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ORIGINAL PAPER

Seawater requirement for the production of lipoxazolidinones by marine actinomycete strain NPS8920

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Abstract A novel marine actinomycete strain NPS8920 produces a new class of 4-oxazolidinone antibiotics lipoxazolidinone A, B and C. Lipoxazolidinone A possesses good potency (1–2 μg/mL) against drug-resistant pathogens methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE). Strain NPS8920 exhibits different morphologies in both agar and submerged cultures. The ability of strain NPS8920 to sporulate on saline-based agar media but not on deionized water-based agar medium supported that strain NPS8920 is a marine actinomycete. While strain NPS8920 does not require seawater for growth, the production of lipoxazolidinones by strain NPS8920 can only be detected in the seawater-based media. The optimal production of lipoxazolidinones was observed in the natural seawater-based medium. Strain NPS8920 produced 10–20% of lipoxazolidinones in the synthetic sea salt Instant Ocean®-based medium and no production in the sodium chloride-based and deionized water-based media.

Keywords Lipoxazolidinones · Marine actinomycete · Antibacterial antibiotic · MRSA · VRE

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Introduction

There is a pressing need to find new sources of antibiotics to combat the drug-resistant pathogens. With the increasing incidences of VRE infections in hospitals [12, 16] and the spread of MRSA in the community [1, 16], where highly virulent strains infect children and young adults [8, 11, 14], we urgently need to find new antibiotics that are active against the above drug-resistant pathogens. The marine environment is a largely untapped source of chemical diversity [2, 7]. The opportunity to discover new microbial species, and therefore, novel chemistry, has increased through the exploration of the marine environment [3, 5, 6, 15]. Recently we reported the isolation of a series of new antibacterial 4-oxazolidinones from a marine actinomycete strain NPS8920 [10]. The most potent member of the chemotype, lipoxazolidinone A, possesses broad spectrum and good potency (1–5 μg/mL) against Gram-positive pathogens, including MRSA and VRE, similar to the commercial antibiotic linezolid [10]. The structures of lipoxazolidinones have some similarity to linezolid, a 2-oxazolidinone (Fig. 1).

The 16S rRNA sequence of strain NPS8920 does not match any of the sequences deposited in GenBank but closely matches those of the newly proposed genus *Marinispora* [6, 9]. In the present study, we demonstrate that marine actinomycete strain NPS8920 produces lipoxazolidinones in seawater-based media but not in deionized water-based or sodium chloride-based media even though the growth rate and yield of strain NPS8920 are similar in the above media. Since lipoxazolidinones are not halogenated metabolites, this finding might suggest that the production of lipoxazolidinones by strain NPS8920 has evolved ecologically from the marine environment.

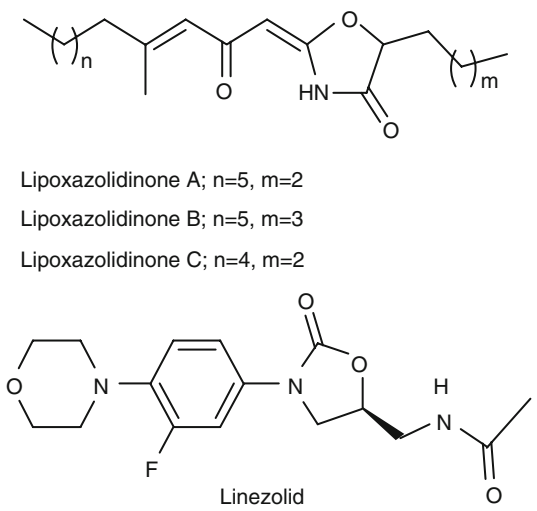

Fig. 1 Structures of lipoxazolidinones and linezolid

Materials and methods

Microorganism

The producing organism, strain NPS8920, was isolated from a sediment sample collected from Cocos Lagoon, Guam, in January 2002. Strain NPS8920 was deposited with the American Type Culture Collection and assigned the accession number PTA-6527. The close to full-length 16S rRNA sequence was submitted to GenBank and assigned the accession number EF470589.

The bacterial pathogens *Staphylococcus aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 43300 (MRSA), *Enterococcus faecalis* ATCC 29212 (VSE) and *Enterococcus faecium* ATCC 700221 (VRE) were obtained from American Type Culture Collection.

Media and culturing conditions

Frozen stock culture of strain NPS8920 was inoculated in 10 mL of seed medium in a 50-mL culture tube. The first seed culture was incubated for 4 days at 28°C on a rotary shaker operating at 250 rpm. The first seed culture of 5 mL was inoculated into 100 mL of the same seed medium in a 500-mL Erlenmeyer flask. The second seed culture was incubated for 2 days at 28°C and 250 rpm on a rotary shaker. 5 mL aliquots of the second seed culture were inoculated into 500-mL Erlenmeyer flasks containing 100 mL of production medium having the same composition as the seed medium.

Media used in this study have the same base composition of starch, 10 g/L; yeast extract, 4 g/L and peptone, 2 g/L. Media were dissolved in deionized water, 24 g/L NaCl solution, 30 g/L synthetic sea salt Instant Ocean® (Aquarium System, Mentor, OH) solution, and natural seawater and designated as A1-DI, A1-NC, A1-IO and A1-SW, respectively. Agar media have the same composition and designation as the liquid media with the addition of 17 g/L of agar as solidifying agent.

Growth analysis

The growth of the culture was determined by centrifuging 10 mL of culture in a 15-mL centrifuge tube at 3,000 rpm for 15 min in a Beckman centrifuge (Allegra model 6). The growth of the culture was expressed as percent packed cell volume defined as the volume of packed cell/volume of culture × 100%.

Extraction and analytical methods

The fermentation sample for high-pressure liquid chromatography (HPLC) analysis was prepared by extracting production culture (3.5 ml) with an equal volume of ethyl acetate for 1 h. A 1-ml aliquot of the extract was evaporated to dryness under a stream of nitrogen and redissolved in 75 μL of dimethyl sulfoxide. 25 μL of the extract was used for HPLC analysis. The amount of lipoxazolidinones in the extract was monitored by Agilent HP1100 HPLC using an ACE C-18 reversed-phase column (4.6 × 150 mm, MacMod, Chadds Ford, PA) and a solvent system consisting of acetonitrile (0.01% TFA) as solvent A and water (0.01% TFA) as solvent B. The elution profile was as follows: 15% solvent A for 1 min followed by a linear gradient to 100% solvent A in 15 min, held at 100% solvent A for 9 min at a flow rate of 1 ml/min with the detector wavelength set at 210 nm and column temperature at 30°C.

Antibiotic activity assay

The minimum inhibitory concentration (MIC) of lipoxazolidinone A was obtained against MSSA, MRSA, VSE and VRE (Table 1) using a conventional broth dilution assay in accordance with standards recommended by the National Committee for Clinical Laboratory Standards, and was described by Macherla et al. [10].

Table 1 Antibacterial activity of lipoxazolidinone A against MSSA, MRSA, VSE and VRE

Pathogen	MIC (μg/mL)
Staphylococcus aureus ATCC 29213 (MSSA)	2
Staphylococcus aureus ATCC 43300 (MRSA)	2
Enterococcus faecalis ATCC 29212 (VSE)	1
Enterococcus faecium ATCC 700221 (VRE)	2

Results and discussion

Strain NPS8920 exhibited different morphologies and sporulation properties on agar media

Strain NPS8920 exhibited different morphologies when plated on natural seawater-based A1-SW agar. Three distinct morphologies are shown in Fig. 2. The 16S rRNA sequences of the colonies from the three morphological variants were determined and found to have the same sequence, and matched exactly the sequence determined from the parent strain deposited in GenBank (accession number EF470589). Comparing the sequence of strain NPS8920 to those deposited in GenBank, strain NPS8920 has 99.98% sequence similarity to strain CNR252 with the proposed genus name of "*Marinispora*" [6, 9]. When plating each morphological variant on the A1-SW agar, all three variants reverted back to the original mixed morphologies appearance.

Strain NPS8920 also exhibited different sporulation properties on the same agar medium (Medium A1 with 17 g/L agar) prepared with different solutions of deionized water (A1-DI), NaCl (24 g/L, A1-NC), synthetic sea salt Instant Ocean® (30 g/L, A1-IO) and natural seawater (A1-SW). Strain NPS8920 grew well with a visually observed similar growth rate and yield on the above agar media. No sporulation was observed when strain NPS8920 was grown in agar medium A1-DI after 14 days of incubation at 28°C. A significant amount of spores was visually observed when strain NPS8920 was grown on agar media A1-NC, A1-IO and A1-SW after 7 days of incubation at 28°C. The production of spores by strain NPS8920 on agar media A1-NC, A1-IO and A1-SW was visually very similar. The growth rate of strain NPS8920 on A1-DI was faster than on A1-NC, A1-IO and A1-SW media while the growth yield was similar on all four agar media by visual observation. The ability of strain NPS8920 to sporulate only on the saline-type media to complete the development cycle on agar media indicated that strain NPS8920 is a marine actinomycete.

Fig. 2 Morphologies of marine actinomycete strain NPS8920 when grown on A1-SW agar

Production of lipoxazolidinones by strain NPS8920 growing in different media by shake flask cultures

The production of lipoxazolidinones by strain NPS8920 in shake flask cultures was compared in liquid media of A1-DI, A1-NC, A1-IO and A1-SW. There was no production of lipoxazolidinones by strain NPS8920 in A1-DI and A1-NC media. Good production of lipoxazolidinones by strain NPS8920 was detected in natural seawater-based A1-SW medium (Fig. 3). The maximal production of lipoxazolidinones was observed at day 3 in A1-SW medium with the peak titer of 115, 37.0 and 19.8 mg/L for lipoxazolidinone A, lipoxazolidinone B and lipoxazolidinone C, respectively. A significant amount (>50% of peak production) of lipoxazolidinones was detected in A1-SW medium as early as day 2. We detected a small amount of lipoxazolidinones by strain NPS8920 growing in synthetic sea salt-based A1-IO medium, ~10–20% of the production in the A1-SW medium. The maximal production of lipoxazolidinones was observed at day 2 in A1-IO medium with the peak titer of 21.9, 5.7 and 2.1 mg/L for lipoxazolidinone A, lipoxazolidinone B and lipoxazolidinone C, respectively (Fig. 3).

The production of lipoxazolidinones by strain NPS8920 growing in the seawater-based medium but not in the NaCl-

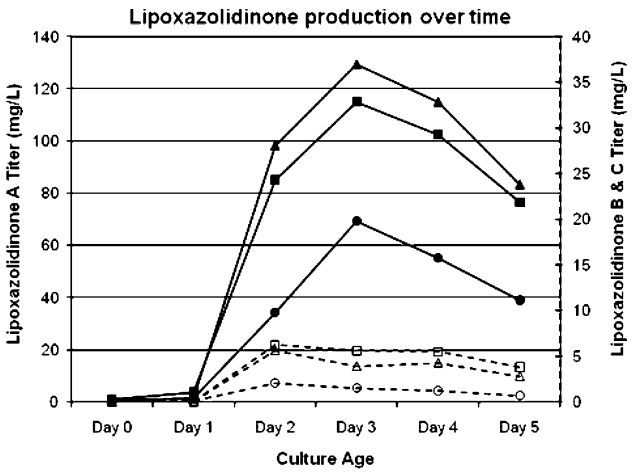

Fig. 3 Production of lipoxazolidinones A (*filled square*, *empty square*), B (*filled triangle*, *empty triangle*) and C (*filled circle*, *empty circle*) by marine actinomycete strain NPS8920 in media containing seawater (closed symbols) and Instant Ocean (open symbols)

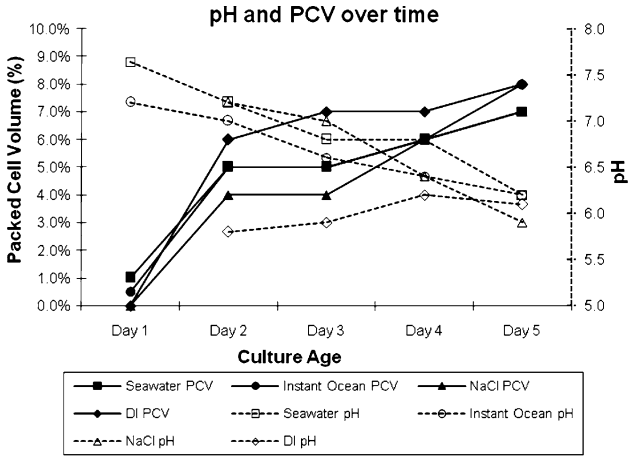

Fig. 4 The growth (packaged cell volume, closed symbols) and pH (open symbols) profiles of marine actinomycete strain NPS8920 in production cultures growing in media containing (a) deionized water (*filled diamond*, *empty diamond*), (b) NaCl (*filled triangle*, *empty triangle*), (c) Instant Ocean (*filled circle*, *empty circle*) and (d) seawater (*filled square*, *empty square*)

and deionized water-based medium is intriguing especially because the growth and pH profiles of strain NPS8920 were very similar in A1-NC, A1-IO and A1-SW media (Fig. 4). The growth rate and yield were slightly higher in the A1-DI medium than the other media but the pH of the A1-DI medium was around a full pH unit lower than that of the A1-SW medium. Even though strain NPS8920 can grow, sporulate and complete the development cycle on A1-NC agar medium, it was not able to produce lipoxazolidinones in the submerged culture growing in A1-NC medium. While NaCl can substitute for seawater in the agar culture to support the sporulation process, it cannot replace natural seawater to support the production of lipoxazolidinones in submerged culture. The synthetic sea salt Instant Ocean®, can mimic natural seawater to a certain extent and can support the production of a small amount of lipoxazolidinones in submerged culture.

Strain NPS8920 exhibited different morphologies in submerged cultures

The morphology of strain NPS8920 grown in A1-SW medium was significantly different from the morphologies grown in A1-DI, A1-NC and A1-IO media. Strain NPS8920 grown in A1-SW exhibited a whitish-gray color and smooth morphology due to fine mycelial growth. Strain NPS8920 grown in A1-DI and A1-NC exhibited a yellowish-brown color with a small pellet-type mycelial growth. In the A1-IO medium, strain NPS8920 exhibited a light yellowish-brown color with fine mycelial growth. Additional data are required to determine whether the production of lipoxazolidinones is related to the whitish-gray fine mycelial growth morphology exhibited by strain NPS8920.

Antibacterial activity (MIC) of lipoxazolidinone A against MRSA and VRE

The antibacterial profile of lipoxazolidinones has been reported by Macherla et al. [10]. Lipoxazolidinone A was the most potent antibiotic among the lipoxazolidinones tested with MIC of 1–5 μg/mL against Gram-positive pathogens, including drug-resistant pathogens such as MRSA and VRE. The antibacterial activity of lipoxazolidinone A against the drug-sensitive MSSA and drug-resistant MRSA and two *Enterococcus* strains VSE and VRE were determined with the MIC values reported in Table 1. Lipoxazolidinone A was active against the drug-sensitive and drug-resistant pathogens at the same concentrations of 1–2 μg/mL and the MIC is in good agreement with the MIC reported by Macherla et al. [10].

Conclusion

Because of the immense biological diversity in the sea, it is increasingly recognized that a large number of novel chemical entities exists in the oceans. Several recent review articles [4, 5, 6, 13] documented the importance of marine natural products as a source for new drug leads. The present study demonstrated another example of the unique characteristics of marine microorganisms. Marine actinomycete strain NPS8920 demonstrated unique morphologies and distinct sporulating patterns on agar cultures. More importantly, the production of a new antibacterial chemotype unprecedented in nature by strain NPS8920 further supports the tremendous therapeutic potential of the marine environment. While it is interesting to observe the seawater requirement by strain NPS8920 to support the production of lipoxazolidinones, the key to the future success of bioprospecting of marine microorganisms is to understand the molecular mechanism governing the seawater requirement for the production of marine natural products. Given the exponential growth in the microbial genomic database, the outlook for unraveling the molecular mechanism of seawater requirement for production of marine natural products is promising.

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