ELSEVIER

Journal of Biotechnology 70 (1999) 27–32

JOURNAL OF Biotechnology

Microbial antagonism: a neglected avenue of natural products research

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Received 19 November 1998; received in revised form 10 December 1998; accepted 22 December 1998

Abstract

Competition amongst microbes for space and nutrients in the marine environment is a powerful selective force which has led to the evolution of a variety of effective strategies for colonising and growing on surfaces. We are particularly interested in the chemical ecology of marine epibiotic bacteria which live on the surfaces of marine algae or invertebrates. Over 400 strains of surface-associated bacteria from various species of seaweed and invertebrate from Scottish coastal waters were isolated and 35% of them shown to produce antimicrobial compounds. This is a much higher proportion than free living marine isolates or soil bacteria. In addition, many strains which did not normally produce antibiotics could be induced to do so by exposing them to small amounts of live cells, supernatants from other bacterial cultures or other chemicals. Thus the number of strains able to produce antibiotics appears to be much higher than previously thought. Induction of antibiotic production was elicited by other marine epibionts and also by terrestrial human pathogens such as *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. An understanding of this type of chemical induction and the factors regulating non-constitutive secretion of antimicrobial compounds will allow more effective strategies for searching for new chemotherapeutic antibiotics to be designed.

Keywords: Marine epibiotic bacteria; Microbial antagonism; Antibiotic

1. Introduction

For the past 50 years antibiotics have revolutionised medicine by providing cures for formerly life-threatening diseases. However, strains of bacteria have recently emerged that are virtually unresponsive to antibiotics. Such multidrug resistance, arising mainly through antibiotic misuse, is now recognised as a global health problem. The situation is exacerbated by the fact that no novel chemical class of antibiotics have been discovered for 20 years. Although many pre-existing antibiotics have been modified to yield new derivatives, bacteria have the potential to mutate known resistance mechanisms to combat these (Hancock, 1997; Knowles, 1997; Levy, 1998). Par-

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0168-1656/99/$ - see front matter
PII: S0168-1656(99)00054-1

ticular strains which are causing problems at the moment are the vancomycin-resistant enterococci (VRE) (Frieden et al., 1993; Leclerc, 1997) and methicillin resistant *Staphylococcus aureus* (MRSA).

It is clear that new classes of antibiotics are urgently needed. Many marine free-living and sediment-inhabiting marine bacteria have been shown to produce secondary metabolites that display antibacterial properties (Burgess et al., 1991). The first antibiotic from a marine bacterium was identified and characterised in 1966 (Burkholder et al., 1966). In addition, bacteria in biofilms on the surfaces of marine organisms have been documented to contain a higher proportion of antibiotic producing bacteria than some other marine environments (Lemos et al., 1986). Marine epiphytic bacteria, associated with nutrient-rich algal surfaces and invertebrates, have also been shown to produce antibacterial secondary metabolites which inhibit the settlement of potential competitors (Jensen and Fenical, 1994; Bernan et al., 1997).

A number of surface-associated marine bacteria have also been found to produce antibiotics. Trischman et al. (1994) isolated a species of *Streptomyces* from the surface of a jellyfish, which produced two new bicyclic peptides (salinamides A and B). These compounds have novel backbones, and exhibit activity against an array of Gram positive organisms. Furthermore, a species of *Bacillus* isolated from a marine worm in Papua New Guinea produced a novel cyclic decapeptide antibiotic, loloatin B, which inhibited the growth of MRSA and VRE (Gerard et al., 1996).

A new bacterium *Pelagiobacter variabilis* from a seaweed *Pocockiella variegata* was found to produce new phenazine antibiotics, termed pelagiomicins which exhibited activity against Gram positive and Gram negative bacteria (Imamura et al., 1997). Recently, the marine bacterium *Alteromonas rava* sp. nov. was found to produce a new antibiotic thiomarinol (Shiozawa et al., 1993). Thus, marine epibiotic bacteria can provide novel antimicrobial compounds.

However, in order to find more novel structures, new ways of screening for these compounds must be applied. We have noticed in our studies that certain strains of bacteria can be induced to produce antibiotics (Mearns-Spragg et al., 1997, 1998). They appear to be doing so in response to chemical signals received from potential competitor strains which elicit an antagonistic response. Such a response, which is inducible rather than constitutive, has been observed in other species of bacteria (Patterson and Bolis, 1997) but remains a little studied phenomenon.

In this work we have screened marine epiphytic bacteria, isolated from the surfaces of seaweeds, starfish and nudibranchs for production of secondary metabolites with antimicrobial activities against multidrug resistant pathogens. We have also investigated the ability of live cells and various chemicals to elicit or enhance antibiotic production in these strains.

2. Materials and methods

2.1. Growth and culture conditions

Clinical isolates were grown on Columbia horse

Table 1
Zone(s) of inhibition against pathogenic bacteria, following antibiotic disc diffusion tests using extracts from epiphytic bacteria isolated from the brittle star

Marine strain	Test strain [zone of inhibition (mm)]			
	EMRSA	VRE	*Acinetobacter* spp.	*P. aeruginosa*
BS-3A	7	0	0	0
BS-4	9	0	0	0
BS-5A	0	7	0	0
BS-8B	8	0	0	0

Table 2
Inhibition of pathogenic bacteria, following antibiotic disc diffusion, using epiphytic bacteria isolated from the common starfish

Marine strain	Test strains [zone of inhibition (mm)]			
	EMRSA	VRE	*Acinetobacter* spp.	*P. aeruginosa*
SV-2	7	0	0	0
SV-6	0	0	0	7
SV-10	0	7	0	0
SV-11	8	0	0	0

blood agar, marine strains were grown on the following media; 50% Marine agar (MA) made with Marine Broth 2216 (Difco, Detroit). Samples of various seaweed and invertebrate species were collected at low tide. For isolation of epiphytic bacteria, the sample was washed three times in sterile seawater to remove loosely attached bacteria. The sample was then placed in another 10 ml of sterile seawater and vortexed to remove more tightly bound epiphytes. Cell suspensions obtained in this way were used to inoculate agar plates of 50% MA. Plates were then incubated (25°C, 5 days) and representative colonies were picked and isolated by successive restreaking.

2.2. Test strains

The following test strains were used; EMRSA (Epidemic MRSA strain) VRE, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Acinetobacter* spp. All test strains were isolated from hospitalised patients and were a kind gift from Edinburgh University Medical Microbiology laboratories. Each of the strains were maintained on Columbia horse blood agar medium at 37°C, and re-plated every 2 weeks.

2.3. Antibiotic assays

All bacterial strains isolated were screened for production of antibacterial substances, using the disc diffusion method, and methods for induction of antibiotic production as previously described with *Bacillus subtilis* used as the test strain in order to test additional Gram positive species (Mearns-Spragg et al., 1998). Out of all tested strains, five strains that showed good antimicrobial activity were selected for these induction studies.

3. Results

3.1. Antimicrobial activity of surface-associated strains

The marine strains MH22, isolated from the seaweed *Fucus vesiculosus*, and FS-25, FS-28 and FS-30, isolated from the seaweed *F. serratus*, displayed good antagonistic activity against VRE and *Enterococcus faecium*. However, only the MH22 strain displayed antibacterial activity against VRE and *P. aeruginosa*, following the disc diffusion test, with 8 mm zones of inhibition. Of the strains of bacteria isolated from the surface of the seaweed *Corallina officinalis*, only one strain, MBCoral-6A, displayed antibiotic activity, with an 8 mm zone of inhibition against the pathogen MRSA.

Strains isolated from the brittle star, *Ophiothrix fragilis* (Table 1), and the common starfish *Asterias rubens* (Table 2), displayed the greatest activity against an epidemic strain of methicillin resistant *S. aureus* (MRSA) and vancomycin resistant enterococcus (VRE). The largest zone of inhibition against MRSA (9 mm) was exhibited by the brittle star strain BS-4. The percentage of antibiotic producing strains from the seaweed, *C. officinalis*, the brittle star and the Common

starfish, was 4.8, 17.4 and 26.7%, respectively (Table 3). In addition to the strains described here, an additional 400 strains were screened and the overall hit rate for antimicrobial activity was 35% (results not shown). Despite this high overall hit rate, isolates from the sea slug, *Archidoris pseudoargus*, did not show any antimicrobial activity (Table 3).

3.2. Characterisation of producer strains

Strain MBCoral 6A grew as a yellow pigmented bacterium on 50% MA and was further characterised as a Gram positive coccus which formed chains. It is also catalase and oxidase positive. Strain BS-4 was a non-pigmented bacterium, forming white pinprick colonies on 50% MA. It was a Gram positive coccus, forming bunches, was catalase positive and oxidase negative. Strain BS-8B was also a non-pigmented bacterium identified as a Gram negative rod, which was oxidase and catalase negative.

3.3. Induction of antibiotic activity by culture supernatants.

Marine epibiotic bacteria enhanced their production of antibiotics, when exposed to bacterial cell-free supernatants. Four out of five strains tested, showed enhanced antimicrobial activity against at least one of the pathogenic organisms, when cell-free supernatants were introduced in the growth medium (data not shown). The induced activity against *B. subtilis* of two marine strains (MH46, a *Fucus* epiphyte, and SSE20, an isolate from the surface of the sponge *Halichondria* sp.) is shown in Fig. 1. Antibacterial activity against *B. subtilis* was enhanced in both strains. In the case of MH46, supernatants from the marine epiphytes MH3, MH2 and MH1 showed the greatest ability to elicit production of antimicrobial compounds. Supernatants from all three marine epiphytes also increased antibiotic production by strain SSE20.

MH46 did not show any antimicrobial activity under control growth conditions, but the addition of bacterial cell-free supernatants induced the production of antibacterial compounds by these strains. SSE20 was active against *B. subtilis* under normal conditions, however this antimicrobial activity was significantly enhanced when exposed to cell-free supernatants from the inducer strains. SSE20 showed enhanced antimicrobial activity against *B. subtilis* when challenged with *Bacillus* cell-free supernatant in their growth medium.

4. Discussion

In this work we have shown that bacteria associated with starfish and seaweed have the ability to produce antimicrobial compounds against other marine bacteria and also against terrestrial pathogenic bacteria. We have also demonstrated that addition of culture supernatants can elicit antibiotic production in marine surface-associated bacteria. It had previously been concluded that antibiotic-producing marine bacteria were often pigmented although this conclusion was reached after mainly testing of pigmented colonies (Lemos et al., 1986). Therefore, as the selection of colonies for testing was not carried out randomly. In this study a random selection of strains were tested, and the most potent antibiotic-producers found to be non-pigmented.

Table 3
Numbers and percentages of the bacterial strains, isolated from different organisms, that display antibiotic activity

Organism	No. of strains isolated	No. of antibiotic producers/strains isolated	% Antibiotic producing strains
A. pseudoargus (sea slug)	19	0/19	0
C. officinalis (seaweed)	21	1/21	4.8
O. fragilis (brittle star)	23	4/23	17.4
A. rubens (common starfish)	15	4/15	26.7

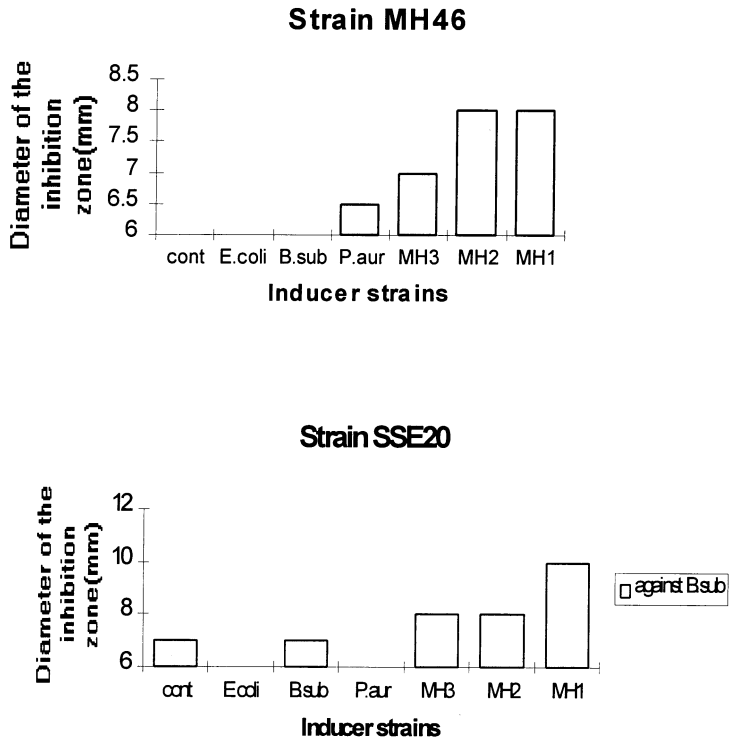

Fig. 1. Effect of cell-free supernatants on antibiotic production by marine strains MH46 and SSE20 after 3 days of incubation with extracts from other species of bacteria. The test organism is *B. subtilis*.

The standard filter paper (Whatman, 6 mm) used for the antibiotic disc assays is composed of cellulose (i.e. β(1–4) linked glucose monomers). The many free hydroxyl groups present on each of the glucose residues, renders the surface of the disc hydrophilic (Braithwaite and Smith, 1990). Thus, if the compounds produced by the marine bacteria were cationic, they would be expected to adsorb to the surface of the disc, and not diffuse into the medium. Consequently, a strain may produce antibiotic compounds that are polar in nature, but which are therefore not noticeably antibacterial by the paper disc diffusion method. Thus the use of different disc materials may allow additional compounds to be discovered.

Echinoderms, such as the brittle star and common starfish, are able to produce antifouling substances that prevent adhesion of micro-organisms, and hence formation of biofilms (McKenzie and Grigolava, 1996). Sub-cuticular bacteria (SCB), the bacteria living in the spaces between the cuticle and epidermis of Echinoderms, have also been isolated (McKenzie and Kelly, 1994). It is interesting to note in this work that some strains appear to be associated with the surfaces of echinoderms, despite the production of antifouling substances by their hosts. In addition to being resistant against these antifouling substances, these strains were shown to possess antimicrobial properties themselves.

In conclusion, in addition to showing that echinoderm and seaweed associated bacteria are able to produce antimicrobial compounds, we have confirmed earlier observations that bacteria can elicit antimicrobial responses in different species in a way which we do not yet understand. It is interesting to speculate that this response represents a chemically induced defence response when the antibiotic producer strain is faced with a potential competing organism in the marine envi-

ronment. Currently we are determining the chemical structure of these antimicrobial compounds.

Acknowledgements

This work was funded in part form a grant from the Natural Environment Research Council, CS was funded by a Wellcome Trust Vacation Scholarship. The authors also thank the British Council (Tokyo) for financial support. We also thank Professors Sebastian Amyes and John Govan of the Department of Medical Microbiology, University of Edinburgh, for provision of clinical isolates, support and advice.

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