Phenotypic Variability of the Sensitivity to Cycloserine of *Klebsiella aerogenes* NCTC 418, Growing in Chemostat Culture

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The susceptibility of *Klebsiella aerogenes* to cycloserine varied according to the growth conditions. In batch culture, cells were less susceptible to the antibiotic when glycine was present in the medium, presumably due to competition between glycine and cycloserine for the uptake system by which glycine, D-alanine and cycloserine are transported into the cell. In the chemostat at average dilution rates, ammonia-limited cultures were more susceptible to the antibiotic than were glucose-limited cultures. Under phosphate-limiting conditions cultures were at least ten times less susceptible. Under ammonia and phosphate limitation the susceptibility increased with increasing growth rate. The sensitivity of glucose-limited cells was independent of the growth rate. A high-affinity uptake system for cycloserine (as measured by D-alanine transport) was present in ammonia- and glucose-limited cells, but not in phosphate-limited cells. Thus, the phenotypically defined alterations in the susceptibility of the bacterium to cycloserine could be correlated with variations in its uptake system for the antibiotic.

INTRODUCTION

Alterations in the composition of the bacterial envelope can involve decreased activities of uptake systems by which antibiotics penetrate the cell and, hence, can lower the susceptibility of the bacteria to these agents (Melling & Brown, 1975). The composition of the envelope can be influenced markedly by the bacterial growth conditions (Brown & Melling, 1969; Robinson & Tempest, 1973) and, consequently, environmental parameters may affect the bacterial sensitivity to antimicrobial agents. For purposes of correlating the antibiotic sensitivity of an organism with its growth conditions, we have used chemostat cultures. With this technique, well-defined growth conditions can be imposed on a culture (Tempest, 1970). The chemostat has already proved to be an excellent tool in studies of the phenotypically defined response of *Pseudomonas aeruginosa* (Finch & Brown, 1975; Gilbert & Brown, 1978), *Escherichia coli* (Koch & Gross, 1979) and *Candida albicans* (Johnson *et al.*, 1978) to various agents.

In this study, the sensitivity of growing cultures of *Klebsiella aerogenes* to cycloserine has been investigated. The inhibition of peptidoglycan synthesis caused by this antibiotic may be regarded as a result of the competitive action between cycloserine and D-alanine for the enzymes alanine racemase and D-alanyl-D-alanine synthetase (Franklin & Snow, 1975). A similar competition has been observed for the uptake system by which the amino acids D-alanine and glycine, as well as the antibiotic cycloserine, are transported into the cell (Wargel *et al.*, 1970). The resistance of *E. coli* (Wargel *et al.*, 1971), *Bacillus subtilis* (Clark & Young, 1977) and *Rhizobium* sp. (Pankhurst & Craig, 1979) to cycloserine has been shown to be coupled with variations in this uptake system. During the course of the present investigation, alterations in the uptake system by which cycloserine may penetrate the cell

have been found to play a major role in the phenotypic variability of the sensitivity of *K. aerogenes* to this antibiotic.

METHODS

Organism. *Klebsiella aerogenes* NCTC 418 was maintained by monthly subculture on nutrient agar.

Growth conditions. Organisms were grown aerobically in nutrient broth or in simple salts media. The latter were basically as described by Evans *et al.* (1970). For batch cultures, the simple salts medium was enriched with 50 mM-NaH₂PO₄ and the pH was adjusted to 7. For continuous cultures, simple salts media were used, the amount of growth-limiting nutrient being adjusted to provide the desired steady state cell concentration. The media contained 1 g glucose l⁻¹ (glucose-limited) or 15 g glucose l⁻¹ (other limitations). Cells were grown in a 600 ml chemostat (L.H. Engineering Co., Stoke Poges); this was termed the growth fermenter. The pH value of the culture was monitored *in situ* and was automatically maintained at 6.8 ± 0.1 by addition of 1 M-NaOH (phosphate limitation) or 4 M-NaOH (other limitations). The temperature was set at 37 °C. Samples were withdrawn when the culture had reached a steady state.

Equipment for susceptibility tests. For susceptibility tests of continuous cultures a 300 ml chemostat was used (Bioflo; NB Scientific, New Brunswick, N.J., U.S.A.); this was termed the test fermenter. Temperature, pH value and dilution rate (*D*) were set at exactly the same values as those imposed on the culture in the growth fermenter. Medium withdrawn from the supply tank of the growth fermenter was used. In addition, a cycloserine stock solution was connected via a delta pump with constant flow. The concentration of this solution was chosen such that with the given flow rate of the delta pump (± 5 ml h⁻¹, measured exactly) and the given dilution rate of the culture the desired final concentration of cycloserine in the test fermenter was maintained. Unequal distribution of medium, NaOH and cycloserine due to foaming of the culture was avoided by adding them through a common tube, by means of which air was also passed into the chemostat. Samples of the overflowing culture were taken immediately after leaving the chemostat and their turbidity was measured at 540 nm (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.).

Susceptibility testing. In batch experiments, cell samples from overnight cultures or chemostat cultures were transferred to side-arm Erlenmeyer flasks with medium and allowed to grow at 37 °C for at least 3 generations. When cultures were in the early-exponential phase of growth, cycloserine was added and the turbidity was measured at 540 nm (Vitatron Scientific, Dieren, The Netherlands). The minimal inhibitory concentration (m.i.c.) of the antibiotic was defined as the lowest concentration causing an eventual cessation of growth.

For susceptibility tests of continuous cultures, samples (350 ml) were withdrawn from the growth fermenter and transferred immediately to the test fermenter. When the turbidity of the overflowing culture of the latter chemostat was constant in time, an amount of stock solution of cycloserine was added (less than 1 ml), depending on the desired final antibiotic concentration in the chemostat. Simultaneously, the cycloserine supply pump was started. The turbidity of the overflowing culture was followed for at least 0.75 of the initial bacterial doubling time (i.e. for at least 3 h). The results were plotted as a fraction of the initial turbidity against the number of generations, thus facilitating a comparison of results obtained from cultures with different steady state turbidities and different growth rates. Since sterilization of the test system was not necessary, successive experiments could be performed relatively frequently.

D-[¹⁴C]Alanine uptake measurements. A 50 ml sample was withdrawn from the growth fermenter, centrifuged without cooling at 12000 *g* for 20 min in a Sorvall RC2-B centrifuge (Ivan Sorvall, Norwalk, Conn., U.S.A.) and the cells were resuspended in 25 ml prewarmed buffer (100 mM-imidazole.HCl, 1% (w/v) glucose, pH 7.0). At *t* = 0, 2.5 ml cell suspension was transferred to a 50 ml Erlenmeyer flask containing 2.5 ml of a D-[U-¹⁴C]alanine (The Radiochemical Centre, Amersham) solution (specific activity 0.1–0.5 GBq ml⁻¹) prewarmed at 37 °C. In general, the specific activity of this mixture was sufficient to give cell samples with an activity of at least 1000 c.p.m. Samples of 100 µl were withdrawn at 30 s intervals for up to 2 min, filtered immediately through a 0.2 µm membrane filter and washed twice with 1 ml 0.9% (w/v) NaCl solution. After drying overnight at 50 °C, samples were counted by liquid scintillation in a toluene/Omnifluor (New England Nuclear, Boston, Mass., U.S.A.) mixture. Initial rates of D-alanine uptake were calculated. A correction was made for the uptake rate that was found for boiled cells or cells that had been treated for at least 10 min with 10 mM-sodium azide.

RESULTS

Susceptibility in batch cultures

The effect of cycloserine, when added to cultures of *K. aerogenes* in the early-exponential phase of growth, was strongly dependent on the composition of the growth medium. In nutrient broth, the m.i.c. of cycloserine was 110 µg ml⁻¹, whereas in minimal salts medium the

Fig. 1. Influence of growth limitation on the susceptibility of *Klebsiella aerogenes* to cycloserine. The turbidity of ammonia-limited (a), glucose-limited (b) and phosphate-limited (c) cultures was measured during exposure to various concentrations of cycloserine, as described in Methods. Applied cycloserine concentrations: in a ($D = 0.4 \text{ h}^{-1}$), $0.5 \mu\text{g ml}^{-1}$ (○), $1 \mu\text{g ml}^{-1}$ (●), $2 \mu\text{g ml}^{-1}$ (□) and $4 \mu\text{g ml}^{-1}$ (■); in b, $1 \mu\text{g ml}^{-1}$ (○, $D = 0.2 \text{ h}^{-1}$), $3 \mu\text{g ml}^{-1}$ (●, $D = 0.3 \text{ h}^{-1}$) and $5 \mu\text{g ml}^{-1}$ (□, $D = 0.3 \text{ h}^{-1}$); in c ($D = 0.3 \text{ h}^{-1}$), $12 \mu\text{g ml}^{-1}$ (○), $40 \mu\text{g ml}^{-1}$ (●) and $80 \mu\text{g ml}^{-1}$ (□). In each case, the wash-out curve according to the dilution rate is represented by the dashed line.

m.i.c. was $11 \mu\text{g ml}^{-1}$. When the minimal salts medium was supplemented with 7 mM-glycine, comparable with the glycine concentration in nutrient broth, the m.i.c. was increased to $128 \mu\text{g ml}^{-1}$. This indicated that glycine probably acted as a competitor for the uptake system by which cycloserine was entering the bacteria.

Susceptibility in chemostat cultures

Influence of growth limitation on the susceptibility to cycloserine. After transfer from the growth fermenter to the test fermenter, cultures usually attained a steady state, as determined by turbidity measurements, within 30 min. In order to evaluate the results of our experiments, the kinetics were determined by which the turbidity of the chemostat culture decreased after a sudden change in the composition of the inflowing medium resulting in bacteriostasis. For this, the bacterial growth was stopped either by omitting the growth limiting nutrient or by supplementing the medium with $40 \mu\text{g}$ chloramphenicol ml^{-1} . This resulted in a rate of wash-out of cells from the chemostat that was not higher than the theoretically predicted wash-out rate (data not shown), indicating the absence of lysis under these conditions.

Susceptibility to cycloserine was examined during growth of *K. aerogenes* under ammonia, glucose and phosphate limitations. Ammonia-limited cultures were the most sensitive: cycloserine added at $0.5 \mu\text{g ml}^{-1}$ to cultures growing at $D = 0.4 \text{ h}^{-1}$ led to a decrease in the turbidity of the culture, and with cycloserine at $2 \mu\text{g ml}^{-1}$ cell lysis took place (Fig. 1 a). Under

Fig. 2. Influence of growth rate on the susceptibility of *Klebsiella aerogenes* to cycloserine. The turbidity of ammonia-limited (a), glucose-limited (b) and phosphate-limited (c) cultures was measured during exposure to 1, 3 and 40 μg cycloserine ml^{-1} , respectively, as described in Methods. Imposed growth rates: in a, 0.2 h^{-1} (○), 0.4 h^{-1} (●) and 0.8 h^{-1} (□); in b and c, 0.1 h^{-1} (○), 0.3 h^{-1} (●) and 0.5 h^{-1} (□). In each case, the wash-out curve according to the dilution rate is represented by the dashed line.

glucose-limited conditions ($D = 0.3 \text{ h}^{-1}$) *K. aerogenes* was slightly less sensitive to cycloserine: 3 μg cycloserine ml^{-1} led to a decrease in the culture turbidity but lysis was not observed until the antibiotic concentration was raised to 5 μg ml^{-1} (Fig. 1b). Cycloserine at 12 μg ml^{-1} in phosphate-limited cultures ($D = 0.3 \text{ h}^{-1}$) caused only a slight decrease in the turbidity of the culture and even 40 μg cycloserine ml^{-1} had no lasting growth inhibiting effect; with 80 μg ml^{-1} there was severe growth inhibition, but without any lysis occurring (Fig. 1c).

Influence of growth rate on the susceptibility to cycloserine. The turbidity of ammonia-limited cultures growing at $D = 0.4 \text{ h}^{-1}$ decreased when the growth medium was supplemented with 1 μg cycloserine ml^{-1} , but subsequently growth restarted (Fig. 1a). However, at a higher dilution rate (0.8 h^{-1}), addition of this concentration of antibiotic led to cell lysis. In contrast, at a lower dilution rate (0.2 h^{-1}), the presence of 1 μg cycloserine ml^{-1} had no effect (Fig. 2a). The same phenomenon was observed under phosphate limitation when 40 μg cycloserine ml^{-1} was added to the growth medium (Fig. 2c). In contrast to these findings, the reaction of glucose-limited cultures upon addition of 3 μg cycloserine ml^{-1} appeared to be independent of the bacterial growth rate (Fig. 2b).

Influence of growth conditions on the activity of the D-alanine uptake system

To investigate a possible correlation between the susceptibility to cycloserine and its uptake by the cells, alanine was used as a probe of the activity of the transport system. The initial rate

Fig. 3. Uptake of D-alanine by *Klebsiella aerogenes* grown under ammonia limitation at $D = 0.1 \text{ h}^{-1}$ (●) and $D = 0.3 \text{ h}^{-1}$ (○), glucose limitation at $D = 0.3 \text{ h}^{-1}$ (▲) and phosphate limitation at $D = 0.1 \text{ h}^{-1}$ (■) and $D = 0.6 \text{ h}^{-1}$ (□).

of uptake, which depended on the input concentration of alanine, varied with the growth conditions of the bacteria (Fig. 3). Ammonia-limited cells exhibited a relatively high uptake rate, while the accumulation of the amino acid by glucose-limited cells and slowly growing phosphate-limited cells ($D = 0.1 \text{ h}^{-1}$) was considerably slower. Phosphate-limited cells growing at a high dilution rate ($D = 0.6 \text{ h}^{-1}$) had an intermediate uptake rate. The D-alanine uptake rate was dependent on the growth rate. Ammonia-limited cells grown at $D = 0.1 \text{ h}^{-1}$ exhibited an uptake rate that was higher than that of cells grown at a higher dilution rate. Conversely, slowly growing phosphate-limited cells had a less active uptake system than fast growing ones.

A transformation of the curves shown in Fig. 3 into Lineweaver–Burk plots yielded straight lines for phosphate-limited cultures, but non-linear curves for ammonia- and glucose-limited cultures (Lineweaver–Burk plots not shown). The latter were interpreted as indicating the occurrence of more than one uptake system with different apparent K_m values. Extrapolation of the plots for ammonia- and glucose-limited cultures yielded a high-affinity apparent K_m value of about $1.4 \times 10^{-6} \text{ M}$ and a low-affinity one in the range $1\text{--}10 \times 10^{-5} \text{ M}$. In phosphate-limited cultures only a low-affinity apparent K_m value was indicated, which also was in the range $1\text{--}10 \times 10^{-5} \text{ M}$.

DISCUSSION

Both the nature of the nutrient which was limiting growth and the growth rate had a striking effect on the response of *K. aerogenes* growing in the chemostat to cycloserine (see Fig. 1, 2). Wargel *et al.* (1970) found both high- and low-affinity systems in batch-grown cultures of *E. coli* for the accumulation of D-alanine, glycine and cycloserine. Our results also indicate the presence of both high- and low-affinity components in the uptake system of *K. aerogenes* cells grown under ammonia or glucose limitation. However, under phosphate-limiting conditions, the high-affinity component was absent. This was coupled with a phenotypically defined decrease in sensitivity to cycloserine that was comparable with the decrease in susceptibility to this antibiotic found in a mutant, *cyc*^{r1}, of *E. coli* which had lost the high-affinity component of the uptake system (Wargel *et al.*, 1970; Curtiss *et al.*, 1965). The increase in susceptibility to cycloserine of ammonia-limited cultures with increasing growth rate could not be correlated

directly with the decreasing D-alanine uptake rate under these conditions. Obviously, the process underlying this response cannot be attributed to alterations in cycloserine uptake alone.

The occurrence of a high-affinity component in the uptake system for D-alanine may be seen as a physiologically relevant process under those conditions when growth is limited by a restricted supply of the nitrogen, carbon or energy source, resulting in a rapid uptake of cycloserine and, as a consequence, a greater sensitivity of the organism to the antibiotic.

A phenotypically defined sensitivity of a bacterial strain to an antibiotic may have serious implications for antimicrobial chemotherapy. Clinical procedures for susceptibility testing are routinely based on in vitro growth conditions of the strain that is to be tested. However, growth conditions in vivo – for instance, in an infected part of the human body – are assumed to be sub-optimal as a result of a limited supply of some essential nutrients (Brown, 1977). The results described in this report show a strong dependency of the bacterial susceptibility to antibiotics on the growth conditions of the culture, and therefore a routine testing system may give only an indication of the in vivo inhibitory concentration.

In our experiments, cycloserine was added to a steady state chemostat culture thereby disturbing the steady state and imposing new, constant growth conditions. The culture either reached a new steady state or was washed-out. A new steady state may have been accompanied by modified yields on nutrients, and a wash-out may be regarded as an expression of a new, lower maximal specific growth rate. However, we have little knowledge of these new parameters and, therefore, of the processes which occur during the transient state. The new parameters may be evaluated by the use of long-term experiments, but these have the disadvantage that the culture might either become 'trained' to a certain concentration of cycloserine (Clark & Young, 1977) or even that resistant mutants may arise.

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