The Effects of Nalidixic Acid on Respiratory Activity of Asynchronous and Synchronous Cultures of *Alcaligenes eutrophus*

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Nalidixic acid inhibited DNA synthesis and cell division during asynchronous growth of *Alcaligenes eutrophus* but treated cells continued to grow as monitored by A_{550} , respiratory activity and cell volume. Differential effects on cell division were seen when the antibiotic was added at different times during synchronous growth. The earlier in the cell cycle the time of addition the greater the inhibition of cell division. These results suggest that chromosome replication is confined to the first half of the cell cycle in *A. eutrophus* grown at 30 °C. Respiration rates of cells selected by continuous-flow centrifugation and immediately treated with nalidixic acid increased in a stepwise fashion. These steps were similar to those observed in untreated control cells and imply that the DNA-division cycle is not the causal factor for the periodicities in respiration rates and ATPase activity that have been previously reported in this bacterium.

INTRODUCTION

Periodic changes in respiratory activity have been reported in synchronous cultures of *Escherichia coli* (Evans, 1975; Poole, 1977), *Alcaligenes eutrophus* (Edwards & Jones, 1977) and *Bacillus subtilis* (Edwards & McCann, 1981). These changes are thought to reflect a changing demand for energy during the cell cycle, and in *A. eutrophus* and *E. coli* have been shown to correlate with periodic rises in ATPase activity (Edwards *et al.*, 1978; Scott *et al.*, 1980). A number of cellular processes could exert a changing energy requirement during the cell cycle. Prime candidates are those events which occur at specific times during the cell cycle. In bacteria, these include events concerned with chromosome replication and cell division, which occur at temporally fixed phases of the cell cycle, providing that the cycle time is sufficient for the completion of a C and D period (James, 1978).

We have investigated respiratory activity in asynchronous and synchronous cultures of *A. eutrophus* in which both chromosome replication and cell division have been inhibited by nalidixic acid. The cells continued to grow as filaments at rates similar to those observed in untreated control cultures. Periodic rises in respiratory activity persisted in cells selected on the basis of size by continuous-flow centrifugation and treated with nalidixic acid. These results imply that in *A. eutrophus* periodic rises in oxygen uptake rates are not coupled to the DNA-division cycle.

METHODS

Maintenance and growth of the organism. *Alcaligenes eutrophus* H16, ATCC 17699 was maintained on plates of nutrient agar. Growth in liquid salts medium, preparation of synchronous cultures by continuous-flow centrifugation and assessment of synchrony were as described previously (Edwards & Jones, 1977).

Analytical methods. Growth was followed by measuring the A_{550} of the culture or by determining cell numbers using a Coulter counter model ZBI fitted with a 30 µm orifice (Coulter Electronics, Harpenden, Herts.) in culture samples which had been appropriately diluted in the electrolyte solution Isoton II (also supplied by Coulter

Electronics). All counts were corrected for coincidental passage. Modal cell volumes were measured using a Channelyzer C-1000 linked to an X-Y plotter. Calibration of the channelyzer readout was done by using latex particles of 1.15 μm diameter.

Oxygen uptake was measured polarographically at 30 °C using a Rank oxygen electrode (Rank Bros, Bottisham, Cambs.) linked to a Vitatron chart recorder (MSE, Crawley, Sussex). Samples (2.4 ml) of undiluted cell suspensions were rapidly transferred from growing cultures into the reaction chamber and oxygen uptake rates followed for approximately 5 min.

Total DNA content in culture samples was determined by the diphenylamine method of Burton (1956).

Chemicals. Stock solutions (2 mg ml⁻¹) of nalidixic acid (Sigma) were made up in 70 mM-NaOH. Appropriate additions of 70 mM-NaOH were made to control cultures and no effects on growth or modal cell volume were observed.

RESULTS

Effects of nalidixic acid during asynchronous growth

During asynchronous growth, control cultures of *A. eutrophus* grew with a doubling time of 75 min as determined by increase in cell numbers and A_{550} . Oxygen uptake rates [nmol O₂ consumed min⁻¹ (ml culture)⁻¹] and the amount of DNA (μg per 20 ml culture) also increased at similar rates. Modal cell volume remained relatively constant at approximately 0.8 μm^3 throughout the growth period (Fig. 1*a*). When nalidixic acid [10 μg (ml culture)⁻¹] was added to exponentially growing cells (time 0), A_{550} and oxygen uptake rates continued to rise at rates similar to those in the control culture. Both cell division and amounts of DNA also continued to rise for approximately 55 min but thereafter stopped and remained constant. Modal cell volume remained at approximately 0.8 μm^3 for the first 55 min after the addition of nalidixic acid but thereafter began to rise such that at 120 min the modal cell volume of the culture was 1.3 μm^3 . This result indicates that although DNA synthesis and cell division are inhibited by nalidixic acid the treated cells continue to grow at rates similar to control cells. Microscopic examination of nalidixic acid treated cultures showed the presence of longer filamentous cells. The distribution of cell volumes 180 min after addition of nalidixic acid is compared with that of untreated cells in Fig. 2. Control cell volumes ranged between 0.5 and 2 μm^3 (modal volume approximately 0.9 μm^3). The nalidixic acid treated culture contained a much broader distribution of cell volumes, between approximately 0.7 and 4.5 μm^3 (modal cell volume approximately 2.2 μm^3). These results confirmed the continued growth of cells after antibiotic treatment.

Effects of nalidixic acid during synchronous growth

The effects of nalidixic acid at different stages of synchronous growth were determined. Cell cycle specific effects were apparent. After selection by the continuous-flow centrifugation method, untreated control cells of *A. eutrophus* divided synchronously after 50 min incubation. Cell numbers increased from 1.5×10^7 to 3×10^7 bacteria ml⁻¹. A second synchronous division occurred at approximately 125 min. The duration of the cell cycle was 75 min, similar to the generation time of exponentially-growing cells. Culture density, as determined by A_{550} , increased exponentially with a doubling time of 75 min. Addition of nalidixic acid at time 0, 20 or 60 min after selection resulted in an increasing percentage of cells dividing (approximately 18, 48 and 72%, respectively, compared with the untreated control). However, there was no second division in the nalidixic acid treated cells, although the A_{550} of all the treated cultures rose in similar fashion to the control cells (Fig. 3). Therefore nalidixic acid inhibited cell division to a greater extent when added in the early stages of the cell cycle.

Measurements of oxygen uptake in cells selected by continuous-flow centrifugation and treated with nalidixic acid

The addition of nalidixic acid to cells immediately after selection by continuous-flow centrifugation almost totally inhibited synchronous division (Fig. 3). It was therefore possible to investigate, in the absence of chromosome replication and cell division, the periodic rises in respiration rates previously reported in *A. eutrophus* (Edwards & Jones, 1977). Addition of the

Fig. 1. Effects of nalidixic acid ($10 \mu\text{g ml}^{-1}$) on asynchronous cultures of *Alcaligenes eutrophus*. (a) Untreated exponentially-growing culture; (b) nalidixic acid-treated exponentially-growing culture. Changes in cell numbers ($\bullet$), A_{550} ($\circ$), oxygen uptake rate [$\text{nmol min}^{-1} (\text{ml culture})^{-1}$] ($\blacksquare$), DNA (μg per 20 ml culture) ($\square$) and modal cell volume (μm^3) ($\blacktriangledown$) are shown.

Fig. 2. Distribution of cell volumes in cells of *A. eutrophus* grown asynchronously in the absence (—) or presence (---) of nalidixic acid ($10 \mu\text{g ml}^{-1}$).

antibiotic to selected cells immediately after continuous-flow centrifugation resulted in continued increase in A_{550} at rates similar to control cells. Approximately 20% of the cells divided after 50 min, thereafter cell numbers remained constant. Oxygen uptake rates rose discontinuously as a series of steps of similar periodicity to those seen in synchronously-growing cells (Fig. 4).

Fig. 3

Fig. 4

Fig. 3. Effect of nalidixic acid added at different times during synchronous growth of *A. eutrophus*. Cell numbers are shown for untreated cultures (●) and for cultures to which nalidixic acid ($10 \mu\text{g ml}^{-1}$) was added at times 0 (▽), 20 (■) or 60 min (□). The increase in A_{550} of untreated cells is also shown (○).

Fig. 4. Oxygen uptake of cells selected by continuous-flow centrifugation and grown in the presence of nalidixic acid ($10 \mu\text{g ml}^{-1}$). ○, A_{550} ; ●, cell numbers; ■, oxygen uptake rates.

DISCUSSION

Nalidixic acid inhibits subunit A of DNA gyrase in *E. coli*. This enzyme catalyses the introduction of negative superhelical turns in closed circular double-stranded DNA in an ATP-dependent reaction. Thus nalidixic acid is a potent inhibitor of chromosome replication in *E. coli* (Gellert *et al.*, 1976; Sugino *et al.*, 1977; Cozzarelli, 1980). Nalidixic acid also inhibits DNA synthesis in *A. eutrophus* which, in cells growing asynchronously, results in the gradual cessation of cell division. This suggests a coupling between chromosome replication and cell division in *A. eutrophus* similar to that observed in *E. coli* (Dix & Helmstetter, 1973). Addition of the antibiotic at different stages of the cell cycle provides evidence for a period during which nalidixic acid has little or no effect on cell division. That is to say, the earlier the time of its addition in the cycle the greater the inhibition of the subsequent cell division. This result is similar to previous data obtained for *E. coli* (Dix & Helmstetter, 1973; James *et al.*, 1975). This suggests that chromosome replication is confined to the early stages of the cell cycle of *A. eutrophus*. From a number of experiments similar to that described in Fig. 3, in which nalidixic acid was added at different times during synchronous growth, we conclude that the C period occupies approximately 0.45 of a cycle. Assuming a cycle time of 75 min then this corresponds to a time of about 35 min.

Respiration rates of nalidixic acid treated cells of *A. eutrophus* increased in stepwise fashion similar to the rises in oxygen uptake rates observed in synchronously-growing cells. These stepped increases occurred with a frequency of approximately 0.5 of a cycle in both antibiotic treated and control cells. We conclude from these results that the respiratory periodicities observed during the cell cycle of *A. eutrophus* are not a function of chromosome replication or cell division. Some other cellular parameter must be exerting a changing energy demand through the cell cycle. It is unlikely that this results from changes in amounts of respiratory proteins such as cytochromes, since in *E. coli* many of these have been shown to increase continuously

throughout the cell cycle (Poole *et al.*, 1980). A possible reason for the observed periodicities might be a change in rates of synthesis of membrane lipids. It has been suggested that membrane lipids could be involved in regulation of membrane-associated enzyme activity. *Rhodopseudomonas*, *Escherichia* and *Bacillus* species all show changes in the rate of lipid synthesis during the cell cycle (Carty & Ingram, 1981). Thus, the fact that many respiratory proteins are membrane-bound, and are intimately associated with lipids, makes the modulation of their activity by changes in membrane lipid composition an attractive possibility.

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