

MB1533 is a Defensin-Like Antimicrobial Peptide from the Intracellular Meristem Endophyte of Scots Pine *Methylobacterium extorquens* DSM13060

Tejesvi MV¹, Andersen B², Antcheva N³, Brinch KS², Koskimäki JJ¹, Kristensen HH², Tossi A³ and Pirttilä AM¹

¹Faculty of Science, Genetics and Physiology, University of Oulu, Finland

²Novozymes AS, DK-2880 Bagsvaerd, Denmark

³Department of Biochemistry, University of Trieste, Trieste, Italy

Abstract

Endophytes induce plant growth and protect host plants against abiotic and biotic stresses, such as phytopathogenic bacteria and fungi. Here we explored the genome of plant growth-promoting, intracellular endophyte *Methylobacterium extorquens* DSM13060, isolated from bud meristems of *Pinus sylvestris* L., for antimicrobial peptides. A defensin-like antimicrobial peptide MB1533 was identified using computational and prediction models from the genome sequence. MB1533 was produced in *Escherichia coli* with a molecular weight of 6160.26 Da. The antimicrobial activity was tested against several strains of bacteria and yeasts, and the peptide had activity against the gram-positive *Staphylococcus aureus* and *Bacillus subtilis* at 128 µg/ml. The potency of the peptide can be improved further by rational-design-techniques. MB1533 is the first defensin-like peptide identified from *Methylobacterium* genus and from bacterial endophytes, in general.

Keywords: Bacterial endophytes; Antimicrobial peptides; Genomics; Antibacterial

Introduction

Plant internal microbes, endophytes, are important for their host due to their ability to protect against biotic and abiotic stresses [1-3] and induction of plant growth [4]. We have earlier identified the endophytic bacterium *Methylobacterium extorquens* DSM13060 inside meristematic cells of Scots pine buds [5,6]. The endophyte *M. extorquens* DSM13060 significantly increases growth of pine seedlings, and the growth effect is comparable to that induced by mycorrhizal fungi [7]. Unlike many other endophytic bacteria, *M. extorquens* DSM13060 does not produce plant hormones to induce host growth [8,9] but this activity can be due to nucleomodulins present in the genome of the intracellular endophyte [9].

Besides growth promotion, bacterial endophytes of Scots pine buds may have a role in protection of the host against pathogens [6,10]. However, antimicrobial products have not been identified in these bacteria. Besides actinomycetes, bacteria are not recognized as such versatile producers of secondary metabolites as endophytic fungi. In general, endophytic fungi that colonize tree leaves produce a large range of various bioactive compounds, such as alkaloids, steroids, peptides, terpenoids, polyketones, flavonoids, quinols, phenols, and chlorinated compounds [11,12].

Although some endophyte-produced secondary metabolites consist of small peptides in the main structure [13], gene-encoded antimicrobial peptides have not been reported in endophytes. Gene-encoded antimicrobial peptides (AMPs) are conserved, often produced by the innate immune system, and not known to develop resistance in bacteria [14]. AMPs can prevent formation of biofilms and kill multi-drug resistant strains with low side effects to the host [14,15]. AMPs interact with microorganisms by electrostatic forces and often cause cell membrane damage [16]. The interaction largely depends on the amino acid composition of the peptide and the cell surface of target organism, and results in formation of pores, disruption of the membrane bilayer and promotion of non-lamellar lipid structure [17].

Recent advances in biotechnology have enabled identification of several AMPs from fungi, and defensin-like peptides from bacteria [15,18,19]. AdDLP, a defensin-like peptide was isolated from *Anaeromyxobacter dehalogenans* with antimalarial activity against *Plasmodium falciparum* [20]. Plectasin, an antimicrobial defensin identified from fungi, shares a genetic origin with animal defensins, having a high degree of structural and sequence similarity [15]. In this work, we explored the genome of the pine meristem endophyte *Methylobacterium extorquens* DSM13060 using computational approaches to find new AMPs, and cloned, expressed, purified and tested antimicrobial activity against various strains of bacteria and yeast.

Materials and Method

Isolation of genomic DNA

Endophytic *Methylobacterium extorquens* strain DSM13060 (NCBI accession: AGJK00000000) was isolated from the buds of mature *P. sylvestris* trees [5]. Bacteria were grown to the late logarithmic phase and cells were harvested by centrifugation at 4000 × g for 5 min at 4°C. The bacterial pellet was ground in liquid nitrogen with mortar and pestle to facilitate the lysis and increase the yield. The genomic DNA was isolated according to the Joint Genome Institute (JGI) standard protocols for bacterial DNA isolation by using CTAB method, and the detailed protocol is reported elsewhere [9].

*Corresponding author: Tejesvi M, Faculty of Science, Genetics and Physiology, University of Oulu, Finland, Tel: +358503501136; E-mail: mvtjesvi@gmail.com

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Genome sequencing and analysis

The draft genome of *M. extorquens* DSM13060 was generated at the JGI using a combination of Illumina [21] and 454 technologies [22]. The genome was constructed and sequenced using Illumina shotgun library, which generated 505,906 reads totaling 113.9 Mb of 454 data. All general aspects of library construction and sequencing performed at the JGI can be found at <http://www.jgi.doe.gov/>. The initial draft assembly contained 734 contigs in 5 scaffolds. The 454 paired end data were assembled together with Newbler, version 2.6 [23]. Illumina data was used to correct potential base errors and increase consensus quality using the software Polisher developed at JGI (Alla Lapidus, unpublished). Automatic annotation was performed using the JGI-Oak Ridge National Laboratory annotation pipeline.

Identification of potential antimicrobial peptides

Identification of antimicrobial, defensin-like peptides was carried out by searching Cysteine residues in the coding regions of the genome. Then, the peptides were tested *in silico* for antimicrobial activity at the CAMP database (<http://www.camp.bicnirrh.res.in/>). The antimicrobial prediction tools available at the CAMP database were used to determine the antimicrobial potential of the peptides [24]. For prediction, four multivariate methods were used such as Support Vector Machines (SVM), Random Forest (RF), Artificial Neural Network (ANN) and Discriminant Analysis (DA) [24]. APD2 database was utilized to check the identities of the peptides to known AMPs [25]. Signal peptides were predicted by SignalP v. 4.0 [26]. Peptides with the highest identities to known AMPs and predicted antimicrobial activities were cloned and expressed in *Escherichia coli*, and tested for antimicrobial activity *in vitro*.

Bioinformatic analysis

Peptide sequences of EAP2_EUCUL (P83597), Galiomycin (P85213) and Heliomycin (FJ546343) were retrieved from Genbank (NCBI) and aligned using Muscle software, a multiple sequence alignments software [27]. The degree of conservation and consensus between all peptide sequences was done using Jalview software. Then, the phylogenetic Neighbour joining tree was constructed using maximum identifies in Jalview [28]. All the sequences were submitted to Genbank with accession numbers KT318397 and KT318398 for MB1533 and MB3338 respectively. The 3D structure of MB1533 was predicted using iTasser server [29].

Cloning, recombinant expression and purification of peptides

The peptides were expressed in *E. coli* as described by Tejesvi et al [30]. Briefly, the MB1533, MB3338 and MB4823 coding sequences were amplified from genomic bacterial DNA using the Roche high-fidelity PCR system and specific primers carrying *KpnI* and *SacI* restriction site overhangs, respectively (Table 1). The PCR products were digested with *KpnI* and *SacI* in NE buffer (New England Biolabs) at the restriction sites in the primer overhangs. The digested DNA fragments were ligated into the plasmid pET32a (+) (Novagen), and the resulting plasmids (pMB1533, pMB3338 and pMB4823) encoded fusion peptides of an N-terminal thioredoxin part, a his-tag, an enterokinase (EK) cleavage site, and the mature peptides MB1533, MB3338 or MB4823.

The plasmid was transformed into *E. coli* DH10B cells, and the insert sequence was verified by sequencing. Then, the plasmid was transformed into the *E. coli* strain BL21 (DE3) competent cells by following the manufacturer's instructions (Invitrogen, USA). A highly expressing clone was selected and grown in 4 l of TB, the culture broth was pelleted

by centrifugation and stored at -20°C until processed. The pelleted cells were lysed by suspending in 1-quarter of the cell culture volume in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.04% lysozyme (Sigma), 0.01% benzonase (Sigma), 1% 3-(N,N-dimethylmyristylammonio) propanesulphonate (SB3-14) (Sigma) and 0.1% 3-(4-heptyl)phenyl-3-hydroxypropyl dimethylammonio propanesulphonate (C_7BzO) (Sigma) for 45 min at room temperature (RT). The resulting lysate was filtered through a 0.22 μm filter (Nalgene super Mach).

Enterokinase digestion and reverse-phase chromatography (RP-HPLC)

To release the mature peptide from the fusion protein, enterokinase (1: 50, v/v) was added and the suspension was incubated at 30°C overnight at 200 rpm. The digest was adjusted with formic acid and loaded onto a Gemini 10 μm C18 250 \times 10 mm column (GE Healthcare) calibrated with 1% formic acid. The bound protein was subsequently eluted with a linear gradient of ethanol (0%–80%, v/v). The fractions containing 6160.26 Da MB1533 were identified on an SDS gel, and the antibacterial activity was verified. The fractions containing MB1533 were pooled, lyophilized, and re-suspended in 0.01% acetic acid. The molecular mass of MB1533 was determined by UPLC-MS using a Q-TOF Premier (Waters).

Antimicrobial activity of peptides

The radial diffusion assay (RDA) was performed following the method described by Tejesvi et al. [30] Briefly, 30 ml of 1/10 Muller-Hinton broth (MHB) supplemented with 1% agarose and 5.0×10^5 CFU/ml of *Staphylococcus aureus* was poured into a single well omnitray (Nunc) and overlaid with a TSP 96-well plate. Gentamicin and vancomycin were included as positive controls, and sterile water was used as the negative control. The gram-positive and gram-negative bacteria, *S. aureus* (ATCC29213), *Bacillus subtilis* (ATCC23857), *Escherichia coli* (ATCC11105), *S. carnosus* (ATCC51365), *Streptococcus pneumoniae* (ATCC49619), *Pseudomonas aeruginosa* (ATCC27853) and *Enterococcus faecalis* (ATCC51575) and the yeasts *Candida glabrata* (ATCC90030), *C. utilis* (ATCC9950) and *C. albicans* (ATCC90028) were tested for susceptibility by determining the MIC using a microbroth dilution assay in duplicates.

Results

Identification of potential antimicrobial peptides

Small peptides from the annotated as well as unannotated amino acid sequence of *M. extorquens* DSM13060 genome were BLAST searched against CAMP database. Six peptides with predicted activity were identified in the screening protocol. The peptides MB1533, MB3338 and MB4823 were selected as the most potent antimicrobial peptides based on prediction by different models (Tables 1 and 2). The full length of MB1533 is 77 amino acids (AA), having a pro-peptide (signal peptide) of 21 AA (1-21AA) and a mature peptide of 56 AA (22-77AA). The peptide MB3338 has a pro-peptide of 21 AA (1-21 AA) in length and a mature peptide of 47 AA (22-68 AA). The third predicted antimicrobial peptide, MB4823, does not contain a pro-peptide, having the length of 38 AA. All of the predicted antimicrobial peptides are cationic and contain a net positive charge of +9, +7 and +6 for MB1533, MB3338 and MB4823 mature peptides, respectively. The peptides were predicted to be antimicrobial using four multivariate methods such as Support Vector Machines (SVM), Random Forest (RF), Artificial Neural Network (ANN) and Discriminant Analysis (DA). The probability values ranged between 0.66 and 0.96 with all

Name	Sequence length	Charge	Cysteine residues	Antimicrobial Prediction Classifiers			
				Support Vector Machine	Random Forest	Artificial Neural Network	Discriminant Analysis
MB1533	56	9	6	0.710	0.7615	AMP	0.953
MB3338	47	7	6	0.913	0.667	NAMP	0.663
MB4823	38	6	2	0.869	0.9665	AMP	0.812

*http://www.camp.bicnirrh.res.in

Table 1: *In silico* prediction of *Methylobacterium* peptides for antimicrobial activity using collection of antimicrobial database*.

SI. No.	Primer Name	Primer Sequence
1	MB1533_fwd	CCCCCCGGTACC GACGACGACGACA AGGCGACAACGGCGATGAGCCG
2	MB1533_rev	CCCCCCGAGCTCTTATTAGAACGACGGCTTATTGCCGA
3	MB3338_fwd	CCCCCCGGTACC GACGACGACGACA AGATGAAGAACCTGATTGCCGA
4	MB3338_rev	CCCCCCGAGCTCTTATTAGGTACGCTTGCTCATGTT
5	MB4823_fwd	CCCCCCGGTACC GACGACGACGACA AGGAGTGCTGCTTTGCCAA
6	MB4823_rev	CCCCCCGAGCTCTTATTACGGAGCCATAGGACGAT

Table 2: Primers used to amplify the defensin like peptides from *Methylobacterium extorquens* DSM13060.

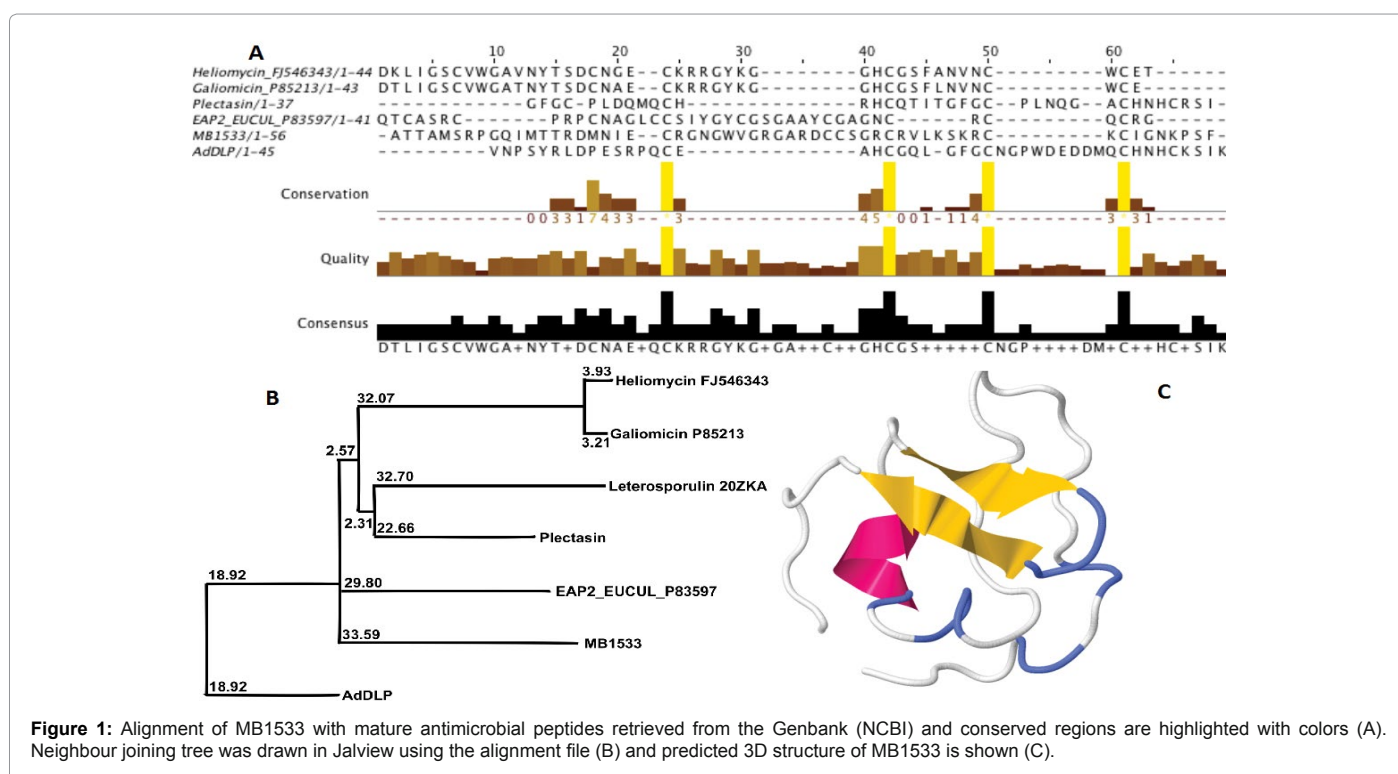


Figure 1: Alignment of MB1533 with mature antimicrobial peptides retrieved from the Genbank (NCBI) and conserved regions are highlighted with colors (A). Neighbour joining tree was drawn in Jalview using the alignment file (B) and predicted 3D structure of MB1533 is shown (C).

four methods used. The higher the value is, the higher probability for antimicrobial activity. There was an exception in the case of MB3338 using ANN, where it was predicted not to be antimicrobial. MB1533 has low homology to EAP2_EUCUL (P83597; *Eucommia ulmoides*), Galiomycin (P85213; *Galleria mellonella*) and Heliomycin (FJ546343; *Heliopsis virescens*) with four cysteine residues conserved among them (Figure 1). MB1533 has α -helices and β -sheets with cysteine bridges as seen in the predicted 3D structure of peptide (Figure 1).

Expression and purification of Trx-MB1533

The plasmids pMB1533, pMB3338 and pMB4823 containing the Trx fusion was transformed into *E. coli* BL21 cells, and after IPTG induction, the peptides were expressed as a fusion protein with thioredoxin. The peptides were released from the fusion partner thioredoxin using enterokinase and purified using RP-HPLC. The size

of pure MB1533 was consistent with SDS – PAGE gel band intensities of 6.166 kDa peptide. Correspondingly, the sizes of MB3338 and MB4823 were 4.665 and 4.460 kDa, respectively (Supplementary Table 1). However, MB3338 and MB4823 could not be purified at optimal levels due to formation of aggregates and were not used in the further tests. The concentration of recombinant MB1533 was determined by RP-HPLC, and the mono-isotopic molecular mass was confirmed by electrospray Q-TOF MS/MS to be 6160.26 Da. Approximately 1.2 mg of purified recombinant MB1533 was produced from IPTG-induced cells from one liter of TB culture medium.

Antimicrobial activity

The antimicrobial activity of MB1533 was tested against both gram-negative and gram-positive bacteria and yeasts using a CLSI MIC assay (NCCLS 2002). MB1533 has activity only against the gram-positive

bacteria, such as *S. aureus* and *B. subtilis*. MB1533 is moderately active at the concentration of 100 µg with the zones of inhibition being 7 and 11 mm against *B. subtilis* and *S. aureus*, compared to, e.g., gentamicin, used as the positive control in this study, that had 23 and 24 mm zones of inhibition at the concentration of 10 µg. MB1533 was active against *S. aureus* and *B. subtilis* with a minimum inhibitory concentration (MIC) value of 128 µg/ml, but no antimicrobial activity was observed against *Escherichia coli*, *Staphylococcus carnosus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* or *Enterococcus faecalis*. The MIC values of the positive control gentamicin were 0.5 and 1 µg/ml against *S. aureus* and *B. subtilis*, respectively. MB1533 had no antifungal activity towards *Candida glabrata*, *C. utilis* or *C. albicans* when tested by the radial diffusion assay (RDA).

Discussion

Antimicrobial peptides (AMPs) are the first line of defense produced by all organisms, including bacteria, and are essential for their survival [31]. In higher animals, AMPs are products of the innate immune system, evolutionarily conserved, and retain their antimicrobial activity for adaptation in various environments [32]. As of July 2015, more than 5040 AMPs are found in the Antimicrobial Peptide database (<http://camp.bicnirrh.res.in/>). Antimicrobial peptides exert various activities including antibacterial, antifungal, antiviral and immunomodulatory [33]. Among AMPs, bacteriocins are produced by bacteria and are quite similar to eukaryotic AMPs with respect to positive charge, hydrophobicity and amphiphilicity. Bacteriocins of *E. coli* and other enterobacteria are often referred to as microcins [34]. Lantibiotics are products of gram-positive bacteria, their size varying between 19-38 amino acids. Nisin, subtilin, lactacin and thuricin are some of the lantibiotics characterized from gram-positive bacteria [35]. Defensins are rich with cysteines, distributed extensively within all organisms, and involved in host defense against pathogens. In many species, β-defensins are the largest group and comprised of 35-50 AA residues, stabilized by three disulphide bridges (Cys1-Cys5, Cys2-Cys-5 and Cys3-Cys6) with antiparallel β-sheets [36].

MB1533 is the first defensin-like peptide identified from the *Methylobacterium* genus and from bacterial endophytes, in general. This AMP is a gene-encoded cationic peptide with a net positive charge of +9 and 6 conserved cysteine residues. The majority of the host defense peptides are cationic by nature due to the presence of basic amino acids and hydrophobicity, and the positive charge has been attributed with antimicrobial activity [37,38]. Cationic peptides often form α-helices or β-sheets, which are essential for the antimicrobial activity [37], and both of these structures are found in the MB1533 together with cysteine bridges (Figure 1).

The defensin-like peptide MB1533 has antibacterial activity against *S. aureus* and *B. subtilis* with an MIC value of 128 µg/ml. The first and the only other defensin-like peptide earlier isolated from bacteria is AdDLP from *Anaeromyxobacter dehalogenans*. This defensin-like AMP does not inhibit bacterial strains even at concentrations as high as 10 and 20 µM. However, AdDLP has antimalarial activity against *Plasmodium falciparum* at 10 µM [20], which suggests that also other significant activities can be found for MB1533. MB1533 has no homology with AdDLP, as can be seen in Figure 1. MB1533 clusters in the phylogenetic tree with EAP2_EUCUL, an antifungal peptide of hardy rubber tree (*Eucommia ulmoides*) [39]. However, the homology between these two antimicrobial peptides is too low for any further analysis.

Whereas we report here the first defensin-like AMP from an

endophytic bacterium, a number of small peptides that are not gene-encoded have been identified from endophytes of many medicinal plants around the world. Endophytic *Pseudomonas viridiflava* isolated from lettuce (*Lactuca sativa*) produces ecomycin, an antifungal compound known to inhibit the growth of *Cryptococcus neoformans* and *Candida albicans* at 4 µg/ml and 31 µg/ml, respectively [40]. Lipopeptides syringomycins A1, E, G and pseudomycins are produced by *Pseudomonas syringae* and exhibit antimycotic activity [41-43]. Recently, two tetrapeptides, Cyclo-(Val-Leu-Val-Leu) and Cyclo-(Leu-Ala-Leu-Ala) were identified from *Castaniopsis fissa* [44]. Furthermore, Talaromins A and B were identified from the endophytic fungus *Talaromyces wortmannii* from *Aloe vera* and had no antibacterial activity against various strains of bacteria at 64 µg/ml [13]. Finally, the endophytic *Epichloe typhina* produces the antifungal peptide Epichlicin, which inhibits *Cladosporium phlei* at an IC50 of 22 nM [45].

To conclude, we discovered by genomic tools the antimicrobial peptide MB1533 from *M. extorquens* DSM13060 with activity towards the gram-positive bacteria *S. aureus* and *B. subtilis*. MB1533 is the first gene-encoded antimicrobial peptide from *Methylobacterium* genus and from bacterial endophytes, in general. Identification of gene-encoded, defensin-like antimicrobial peptide from the bacterial endophyte opens completely new avenues for, e.g., safe biocontrol of phytopathogens. For applied use, the antimicrobial activity of the peptide MB1533 can be improved by using rational design techniques. For example, it has been shown that by modifying the amino acids of the peptide, or using peptide conjugates, peptide congeners, peptide mimetics, or hybrid peptides, the activity can be improved several folds [15,46].

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