ORIGINAL ARTICLE

Microbial degradation of cyclic peptides produced by bacteria

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Bacterial strain, B-9, isolated from Lake Tsukui, Japan, and characterized as genus *Sphingosinicella* sp., possesses hydrolytic enzymes capable of degrading various toxic and non-toxic cyanobacterial cyclic peptides, such as microcystins, nodularin, microviridin, microcyclamide and aeruginopeptin. In this study, the degradation activities of the cell extract of B-9 against bacterial cyclic peptides, bacitracin, colistin, polymyxin, mikamycin, thiopeptin and WAP-8294A2, were investigated and the degradation products were analyzed using HPLC and liquid chromatography/ion trap tandem mass spectrometry (LC/ITMS). As a result of extensive experiments, it was confirmed that B-9 could also degrade these bacterial cyclic peptides by hydrolysis of their peptide or ester bonds, except for WAP-8294A2. These results indicated that the functions of the bacterium with its enzymes were further extended and offered the possibility of degrading other types of compounds.

The Journal of Antibiotics (2009) 62, 181–190; doi:10.1038/ja.2009.8; published online 13 February 2009

Keywords: B-9 strain; biodegradation; cyclic peptide antibiotics; microcystin

INTRODUCTION

In the environment, there are many bacteria with degradation activities against hazardous and harmful compounds. Microcystins (MCs) are such typical compounds produced by cyanobacteria, such as *Microcystis*, *Anabaena* and *Planktothrix*, because they are cyclic heptapeptides showing a potent hepatotoxicity and tumor promoting activity.¹ The strain, B-9, was isolated from Lake Tsukui, Japan as an MC-degrading bacterium in 1997.² This strain showed a promising potential for the degradation of MC-related compounds and nodularin.³ Such an MC-degrading bacterium was first isolated in Australia and was identified as one of the *Sphingomonas* strains (ACM-3962) in 1994.⁴ Phenotypically, similar bacteria capable of degrading MC have been reported subsequently all over the world.^{5–10} B-9 is 99% similar to the *Sphingosinicella microcystinivorans* strain, Y2, one of these MC-degrading bacteria, based on the 16S rDNA sequence (GenBank accession no. AB084247).^{2,11} In a pilot-scale study, the ACM-3962 effectively degraded the microcystin-LR (MCLR) during slow sand-filtration,¹² and a feasible bioreactor, using a B-9 strain immobilized in polyester resin, rapidly removed MC from the lake water.²

In an earlier study, we applied B-9 to various cyclic peptides, such as aeruginopeptin, nostophycin, microcyclamide and microviridin, derived from cyanobacteria.¹³ As a result of the investigation, it was confirmed that the enzymes included in B-9 could also hydrolyze peptide bonds of several cyanobacterial cyclic peptides that were structurally different from the MCs and nodularin. In addition, the use of the cell extract suggested that all MC-degrading bacteria, including B-9, inherently possessed these capabilities. Although the

role of these enzymes for bacteria has not been verified completely, the abilities of these enzymes from microorganisms existing in the natural environment may contribute to environmental self-purification.

A molecular study of the *Sphingomonas* strain, ACM-3962, revealed the presence of three hydrolytic enzymes involved in the degradation. Microcystinase (MlrA) catalyzes the initial ring opening of MCLR at a unique β -amino acid, (2S,3S,8S,9S)-3-amino-9-methoxy-2,4,6-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Adda)-Arg peptide bond to give the linearized MCLR, which is further degraded to a tetrapeptide by MlrB. The third enzyme, MlrC, hydrolyzes the tetrapeptide into smaller peptides and amino acids. The putative protein, MlrD, provided the transport of MCLR and its degradation products across the bacterial cell wall.^{14,15} The effective use of an appropriate protease inhibitor could accumulate the following degradation intermediates: the use of EDTA (ethylenediaminetetraacetic acid) accumulated the linearized MCLR (Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg) and tetrapeptide (Adda-Glu-Mdha-Ala), which allowed the MlrA and MlrC to be classified as two metalloproteases, whereas the use of 1,10-phenanthroline characterized MlrB as a possible serine protease.^{14,15} Furthermore, the intact Adda was first isolated from MCLR by degradation using a B-9 strain as one of the final products.¹⁶

As mentioned above, cyanobacteria produce a wealth of bioactive peptide derivatives with a broad range of biological activities and pharmacological properties.¹⁷ Many of these are synthesized on non-ribosomal peptide synthetases (NRPSs).^{18–21} Bacteria and fungi also produce structurally characteristic peptides by the NRPS pathway, in which they have various bioactivities, such as anti-microbial and

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Received 9 October 2008; accepted 21 January 2009; published online 13 February 2009

immunosuppressive activities.^{21–23} The cyclic peptide is one of the typical products by way of the NRPS pathway and is usually stable against various hydrolytic enzymes, such as trypsin, because of its cyclic structure and unusual constituent amino acids.^{4,24} Therefore, it is laborious to reproducibly obtain partially hydrolyzed products in the usual manner. This study focused on the further hydrolytic activity and universality of B-9, therefore; it was applied to representative bacterial cyclic peptides.

MATERIALS AND METHODS

Reagents

The Tris-Cl buffer (pH 7.6), dithiothreitol, HPLC grade methanol and formic acid for column chromatography were purchased from Nacalai Tesque (Kyoto, Japan). The distilled water used for the HPLC and LC/MS mobile phases was purchased from Wako Pure Chemicals (Osaka, Japan).

Bacterial cyclic peptides

Bacitracin, mikamycin, thiopeptin and polymyxin were kindly provided by Dr Ikai (Aichi Prefectural Institute of Public Health, Japan). Colistin was purchased from Wako Pure Chemicals (Osaka, Japan). WAP-8294A2 was provided by Wakamoto Pharmaceutical Co., Ltd. (Tokyo, Japan).

Cell extract of MC-degrading bacterium

The bacterial strain, *Sphingococcinella* B-9, isolated from the surface water of Lake Tsukui, Kanagawa, Japan,² was inoculated in a flask containing 100 ml of Sakurai medium composed of 0.2% peptone, 0.1% yeast extract and 0.05% glucose² and was incubated at 27 °C and 200 r.p.m. for 3 days. After centrifuging at 3000×g for 30 min at 20 °C, the cells were harvested as a pellet and then re-suspended in 20 ml of 50 mM Tris-Cl buffer (pH 7.6) containing 0.5 mM dithiothreitol. The suspension was next extracted using a French pressure press at 10000 psi and harvested as the cell extract. The absence of resting cells in the cell extract was determined using a microscope.

Degradation of cyclic peptides

Fifty micrograms of the cyclic peptides, bacitracin-A, mikamycin, thiopeptin, colistin, polymyxin and WAP-8294A2 was dissolved in water at a concentration of 1.0 mg ml⁻¹, added to 500 µl of the cell extracts of B-9 and incubated at 27 °C for 24, 48 and 96 h. In addition, the bacitracin, polymyxin and colistin were monitored at 2, 6 and 12 h. MCLR was used as a positive control to confirm the activity of B-9. After incubation, 50 µl of these mixtures was added to 50 µl of methanol containing 0.2% formic acid and centrifuged at 15 000×g for 10 min at 20 °C to stop the degradation and to eliminate the proteins. Next, each supernatant was analyzed using HPLC and liquid chromatography/ion trap tandem mass spectrometry (LC/ITMS).

HPLC

The cyclic peptides were analyzed using a high-performance liquid chromatograph equipped with a photodiode array detector. The system consisted of two liquid chromatography (LC) 10A VP pumps, a DGU 12A degasser, a CTO 6A column oven, an SPD 10A VP photodiode array detector and an SCL 10A VP system controller (Shimadzu, Kyoto, Japan). Five microliters of the sample was filtered using an Ultrafree-MC membrane centrifuge filtration unit (hydrophilic PTFE, 0.20 µm, Millipore, Bedford, MA, USA) and loaded onto a TSK-gel ODS-80Ts column (5 µm, 2.0×150 mm, TOSOH, Tokyo, Japan) at 40 °C. The mobile phase was water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The flow rate was 200 µl min⁻¹ and the gradient conditions were initially 40–90% B at 20 min.

LC/ITMS

The cyclic peptides were analyzed using LC/ITMS. The LC separation was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The sample preparation, column, mobile phase and gradient conditions were the same as those in the HPLC analysis. The entire eluent was directed into the mass spectrometer, in which it was diverted to waste at

2.5 min after injection to avoid any introduction of salts into the ion source. The MS analysis was accomplished using a Finnigan LCQ Deca XP plus ITMS (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface. The ESI conditions in the positive ion mode were as follows: capillary temperature, 300 °C; sheath gas flow rate, 35 (arbitrary unit); ESI source voltage, 5000 V; capillary voltage, 43 V; and tube lens offset, 15 V·MSⁿ ($n=1–4$). Various scan ranges were used according to the molecular weights of the analytes. The MS³ and MS⁴ spectra were acquired, if possible and if necessary, for the structural analysis study of the degradation products.

RESULTS

Cyclic peptides selected

The bacterial cyclic peptides used in this study are classified structurally into the following two groups: cyclic oligopeptides and cyclic depsipeptides. The former includes bacitracin, colistin and polymyxin, whereas mikamycin, thiopeptin and WAP-8294A2 are included in the latter group, as shown in Figure 1. Bacitracin-A (**1**) is the main component of a mixture of the cyclic peptides containing a dihydrothiazole ring and was used after purification by high-speed countercurrent chromatography.²⁵ Colistins A (**2A**) and B (**2B**) and polymyxins B1 (**3B1**) and B2 (**3B2**) were used as mixtures.^{26–29} A sample containing mikamycins A (**4A**) and B (**4B**), which possess characteristic structures with a different skeleton, also has other related components.³⁰ Thiopeptin B (**5**) is the main component among the related compounds and is characterized structurally as having several thiazole rings.³¹ WAP-8294A2 (**6**) was isolated as an anti-methicillin-resistant *Staphylococcus aureus* agent and is being developed clinically.^{32,33}

Degradation behavior

Degradation of these peptides was monitored using HPLC and LC/MS. Figure 2a shows the degradation manner of the cyclic peptides observed using HPLC in the presence of the B-9 cell extract. Three cyclic peptides, bacitracin-A (**1**), colistins A (**2A**) and B (**2B**) and polymyxin B1 (**3B1**), showed similar degradation behavior such that they decomposed smoothly within 12 h as shown in Figure 2a. Polymyxin B2 (**3B2**) is not shown in Figure 2a because it was difficult to observe using HPLC. Figure 3 shows the mass chromatograms monitored at calculated m/z of the degradation products of bacitracin-A (**1**) for 48 h. The antibiotic was observed as the doubly charged protonated molecule (**1**) at m/z 712.3 in the mass chromatograms, which disappeared completely within 6 h, and the degradation products were detected sequentially. The degraded ions, (**1a**) and (**1b**), were found initially at m/z 721 and 647, respectively. The detected intensity of **1a** reached a maximum at 1 h and disappeared by 12 h, and **1b** was observed maximally at 6 h and had disappeared by 12 h. Furthermore, the peaks at m/z 521 (**1c**) and 464 (**1d**) were observed at almost the same retention time and were found intensely as the doubly charged ion from 6 h. The ion (**1e**) formed from **1d** was detected at 12 h, and the intensity continued to increase by 48 h.

Colistins A (**2A**) and B (**2B**), whose doubly charged protonated molecules were observed at m/z 585.5 and 578.5, respectively, in the spectrum, were degraded quickly and the hydrolyzed products (m/z 594 and 587, respectively) were formed within 2 h (data not shown). However, no further degraded products were detected. Polymyxins B1 (**3B1**) and B2 (**3B2**) also provided the doubly charged protonated molecules at m/z 602.4 and 595.3, respectively, in the mass spectrum (data not shown). Although the hydrolyzed product of B1 was detected clearly at m/z 611.4, the corresponding ion was not detected in the case of B2 (data not shown).

Figure 1 Structures of tested bacterial cyclic peptides.

Figure 2 Degradation process of the cyclic peptides observed using HPLC in the presence of the cell extract of B-9. **(a)** Cyclic oligopeptides and **(b)** cyclic depsipeptides.

Figure 3 Mass chromatograms of 1 and its expected degradation products (theoretical $m/z \pm 1.5$ Da) at 0, 0.5, 1, 2, 6, 12, 24 and 48h after the beginning of degradation.

For the depsipeptides, mikamycins, thiopeptin and WAP-8294A2, a different degradation behavior was observed as shown in Figure 2b. Although the protonated molecule at m/z 526 from mikamycin A (**4A**)

was almost degraded within 24h, no definite degradation products were detected (data not shown). The hydrolyzed ion $(M+H+H_2O)^+$ (**4Ba**) at m/z 885.3 from mikamycin B (**4B**) increased with the

Figure 4 Proposed degradation products of the tested cyclic peptides and their monoisotopic molecular weights. (a) Cyclic oligopeptides and (b) cyclic depsipeptides.

Figure 4 Continued.

Figure 5 MS spectra of **1** (a) and its degradation products (b: **1a**, c: **1b**, d: **1c** and **1d**, e: **1e**).

decrease in the protonated molecule at m/z 867.4 within 12 h (data not shown). After 6 h, a characteristic ion (**4Bb**) appeared at m/z 752.3 and another ion (**4Bc**) was also found at m/z 627.5 by 96 h (data not shown). Thiopeptin B (**5**) was relatively resistant to this bacterium, and the protonated molecule (**5**) at m/z 1669.7 was observed even after 24 h (data not shown). However, the ion (**5a**) at m/z 1600.2 began to appear after 24 h and continued even at 96 h (data not shown). WAP-8294A2 (**6**) was very stable toward this strain and no degradation occurred during the experimental period.

Structural analysis of degradation products by LC/ITMS

The degradation products were detected using total ion current chromatography. When a degradation product was found, the structure was determined using the mass spectrum, mass chromatogram and MS^n technique. The proposed degradation products of the tested cyclic peptides are summarized in Figure 4. Figure 5 shows the mass spectra of the degradation products of bacitracin-A (**1**). The doubly charged ion (**1a**) at m/z 721.1 was formed by hydrolysis of the Phe–His peptide bond of **1**. The formation of the ion **1b** at m/z 647.5 was because of the cleavage at the Ile–Phe peptide bond of **1a**. The characteristic doubly charged ion (**1c**) at m/z 521.5 was formed by hydrolysis of the His–Asp peptide bond of **1b**. The subsequent degradation product at m/z 464.6, as the doubly charged ion that has almost the same retention time as **1d**, was decomposed to several product ions, m/z 796.5, 682.4, 554.4 and 441.1, under MS^2 condi-

tions, indicating that a degradation product with the monoisotopic molecular weight of 926.6 was **1d** (Figure 6). The doubly charged ion at m/z 407.7 (**1e**) was formed by hydrolysis of the Ile–Orn peptide bond of **1d** (Figure 5). Although colistins A (**2A**) and B (**2B**) were firmly hydrolyzed, no definite degradation products were detected. Polymyxins B1 (**3B1**) and B2 (**3B2**) also showed a behavior similar to that of colistins (**2A** and **2B**) and no degradation product was found, except for the formation of (**3B1**+H₂O) (Figure 4a).

As mentioned above, mikamycin A (**4A**) was almost degraded within 24 h, and no definite degradation products were detected. Mikamycin B (**4B**) provided two degradation products, **4Ba** and **4Bb**. The MS^2 experiments of **4Bb** at m/z 752.4, MS^3 at m/z 609.2 and MS^4 at m/z 405.1 indicated that **4Bb** was the degradation product shown in Figure 7. The compound **4Bc** is a further degraded compound with the monoisotopic molecular weight of 626.3, as shown in Figure 4b. Thiopeptin B (**5**) was relatively stable, and a degradation product was detected as **5a** that corresponds to the loss of the dehydroalanine moiety from the parent compound. WAP-8294A2 (**6**) did not provide any degradation product (Figure 4b).

DISCUSSION

To extend the hydrolytic capability of the MC-degrading enzymes of B-9, we investigated the degradation behavior of the bacterial cyclic peptides in the presence of the B-9 cell extract. The examined peptides were divided structurally into two groups, that is, cyclic oligopeptide

Figure 6 (a) LC/MS spectrum of **1d** and (b) MS² spectrum of m/z 464.8 ($M+2H$)⁺⁺.

and depsipeptide, which showed individual characteristic features in the degradation behavior. The former, including bacitracin-A (**1**), colistin (**2**) and polymyxin B (**3**), showed a common degradation behavior in which these peptides are first hydrolyzed in the ring moiety to give linearized peptides, followed by hydrolysis of the peptide bonds. In this study, the linearized product (**1a**) and the subsequent hydrolyzed products (**1b**, **1c**, **1d**, and **1e**) of bacitracin-A (**1**) were identified. On the other hand, mikamycin (**4**), thiopeptin B (**5**) and WAP-8294A2 (**6**) in the latter group are depsipeptides with an ester bond but showed different degradation behavior. Usually, the B-9 strain first attacks the ester bond, and the resulting linearized peptide is then subjected to further hydrolysis. The case of mikamycin-B (**4B**) was a typical example, and two degradation products, **4Bb** and **4Bc**, were identified. However, the B-9 strain did not hydrolyze the ester bond, rather it hydrolyzed a peptide bond at the dehydroalanine-dehydroalanine in the C-terminus of thiopeptin B (**5**). This result may be caused by the steric exclusion of the enzymes and WAP-8294A2 (**6**) was strongly subjected to this effect but did not provide any degradation product.

B-9 is a bacterial strain isolated from a eutrophic lake and belongs to *Sphingosinicella* sp., which can hydrolyze MCs and nodularin using its intracellular enzymes.³ An earlier study was carried out

to investigate further the hydrolytic capabilities of the MC-degrading enzymes of B-9.¹³ As a result of this investigation, it was confirmed that the enzymes can also hydrolyze the peptide bonds of several cyanobacterial cyclic peptides that were structurally different from the MCs and nodularin.¹³ In this study, the B-9 strain was also applied to the bacterial cyclic peptides, including cyclic oligopeptides and depsipeptides, and interesting results were obtained. On the basis of these results, the hydrolytic behavior using this strain is summarized as follows: (1) The reaction basically occurs at a peptide bond in a cyclic peptide moiety to give a linearized peptide. (2) B-9 initially hydrolyzes an ester bond in a depsipeptide and the resulting peptide is further hydrolyzed. (3) A peptide possessing a bulky ring is hydrolyzed at the acyclic part and no other reaction occurs. (4) The resulting linearized peptide is more quickly hydrolyzed compared with the original one. In some cases, it is difficult to detect the degraded peptides or amino acids.

As mentioned above, B-9 possesses at least three hydrolytic enzymes, namely, MlrA, MlrB and MlrC.¹⁵ Very recently, it was found that another MC-degrading bacterium has additional enzymes that can also hydrolyze peptide bonds (personal communication). It is considered that the degradation capabilities of B-9 are not induced by the degradation substrates but are inherent, because we always used a

Figure 7 (a) LC/MS spectrum of 4Bb, (b) MS² spectrum of *m/z* 752.4, (c) MS³ spectrum of *m/z* 609.2 and (d) MS⁴ spectrum of *m/z* 405.1.

bacterial cell extract instead of growing bacteria, and the degradation began without any lag time. These results suggested that the substrate specificity of the hydrolytic enzymes of B-9 is relatively wide and that B-9 can be applied successfully to other cyclic peptides as shown in this study. How many hydrolytic enzymes does B-9 possess and how does B-9 use the inherent enzymes against substrates? It is likely that B-9 has further potential to hydrolyze other types of compounds.

It is laborious to obtain the partially hydrolyzed product of cyclic peptides produced using the NRPS pathway.^{18–21} It is known that nagarse and colistinase hydrolyze some cyclic peptides, which are produced by bacteria and belong to the subtilisin family.²⁷ Subtilisin is a serine protease and cleaves a peptide bond at the C-terminus side of hydrophobic amino acids.²⁷ To the best of our knowledge, no detailed result has been reported regarding the degradation behavior of cyclic peptides by subtilisin. It is considered that the hydrolytic enzymes from B-9 possess wider substrate specificities similar to that of subtilisin. Therefore, the B-9 strain can be one of the most effective tools for the partial hydrolysis of a cyclic peptide. Indeed, we have succeeded in preparing an immobilized B-9 with a polyester resin,² which would be an appropriate and effective way to degrade cyclic peptides.

Conclusions

This study was carried out to further investigate the hydrolytic capabilities of the MC-degrading enzymes of B-9. As a result of this investigation, it was confirmed that the enzymes can also hydrolyze the peptide bonds of several bacterial cyclic peptides. In addition, the use of the cell extract suggested that these unique activities were present inherently in all MC-degrading bacteria, including B-9. Although the role of these enzymes in bacteria is not clarified fully, the abilities of these enzymes in microorganisms existing in the natural environment may contribute to environmental self-purification. On the other hand, this study also indicates the possibility that a technique using B-9 would be a practical method for natural product chemistry.

ACKNOWLEDGEMENTS

The authors thank Dr Yoshitomo Ikai and Mr Azusa Kato for providing several cyclic peptides and WAP-8294A2, respectively.

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