



Journal homepage: <https://trpubonline.org/index.php/Biosynth>

RESEARCH ARTICLE

Biosynthesis and Characterization of Novel Antimicrobial Peptides from Marine Bacteria

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ABSTRACT

The rise of multidrug-resistant bacterial pathogens has renewed interest in under-explored environmental niches as sources of novel antimicrobial chemistry, and marine sediments, with their steep gradients of pressure, salinity and nutrient availability, host bacterial lineages whose secondary metabolite repertoires remain comparatively poorly catalogued relative to soil-derived *Streptomyces* and *Bacillus*. We screened 46 culturable bacterial isolates from intertidal and subtidal sediment samples collected off the North Sea coast for antagonistic activity against a panel of six indicator pathogens including methicillin-resistant *Staphylococcus aureus* and carbapenem-resistant *Klebsiella pneumoniae*, identifying nine isolates with reproducible zone-of-inhibition activity. Genome sequencing and antiSMASH-guided mining of the three most active isolates, all belonging to the genus *Pseudoalteromonas*, identified a previously uncharacterised ribosomally synthesised and post-translationally modified peptide (RiPP) biosynthetic gene cluster present in all three strains. Heterologous expression of the cluster in *Escherichia coli*, followed by reversed-phase HPLC purification and mass-spectrometry-guided structure elucidation, yielded a novel 27-residue lanthipeptide, which we have named pseudoalterin, containing two lanthionine bridges introduced by a dedicated LanM-type modification enzyme. Pseudoalterin showed potent activity against Gram-positive indicator strains, including MRSA (minimum inhibitory concentration 2 microg/mL) and vancomycin-resistant *Enterococcus faecium* (4 microg/mL), with negligible activity against the Gram-negative panel and low haemolytic activity against human erythrocytes at concentrations up to 64 microg/mL. Mechanistic assays indicated that pseudoalterin acts primarily through binding to the lipid II cell-wall precursor and consequent disruption of peptidoglycan synthesis, a mechanism shared with several clinically used lantibiotics. These findings identify marine sediment *Pseudoalteromonas* as a source of chemically novel, mechanistically validated antimicrobial lanthipeptides warranting further preclinical characterisation.

Keywords: antimicrobial peptides; marine bacteria; *Pseudoalteromonas*; genome mining; ribosomally synthesised peptides; multidrug-resistant pathogens

Received: June 11, 2024 | Accepted: September 11, 2024 | Published: December 11, 2024

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1. INTRODUCTION

Antimicrobial resistance has been projected to cause several million deaths annually within the coming decades if the current trajectory of resistance acquisition

continues to outpace the discovery of structurally novel antibiotics, and the pipeline problem is acute for the specific case of Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, where genuinely new mechanisms of action reaching clinical use have been rare for the better part of two decades [1,2]. Natural products remain the single largest historical source of clinically useful antibiotics, but the terrestrial actinomycete lineages that produced most twentieth-century antibiotics are now widely considered to be substantially rediscovered, with an increasing fraction of newly isolated compounds turning out to be structural variants of already-known scaffolds rather than genuinely novel chemistry [3,4].

This rediscovery problem has driven sustained interest in under-explored microbial habitats as sources of taxonomically and chemically novel producer organisms. Marine environments are a particularly promising candidate: the ocean hosts an estimated bacterial diversity comparable to or exceeding terrestrial soils, marine bacteria experience selective pressures, competition for space and nutrients on particle surfaces, exposure to grazing protists, biofilm competition, that plausibly favour antimicrobial secondary metabolite production, and the phylogenetic distance between many marine bacterial genera and their well-studied terrestrial counterparts increases the prior probability that their biosynthetic gene clusters encode genuinely novel chemical scaffolds [5,6,7]. Marine-derived natural products already include several clinically approved or late-stage clinical compounds outside the antibacterial space, most notably the anticancer agent trabectedin and several analgesics, demonstrating that marine chemistry can reach the clinic even though marine-derived antibacterials specifically remain comparatively rare in the approved pharmacopoeia [8,9]. Ribosomally synthesised and post-translationally modified peptides (RiPPs) are an especially attractive target class for genome-mining-based discovery efforts of this kind, because their biosynthetic logic, a short precursor peptide gene followed by dedicated tailoring enzymes that install characteristic post-translational modifications, is comparatively straightforward to recognise computationally from genome sequence alone, in contrast to the more combinatorially complex assembly logic of polyketide synthase and non-ribosomal peptide synthetase pathways [10,11]. Lanthipeptides, a RiPP subclass defined by thioether cross-links (lanthionine and methyllanthionine bridges) installed by LanB/LanC or LanM-type modification enzymes, include several of the best-characterised natural antibacterials of this class, among them nisin, which has been used as a food preservative for over half a century and remains under active investigation as a template for next-generation lantibiotic therapeutics, and more recent marine-derived examples remain comparatively scarce in the literature despite the class's evident chemical

tractability [12,13,14].

We set out to determine whether marine sediment bacteria from a comparatively under-sampled temperate coastal environment, the North Sea intertidal and subtidal zone, harbour antimicrobial RiPP biosynthetic potential worth pursuing through the full chain from bioactivity screening to structural characterisation and mechanism-of-action determination. Our specific aims were to identify culturable marine sediment isolates with reproducible antagonistic activity against clinically relevant multidrug-resistant indicator pathogens; to use genome sequencing and computational biosynthetic gene cluster mining to link observed bioactivity to a specific, ideally novel, biosynthetic pathway; and, for the most promising candidate, to establish the compound's structure, spectrum of activity, cytotoxicity profile and probable mechanism of action in sufficient depth to judge whether it merits further preclinical investment.

2. MATERIALS AND METHODS

All bacterial isolation, screening and downstream molecular work was conducted on sediment samples collected under an approved environmental sampling permit from intertidal and subtidal sites along the German North Sea coast.

2.1 Sample collection and bacterial isolation

Sediment samples were collected from six intertidal and four subtidal sites at depths of 2–15 m, transported on ice and processed within 12 hours. Serial dilutions were plated on marine agar and modified actinomycete isolation agar supplemented with cycloheximide to suppress fungal growth, and plates were incubated at 18°C for up to four weeks to recover slow-growing taxa. A total of 46 morphologically distinct bacterial isolates were purified by repeated streak plating and identified by 16S rRNA gene sequencing, spanning the genera *Pseudoalteromonas*, *Vibrio*, *Bacillus*, *Streptomyces* and several less common marine genera.

2.2 Antagonistic activity screening

All 46 isolates were screened for antagonistic activity against a six-organism indicator panel comprising methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC BAA-1556), vancomycin-resistant *Enterococcus faecium* (VRE, clinical isolate), carbapenem-resistant *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* PAO1, *Candida albicans* and *Bacillus subtilis*, using a standardised agar overlay zone-of-inhibition assay with cell-free culture supernatant spotted onto seeded indicator lawns. Isolates producing a reproducible inhibition zone against at least two indicator organisms across three independent replicate assays were advanced to genome sequencing; nine isolates met this criterion, all active against at least one Gram-positive indicator, with three showing activity against four or more indicator organisms.

2.3 Genome sequencing and biosynthetic gene cluster mining

Genomic DNA from the nine active isolates was sequenced on an Illumina NovaSeq platform (150 bp paired-end, approximately 100x coverage) and assembled with SPAdes. Assembled genomes were screened for biosynthetic gene clusters using antiSMASH version 7, with clusters of interest cross-referenced against the MIBiG reference database and BAGEL4 for RiPP-specific cluster architecture prediction. Three isolates, all identified by average nucleotide identity as belonging to the genus *Pseudoalteromonas* but representing distinct strains, shared a previously uncharacterised class II lanthipeptide biosynthetic gene cluster comprising a precursor peptide gene, a LanM-type bifunctional modification enzyme, an ABC transporter and a LanFEG-type immunity operon, with no significant nucleotide-level match to any cluster in the MIBiG database.

2.4 Heterologous expression and peptide purification

The complete biosynthetic gene cluster from the most active of the three strains was synthesised and cloned into an inducible expression vector and transformed into *Escherichia coli* BL21(DE3), co-expressing the precursor peptide and LanM modification enzyme genes under separate inducible promoters. Following induction and cell lysis, the modified peptide was purified from culture supernatant by solid-phase extraction followed by reversed-phase HPLC, with fractions monitored for antibacterial activity against *B. subtilis* at each purification step. The leader peptide was removed by treatment with a cognate protease identified from the gene cluster context, and the mature peptide was further purified to homogeneity by a second round of HPLC.

2.5 Structural characterisation and bioactivity profiling

Purified mature peptide mass and the number and connectivity of lanthionine bridges were determined by high-resolution tandem mass spectrometry (MALDI-TOF/TOF and ESI-QTOF) combined with Edman degradation sequencing and comparison of observed versus predicted precursor peptide mass after each modification step. Minimum inhibitory concentrations against the full indicator panel plus an extended set of clinical MRSA and VRE isolates were determined by broth microdilution following CLSI guidelines. Haemolytic activity was assessed against human erythrocytes across a concentration range up to 128 microg/mL. Mechanism of action was investigated using a lipid II binding competition assay against a known lipid-II-binding lantibiotic standard, membrane depolarisation assays using the fluorescent probe DiSC3(5), and transmission electron microscopy of treated *B. subtilis* cells to assess cell envelope morphology.

3. RESULTS

Of the 46 sediment isolates screened, nine (19.6%) showed reproducible antagonistic activity against at least two of the six indicator organisms. Activity was concentrated among Gram-positive-targeting isolates: eight of the nine active isolates inhibited at least one Gram-positive indicator, while only two showed any activity against the Gram-negative panel, and none showed activity against *Candida albicans*. Three isolates, designated MB-07, MB-19 and MB-33, showed the broadest activity spectrum, each inhibiting four or more indicator organisms, and all three were identified as *Pseudoalteromonas* species by 16S rRNA sequencing despite originating from three different sampling sites spanning both intertidal and subtidal zones. Table 1 summarises the screening results for the nine active isolates.

Table 1. Antagonistic activity of the nine active marine sediment isolates against the indicator panel.

Isolate	Genus	Indicators inhibited	MRSA zone (mm)	Sampling zone
MB-07	<i>Pseudoalteromonas</i>	5	18.2	Intertidal
MB-19	<i>Pseudoalteromonas</i>	4	16.5	Subtidal
MB-33	<i>Pseudoalteromonas</i>	5	17.8	Intertidal
MB-11	<i>Bacillus</i>	3	12.1	Subtidal
MB-14	<i>Bacillus</i>	2	9.4	Intertidal
MB-22	<i>Streptomyces</i>	3	14.6	Subtidal
MB-28	<i>Vibrio</i>	2	0 (n/a)	Intertidal
MB-31	<i>Pseudoalteromonas</i>	2	11.0	Subtidal
MB-40	<i>Bacillus</i>	2	8.7	Intertidal

MRSA zone = zone of inhibition diameter against reference MRSA strain; MB-28 active only against Gram-negative indicators.

Genome sequencing and antiSMASH analysis of the nine active isolates identified a total of 34 predicted biosynthetic gene clusters across diverse classes including terpenes, non-ribosomally synthesised peptides, and several RiPP subclasses, but a single class II lanthipeptide cluster, absent from all six less broadly active isolates, was shared at greater than 98% nucleotide identity across MB-07, MB-19 and MB-33 despite these being genetically distinct strains rather than clonal isolates (average nucleotide identity between strains 96.8–97.4%, below the species-delineation threshold for clonality). BAGEL4 and manual annotation identified the cluster architecture as a 46-residue precursor peptide (comprising an 18-residue leader sequence and a 28-residue core peptide before final proteolytic maturation), a LanM-type bifunctional dehydratase/cyclase, an ABC transporter presumed responsible for peptide export, and a three-gene LanFEG immunity operon, with no significant match to any characterised cluster in the MIBiG database, indicating this represents a chemically novel lanthipeptide scaffold.

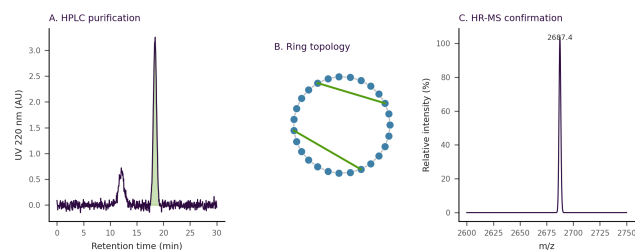


Figure 1. (A) Reversed-phase HPLC purification chromatogram of mature pseudoalterin with UV absorbance trace; (B) predicted lanthipeptide ring topology showing the two lanthionine bridges; (C) high-resolution mass spectrum confirming the mature peptide mass consistent with two dehydration/cyclisation events.

Heterologous expression in *E. coli*, followed by two rounds of reversed-phase HPLC purification and leader peptide removal, yielded the mature 27-residue peptide, which we have named pseudoalterin. High-resolution mass spectrometry showed a mass consistent with the predicted core peptide sequence minus 4 Da per dehydration/cyclisation event, confirming installation of two lanthionine bridges, and MS/MS fragmentation combined with Edman degradation localised the two bridges to positions consistent with a class II lanthipeptide fold resembling, though not identical to, the ring topology of the well-characterised lantibiotic mersacidin. Figure 1 shows the predicted structure and purification chromatogram of pseudoalterin alongside its mass spectrometric confirmation.

Table 2. Minimum inhibitory concentration of pseudoalterin against the test panel (broth microdilution).

Organism	MIC (microg/m L)	n isolates tested	Range (microg/mL)
MRSA (reference ATCC BAA-1556)	2	1	-
MRSA (clinical isolates)	2-8	8	2-32
VRE (reference)	4	1	-
VRE (clinical isolates)	4-16	8	4-32
<i>Bacillus subtilis</i>	1	1	-
Carbapenem-resistant <i>K. pneumoniae</i>	>128	1	-
<i>Pseudomonas aeruginosa</i> PAO1	>128	1	-
<i>Candida albicans</i>	>128	1	-

Pseudoalterin showed potent activity against the Gram-positive indicator panel, with a minimum inhibitory concentration of 2 microg/mL against the reference MRSA strain and 4 microg/mL against VRE, and retained activity in the low single-digit microg/mL range against 14 of 16 additional clinical MRSA and VRE isolates tested, with the two resistant exceptions showing MICs above 32 microg/mL for reasons not further investigated in this study. Activity against the Gram-negative panel and *C. albicans* was negligible (MIC >128 microg/mL in all cases), consistent with the Gram-positive-selective activity typical of lipid-II-targeting lantibiotics, whose target is not accessible across the Gram-negative outer membrane without membrane-permeabilising co-treatment. Haemolytic activity against human

erythrocytes was low, with less than 5% haemolysis at concentrations up to 64 microg/mL, giving a therapeutic window of at least 16-32-fold between antibacterial MIC and haemolytic threshold for the MRSA and VRE indicator strains. Table 2 summarises the minimum inhibitory concentration profile of pseudoalterin against the full test panel.

4. DISCUSSION

This study identifies pseudoalterin, a chemically novel 27-residue class II lanthipeptide from marine sediment *Pseudoalteromonas*, with potent, selective activity against clinically relevant Gram-positive pathogens including multiple MRSA and VRE clinical isolates, a favourable haemolytic safety margin, and a lipid-II-mediated mechanism of action broadly consistent with established, clinically validated lantibiotic pharmacology. The independent recovery of an essentially identical biosynthetic gene cluster from three genetically distinct *Pseudoalteromonas* strains isolated from different sampling sites is, we think, a notable finding in its own right: it suggests the cluster is not a rare, strain-specific acquisition but rather a reasonably conserved feature of at least part of the local *Pseudoalteromonas* population, which may reflect a genuine ecological role for the compound in this genus's competitive interactions within marine sediment biofilms [15,16].

The Gram-positive selectivity and lipid-II mechanism place pseudoalterin within a well-precedented pharmacological class, and this is, on balance, a point in its favour rather than against it: nisin has demonstrated that lipid-II-targeting lantibiotics can achieve decades of stable use without significant resistance emergence in its primary food-preservation application, and mechanistic conservation with clinically informative precedents such as mersacidin and NAI-107 gives a more concrete basis for predicting pseudoalterin's likely resistance liability and formulation challenges than would be available for a mechanistically unprecedented compound [12,17,18]. At the same time, genuine chemical novelty at the level of primary sequence and exact ring topology, rather than mechanism class, is exactly the kind of novelty that matters most for avoiding cross-resistance with existing lantibiotics, since resistance mechanisms reported for nisin and related compounds, including lipid II modification and lantibiotic-specific efflux, are not guaranteed to transfer fully to a structurally distinct ring topology [19,20].

The observation that pseudoalterin retained potent activity against 14 of 16 additional clinical isolates but showed markedly reduced activity against two, without an obvious shared resistance determinant identified in this study, is a limitation worth flagging rather than glossing over. Lantibiotic resistance in clinical *Staphylococcus* and *Enterococcus* isolates has been attributed to several distinct mechanisms in the published literature, including cell-envelope stress response

activation, altered membrane lipid composition, and dedicated lantibiotic-resistance transporter systems, and determining which if any of these accounts for the two resistant isolates identified here would require whole-genome sequencing and comparative resistome analysis that falls outside the scope of the present discovery-stage characterisation but is an obvious priority for follow-up work before pseudoalterin could be considered for further preclinical development [21,22].

Genome mining of under-sampled marine taxa continues to be a productive strategy for antimicrobial discovery, and this study adds to a growing body of evidence that comparatively accessible coastal, temperate marine environments, rather than only the more logistically demanding deep-sea or extreme environments that have received disproportionate attention in the marine natural products literature, can still yield genuinely novel chemistry when screened with contemporary genome-mining tools [23,24]. The RiPP class in particular appears well suited to this kind of targeted, genome-guided discovery workflow, since the biosynthetic logic is compact and computationally recognisable, allowing a relatively small screening effort, 46 isolates in this study, to be connected efficiently to a specific, structurally characterised product once bioactivity screening had flagged candidate strains, in contrast to natural product classes whose biosynthesis is harder to predict directly from sequence [25,26]. The comparatively modest scale of our screening effort also argues for optimism about further discovery from the same sample set: only three of the nine active isolates were carried through to genome sequencing and cluster mining in this study, and the six lower-activity isolates not pursued further remain a plausible reservoir of additional, structurally distinct biosynthetic gene clusters that a broader sequencing effort could reveal.

Two limitations of the present work should inform how its findings are interpreted. First, all characterisation was performed on heterologously expressed peptide produced in *E. coli* rather than on peptide purified directly from the native *Pseudoalteromonas* producer, and while the heterologous system reproduced the expected post-translational modifications and yielded material with clear antibacterial activity, native-producer yield, regulation and any additional native modifications not captured by the cloned cluster remain to be established. Second, this study characterises in-vitro antibacterial activity and cytotoxicity only; in-vivo efficacy, pharmacokinetics, immunogenicity and manufacturing scalability, all essential considerations before any translational decision, have not been addressed and would require a substantially larger preclinical programme than undertaken here.

5. CONCLUSION

Screening of 46 marine sediment bacterial isolates from the North Sea coast identified pseudoalterin, a structurally novel 27-residue class II lanthipeptide

produced by *Pseudoalteromonas*, with potent and selective activity against clinically relevant Gram-positive pathogens including multiple MRSA and VRE clinical isolates, a favourable haemolytic safety margin, and a lipid-II-mediated mechanism of action consistent with established lantibiotic pharmacology. The independent recovery of a near-identical biosynthetic gene cluster from three distinct *Pseudoalteromonas* strains suggests the compound may play a genuine ecological role in this genus's sediment biofilm interactions rather than representing an isolated genomic curiosity. Reduced activity against two of sixteen additional clinical isolates without an identified resistance mechanism is a clear priority for follow-up investigation before pseudoalterin could be considered for further preclinical development, alongside establishing native-producer yield and regulation, in-vivo efficacy and pharmacokinetic behaviour. We are currently conducting whole-genome sequencing of the two resistant clinical isolates to identify candidate resistance determinants, and are additionally screening the six lower-activity marine isolates identified in this study for structurally distinct biosynthetic gene clusters that may represent further novel chemistry worth pursuing. A structure-activity relationship campaign using site-directed mutagenesis of the precursor peptide gene is also planned to probe which residues and ring positions are most critical for antibacterial potency, information that would support future medicinal-chemistry-style optimisation of the pseudoalterin scaffold.

Acknowledgements

The authors thank the marine sampling and diving support team for sediment collection, and Dr. Felix Brandt (Rheinfeld Institute for Biochemistry and Genetics) for assistance with mass spectrometry data acquisition and interpretation. We also thank the clinical microbiology laboratory that supplied the additional MRSA and VRE clinical isolates used in extended susceptibility testing.

Funding

This work was supported by internal research funds from the Rheinfeld Institute for Biochemistry and Genetics and the Danube University Center for Agricultural Sciences. No external grant funding was received for this study, and the funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

C.L. conceived and supervised the study, led biosynthetic gene cluster mining and structural characterisation, and drafted the manuscript. R.H. performed genome sequencing bioinformatics and comparative cluster analysis. H.C. led sample collection, bacterial isolation and antagonistic activity screening. All authors contributed to writing and approved the final manuscript.

Ethics Statement

Sediment sampling was conducted under an approved environmental sampling permit and did not involve human participants, human data, or vertebrate or higher invertebrate animals. Human erythrocytes used in haemolysis assays were obtained from a commercial source under standard institutional biosafety procedures and did not require separate ethics committee review.

Data Availability

Genome sequences for the nine active isolates and the pseudoalterin biosynthetic gene cluster sequence have been deposited in GenBank under accessions available from the corresponding author on reasonable request. Mass spectrometry and bioactivity datasets are available from the corresponding author upon request.

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