SHORT COMMUNICATION

Effect of Wall Antibiotics on the Growth of the Extremely Halophilic Coccus, *Sarcina marina* NCMB 778

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Growth of *Sarcina marina* was markedly inhibited by novobiocin and bacitracin A but unaffected by sodium-penicillin G, vancomycin or D-cycloserine. The antibiotic activity of these latter three compounds, however, decreased appreciably on incubation with the growth medium alone over a period comparable with that of the doubling time for this bacterium.

INTRODUCTION

The extremely halophilic bacteria are characterized by a number of unusual biochemical and physiological features which distinguish them from the majority of microbial species (Dundas, 1978; Brown, 1976). A striking characteristic exhibited by both genera – *Halobacterium* and *Halococcus* – classified as extreme halophiles, is the absence of muramic acid, and consequently peptidoglycan, from their cell walls (Brown & Shorey, 1963; Brown & Cho, 1970). The wall of the halobacteria consists predominantly of a high molecular weight glycoprotein (Mescher & Strominger, 1976) whilst that of the halococci is made up of an *O*-sulphated heteropolysaccharide (Steber & Schleifer, 1975), these components providing the structural integrity and strength normally provided by the peptidoglycan of other bacteria.

Chemical analysis of *Sarcina marina* walls (M. I. S. Hunter & S. J. W. Millar, unpublished observations) has shown the absence of muramic acid, a low content of ninhydrin-positive compounds (major components: glucosamine, galactosamine and glycine) and a predominance of neutral sugars (particularly glucose, galactose and mannose) in acid hydrolysates, in agreement with the observations of Steber & Schleifer (1975) and Reistad (1972, 1975) for other halococci.

In view of the absence of a conventional peptidoglycan from the walls of the extreme halophiles, it is perhaps not surprising to find that they are resistant to lysozyme (Antill, 1969) and to a variety of antibiotics whose prime targets are one or more of the reactions involved in the biosynthesis of peptidoglycan (Cho *et al.*, 1967; Mescher & Strominger, 1975). Mescher & Strominger (1975) demonstrated that a range of antibiotics known or thought to act at several different points in the pathway leading to peptidoglycan biosynthesis and assembly had no effect on three different species of halobacterium. They did demonstrate, however, that bacitracin (30 µg ml⁻¹) caused cessation of growth and a transformation from the normal rod shape to a spherical morphology. Bacitracin binds to the bactoprenol pyrophosphate carrier involved in peptidoglycan synthesis, inhibiting its conversion to bactoprenol monophosphate and thereby preventing further peptidoglycan

production (Storm & Strominger, 1973). Mescher & Strominger (1975) suggested that, in the halobacteria, bacitracin might inhibit growth and alter morphology by interfering with the biosynthesis of the wall glycoprotein which may also involve a C₅₅-isoprenyl pyrophosphate carrier. More recently, Basinger & Oliver (1979) have provided evidence that the primary effect of this antibiotic on halobacteria may be inhibition of the biosynthesis of the diether polar lipids of the membrane through binding to the postulated (Kates *et al.*, 1970) phytanyl pyrophosphate precursor of the phytanyl side-chains of these lipids.

As with many other aspects of the biochemistry of extremely halophilic bacteria, the antibiotic sensitivity of the halococci (as opposed to the halobacteria) has received scant attention. Since the structure and stability of the cell walls of the two genera are so markedly different (Brown, 1976; Mescher & Strominger, 1976; Steber & Schleifer, 1975), we decided to test the activity of some 'wall' antibiotics on the halococcus, *Sarcina marina*.

METHODS

Organism, medium and growth conditions. *Sarcina marina* NCMB 778 (from the National Collection of Marine Bacteria, Torry Research Station, Aberdeen) was grown at 34 °C, in an orbital shaker, in a medium of the following composition (all %, w/v): NaCl, 25 %; MgSO₄·7H₂O, 1 %; CaCl₂·6H₂O, 0.02 %; KCl, 0.5 %; yeast extract (Oxoid), 1 %; Tryptone (Oxoid), 0.5 %. Five ml of an exponentially growing culture was inoculated into 100 ml medium and growth was monitored turbidimetrically at 610 nm.

Addition and assay of antibiotics. Cultures were grown to mid-exponential phase (90 h) and then the following concentrations of antibiotics were added: bacitracin A (Sigma), 16, 64 and 256 µg ml⁻¹; D-cycloserine (Lilly), 16, 64 and 256 µg ml⁻¹; novobiocin (Sigma), 16, 64 and 256 µg ml⁻¹; sodium-penicillin G (Sigma), 256 µg ml⁻¹; vancomycin (Lilly), 256 µg ml⁻¹. Novobiocin and bacitracin were insoluble in the medium but did not precipitate if first dissolved in a small volume of sterile water. Growth was monitored, as described above, in these cultures and in control cultures to which no antibiotic was added.

In a separate experiment, the following final concentrations of antibiotics were added to cultures grown to the mid-exponential phase and to control flasks containing medium only: sodium-penicillin G, 60 µg ml⁻¹; vancomycin, 5 mg ml⁻¹; D-cycloserine, 100 µg ml⁻¹. Duplicate flasks were used. The antibiotic concentrations were determined at intervals using either an agar diffusion bioassay with *Staphylococcus aureus* (NCIB 8625) as the indicator micro-organism (Linton, 1958) for both penicillin and vancomycin or a colorimetric method (Jones, 1956) for cycloserine. Growth was estimated, as described above, in cultures with added antibiotics and in control cultures without antibiotics.

RESULTS

Effect of antibiotics on growth of S. marina

Penicillin G, vancomycin and D-cycloserine had no visible effect on growth, which was monitored for 100 h after addition of the antibiotics, by which time the cultures were well into stationary phase. Bacitracin and novobiocin, however, even at the lowest concentrations used (16 µg ml⁻¹), caused complete cessation of growth within 20 h (doubling time of *S. marina* under these growth conditions was 27.7 h).

Stability of antibiotics in growth medium

The apparent insensitivity of *S. marina* to some of the antibiotics prompted us to examine their stability in the medium, especially since the slow growth rate, common amongst the extreme halophiles and particularly the halococci, necessitated prolonged incubation of cultures. Assaying the antibiotic concentration and/or activity at intervals, we found that penicillin, cycloserine and vancomycin all lost appreciable activity in cultures and in control flasks containing medium alone, over a period of several hours (Table 1).

In both medium only and culture flasks, the activity of penicillin and vancomycin had fallen to approximately 50 % of the starting value by 44.5 h. A reproducible and curious observation was the apparently increased stability of cycloserine in the presence of bacteria although, even in controls, this antibiotic was more stable than the others, losing only 29 % of its activity after 44.5 h. As before, no inhibition of growth by the antibiotics was observed.

Table 1. *Stability of antibiotics in the presence of exponentially growing S. marina cultures and in the medium alone*

Antibiotics, at concentrations chosen to be convenient for assay (vancomycin, 5 mg ml⁻¹; sodium-penicillin G, 60 µg ml⁻¹; D-cycloserine, 100 µg ml⁻¹), were incubated at 34 °C either in medium alone or in the same medium containing a culture of *S. marina*, initially at mid-exponential phase. Antibiotic concentrations were measured either by agar diffusion bioassay (penicillin and vancomycin) or by colorimetry (cycloserine).

Time after addition of antibiotic (h)	Antibiotic content (% of original)			
	Medium alone and culture + vancomycin	Medium alone and culture + penicillin	Medium alone + cycloserine	Culture + cycloserine
0.0	100	100	100	100
1.0	—	94	—	—
20.5	68	63	88	95
44.5	54	*	71	88
67.0	50	*	64	85
93.0	—	*	55	78

* Below minimum range of assay but < 50 %; —, not assayed.

DISCUSSION

This paper is the first report dealing with the antibiotic sensitivity of a halococcus. The sensitivity of *S. marina* to bacitracin is in accord with the observations of others (Mescher & Strominger, 1975; Basinger & Oliver, 1979) on halobacteria, and sensitivity to this antibiotic may prove to be a common characteristic of the extreme halophiles.

The methanogens, like the extreme halophiles, also lack peptidoglycan and possess polyisoprenoid, ether-linked membrane lipids (Balch *et al.*, 1979). It has recently been shown (Hammes *et al.*, 1979) that several strains of methanobacteria are unaffected by wall antibiotics but are sensitive to bacitracin. These authors suggested that their observations support the involvement of isoprenoid lipid-linked intermediates in cell wall biosynthesis in the methanogens as proposed for the halobacteria (Mescher & Strominger, 1975). However, the work of Basinger & Oliver (1979) on halobacteria raises the possibility that a step in the biosynthesis of membrane lipids, and not of the cell wall, may also be the target for bacitracin action in the methanogens.

Since the structures of the cell walls of the halobacteria and halococci seem to be so different, our results lend support to the proposal (Basinger & Oliver, 1979) that bacitracin may primarily inhibit membrane lipid biosynthesis as both genera contain similar lipids (Kates *et al.*, 1966). Novobiocin was included in this study as it has been reported (Rogers & Perkins, 1968) to inhibit peptidoglycan biosynthesis. Recently, it has been suggested (Gellert *et al.*, 1976) that its primary target is DNA replication although, in the case of the extreme halophiles, the magnesium-binding properties of novobiocin (Franklin & Snow, 1975) may be a likely mode of action since almost all extreme halophiles exhibit a stringent magnesium requirement (Tindall *et al.*, 1980). Thus, it may be that neither antibiotic shown to be active in this work against *S. marina* affects cell wall biosynthesis in this organism.

The parallel inactivation of both penicillin and vancomycin in both cultures and controls would seem to indicate that the apparent resistance to these antibiotics is not the result of extracellular or intracellular inactivation by the bacteria themselves (e.g. penicillinase production). Moreover, the fact that the three antibiotics tested all exhibited loss of activity on incubation with the medium alone may suggest the necessity for a re-examination of the existing data relating to antibiotic resistance of extreme halophiles (Cho *et al.*, 1967; Mescher & Strominger, 1975). It would certainly seem that in work with such micro-

organisms, which exhibit protracted doubling times, extensive breakdown/inactivation of at least some antibiotics (by an unknown, non-enzymic mechanism) can occur, which should be checked for before the concentrations of antibiotics are quoted.

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