

NOTE

Mode of action and structure–activity relationship studies of geobacillin I

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Lanthipeptides are lanthionine- and methylanthionine-containing peptides that are ribosomally synthesized and post-translationally modified.¹ Lanthipeptides that possess antimicrobial activity are called lantibiotics.² Lanthionines consist of two alanine residues that are linked through a thioether that connects their β -carbons, and methylanthionines contain an additional methyl group (Figure 1a). Nisin is the best studied and longest known lantibiotic and has been used as a food preservative for over 50 years.^{3,4} Nisin displays antibacterial activity against clinically important pathogens such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant Enterococci, *Streptococcus pneumoniae*, and food-borne pathogens such as *Clostridium botulinum*, and *Listeria monocytogenes*.^{5–7} Despite its use for over 50 years, reports of resistance against nisin have been scarce.^{8–12} The slow development of resistance may stem from the dual mode of action of nisin. Nisin exhibits antimicrobial activity by binding to the pyrophosphate moiety of lipid II (Figure 1b),^{13,14} a membrane-bound advanced intermediate involved in the biosynthesis of the cell wall. By doing so, nisin inhibits the transglycosylation step in cell wall biogenesis and sequesters lipid II from its functional location.^{15,16} Furthermore, the nisin–lipid II complex leads to the formation of pores in the membrane, causing cell death.¹⁷

Recently, we characterized two lanthipeptides, geobacillin I and geobacillin II, from the thermophilic bacterium *Geobacillus thermodenitrificans* NG80-2.¹⁸ Geobacillin I contains seven thioether bridges, one dehydroalanine (Dha) and one dehydrobutyrine (Figure 1c). The N-terminal A and B rings of geobacillin I are very similar to the corresponding rings of nisin; however, the C-terminal structures are very different (Figure 1c). The nisin A and B rings are involved in lipid II binding,¹³ and hence we anticipated that geobacillin I might also bind lipid II. The three amino-acid linker peptide between the C

and D rings of nisin has been shown to be indispensable for pore formation activity.^{17,19–21} For instance, the Δ N20 Δ M21 and N20P/M21P mutants of nisin lost pore formation ability against Gram-positive bacteria.^{17,19} Geobacillin I has only a single amino acid between the C and D rings, similar to the Δ N20 Δ M21 mutant of nisin. Thus, based on the available data on nisin, we anticipated that geobacillin I would bind to lipid II but not form pores in the membrane of Gram-positive bacteria. In this work, we tested these expectations experimentally.

We first conducted antimicrobial activity assays in liquid medium. In these assays, geobacillin I exhibited a fourfold higher MIC against *Bacillus subtilis* ATCC 6633 compared with nisin (Table 1). Flow cytometry was then used to examine changes in the polarization of the bacterial membrane of *B. subtilis* ATCC 6633 upon incubation with geobacillin I using the membrane potential-sensitive dye 3,3'-diethyloxacarbocyanine iodide.²² Incubation with geobacillin I resulted in a significant decrease in the mean fluorescence intensity, similar to the observations when the same experiments were carried out with nisin (Figure 2a and Supplementary Figures S1 and S2). The unexpected ability of geobacillin I to form pores despite the single amino-acid linker between rings C and D may be a consequence of the overall differences between the C-terminal region of geobacillin I and nisin (Figure 1c).

We also investigated the efficiency of pore formation by geobacillin I using propidium iodide (PI)—a membrane impermeable fluorescent dye. Upon pore formation or membrane disruption, PI can enter the cell, resulting in an increase in fluorescence intensity because of the interaction of PI with nucleic acids. PI uptake was monitored at nine different concentrations with each experiment conducted in triplicate (Figure 2b and Supplementary Figure S3). The data showed only twofold lower efficiency in PI uptake for geobacillin I, with IC₅₀

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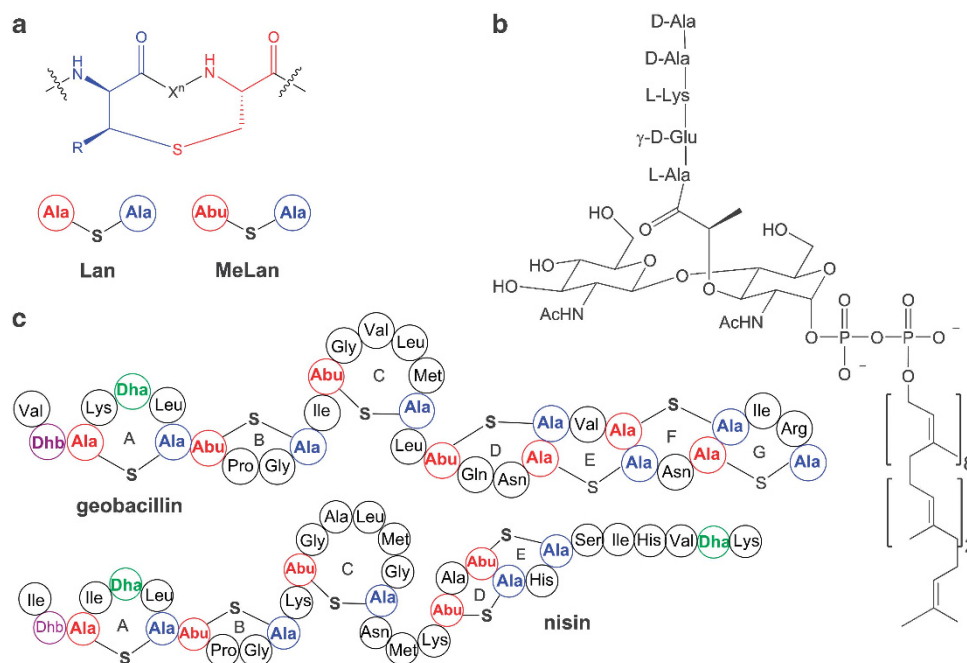


Figure 1 Structures of (a) lanthionine and methylanthionine, (b) lipid II, and (c) geobacillin I and nisin. The shorthand notation for lanthionine (Lan) and methylanthionine (MeLan) depicted in a is used in c.

Table 1 Specific activity of nisin, geobacillin I and the geobacillin I analogs generated in this study against *B. subtilis* ATCC 6633

Peptide	IC ₅₀ (μM)	MIC (μM)
Nisin	0.12 ± 0.01	0.25
Geobacillin I	0.56 ± 0.01	1.0
Geobacillin-L19P	1.00 ± 0.03	2.0
Geobacillin-L19NVA	5.70 ± 0.08	8.0
Geobacillin-Dha5F/L6I	0.45 ± 0.09	1.0

Abbreviation: Dha, dehydroalanine.

values for nisin at 0.3 μM compared with 0.6 μM for geobacillin I (Figure 2b and Supplementary Figure S3).

Previously, site-saturation mutagenesis was performed on the amino acids in the linker between the C and D rings of nisin. The antibiotic activities of the nisin mutants N20P, M21V, K22T and K21A were increased against several pathogenic bacteria.²³ Conversely, introduction of a double Pro in this region (N20P/M21P) strongly decreased pore formation;¹⁷ this double mutant as well as the single M21P mutant also had strongly reduced antimicrobial activities.^{17,23} To evaluate how such mutations would affect the activity of geobacillin I, site-directed mutagenesis was used in this study to replace the naturally occurring Leu19 in geobacillin I with Pro and to introduce the tripeptide AsnValAla as linker between the C and D rings, thus generating a linker sequence that combines two of the mutations in the nisin variants with improved activity. These analogs were generated by co-expression of mutants of the precursor peptide GeoAI with the modification enzymes GeoB and GeoC in *Escherichia coli* as previously reported for the production of wild-type geobacillin I (Supplementary Figure S4; Tables S1 and S2).¹⁸ Compared with wild-type geobacillin I, the analogs with NVA and P as the linker between the C and D rings had eightfold and twofold increased MIC values, respectively (Table 1). The ability to induce pore formation by these

analog was also investigated. Although the efficiency of pore formation was strongly reduced, replacement of Leu19 with Pro did not abolish this activity (Figure 2b). Introduction of the amino-acid residues NVA in this region also greatly reduced the formation of pores in the bacterial cell membrane by geobacillin I. Thus, mutations in the linker peptide between the C and D rings affect the activities of nisin and geobacillin I quite differently, suggesting that the detailed mechanism of pore formation by geobacillin I differs from that of nisin. These findings also suggest that the structure of the C-terminus of class I lantibiotics may vary significantly while retaining pore formation activity.

The ability of geobacillin I to bind lipid II was investigated next using *in vitro* inhibition of the transglycosylation reaction catalyzed by penicillin-binding protein 1b from *E. coli*. Penicillin-binding protein 1b uses lipid II as a substrate for glycan polymerization.²⁴ Geobacillin I inhibited Penicillin-binding protein 1b-catalyzed peptidoglycan formation using 4 μM heptaprenyl lipid II with a half-maximal inhibitory concentration (IC₅₀) of 4.6 ± 0.8 μM (Figure 2c). For comparison, inhibition by nisin under the same conditions displayed an IC₅₀ of 2.9 ± 0.6 μM (Figure 2c). Thus, the inhibitory activity of the two peptides is very similar.

Geobacillin I has higher stability at physiological pH compared with nisin.¹⁸ Although higher stability for a compound from a thermophile is not unexpected, the higher stability was somewhat surprising because nisin degradation at neutral pH is believed to be caused by non-enzymatic hydrolysis at Dha5,²⁵ a residue that is also present in geobacillin I. We wondered whether the stability and hence antimicrobial activity of geobacillin I could be further improved by mutation of Dha5 in light of a previous report that the nisin analog I4K/Dha5F/L6I had higher antimicrobial activity against various bacteria.²⁶ Geobacillin I already has a Lys at position 4, and hence the mutant Dha5F/L6I was generated; however, it proved to be only slightly more stable than the wild-type geobacillin I (Supplementary Figure S4) while displaying similar MIC values (Table 1).

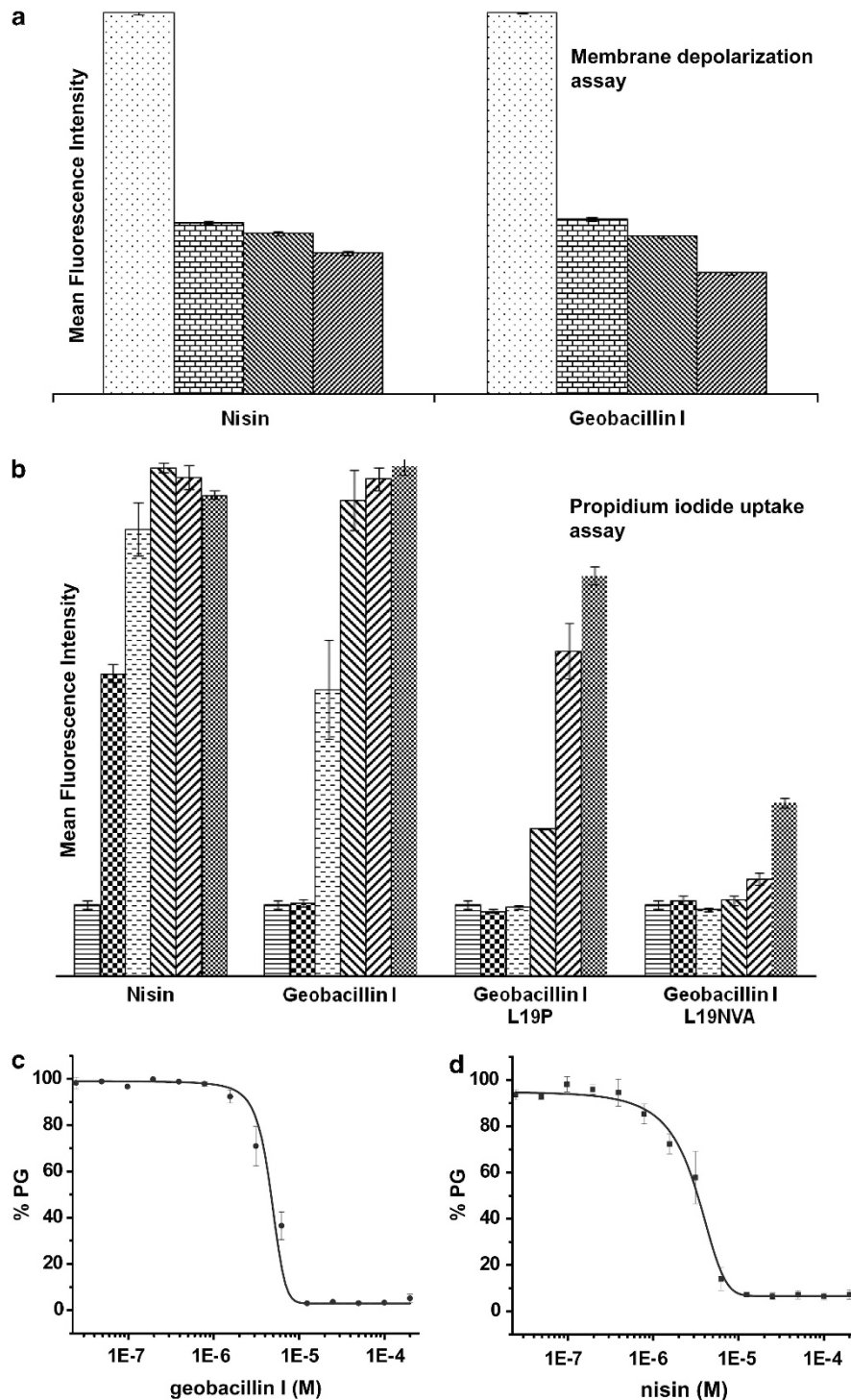


Figure 2 The effect of geobacillin I and nisin on the membrane integrity of *B. subtilis* ATCC 6633. (a) Average mean fluorescence intensity (MFI) of triplicate flow cytometry measurements with different concentrations of nisin and geobacillin I using 3,3'-diethyloxycarbocyanine iodide as the indicator of membrane potential. The different bars represent concentrations of 0, 0.2, 2 and 20 μM (left to right). For a representation using the ratio of fluorescence at 610 and 530nm, see Supplementary Figure S2. (b) Increase in MFI resulting from propidium iodide uptake by *B. subtilis* ATCC 6633 in response to treatment with nisin and geobacillin I (average of three measurements). The different bars represent concentrations of 0, 0.31, 1.25, 2.5, 5 and 10 μM (left to right). (c) Inhibition of penicillin-binding protein 1b (PBP1b)-catalyzed peptidoglycan (PG) formation by geobacillin I and by nisin (d) at a lipid II concentration of 4 μM and a PBP1b concentration of 100 μM . Error bars represent the s.d. from triplicate experiments.

Compared with nisin, ring C of geobacillin I is reduced in size by one amino acid, the region between rings C and D is reduced in length by two amino acids and ring E is a lanthionine ring as opposed

to a methylanthionine ring (Figure 1c). Furthermore, geobacillin I contains two additional thioether bridges at its C-terminus. However, the antimicrobial activity and the overall mode of action of the two

lantibiotics appear to be quite similar: binding to lipid II and pore formation.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)