (doubling times) for batch cultures were calculated from the slope of the linear (exponential) portions of plots of log OD₆₀₀ against time. For growth in continuous culture, aminoglycoside uptake medium (AUM; Bryan & Van Den Elzen, 1971) was used as previously described (Muir et al., 1984). Growth was limited by availability of either carbon [1, 2 or 5 mM-glucose plus 0.1% (w/v) Casamino acids (Difco)] or KH₂PO₄ (0.1 mM) since increasing the concentration of either glucose (or Casamino acids) or KH₂PO₄ in the carbon-limited and phosphate-limited chemostats respectively, resulted in higher cell densities. The pH of chemostat cultures in steady-state at the dilution rates used was the same as that of the uninoculated medium.

For anaerobic growth, the complete chemostat apparatus including a flask containing 400 ml sterile AUM was autoclaved at 0.069 MPa for 25 min in order to dispel oxygen from the medium and sterilize the apparatus. When the medium had cooled to about 50°C, nutrient solutions were added aseptically. The chemostat was then inoculated with 3 ml culture, and the apparatus assembled and flushed with nitrogen (instrument grade; CIG, Australia) for several hours. After equilibration of culture and medium had occurred in the oxygen-free environment, medium flow to the chemostat was commenced. Nitrogen was bubbled continuously through the sparger to mix cells and maintain the anaerobic environment. Syringes for sample withdrawal were flushed with nitrogen before and after use. The absence of oxygen in the chemostat was confirmed using a Clark-type oxygen electrode. The chemostat apparatus and medium for the aerobic (control) experiment were prepared in an identical manner except that air was supplied instead of nitrogen and medium flow was initiated as soon as the chemostat was inoculated. Growth was monitored by OD measurements in a Spectronic 20 spectrophotometer at 600 nm or in a Klett-Summerson colorimeter fitted with a blue filter.

Streptomycin accumulation experiments. Streptomycin was mixed with [3H]dihydrostreptomycin (Amersham) to give a specific activity of 169 d.p.m. (ng streptomycin)⁻¹. The final concentration of streptomycin in all experiments was 20 μg ml⁻¹. Accumulation experiments in batch and continuous cultures were done as previously described (Muir et al., 1984).

Measurement of respiration rates in chemostat cultures. Respiration rates were measured by trapping and counting the 14CO₂ that was evolved from [14C]glucose. The concentration of glucose in the medium was 10 mM (0.1 μCi; 3.7 kBq) ml⁻¹ and 2 mM (0.02 μCi; 0.74 kBq) ml⁻¹ for phosphate- and carbon-limited cultures respectively. Gases from the chemostat vessel passed through an acid reservoir to the CO₂ trapping system which consisted of a sealed scintillation vial containing ethanolamine (2 ml) and methanol (4 ml). The seal on the scintillation vial was penetrated by a narrow necked glass tube which allowed bubbling of gases through a liquid column of ethanolamine and methanol. The CO₂ was collected for 1 h after which time the glass tube was washed twice with methanol (2 ml) into the scintillation vial. A fresh vial and trap was connected at hourly intervals and collection continued until a constant evolution rate of CO₂ was established (usually five or six samples). Aquassure scintillation fluid (5 ml; New England Nuclear) was added and the vials were counted in a Packard Tricarb liquid scintillation spectrometer (model 300). The five or six values for d.p.m. were averaged for calculation of respiration rates which are expressed as d.p.m. in CO₂ evolved h⁻¹ (mg dry wt bacteria)⁻¹.

The efficiency of the CO₂ trapping system was tested by adding NaH¹⁴CO₃ to the chemostat vessel which contained 1 m-HCl: 90% of the radioactive bicarbonate was recovered as ¹⁴CO₂.

Estimation of quinones in whole cells. Bacteria grown in batch cultures of nutrient broth supplemented with 0.5% (w/v) brain heart infusion were extracted with acetone and the concentration of quinones, was determined by thin-layer chromatography on silica gel G plates as described previously (Muir et al., 1981).
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Fig. 1. Streptomycin accumulation in chemostat cultures of E. coli. strains JP2140, NSW60 (ubiA menA) and NSW62 (ubiA menA) under either carbon or phosphate limitation. Strain JP2140 was grown in chemostats in AUM with limiting phosphate at \( D = 0.25 \text{ h}^{-1} \) (●) and \( D = 0.6 \text{ h}^{-1} \) (○), or with limiting carbon at \( D = 0.25 \text{ h}^{-1} \) (■) and \( D = 0.6 \text{ h}^{-1} \) (□). Strains NSW60 (▲) and NSW62 (△) were grown similarly under phosphate limitation and at \( D = 0.25 \text{ h}^{-1} \). The medium for strain NSW60 contained p-hydroxybenzoate (0.1 mM). When steady-state growth was reached streptomycin (20 µg ml\(^{-1}\)) was added to the culture vessel and medium reservoir and samples were taken and measured for accumulation of streptomycin (see Methods).

Table 1. Respiration rates in carbon- or phosphate-limited chemostats of E. coli strains JP2140, NSW60 and NSW62

<table>
<thead>
<tr>
<th>Strain</th>
<th>( D ) (h(^{-1}))</th>
<th>Phosphate-limited</th>
<th>Carbon-limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP2140</td>
<td>0.25</td>
<td>1.78 ± 0.12 × 10^6</td>
<td>6.66 ± 0.12 × 10^5</td>
</tr>
<tr>
<td>JP2140</td>
<td>0.6</td>
<td>1.95 ± 0.10 × 10^6</td>
<td>1.22 ± 0.03 × 10^5</td>
</tr>
<tr>
<td>NSW60*</td>
<td>0.25</td>
<td>6.52 ± 0.52 × 10^5</td>
<td>NT</td>
</tr>
<tr>
<td>NSW62</td>
<td>0.25</td>
<td>1.12 ± 0.14 × 10^6</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, Not tested.
* Medium contained p-hydroxybenzoate (0.1 mM).

RESULTS

Respiration rates and accumulation of streptomycin in carbon-limited and phosphate-limited chemostat cultures

As expected from previous results (Neijssel & Tempest, 1975), respiration rate, as measured by evolution of CO\(_2\), increased with increasing dilution rate in carbon-limited chemostat cultures of E. coli strain JP2140 (Table 1). Accumulation of streptomycin was also greater in faster growing cultures (Fig. 1; Muir et al., 1984). At a lower dilution rate (\( D = 0.25 \text{ h}^{-1} \)), CO\(_2\) evolution in a phosphate-limited chemostat culture was approximately three times greater than that in a carbon-limited culture (Table 1). This result is consistent with that of Neijssel & Tempest (1976). Though the respiration rate in the phosphate-limited culture was almost threefold greater than in the carbon-limited culture, the rates of accumulation of streptomycin were the same for both (Fig. 1).
Respiration rates and accumulation of streptomycin in ubiA menA strains of E. coli

To study the effects of a decrease in cellular ubiquinone on respiration and on accumulation of streptomycin, we measured the rates of evolution of CO₂ and of streptomycin accumulation in a ubiquinone-deficient strain of E. coli (NSW60; ubiA menA) and its parent (NSW62; ubi+ menA). We chose the menA strain so that menaquinone and demethylmenaquinone, which would have been present in men⁺ strains, could not influence the results. It had previously been found (M. Muir, unpublished results) that removal of menaquinone and demethylmenaquinone had no effect on aerobic respiration and only a slight negative effect on accumulation of streptomycin. In phosphate-limited chemostat cultures of strain NSW62 (ubi⁺ menA) the respiration rate was 1.7 times that of strain NSW60 (ubiA menA) at D = 0.25 h⁻¹ (Table 1) whereas the rates of accumulation of streptomycin in the two strains were identical (Fig. 1). In batch culture, however, accumulation of streptomycin was much greater for strain NSW62 than for strain NSW60 (Fig. 2). Generation times for batch cultures of strains NSW60 and NSW62 were 130 min and 25 min respectively.

Both batch and chemostat cultures of strain NSW60 were supplemented with p-hydroxybenzoate (0.1 mM) to allow the production of some ubiquinone (Wallace & Young, 1977). When harvested from batch cultures, strains NSW60 and NSW62 contained 26 and 226 ng ubiquinone (mg dry wt bacteria)⁻¹ respectively. No menaquinone or demethylmenaquinone could be detected in either strain.

Accumulation of streptomycin in an anaerobic chemostat culture of E. coli

The finding that a ubiquinone-deficient strain with a lower respiration rate accumulated streptomycin at the same rate as a ubi⁺ strain provided that growth rates were taken into account (Fig. 1) prompted us to consider the possibility that the lowered accumulation of aminoglycosides by facultative anaerobes growing anaerobically might be accounted for (at least partially) by slower growth rates. For example, a batch culture of strain JP2140 growing...
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Respiration and streptomycin uptake by E. coli aerobically in AUM with glucose and Casamino acids had a generation time of 36 min whereas anaerobically its generation time was 72 min. To avoid effects that differences in growth rates might have, we measured the accumulation of streptomycin in chemostat cultures growing aerobically and anaerobically at the same dilution rate. To eliminate the menaquinone-dependent electron transport to fumarate that may occur under anaerobic conditions (Kroger, 1978), strain NSW62 (menA) was used for these experiments. AUM was modified by omitting nitrate (NH₄Cl replaced NH₄NO₃) which, if present, might act as an electron acceptor. Uracil (0.2 mM) and KHC0₃ (20 mM) were also added to aerobic and anaerobic cultures. In addition to Casamino acids (0.1%, w/v), the anaerobic culture contained glucose (5 mM) (aerobic, 1 mM) in order to provide similar cell densities at steady-state in both cultures. The pH of aerobic and anaerobic cultures was 7.0, indicating that no pH decrease was associated with anaerobic growth at the glucose concentrations used.

Accumulation of streptomycin occurred at similar rates in aerobic and anaerobic chemostat cultures of strain NSW62 (D = 0.34 h⁻¹; Fig. 3). However, accumulation in the anaerobic culture was preceded by a 1.5 h lag, whereas no lag was evident for the aerobic culture. Accumulation of streptomycin under anaerobic conditions was abolished by carbonyl cyanide m-chlorophenylhydrazone (CCCP; 10 µM) added 180 min after the streptomycin.

**DISCUSSION**

In this study we dissociated growth rates from respiration rates by three means. The rate of respiration was higher in chemostat cultures at a fixed dilution rate when phosphate rather than carbon was the growth-limiting nutrient. Decreases in respiration rates were accomplished using chemostat cultures of a respiratory mutant and by growth of a non-respiratory mutant under anaerobic conditions. In all cases the rate of accumulation of streptomycin remained unaffected by changes in respiratory activity. It must be noted however that accumulation under anaerobic conditions was preceded by a lag that was not apparent in the corresponding aerobic culture. This lag may explain the heightened resistance to streptomycin that is characteristic of facultative anaerobes under anaerobic conditions (Kogut et al., 1965).

Muir et al. (1981) reported the isolation and characterization of a respiratory mutant of E. coli K12 (strain NSW77) with decreased accumulation of aminoglycosides in batch culture. This strain harboured a mutation at ubiF, a gene encoding the enzyme that catalyses the penultimate step of ubiquinone biosynthesis (Young et al., 1973). Another ubiquinone-deficient strain (ubiD) and a haem-deficient strain of E. coli also had decreased accumulation of aminoglycosides (Campbell & Kadner, 1980; Bryan & Van Den Elzen, 1977). Mutant strains of other genera of bacteria possessing both low level resistance to aminoglycosides and defective respiratory metabolism have been isolated. Thus, cytochrome-deficient strains of Pseudomonas aeruginosa (Bryan et al., 1980; Bryan & Kwan, 1981), menaquinone-deficient strains of Bacillus subtilis (Taber et al., 1981; Taber & Halfenger, 1976) and haem mutants of Staphylococcus aureus (Tien & White, 1968; Kaplan & Dye, 1976; Miller et al., 1980) were isolated from wild-type populations by selection for increased resistance to aminoglycoside antibiotics. These observations, in addition to those indicating that aminoglycoside uptake and kill are decreased in the presence of respiratory inhibitors such as azide and cyanide, and under anaerobic conditions, have led to the view that aminoglycoside accumulation is influenced by respiratory activity.

For the ubiA strain (NSW60; Fig. 1) and the ubiF strain (NSW77; M. Muir, unpublished results) the accumulation of streptomycin in chemostat cultures of mutant and parent was identical provided that dilution rates were the same. It seems possible that the previously observed decreases in aminoglycioside accumulation by various respiratory-deficient mutants, and by batch cultures of facultative anaerobes growing anaerobically, can be accounted for by differences in growth rates between parent and mutant strains, and between aerobic and anaerobic cultures. For example, the ubiD mutant with a decreased uptake of streptomycin in batch cultures (Bryan & Van Den Elzen, 1977) has a generation time of 2.5 h compared with 1.25 h for the wild-type strain AB2154 (Newton et al., 1972). A twofold difference in growth rate
between parent and mutant could explain the decreased uptake of streptomycin observed. With B. subtilis, which contains menaquinone but not ubiquinone, a menaquinone requirement for gentamicin uptake was manifest when the menaquinone content of cells was decreased to less than 10% of the normal value (Taber et al., 1981). It is probable that loss of more than 90% of the cellular menaquinone had a deleterious effect on growth rate which would serve to decrease accumulation of the aminoglycoside. Campbell & Kadner (1980) found that uptake of dihydrostreptomycin could be partially restored in anaerobic batch cultures by the addition of nitrate. This restoration might result from a stimulation in growth rate due to an increase in electron transport activity.

It has been proposed that the bacterial quinones ubiquinone and menaquinone play a role in aminoglycoside accumulation as membrane carriers of the antibiotic in E. coli (Bryan & Van Den Elzen, 1977) and B. subtilis (Taber et al., 1981) respectively. The results of this paper suggest that neither ubiquinone nor menaquinone are directly involved in streptomycin accumulation in E. coli. Thus when the concentration of ubiquinone was substantially decreased in the ubiA strain (and the menaquinone and demethylmenaquinone removed entirely), the respiration rate was decreased but no effect on streptomycin accumulation was observed. Complete replacement of ubiquinone in the ubiF strain by a structural analogue, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, though causing a decrease in respiration rate (Muir et al., 1981), did not affect the accumulation of streptomycin in chemostat cultures. This suggests either that ubiquinone is not involved in streptomycin accumulation or that the analogue can take over the role of ubiquinone without loss of efficiency.

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REFERENCES


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