

The Rate of Killing of *Escherichia coli* by β -Lactam Antibiotics Is Strictly Proportional to the Rate of Bacterial Growth

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Nongrowing bacteria evade the bactericidal activity of β -lactam antibiotics. We sought to determine if slow growth rate also alters bactericidal activity. The bactericidal activity of two β -lactams on *Escherichia coli* grown in glucose limited chemostats was compared for generation times ranging from 0.7 to 12 h. The degree of killing varied with drug structure and with *E. coli* strain. However, all killing rates were a constant function of the bacterial generation time: slowly growing bacteria became progressively more phenotypically tolerant to β -lactam antibiotics as the generation time was extended.

INTRODUCTION

The earliest studies on the mechanism of action of penicillin showed that the bactericidal activity of β -lactam antibiotics depends on bacterial growth (Hobby *et al.*, 1942; Hobby & Dawson, 1944). This is best illustrated by the inability of penicillin to kill nongrowing bacteria in contrast to the rapid killing and lysis of the same cells during exponential growth. The insensitivity of nongrowing cells to the bactericidal and lytic effect of antibiotics is a property shared by all bacteria and has been termed phenotypic tolerance (Tuomanen, 1986). It should be distinguished from genotypic tolerance, which is also associated with markedly slowed bacterial killing by β -lactams, but which is manifest even during rapid growth and is limited to bacterial strains with defective autolytic activity (Tomasz *et al.*, 1970). While its mechanism is unknown, phenotypic tolerance of nongrowing bacteria is the basis of penicillin selection procedures for auxotrophic mutants (Davis, 1949; Lederberg & Zinder, 1948).

Here, we report the results of our investigation of this phenomenon, using bacterial cultures grown in the chemostat with reduced growth rates.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains 205 and W7 (Lys⁻Dap⁻) were grown in M9 minimal salts medium (Goodell & Tomasz, 1980), supplemented as required with 50 μ M-L-histidine, 25 μ g L-lysine ml⁻¹ and 5 μ g diaminopimelic acid ml⁻¹. Batch cultures were maintained at 37 °C in a shaking water bath. Under these conditions the generation time was 40 min. To halt growth, bacteria were collected on a membrane filter (0.45 μ m, Millipore) and resuspended in medium without glucose.

Reductions in growth rate were achieved by growing the bacteria in 50 ml volume, all glass, aerated chemostats at 37 °C as described previously (Cozens *et al.*, 1986; Gilbert & Stuart, 1977). Growth medium was as previously described (Cozens *et al.*, 1986) except that glycerol (1 mM) replaced glucose as the limiting nutrient. Generation times ranged from 1.5 to 23 h. Under these conditions the culture was allowed to stabilize for at least 10 generations before the start of an experiment, at which time the population was $1-5 \times 10^8$ c.f.u. ml⁻¹.

Assessment of the antibacterial activity of β -lactam antibiotics against exponentially growing and nongrowing bacteria in batch culture. Antibiotics were added to culture samples in mid-exponential phase or 10 min after suspension in medium without glycerol. Cultures were assayed for OD₄₂₀ (Pye Unicam SP550 spectrophotometer) and for c.f.u.

Abbreviation: PBP, penicillin binding protein.

on nutrient agar plates and on M9 media plates supplemented as described. Antibiotics tested included: benzylpenicillin (Eli Lilly), cefonicid (Smith Kline & French), and a new cephalosporin, CGP 17520 (7 β -[D(-)-4'-(D-serine-O-carboxylamino)- α -hydroxy-phenylacetamidol]-3-[(1-methyl-1H-tetrazol-5-yl)-thiomethyl]-ceph-3-em-4-carboxylic acid, sodium salt) (Ciba-Geigy) (Zak *et al.*, 1984). MICs, determined in complete M9 medium, were defined as the concentration of drug inhibiting growth of 10^8 c.f.u. ml⁻¹ of bacteria over 24 h. These values (μ g ml⁻¹) were as follows for *E. coli* W7 and 205, respectively: benzylpenicillin 5, 4; cefonicid 1, 1; CGP 17520 0.8, 1.

Assessment of the rate of killing by β -lactam antibiotics in chemostat cultures. All experiments were done at least in duplicate. To assess the rate of killing, antibiotic (cefonicid or CGP 17520) was added simultaneously to the chemostat and the medium reservoir at time 0 to achieve concentrations of 1 or $10 \times$ the MIC. Thereafter, samples were taken from the culture at suitable time intervals for the following determinations: OD₄₂₀ (Pye Unicam SP550 spectrophotometer), total cell count (Coulter counter), viability (c.f.u. on nutrient agar plates) and light microscopic appearance of the cells (Zeiss Research Microscope, Leitz Dialus 20 microscope). Upon addition of the antibiotic, viable titre started to decline in all cultures within less than 0.25 generation. The concentration of bacteria in the chemostats fell as a result of two processes: (i) the washout rate of the system, independent of bacterial death, and (ii) loss of viability due to killing by the antibiotic. Thus, the true degree of killing was determined at each time point by subtracting the theoretical counts lost (calculated from the flow rate; Cozens *et al.*, 1986) from the actual counts lost.

Assessment of the rate of lysis of E. coli W7 by CGP 17520. Rate of lysis was compared to rate of killing by adapting the chemostat as follows. *E. coli* W7 was grown in pairs of chemostats at each generation time of 1.5, 3.5, 7 and 12.6 h for 8 generations. Glycerol (1 mM) was substituted for glucose in the growth medium to promote uptake of radiolabelled *N*-acetylglucosamine. The medium was then supplemented with 0.3 μ Ci (11 kBq) *N*-acetyl[³H]-glucosamine ml⁻¹ (New England Nuclear) to radiolabel the cell wall. After 2 generations, the medium was switched to unlabelled medium. Immediately, one of the pair of chemostats of the same generation time received $10 \times$ MIC CGP 17520; the other remained as a control. Samples were taken as described above; at the same time 200 μ l samples were collected on ice, frozen and then processed for cell wall associated radiolabel by hot 4% (w/v) SDS extraction as described previously (Schwarz, 1975). The amount of cell wall degradation due to antibiotic induced lysis was determined by subtracting the amount of label remaining in cell wall in control cultures from that in drug treated cultures.

Penicillin binding proteins (PBPs). The effect of growth rate on the PBP pattern of *E. coli* W7 was assessed by both *in vivo* (whole cell) and *in vitro* (membrane) titrations. For *in vivo* assays the method of Broome-Smith & Spratt (1982) was used. Multiple 1 ml (10^8 c.f.u. ml⁻¹) samples were taken from batch cultures or chemostats grown at generation times of 1.5, 3, 6, 12 h and were frozen quickly. After five freeze-thaw cycles, samples were exposed to 0.1 – $10 \times$ MIC [³H]benzylpenicillin (Merck, Sharp & Dohme; 25 Ci mmol⁻¹, 925 GBq mmol⁻¹) for 10 min at 32 °C, and prepared for SDS polyacrylamide gel electrophoresis as described by Broome-Smith & Spratt (1982). For *in vitro* determinations, 35 ml samples from the same batch or chemostat cultures just described were harvested, and the cells were washed in saline and frozen. Membranes were prepared from each sample by standard procedures (Spratt, 1977) and samples containing 200 μ g protein were titrated with [³H]benzylpenicillin as described by Spratt (1977). These two assays were used to assess the number and relative affinity of *E. coli* PBPs in cells growing at different generation times.

RESULTS

Rate of bacterial killing as a function of generation time

The responses of rapidly growing (0.75 h) and nongrowing batch cultures of *E. coli* W7 to $10 \times$ MIC benzylpenicillin were determined to serve as a comparison to the behaviour of chemostat grown cultures in Fig. 1. Cells were rapidly killed in the exponentially growing culture (3 log kill in 1 h). In contrast, nongrowing cells (deprived of glucose) were not killed by the same concentration of antibiotic, i.e. the cells were phenotypically tolerant (0.5 log kill in 4 h). No decline in the viable titre was observable even after prolonged drug exposures (24 h). This response was also found upon treatment with cefonicid or CGP 17520 (results not shown).

Fig. 1 depicts the rate of bacterial killing by CGP 17520 of *E. coli* W7 cultures grown in the chemostat at four different long generation times between 1.5 and 12.6 h. The rate of loss of bacterial viability was slower in the slowly growing cultures (Fig. 1*a*). Replotting the viability data as a function of the number of generations resulted in nearly superimposable curves, indicating that the rate of loss of viability was constant with respect to the generation time (T_G) (Fig. 1*b*). For example, cultures with $T_G = 1.5$ h demonstrated a 3 log kill in 1.2 generations (i.e. ~ 2 h); bacteria growing with $T_G = 12.6$ h demonstrated a 3 log kill in 1.1 generations (i.e.

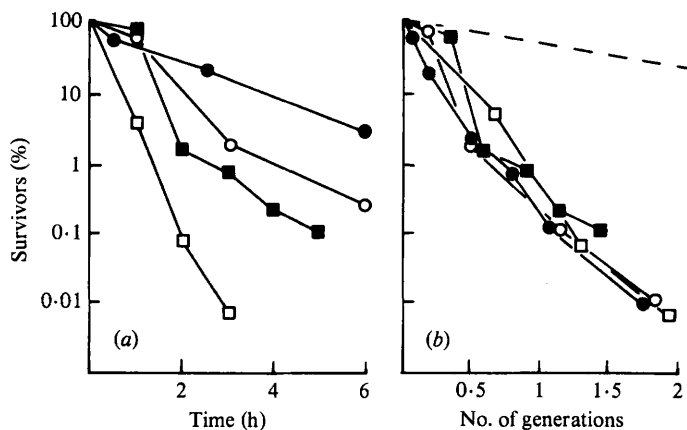


Fig. 1. Rate of killing by $10 \times$ MIC CGP 17520 of *E. coli* W7 growing at various generation times, under glycerol limitation. Pairs of chemostats containing 10^8 c.f.u. ml⁻¹ were equilibrated at four generation times: 1.5 (\square), 3.4 (\blacksquare), 5.9 (\circ) or 12.6 (\bullet) h (the normal generation time is 40 min under these conditions). $10 \times$ MIC CGP 17520 was then added to the cultures and incoming medium and viability determined over two generations. The addition of antibiotic halts growth, leading to a theoretical washout rate as shown (---). The degree of killing decreased as the generation time was extended (a). However, the rate of killing expressed as a function of number of generations was constant (b). The data are representative of experiments done at least three times.

Table 1. Rate of bacterial killing as a function of generation time, antibiotic and strain of *E. coli*

Strain	Antibiotic*	Log kill per generation†	Generation times tested (h)
<i>E. coli</i> 205	CGP 17520	1.20 ± 0.07	12.6, 7.2, 4.0
	Cefonicid	0.22 ± 0.05	11.8, 7.4, 3.9, 1.8
<i>E. coli</i> W7	CGP 17520	0.93 ± 0.20	16.9, 7.3, 2.6, 1.9
	Cefonicid	0.91 ± 0.23	17.3, 6.8, 1.8

* Antibiotics were used at $1 \times$ MIC.

† Means \pm SD, $n = 3$.

~ 14 h). This relationship was the same as found in the rapidly growing batch cultures (i.e. 3 log kill in 1.2 generations).

In order to test the general validity of these findings for other strains and other β -lactam antibiotics, the rate of killing as a function of generation time for *E. coli* strains 205 and W7 treated with $1 \times$ MIC cefonicid or CGP 17520 was compared (Table 1). For each condition the mean log kill per generation time was remarkably constant over a wide range of generation times. However, the absolute rate of killing varied considerably with the bacterial strain and the drug; for example, the degree of killing of *E. coli* W7 per generation was more than four times that of *E. coli* 205 with cefonicid at each generation time. While CGP 17520 and cefonicid produced similar rates of killing in *E. coli* W7, CGP 17520 was far superior to cefonicid in killing *E. coli* 205 at all generation times tested. An increase in the concentration of CGP 17520 from 1 to $10 \times$ MIC resulted in an increase in the log kill of *E. coli* W7 per generation from 0.9 to 3. Thus, while the rate of killing per generation was constant under all conditions tested, the absolute rate depended on the structure and concentration of the drug and on the bacterial strain.

Rate of bacterial lysis as a function of generation time

Killing of *E. coli* by cell wall active antibiotics almost always involves cell lysis as well. It was therefore important to determine whether the rate of lysis of *E. coli* bore a similar constant relationship to generation time as did bacterial killing.

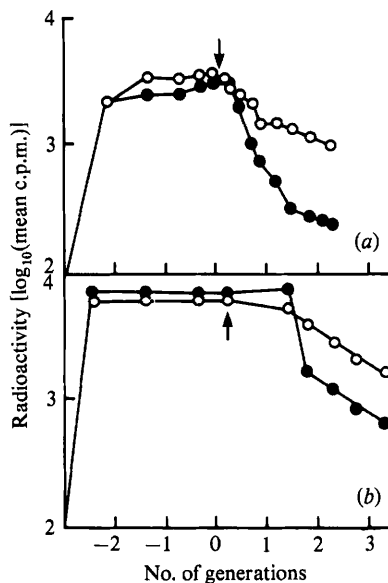


Fig. 2. Rate of cell wall degradation following addition of $10 \times$ MIC CGP 17520 to *E. coli* W7 cultures growing at generation times of 12.6 h (a) or 3.5 h (b) under glycerol limitation. As described in the text, *N*-acetyl[^3H]glucosamine was added to the chemostat for 2 generations (-2 to 0), resulting in a stable rate of incorporation of radiolabel into cell wall (hot SDS precipitable material). The medium was then switched back to unlabelled medium and within 1 generation the rate of loss of label in control cultures (O) approached the theoretical dilution rate calculated for the flow through the chemostat. For each control chemostat a second chemostat growing at the same rate received $10 \times$ MIC CGP 17520 (●) at the arrow. The amount of cell wall associated radiolabel was followed (c.p.m. remaining in hot SDS precipitable material, mean of two experiments). Drug induced cell wall degradation was calculated as the difference in c.p.m. of control versus drug treated cultures. The rate of degradation per generation was constant at 27% in (a) and 26% in (b). The data shown are representative of experiments repeated at least twice for each generation time.

The rate of bacterial lysis, measured as loss of cell wall associated radiolabel, was determined for *E. coli* W7 exposed to CGP 17520 when growing in batch cultures and at four extended generation times between 1.5 and 12.6 h. An example of such an experiment is shown in Fig. 2. *E. coli* W7 growing at a wide variety of generation times released a relatively constant fraction of the total cell wall per generation in response to CGP 17520. For growth rates of 0.75, 1.5, 3.4, 6 and 12.6 h, the loss of cell wall associated radiolabel per generation was 22, 31, 26, 27 and 27%, respectively. Thus, both the rates of bacterial killing and lysis appear to be linked to generation time.

It can be estimated from Fig. 2 that the control cultures continued to incorporate [^3H]glucosamine into the cell wall for only a small fraction of a generation time after the switch to nonradiolabelled medium, a finding consistent with the relatively small pool size of [^3H]glucosamine in *E. coli* (Spratt, 1977). The addition of antibiotic resulted in a rapid onset of cell wall degradation and the rate of degradation remained constant over several subsequent generations.

PBPs of slowly growing cells

In order to determine whether alterations in growth rate due to glucose limitation lead to alterations in PBPs, PBP titrations *in vivo* and in membrane preparations were performed on cultures growing at slow rates and were compared to the PBP profiles found in batch cultures. The results of densitometric tracings of PBP profiles are summarized in Table 2. The relative amount of radioactive penicillin bound to the PBP 1 complex steadily decreased as the

Table 2. Alteration of PBP profile in membrane preparations of *E. coli* W7 as a function of variations in growth rate

PBP	PBP profile*		
	Generation time (h):		
	0.75	3	6
1a/b	633	181	45
3	420	473	457
4	1460	1528	459
5/6	4500	5193	4029

* Densities expressed in arbitrary units as determined by scanning a representative autoradiogram in which membrane preparations were placed in contiguous lanes. Experiments were done in triplicate; the results shown are from one typical experiment.

generation time increased. At the longest generation times, binding of penicillin to PBP 4 also markedly decreased. Relatively little, if any, change in the binding to the other PBPs was detected. The decreased antibiotic binding to PBPs 1 and 4 was found both in whole cells and in membranes prepared from the slow growing bacteria.

DISCUSSION

The new major finding of this report is that the rate of bacterial killing and the rate of lysis following exposure to cell wall inhibitory antibiotics appear to be a direct function of the rate of bacterial growth prior to antibiotic addition. The fact that nongrowing bacteria evade the bactericidal activity of antibiotics is well documented (see Introduction). It has also been shown that the time from drug addition to the onset of bacterial lysis is short in rapidly growing cells but prolonged in slowly growing cells (Boman & Eriksson, 1963). It has been suspected that rapid bacterial growth is associated with rapid death following antibiotic exposure, since enrichment of growth media with blood, for example, enhances both growth and death rates (Hobby & Dawson, 1944; Hobby *et al.*, 1942). However, it is not clear whether these effects were related to differences in growth rates or to differences in the medium composition (and thus in pH or ionic strength, which can profoundly alter bactericidal and lytic activity; Lopez *et al.*, 1976). In our experiments we used chemostat cultures in which different growth rates were produced by limitation of a single nutrient (glucose), so we were able to study the actual rate of the bactericidal and lytic activities of antibiotics as a function of generation time in comparable cultures.

Thus, while the rate of bacterial death varied greatly in terms of absolute time, it remained a constant function of the number of generations. This same rate prevailed not only for the first 'generation of death' but also for the succeeding second and third generation times. Thus, it appears that cells have a mechanism whereby the timing of bactericidal and bacteriolytic events is set as a constant function of the previous rate of bacterial growth. Importantly, the absolute value of the rate of death per generation can be altered by the nature of the drug, or the concentration of the drug. For example, cefonicid kills *E. coli* faster per generation than CGP 17520. However, a given drug kills *E. coli* growing at a wide variety of generation times at the same rate per generation.

The mechanism whereby the culture growth rate determines the rate of bacterial death and lysis is unknown. In nongrowing cells deprived of a required amino acid it has been suggested that phenotypic tolerance is related to the activity of the *relA* locus (stringent response) (Goodell & Tomasz, 1980). Two observations argue against this mechanism in slowly growing cells. The characteristics of the stringent response have not been shown to occur under glucose limited conditions (Lazzarini & Cashel, 1971). Secondly, a recent study of bacterial chemostat cultures limited by glucose indicated that a stringent response affecting growth yield can only be detected at generation times > 50 h (Van Verseveld *et al.*, 1984). In our experiments, bacteria did not

suddenly become completely tolerant at longer generation times. Instead, the rate of antibiotic-induced death (and lysis) appeared to decrease gradually in direct proportion to the decrease in growth rates.

The striking relationship found between the rate of antibiotic induced killing and the growth rate raises several important points of discussion. At first glance, the constant relationship between number of generations and rate of death, at all growth rates, suggests a link between cell cycle events and death. Such relationships have been suggested (Schwarz, 1975; Olsen *et al.*, 1974). However, several findings argue against such an interpretation. It has been clearly shown that bacterial death does not require completion of a cell cycle (Lark, 1958). In fact, nongrowing cells die upon exposure to certain drugs (Tuomanen, 1986). Examination of the early time points of Figs 1 and 2 indicates that the multiplication of bacteria stopped well within a quarter of a generation time after antibiotic addition. Thus, the progression of subpopulations of cells towards a later cell cycle event in which cell division is required for cell death is unlikely. Furthermore, it would be difficult to reconcile such a cell cycle dependent event with the accelerated rate of death observed when the drug concentration is increased. Therefore, the rate of killing must not simply be the rate of progression towards a cell cycle event. Clearly, the killing rate does, however, precisely titrate something that is happening or is present in a concentration proportional to the pre-drug growth rate. Several possibilities are argued.

Since the rate of cell wall synthesis is regulated by the growth rate it is conceivable that the growth rate dependent loss of viability is a reflection of the frequency with which a particular cell wall component is incorporated into the sacculus of the antibiotic-treated bacteria (e.g. attachment sites for autolysin molecules; Tomasz *et al.*, 1970). Variations in medium composition and growth phase are known to affect the composition and properties of the cell wall (Mengin-Lecreulx & Van Heijenoort, 1985; Pisabarro *et al.*, 1985; Rosenthal *et al.*, 1985; Horne & Tomasz, 1985; Goodell & Tomasz, 1980). Alternatively, a protein (e.g. cell divisional or DNA replication associated protein or autolysin) may be synthesized as a function of generation time, as has been reported in other systems (Baker & Wolf, 1984). If it is assumed that killing and lysis rates are determined by the absolute concentration of this protein, then the rate of kill would decrease in a slowly growing cell. In this context it is interesting that in glucose limited chemostat cultures, the amounts of penicillin bound to PBPs 1a/b decreased in proportion to the growth rate. It is not known if this resulted from a decreased cellular concentration, a decreased affinity or an increased deacylation rate of the PBP 1 complex. This decreased binding is intriguing since selective interference with the functions of PBPs 1a and 1b results in rapid lysis and killing of *E. coli* (Kitano & Tomasz, 1979; Chase *et al.*, 1981; Suzuki *et al.*, 1978). While this mechanism does not appear to extend to all conditions of growth limitation, in that PBP alterations are not seen in amino acid starved *E. coli* (Tuomanen, 1986), precedent for alterations in PBPs with growth conditions exists, including amino acid starved pneumococci (E. Tuomanen, unpublished observations), *Streptococcus faecalis* (Fontana *et al.*, 1980), stationary versus exponential phase *E. coli* (Buchanan & Sowell, 1982; de la Rosa *et al.*, 1983), *Bacillus subtilis* (Neyman & Buchanan, 1985), *Haemophilus influenzae* (P. M. Mendelman & D. O. Chaffin, unpublished observations), and *Bordetella pertussis* (E. Tuomanen, unpublished observations). Since the decrease in PBPs 1a/b is observed in membrane preparations as well as whole cells, and the titration of other PBPs is not similarly altered with generation time, alterations in drug permeability to the cytoplasmic membrane targets cannot explain growth rate dependent killing. This is important in the light of alterations known to occur in membrane properties with generation time (Brown & Williams, 1985). This is consistent with recent data demonstrating that a wide range of antibiotics with differing membrane permeabilities still show a growth rate dependent killing constant (Cozens *et al.*, 1986). The effects of drug structure, permeability, susceptibility to β -lactamases, etc., are likely to be reflected in the absolute value of the killing constant rather than the constancy itself.

It has recently been demonstrated that nongrowing bacteria are phenotypically tolerant to the killing activity of β -lactam antibiotics not only *in vitro* but in *in vivo* environments (in animals) as well (Tuomanen, 1986). Yet it is likely that the more common situation *in vivo* is not total lack of growth but rather a markedly reduced rate of growth, similar to the slow growth rates produced

in our chemostat cultures (Zak & Sande, 1982). Our findings suggest that such slowly growing cells would die proportionately more slowly *in vivo* also. Such slow death may not be adequate in the therapy of serious infectious diseases, particularly in states of deficient host defences. In this setting, our finding that β -lactam antibiotics differ greatly in their absolute rate of bactericidal activity against slowly growing cultures will be important to explore.

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REFERENCES

- BAKER, H. V., II & WOLF, R. E., JR (1984). Essential site for growth-rate dependent regulation within *Escherichia coli* *gnd* structural gene. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 7669–7673.
- BOMAN, H. G. & ERIKSSON, K. G. (1963). Penicillin induced lysis in *Escherichia coli*. *Journal of General Microbiology* **31**, 339–352.
- BROOME-SMITH, J. K. & SPRATT, B. G. (1982). Deletion of the penicillin-binding protein 6 gene of *Escherichia coli*. *Journal of Bacteriology* **152**, 904–906.
- BROWN, M. R. W. & WILLIAMS, P. (1985). The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology* **39**, 527–556.
- BUCHANAN, C. E. & SOWELL, M. O. (1982). Synthesis of penicillin-binding protein 6 by stationary-phase *Escherichia coli*. *Journal of Bacteriology* **151**, 491–494.
- CHASE, H. A., FULLER, C. & REYNOLDS, P. E. (1981). The role of penicillin-binding proteins in the action of cephalosporins against *Escherichia coli* and *Salmonella typhimurium*. *European Journal of Biochemistry* **117**, 301–310.
- COZENS, R., TUOMANEN, E., TOSCH, W., ZAK, O., SUTER, J. & TOMASZ, A. (1986). Evaluation of the bactericidal activity of beta lactam antibiotics on slowly growing bacteria cultured in the chemostat. *Antimicrobial Agents and Chemotherapy* (in the Press).
- DAVIS, B. D. (1949). Isolation of biochemically deficient mutants of bacteria by penicillin. *Proceedings of the National Academy of Sciences of the United States of America* **35**, 1–10.
- FONTANA, R., CANEPARI, P., SATTÀ, G. & COYETTE, J. (1980). Identification of the lethal target of benzylpenicillin in *Streptococcus faecalis* by *in vivo* penicillin binding studies. *Nature, London* **287**, 70–72.
- GILBERT, P. & STUART, A. (1977). Small scale chemostat for the growth of mesophilic and thermophilic microorganisms. *Laboratory Practice* **26**, 627–628.
- GOODELL, W. & TOMASZ, A. (1980). Alteration of *Escherichia coli* murein during amino acid starvation. *Journal of Bacteriology* **144**, 1009–1016.
- HOBBS, G. L. & DAWSON, M. H. (1944). Effect of rate of growth of bacteria on action of penicillin. *Proceedings of the Society for Experimental Biology and Medicine* **56**, 181–184.
- HOBBS, G. L., MEYER, K. & CHAFFEE, E. (1942). Observation on the mechanism of penicillin. *Proceedings of the Society for Experimental Biology and Medicine* **50**, 281–285.
- HORNE, D. & TOMASZ, A. (1985). Factors affecting sensitivity of group B streptococci to an exogenous murein hydrolase. *Canadian Journal of Microbiology* **31**, 417–422.
- KITANO, K. & TOMASZ, A. (1979). Triggering of autolytic cell wall degradation in *Escherichia coli* by beta-lactam antibiotics. *Antimicrobial Agents and Chemotherapy* **16**, 838–848.
- LARK, K. G. (1958). Variation during the cell-division cycle in the penicillin induction of protoplast-like forms of *Alcaligenes faecalis*. *Canadian Journal of Microbiology* **4**, 179–189.
- LAZZARINI, R. A. & CASHEL, M. (1971). On the regulation of guanosine tetraphosphate levels in stringent and relaxed strains of *Escherichia coli*. *Journal of Biological Chemistry* **246**, 4381–4385.
- LEDERBERG, J. & ZINDER, N. (1948). Concentration of biochemical mutants of bacteria with penicillin. *Journal of the American Chemical Society* **70**, 4267–4268.
- LOPEZ, R., RONDA-LAIN, C., TAPIA, A., WAKS, S. B. & TOMASZ, A. (1976). Suppression of the lytic and bactericidal effects of cell-wall inhibitory antibiotics. *Antimicrobial Agents and Chemotherapy* **10**, 697–706.
- MENGIN-LECREULX, D. & VAN HEIJENOORT, J. (1985). Effect of growth conditions on peptidoglycan content and cytoplasmic steps of its biosynthesis in *Escherichia coli*. *Journal of Bacteriology* **163**, 208–212.
- NEYMAN, S. L. & BUCHANAN, C. E. (1985). Restoration of vegetative penicillin-binding proteins during germination and outgrowth of *Bacillus subtilis* spores: relationship of individual proteins to specific cell cycle events. *Journal of Bacteriology* **161**, 164–168.
- OLSEN, W., HEIDRICH, H., HANNIG, K. & HOFSCHEIDER, P. H. (1974). Deoxyribonucleic acid-envelope complexes isolated from *Escherichia coli* by free-flow electrophoresis: biochemical and electron microscopic characterization. *Journal of Bacteriology* **118**, 646–653.
- PISABARRO, A. G., DE PEDRO, M. A. & VAZQUEZ, D. (1985). Structural modifications in the peptidoglycan of *Escherichia coli* associated with changes in the state of growth of the culture. *Journal of Bacteriology* **161**, 238–242.
- DE LA ROSA, E. J., DE PEDRO, M. & VAZQUEZ, D. (1983). Growth dependent modifications of the penicillin binding proteins of *Escherichia coli*: information supporting the generality of the process and the multiplicity of mechanisms. In *The Target of Penicillin*, pp. 465–489. Edited by R. Hakenbeck, J.-V. Høltje & H. Labischinski. Berlin: Walter de Gruyter.
- ROSENTHAL, R. S., GFELL, M. A. & FOLKENING, W. J. (1985). Influence of protein synthesis inhibitors on

- regulation of extent of *O*-acetylation of gonococcal peptidoglycan. *Infection and Immunity* **49**, 7-13.
- SCHWARZ, U. (1975). Process of cellular division in *Escherichia coli*: differentiation of growth zones in the sacculus. *Journal of Molecular Biology* **98**, 749-759.
- SPRATT, B. G. (1977). Properties of the penicillin-binding proteins of *Escherichia coli* K12. *European Journal of Biochemistry* **72**, 341-352.
- SUZUKI, H., NISHIMURA, Y. & HIROTA, Y. (1978). On the process of cellular division in *Escherichia coli*, a series of mutants of *Escherichia coli* altered in the penicillin binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 664-668.
- TOMASZ, A., ALBINO, A. & ZANATI, E. (1970). Multiple antibiotic resistance in a bacterium with suppressed autolytic system. *Nature, London* **227**, 138-140.
- TUOMANEN, E. (1986). Phenotypic tolerance: the search for beta-lactam antibiotics which kill non-growing bacteria. In *Proceedings of the International Symposium on Evaluation of New Beta-Lactam Antibiotics, May 20-22, 1985, Visby, Gotland, Sweden. Reviews of Infectious Diseases July-August 1986 Supplement*.
- VAN VERSEVELD, H. W., CHESEBRO, W. R., BRASTER, M. & STOUTHAMER, A. H. (1984). Eubacteria have three growth modes keyed to nutrient flow. *Archives of Microbiology* **137**, 176-184.
- ZAK, O. & SANDE, M. A. (1982). Correlation of *in vitro* antimicrobial activity of antibiotics with results of treatment in experimental animal models and human infection. In *Action of Antibiotics in Patients*, pp. 55-67. Edited by L. D. Sabath. Bern: Huber Publishers.
- ZAK, O., TOSCH, W. & KUNZ, S. (1984). Comparative efficacy of a new cephem CGP 17520, cefonicid and ceftriazone *in vivo*. In *Proceedings of the International Conference on Antimicrobial Agents and Chemotherapy, October 8-10, 1984, Washington, DC*, p. 216, abstract no. 725. Washington DC: American Society for Microbiology.