Bacitracin Induces Sphere Formation in *Halobacterium* Species which Lack a Wall Peptidoglycan

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(Received 27 January 1975; revised 23 April 1975)

Bacteria of the genus *Halobacterium* lack the peptidoglycan layer characteristic of the walls of most prokaryotic organisms (Larsen, 1967). The major carbohydrate-containing component of the envelope of *H. salinarium* is a high molecular weight glycoprotein (Mescher, Strominger & Watson, 1974) which may play a role in maintaining the structural integrity of the envelope (Mescher *et al.* 1974). Similar glycoproteins appear to be present in the envelopes of *H. cutirubrum* (unpublished results) and *H. halobium* (Konciewicz, 1972). Conflicting reports have appeared regarding inhibition of the growth of *Halobacteria* by penicillin G (Cho, Doy & Mercer, 1967; Mohr & Larsen, 1963), a specific inhibitor of the transpeptidase and carboxypeptidase activities of peptidoglycan synthesis (Strominger, 1970). Since *Halobacteria* lack peptidoglycan, the ability of Na-penicillin G to inhibit their growth was re-examined. Ampicillin and cephalosporin C, which inhibit these same activities (Gale *et al.* 1972), were also tested together with several antibiotics which interfere with other steps in the pathway of peptidoglycan synthesis. These included: D-cycloserine, which inhibits the conversion of L-alanine to D-alanine and the formation of D-alanyl-D-alanine (Gale *et al.* 1972); bacitracin, which inhibits the dephosphorylation of C₅₅-isoprenyl pyrophosphate (Siewert & Strominger, 1967); and vancomycin, ristocetin (Gale *et al.* 1972; Strominger, 1970), moenomycin (Lugtenberg, van Schijndel-van Dam & van Belle-gem, 1971) and janiomycin (Linnett & Strominger, 1973), all of which appear to inhibit the transfer of the disaccharide-pentapeptide unit from the lipid intermediate to the acceptor.

METHODS

The organisms used were *Halobacterium salinarium*, strain I (ATCC19700), *H. halobium* (NRC34003) and *H. cutirubrum* (NRC34001). All strains were grown in the liquid medium previously described for growth of *H. salinarium* (Mescher *et al.* 1974). Growth was measured with a Klett photometer using a red filter. The antibiotics used were obtained from The Squibb Institute for Medical Research, New Brunswick, New Jersey, U.S.A. (Na-penicillin G, ampicillin, janiomycin); Abbot Laboratories, North Chicago, Illinois, U.S.A. (ristocetin); Eli Lilly & Co., Indianapolis, Indiana, U.S.A. (vancomycin, D-cyclo-serine, cephalosporin C); The Upjohn Co., Kalamazoo, Michigan, U.S.A. (bacitracin); and Hoechst Pharmaceutical Co., Frankfurt, Germany (moenomycin).

RESULTS AND DISCUSSION

The concentrations of the antibiotics required to give 50 % inhibition of growth are shown in Table 1. All of the antibiotics tested, with one exception, either caused no inhibition at

Table 1. Antibiotic concentrations required to inhibit growth by 50 %

Growth inhibition was determined by comparing the extinctions of cultures grown at several concentrations of antibiotic to the extinctions of control cultures (no antibiotic) at the end of exponential phase. Cultures were started using a 5 % inoculum of organisms grown for 36 h in liquid medium.

Antibiotic	Concentration required for 50 % growth inhibition ($\mu\text{g/ml}$)		
	<i>H. salinarium</i>	<i>H. cutirubrum</i>	<i>H. halobium</i>
Na-penicillin G	1400	2025	1300
Ampicillin	6300	6800	5400
Cephalosporin C	> 1500	> 1500	> 1500
D-Cycloserine*	500	450	650
L-Cycloserine*	150	225	150
Bacitracin	5	1	12
Vancomycin	> 2000	> 2000	> 2000
Ristocetin	> 2000	1200	1200
Moenomycin	> 1000	> 1000	60
Janiemycin	> 1000	> 1000	> 1000

* Both isomers of cycloserine may inhibit growth by reacting non-enzymically with pyridoxal phosphate (Roze & Strominger, 1963). If inhibition by D-cycloserine were specific, as in peptidoglycan synthesis, it would be expected to inhibit growth at lower concentrations than does L-cycloserine.

the maximum concentration tested or caused inhibition at concentrations 100 to 10000 times greater than those required for inhibition of bacteria possessing peptidoglycan. The inhibition observed at these extremely high concentrations was probably non-specific and unrelated to the specific effects of the antibiotics on peptidoglycan synthesis in normal bacteria.

The striking exception was bacitracin, which inhibited the growth of *Halobacteria* at concentrations comparable to those required for normal bacteria. Further examination of the effect of bacitracin showed that it caused a morphological change in the bacteria. Addition of bacitracin (30 $\mu\text{g/ml}$) to cultures which had entered the exponential phase of growth decreased the growth rate and growth stopped after approximately one doubling (as measured by a Klett photometer) whereas control cultures without added bacitracin continued to grow for more than two doublings. Cultures were examined by phase contrast microscopy at various times after addition of bacitracin (Fig. 1). Organisms in the control culture (no bacitracin) were rod-shaped at all times examined (Fig. 1*a*). Organisms inhibited by bacitracin gradually changed from the normal rod shape to spherical forms. Complete conversion to spherical forms occurred only after growth in bacitracin for approximately one doubling time (about 30 h). Similar results were obtained with both *H. halobium* and *H. cutirubrum*.

While several effects of bacitracin on bacterial membranes have been reported (Weinberg, 1967), Storm & Strominger (1973, 1974) have clearly shown that it specifically forms a complex with the C_{55} -isoprenyl pyrophosphate, thus inhibiting peptidoglycan synthesis, and have strongly suggested that this is its primary effect in the inhibition of bacterial growth. The low concentration of bacitracin required to inhibit *Halobacteria* and its effect on their morphology suggest that it may act by inhibiting the synthesis of a structural component of their envelope. There is now considerable evidence that the synthesis of at least some of the glycoproteins in eukaryotic organisms involves polyisoprenol phosphate intermediates (Behrens, 1974). Moreover, preliminary results suggest that the synthesis of the *H. salinarium* envelope glycoprotein also involves a polyisoprenyl pyrophosphate-linked intermediate, and bacitracin may therefore inhibit growth of *Halobacteria* and cause

Fig. 1. Phase-contrast photomicrographs of normal and bacitracin-inhibited cultures. (a) Control culture. Bacteria appeared identical at all times, and therefore only one photograph (taken 24 h after inoculation) is shown. (b), (c), (d) Cultures 12.5, 24.0 and 36.5 h after addition of bacitracin, respectively.

conversion to spherical forms by inhibiting the synthesis of this glycoprotein as a consequence of forming a complex with polyisoprenyl pyrophosphate. Bacitracin may also alter the lipid composition of the membrane to some extent by inhibiting synthesis of saturated isoprenoid chains of the type known to be present in at least one of the lipids of *H. cutirubrum* (Kates, Yengoyan & Sastry, 1965).

These results show that a number of antibiotics thought specifically to affect peptidoglycan synthesis at several different points in the biosynthetic pathway do not inhibit the growth of *Halobacteria*. An exception to this rule is bacitracin; the morphological changes which accompany growth inhibition and the nature of the components of the *Halobacteria* envelope suggest that the mode of action of bacitracin in inhibiting these bacteria may to some extent be similar to its mode of action in bacteria possessing peptidoglycan.

This investigation was supported by research grants from the U.S. Public Health Service (AM-13230) and from the National Science Foundation (GB-29747).

REFERENCES

- BEHRENS, N. H. (1974). Polyprenol sugars and glycoprotein synthesis. In *Biology and Chemistry of Eucaryotic Cell Surfaces*, pp. 159–180. Edited by E. Y. C. Lee and E. E. Smith. New York: Academic Press.
- CHO, K. Y., DOY, C. H. & MERCER, E. H. (1967). Ultrastructure of the obligate halophilic bacterium *Halobacterium halobium*. *Journal of Bacteriology* **94**, 196–201.
- GALE, E. F., CUNDLIFFE, E., REYNOLDS, P. E., RICHMOND, M. H. & WARING M. J. (1972). *The Molecular Basis of Antibiotic Action*, pp. 49–120. London: John Wiley.
- KATES, M., YENGOYAN, L. S. & SASTRY, P. S. (1965). A diether analog of phosphotidyl glycerophosphate in *Halobacterium cutirubrum*. *Biochimica et biophysica acta* **98**, 252–268.
- KONCEWICZ, M. A. (1972). Glycoproteins in the cell envelope of *Halobacterium halobium*. *Biochemical Journal* **128**, 124P.
- LARSEN, H. (1967). Biochemical aspects of extreme halophilism. *Advances in Microbial Physiology* **1**, 97–132.
- LINNETT, P. E. & STROMINGER, J. L. (1973). Additional antibiotic inhibitors of peptidoglycan synthesis. *Antimicrobial Agents and Chemotherapy* **4**, 231–236.
- LUGTENBERG, E. J. J., VAN SCHUNDEL-VAN DAM, A. & VAN BELLEGEM, T. H. M. (1971). *In vivo* and *in vitro* action of new antibiotics interfering with the utilization of *N*-acetyl-glucosamine-*N*-acetyl-muramyl-peptide. *Journal of Bacteriology* **108**, 20–29.
- MESCHER, M. F., STROMINGER, J. L. & WATSON, S. W. (1974). Protein and carbohydrate composition of the cell envelope of *Halobacterium salinarum*. *Journal of Bacteriology* **120**, 945–954.
- MOHR, V. & LARSEN, H. (1963). On the structural transformation and lysis of *Halobacterium salinarum* in hypotonic and isotonic solutions. *Journal of General Microbiology* **31**, 267–280.
- ROZE, U. & STROMINGER, J. L. (1963). The non-enzymatic reaction between D-cycloserine and pyridoxal phosphate. *Federation Proceedings* **22**, 465.
- SIEWERT, G. & STROMINGER, J. L. (1967). Bacitracin, an inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in biosynthesis of bacterial cell walls. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 767–773.
- STORM, D. R. & STROMINGER, J. L. (1973). Complex formation between bacitracin peptides and isoprenyl pyrophosphates. *Journal of Biological Chemistry* **248**, 3940–3945.
- STORM, D. R. & STROMINGER, J. L. (1974). Binding of bacitracin to cells and protoplasts of *Micrococcus lysodeikticus*. *Journal of Biological Chemistry* **249**, 1823–1827.
- STROMINGER, J. L. (1970). Penicillin-sensitive enzymatic reactions in bacterial cell wall synthesis. *Harvey Lectures* **64**, 179–213.
- WEINBERG, E. D. (1967). Bacitracin. In *Antibiotics*, vol. 1, pp. 90–101. Edited by D. Gottlieb and P. D. Shaw. New York: Springer-Verlag.