ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1990, p. 1865–1868
0066-4804/90/101865-04$02.00/0

Vol. 34, No. 10

MINIREVIEW

Influence of Growth Rate on Susceptibility to Antimicrobial Agents: Biofilms, Cell Cycle, Dormancy, and Stringent Response

PETER GILBERT,[1] PHILLIP J. COLLIER,[2] AND MICHAEL R. W. BROWN[2]*

Pharmaceutical Sciences Institute, Department of Pharmaceutical Sciences, Aston University, Aston Triangle, Birmingham B4 7ET,[2] and Department of Pharmacy, University of Manchester, Manchester M13 9PL,[1] United Kingdom

BIOFILMS, MICROCOLONIES, AND THE GLYCOCALYX

Many chronic infections, particularly those involving medical implants and prosthetic devices, involve bacterial consortia which grow as adherent biofilms within extended polysaccharide glycocalices (3, 5, 7). Such microcolonies can be regarded as functional consortia which condition their environment through the concentration of enzymes and metabolic products (7, 9). The glycocalyx, acting as a barrier, isolates the enclosed cells from fluctuations in the surrounding environment. In these respects, the physiological properties of sessile biofilm populations are distinct from those of their planktonic counterparts and contribute to survival within infected hosts.

There have been relatively few direct studies of antibiotic susceptibility in biofilms. Recent reports have suggested that such populations are profoundly resistant to many antibiotics and that this resistance contributes to the recalcitrance of infections involving medical implants and prosthetic devices. Tobramycin resistance of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* and vancomycin resistance of *S. epidermidis* were increased 20- to 100-fold for biofilms relative to equivalent planktonic populations (15, 18, 28, 36). Although the importance of the biofilm mode of growth in vivo is unequivocal, observations relating to antibiotic susceptibility of biofilm populations have often been made with in vitro devices which utilize complex media, are uncontrolled with respect to the growth rate, and compare the antibiotic susceptibility of established biofilm populations with that of planktonic populations. Growth rate differences have been indicated as a possible cause of susceptibility changes (2, 15, 21).

Recently, techniques which allow for the control of the growth rate in adherent populations have been developed (17). It has been shown that much of the resistance to antibiotics such as tobramycin (14), quaternary compounds, and quinolones (D. J. Evans, M. R. W. Brown, D. G. Allison, and P. Gilbert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, A152, p. 26) shown by biofilm-associated cells relates to their slow growth rate rather than to any innate properties of the glycocalyx (8, 9). The growth rate also appears to modulate the hydrophobicity of the cell surface and thereby to influence the colonization of surfaces (1; D. G. Allison, D. J. Evans, M. R. W. Brown, and P. Gilbert, FEMS Microbiol. Lett., in press).

* Corresponding author.

GROWTH RATE AND CELL CYCLE

Asynchronous populations contain organisms at all stages of the division cycle. At any time, presuming balanced growth, a constant proportion is at any given stage. Mitchison (25) demonstrated that enzyme levels undergo a series of ordered changes during the cellular division cycle. Some cellular enzyme activities are continuous throughout the division cycle, yet others double at particular points to allow for equality in the daughter cells. Other enzymes may be proportional to cell mass (exponential increase throughout the division cycle) or synthesized periodically (25). For chemically stable enzymes, the latter results in stepped increases in activity for individual cells, whereas for unstable enzymes, peaks of activity result. The timing of peaks and steps of biosynthetic activity with respect to the division cycle and changing growth rate have received little direct study. For peak enzymes, rates of degradation relate more to pH and temperature than to the specific growth rate. Thus, the faster the division rate, the more frequent the biosynthetic period and the higher the mean level of activity in heterogeneous populations. Events such as DNA synthesis, septation, and constriction are well documented (12, 13, 19) and occupy finite periods of the division cycle irrespective of the growth rate (6, 20). In such instances alterations in the specific growth rate alter the proportion of the cell cycle over which these events occur and also the proportion of the population expressing such activity at any time. When such properties affect drug susceptibility, the level of drug susceptibility expressed by the asynchronous population will vary. Drug susceptibility may be greatly influenced by the presence of small fractions of cells undergoing particular sensitizing events. Thus, the fraction of cells undergoing DNA replication may be especially susceptible to drugs such as mitomycin or phenylethanol. Different fractions of the populations may be quite resistant to these agents yet display an increased susceptibility to penicillins and cephalosporins because of their synthesis of polar cap material. In this respect it is interesting to note that although susceptibility to β-lactams was shown to increase with the growth rate, it was invariant when plotted against the number of generations (11, 42). The susceptibility of *Escherichia coli* to tobramycin is directly related to the growth rate (14; Evans et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1990). Studies with synchronous populations showed that this susceptibility occurred at and immediately after cell division and was related to peaks of resistance late in the division cycle. Clearly, wherever a marked dependence of susceptibility on the growth rate is demonstrated, the possibility of sensitization within the division cycle cannot be overlooked.

DORMANCY AND STRINGENT RESPONSE

The growth of heterotrophic bacteria in natural environments is inhibited by periods of insufficient levels of energy and nutrients (41). Such inhibition may reduce the growth rate of the bacteria to such an extent that they may be considered to have growth rates that approximate zero. Moyer and Morita (27) hypothesized that cell populations exhibiting very low growth rates or a growth rate of zero are most closely representative of cells found in oligotrophic marine environments and that it is cells in these environments and similar ones that exhibit the phenomena associated with starvation and dormancy. Such studies may well cast light on cell properties in chronic infections.

The survival strategies of bacteria in their natural environments under starvation conditions have been identified (39) and suggest that bacterial cultures undergo a series of physiological or phenotypic changes which enable the survival of some of the cells. Rapid multiple divisions of starved cells which lead to the formation of ultramicrobacteria (<0.3 μm in diameter) have been observed (29). The presence of large, "normal" marine bacterial cells has been observed only at interfaces at which nutrients were readily available (22); presumably the rest of the bacterial cells were starved and were therefore dormant ultramicrobacteria. It is assumed that such a reduction division (29) in response to starvation improves the chances of individual genomes surviving by the rapid formation of multiple copies. The reduction division is followed by a progressive reduction in the viability of the cultures until only 0.3% of the original cell number is found to be viable after 70 weeks of starvation (27, 30). Novitsky and Morita (31) showed that because of the initial multiplication of cell numbers, over 15 times the original cell number was viable, after starvation, as ultramicrobacteria. Roszak and Colwell (38) suggested that ultramicrobacteria were exogenously dormant forms, responding to unfavorable environmental conditions, and sporelike or "somnicell" stages of nonsporeforming bacteria. Morita (26) also suggested that ultramicrobacteria were the dominant or normal state of bacterial cells in marine, aquatic, and terrestrial environments.

The similarity in the responses to nutrient starvation or nutrient limitation conditions of both sporeforming and nonsporeforming bacterial species may be due to the possession by both groups of the stringent response (SR) gene, *relA*. The SR is a phenotypic adaptation to conditions of amino acid limitation (4). The gene product of *relA* is ATP:GTP 3'-pyrophosphotransferase [(p)ppGpp synthetase I], which phosphorylates GDP and GTP to the polyphosphorylated ppGpp and pppGpp forms [referred to together as (p)ppGpp]. It is thought that the decrease in available GDP and GTP is responsible for the SR effect of protein regulation, as opposed to the creation of (p)ppGpp. However, it has been suggested that (p)ppGpp acts as a suppressor of ribosome or protein activity by direct binding to the ribosome (24).

relA activity can also induce sporulation in endosporeforming bacterial species (16, 32), although it is unclear whether this gene can be considered the primary gene in the sporulation process. It is more generally accepted that the *spo0A* gene is the primary gene in sporulation. It has been shown that the *spo0A* gene is responsible for the formation of highly phosphorylated nucleotides, ppApp and pppApp [referred to together as (p)ppApp] (35, 37). The mechanisms of action and induction of these two genes appear to be very similar. Indeed, the activity of a single gene, *abrB*, has been shown to suppress partially the effects of both *relA* and *spo0A* activities (16).

Relationships between sporulation and the SR and between antibiotic production and susceptibility have been elucidated (23, 33, 35, 40). For example, the SR appears to be essential for self-resistance antibiotics produced by *Bacillus subtilis* (34). Chloramphenicol (1 μg/ml) was used to inhibit bacterial synthesis of (p)ppGpp, and the subsequent antibiotic production was demonstrably reduced (34). It was determined that the SR was indispensable for the initiation of antibiotic production by *B. subtilis* and that this antibiotic production led in turn to self-resistance. It was also shown that antibiotic-proficient mutants lacking the SR (*relA*) could also become self-resistant, indicating that the SR is essential for antibiotic production in *B. subtilis* but not for subsequent self-resistance.

Tuomanen and Tomasz (43) observed that unless the SR was relaxed, all nongrowing bacteria rapidly developed resistance to autolysis induced by a variety of agents, including various types of cell wall synthesis inhibitors. Cozens et al. (10) also remarked on the lack of susceptibility of slowly growing or nongrowing bacteria to β-lactam antibiotics and subsequent autolysis. The latter did, however, find that a novel carbapenem antibiotic, imipenem, could induce autolysis in *E. coli*. Matin et al. (24) also suggested that nutrient-deprived cells were more resistant to disinfectant agents and to osmotic stress.

Stenstrom et al. (41) examined the effects of several antibiotics on bacterial cultures undergoing starvation. They found that some inhibitors significantly reduced the viability of long-term-starved cells. They also observed, however, that *Salmonella typhimurium* exhibited reduced susceptibility to tetracycline (an inhibitor of protein synthesis) after 20 days of starvation, as compared with its susceptibility at 12 days. Starved *S. typhimurium* also exhibited little or no susceptibility to inhibitors of cell wall or DNA synthesis. It was suggested that long-term-starved cells may express different phenotypes which may include changes in surface structures and binding and uptake of antibiotics (41). It is apparent that *relA*-competent cells possess unusual antibiotic susceptibilities and relationships in that they appear, in general, to have an enhanced resistance to many antibiotics. This resistance may be due solely to reduced rates of anabolism, which could explain the lack of susceptibility to cell wall- and DNA-active antibiotics observed by Stenstrom et al. (41). Alternatively, some product or products of the SR process may serve to protect intracellular targets from the action of antibiotics. In particular, the known binding affinity of (p)ppGpp for ribosomes may prevent the action of aminoglycoside antibiotics.

Tuomanen (42) observed that the metabolic state of bacteria subjected to conditions of amino acid limitation differed from that of growing cells by a pronounced and rapid decrease in the rates of protein, RNA, and cell wall syntheses. Tuomanen (42) posed the following question: is phenotypic tolerance obligatory in dormant cells? She hypothesized that if this were so, then two criteria must be met: (i) *relA* mutants should not be phenotypically tolerant and (ii) in the presence of the SR of all wild-type bacteria, antibiotics should fail to overcome phenotypic tolerance. The first case was found to be true (42), but the second proved false. Phenotypic tolerance of antibiotics is not absolute in all *relA*$^+$ cells (41), indicating that *relA*$^+$ cells are susceptible to antibiotics which act as "relaxing" agents or that some other *relA*-related process may be inhibited with lethal effect. The observed reduction in susceptibility to some antibiotics,

such as cell wall and DNA synthesis inhibitors, of viable but nongrowing cells raises the issue of the effectiveness of accepted MICs in both natural ecosystems (41) and chronic infections.

ACKNOWLEDGMENTS

We are grateful to the Medical Research Council (United Kingdom) and to the Cystic Fibrosis Research Trust (United Kingdom), who supported much of the work reported from our laboratories. Also gratefully acknowledged is help from Bayer and Ciba-Geigy.

In addition, we are grateful for critical discussions with H. Anwar, M. Barza, J. W. Costerton, R. M. Cozens, A. Dalhoff, P. Kroll, J. Melling, M. A. Sande, H. Smith, D. W. Tempest, E. Tuomanen, P. Williams, O. Zak, H.-J. Zeiler, and many other colleagues at the Centre for Applied Microbiology Research, Salisbury, United Kingdom, who pioneered much of this work several decades ago.

LITERATURE CITED

1. **Allison, D. G., D. J. Evans, M. R. W. Brown, and P. Gilbert.** 1990. Possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. J. Bacteriol. **172**:1667–1669.
2. **Brown, M. R. W., D. G. Allison, and P. Gilbert.** 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrob. Chemother. **22**:777–780.
3. **Brown, M. R. W., and P. Williams.** 1985. The influence of environment on envelope properties affecting survival of bacteria in infections. Annu. Rev. Microbiol. **39**:527–556.
4. **Cashel, M., and K. E. Rudd.** 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
5. **Charaklis, W. G., and P. A. Wilderer (ed.).** 1989. Structure and function of biofilms. Dahlem Workshop Reports. Wiley, Chichester, United Kingdom.
6. **Cooper, S.** 1979. A unifying model for the G1 period in prokaryotes and eukaryotes. Nature (London) **280**:17–19.
7. **Costerton, J. W., K. J. Cheng, K. G. Geesey, P. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
8. **Costerton, J. W., R. T. Irwin, and K. J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35**:399–424.
9. **Costerton, J. W., T. J. Marrie, and K. J. Cheng.** 1985. Phenomena of bacterial adhesion, p. 3–43. *In* D. C. Savage and M. Fletcher (ed.), Bacterial adhesion. Plenum Publishing Corp., New York.
10. **Cozens, R. M., Z. Markiewicz, and E. Tuomanen.** 1989. Role of autolysins in the activities of imipenem and CGP 31608, a novel penem, against slowly growing bacteria. Antimicrob. Agents Chemother. **33**:1819–1821.
11. **Cozens, R. M., E. Tuomanen, W. Tosch, O. Zak, J. Suter, and A. Toomasz.** 1986. Evaluation of the bactericidal activity of β-lactam antibiotics upon slowly growing bacteria cultured in the chemostat. Antimicrob. Agents Chemother. **29**:797–802.
12. **Donachie, W. D., and K. J. Begg.** 1970. Growth of the bacterial cell. Nature (London) **227**:1220–1224.
13. **Donachie, W. D., K. J. Begg, and M. Vincente.** 1976. Cell length, cell growth and cell division. Nature (London) **264**:328–332.
14. **Evans, D. J., M. R. W. Brown, D. G. Allison, and P. Gilbert.** 1990. Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in division cycle. J. Antimicrob. Chemother. **25**:585–591.
15. **Evans, R. C., and C. J. Holmes.** 1987. Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. Antimicrob. Agents Chemother. **31**:889–894.
16. **Freese, E., and J. Heinze.** 1983. Metabolic and genetic control of bacterial sporulation, p. 101–172. *In* A. Hurst and G. W. Gould (ed.), The bacterial spore. Academic Press, Inc. (London), Ltd., London.
17. **Gilbert, P., D. G. Allison, D. J. Evans, P. S. Handley, and M. R. W. Brown.** 1989. Growth rate control of adherent bacterial populations. Appl. Environ. Microbiol. **55**:1308–1311.
18. **Gristina, A. G., C. D. Hobgod, L. X. Webb, and Q. N. Myrvik.** 1987. Adhesive colonisation of biomaterials and antibiotic resistance. Biomaterials **8**:423–426.
19. **Helmstetter, C. E.** 1967. Rates of DNA synthesis during the division cycle of *Escherichia coli* B/r. J. Mol. Biol. **24**:417–427.
20. **Helmstetter, C. E., and S. Cooper.** 1968. DNA synthesis during the division cycle of rapidly growing *Escherichia coli* B/r. J. Mol. Biol. **31**:507–518.
21. **Holmes, C. J., and R. C. Evans.** 1989. Resistance of bacterial biofilms to antibiotics. J. Antimicrob. Chemother. **24**:84.
22. **Kjelleberg, S., B. A. Humphrey, and K. C. Marshall.** 1982. Effects of interfaces on small, starved marine bacteria. Appl. Environ. Microbiol. **43**:1166–1172.
23. **Koch, A. L., and G. H. Gross.** 1979. Growth conditions and rifampin susceptibility. Antimicrob. Agents Chemother. **15**:220–228.
24. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in non-differentiating bacteria. Annu. Rev. Microbiol. **43**:293–316.
25. **Mitchison, J. M.** 1969. Enzyme synthesis in synchronous cultures. Science **165**:657–663.
26. **Morita, R. Y.** 1986. Starvation survival: the normal mode of most bacteria in the ocean, p. 242–248. *In* Proceedings of the 4th International Symposium on Microbiology and Ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
27. **Moyer, C. L., and R. Y. Morita.** 1989. Effect of growth rate and starvation-survival on the viability and stability of a psychrophilic marine bacterium. Appl. Environ. Microbiol. **55**:1122–1127.
28. **Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary tract catheter materials. Antimicrob. Agents Chemother. **27**:619–624.
29. **Novitsky, J. A., and R. Y. Morita.** 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio. Appl. Environ. Microbiol. **32**:617–622.
30. **Novitsky, J. A., and R. Y. Morita.** 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. **33**:635–641.
31. **Novitsky, J. A., and R. Y. Morita.** 1978. Possible strategy for the survival of marine bacteria under starvation conditions. Mar. Biol. **48**:289–295.
32. **Ochi, K., J. Kandala, and E. Freese.** 1981. Initiation of *Bacillus subtilis* sporulation by the stringent response to partial amino acid deprivation. J. Biol. Chem. **256**:6866–6875.
33. **Ochi, K., and S. Ohsawa.** 1984. Initiation of antibiotic production by the stringent response of *Bacillus subtilis* Marburg. J. Gen. Microbiol. **130**:2473–2482.
34. **Ochi, K., and M. Yamashita.** 1984. Indirect involvement of the stringent response of *Bacillus subtilis* in the development of self-resistance to its own antibiotic. J. Antibiot. **37**:1085–1087.
35. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40**:908–962.
36. **Prosser, B. L. T., D. Taylor, B. A. Dix, and R. Cleeland.** 1987. Method of evaluating effects of antibiotics on bacterial biofilm. Antimicrob. Agents Chemother. **31**:1502–1506.
37. **Rhaese, H. J., H. Dichtelmüller, R. Grade, and R. Groscurth.** 1975. High phosphorylated nucleotides involved in regulation of sporulation in *Bacillus subtilis*, p. 335–340. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
38. **Roszak, D. B., and R. R. Colwell.** 1987. Metabolic activity of bacterial cells enumerated by direct viable count. Appl. Environ. Microbiol. **53**:2889–2893.
39. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of

bacteria in the natural environment. Microbiol. Rev. **51**:365–379.
40. **Schaeffer, P.** 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol. Rev. **33**:48–71.
41. **Stenstrom, T.-A., P. Conway, and S. Kjelleberg.** 1989. Inhibition by antibiotics of the bacterial response to long-term starvation of *Salmonella typhimurium* and the colon microbiota of mice. J. Appl. Bacteriol. **67**:53–59.
42. **Tuomanen, E.** 1986. Phenotypic tolerance: the search for β-lactam antibiotics that kill non-growing bacteria. Rev. Infect. Dis. **8**(Suppl.):S279–S291.
43. **Tuomanen, E., and A. Tomasz.** 1986. Induction of autolysis in nongrowing *Escherichia coli*. J. Bacteriol. **167**:1077–1080.