

# New Type of Antimicrobial Protein Produced by the Plant Pathogen *Clavibacter michiganensis* subsp. *michiganensis*

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It has previously been shown that the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* secretes a 14-kDa protein, *C. michiganensis* subsp. *michiganensis* AMP-I (*CmmAMP-I*), that inhibits growth of *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot of potato. Using sequences obtained from tryptic fragments, we have identified the gene encoding *CmmAMP-I* and we have recombinantly produced the protein with an N-terminal intein tag. The gene sequence showed that *CmmAMP-I* contains a typical N-terminal signal peptide for Sec-dependent secretion. The recombinant protein was highly active, with 50% growth inhibition ( $IC_{50}$ ) of approximately 10 pmol, but was not toxic to potato leaves or tubers. *CmmAMP-I* does not resemble any known protein and thus represents a completely new type of bacteriocin. Due to its high antimicrobial activity and its very narrow inhibitory spectrum, *CmmAMP-I* may be of interest in combating potato ring rot disease.

Many bacteria produce antimicrobial compounds called bacteriocins. These are ribosomally synthesized peptides or proteins that most often act on species that are closely related to the producer strain. Bacteriocins are thought to participate in the competition between similar species occupying the same ecological niche (1–3). Bacteriocins produced by phytopathogens are of great interest because they may contribute to determining the composition of microbial ecosystems in, e.g., the rhizosphere, which again may affect the emergence and severity of plant disease outbreaks. Furthermore, such compounds are of interest because they may provide new tools for combating plant pathogens. In this respect, it is helpful that bacteriocins tend to have a narrow target cell spectrum and lack a “general” antibiotic activity that could hamper their potential application. Despite their obvious importance and potential, relatively little is known about bacteriocins from plant pathogens (4).

We have previously shown that the Gram-positive tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis*, an actinomycete, produces two proteinaceous antimicrobial compounds: a heat-stable 2,145-Da posttranslationally modified peptide called michiganin A (5) and a heat-sensitive antimicrobial protein with a molecular mass of approximately 14 kDa called *C. michiganensis* subsp. *michiganensis* AMP-I (*CmmAMP-I*) (6). Michiganin A was studied in detail and turned out to be a globular posttranslationally modified peptide bacteriocin belonging to the so-called type B lantibiotics. The *CmmAMP-I* protein was purified to apparent homogeneity, but Edman degradation for protein sequencing did not yield sufficiently clear results for protein identification. The antimicrobial activity of *CmmAMP-I* was confirmed in microtiter plate-based bacteriocin assays. Furthermore, upon overlaying renatured SDS-PAGE gels with indicator cells, the lane containing purified *CmmAMP-I* showed a clear halo at approximately 14 kDa (6). Most interestingly, so far, the only identified organism sensitive to both Michiganin A and AMP-I is *C. michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot in potatoes. Since potato ring rot is a regulated quarantine disease causing economic losses in North America, Asia, and Eu-

rope, natural compounds that inhibit *C. michiganensis* subsp. *sepedonicus* may be of value from a biocontrol perspective.

Michiganin A belongs to a class of bacteriocins that require a variety of posttranslational modifications and dedicated secretion machinery (7, 8). Thus, exploiting michiganin A is not straightforward, as both industrial-scale synthesis of the peptide and expression of the peptide in, e.g., transgenic plants would require large and complex genetic manipulations. On the other hand, *CmmAMP-I* could in principle be a normal secreted protein that would be easier to produce and exploit.

Here we describe the identification of the structural gene for *CmmAMP-I* via mass spectrometry (MS)-based sequence analysis of tryptic fragments of the purified protein. The gene encoding *CmmAMP-I* was identified in the genome of *C. michiganensis* subsp. *michiganensis* (9) and successfully expressed using an Impact system in *Escherichia coli*. Using purified heterologously expressed *CmmAMP-I*, we showed that the protein kills *C. michiganensis* subsp. *sepedonicus* at low concentrations and thus presents an interesting potential tool for developing new control strategies for bacterial ring rot in potatoes. Interestingly, *CmmAMP-I* does not resemble any known protein and thus represents a completely new type of bacteriocin.

## MATERIALS AND METHODS

**Bacterial strains and media.** *C. michiganensis* subsp. *michiganensis* NCPPB 1468 and *C. michiganensis* subsp. *sepedonicus* NCPPB 2136 (Na-

Received 8 April 2013 Accepted 8 July 2013

Published ahead of print 12 July 2013

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doi:10.1128/AEM.01065-13

tional Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom) and Bioforsk strains 1977-2, 1979-9, 1982-4, 1982-11, and 2006-A55 (all from the Norwegian county of Akershus; the year of collection is indicated by the first part of the strain designation) were grown in NBY medium (10) with shaking at 100 rpm and at 20°C. *Escherichia coli* TOP10 (Invitrogen), used as the host cell for plasmid construction, and *E. coli* strain C3016 (New England BioLabs), used as the host cell for protein expression, were grown in 2× TY medium with ampicillin (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, 150 mg/liter ampicillin) at 37°C with shaking. For plates, the media were solidified with 15 g/liter agar.

Strains tested for AMP-I sensitivity included the following: *Bacillus subtilis* BAB-1, *Enterococcus faecalis* V583, *E. coli* Top10, *E. coli* C3016, *Lactobacillus plantarum* NC8, *Lactobacillus sakei* Lb790, *Listeria innocua* 4202, *Pediococcus pentosaceus* LMG2001, *Serratia marcescens* BJL200, *Streptococcus faecalis* NCDO581, and 6 strains of *Streptomyces* spp. from a local culture collection numbered 1-A, 1-B, 5-A, 5-B, 6-A, and 10-A. *L. plantarum*, *L. sakei*, *S. faecalis*, and *P. pentosaceus* were grown in MRS medium (Oxoid Ltd., Hampshire, England) at 30°C. *L. innocua* was grown in BHI (brain heart infusion) medium (Oxoid Ltd., Hampshire, England) at 30°C. *E. faecalis* was grown in BHI medium at 37°C. *S. marcescens* and *E. coli* were grown in LB medium (10 g/liter Bacto-tryptone, 5 g/liter Bacto-yeast extract, 10 g/liter NaCl) at 30°C. *Streptomyces* spp. were grown in GYM *Streptomyces* medium (4 g/liter glucose, 4 g/liter yeast extract, 10 g/liter malt extract, 2 g/liter CaCO<sub>3</sub>, pH 7.2) at 30°C. The media were solidified by adding 15 g/liter agar.

**Tryptic mapping and sequence analysis of *CmmAMP-I*.** *CmmAMP-I* was produced and purified from a culture supernatant of *C. michiganensis* subsp. *michiganensis* NCPPB 1468 as described previously (6). Purified protein was dried in a vacuum and redissolved in 50 μl 100 mM (NH<sub>4</sub>)HCO<sub>3</sub>. For reduction and alkylation of cysteine residues, 2.5 μl 200 mM dithiothreitol (DTT)–100 mM (NH<sub>4</sub>)HCO<sub>3</sub> was added and the solution was incubated at 56°C for 30 min. After cooling to room temperature, 5 μl 0.55 M iodoacetamide–100 mM (NH<sub>4</sub>)HCO<sub>3</sub> was added, and the solution was incubated at room temperature in the dark for 30 min. Finally, 3 μl 1 M DTT–100 mM (NH<sub>4</sub>)HCO<sub>3</sub> was added, and the solution was incubated for an additional 15 min.

Tryptic digestion was performed overnight (16 h) by adding sequencing-grade modified trypsin (Promega, Madison, WI) to reach a final concentration of 5 ng/μl (giving molar trypsin/protein ratios of 1:50 to 1:100). The reaction was terminated by adding trifluoroacetic acid to reach a final concentration of 0.5% (vol/vol), and the tryptic peptides were purified using a STAGE column (11).

Tryptic fragments were analyzed using a matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) instrument (Ultraflex; Bruker Daltonics) as described previously (12). For *de novo* sequencing, an Ettan chemically assisted fragmentation-MALDI (CAF-MALDI) sequencing kit (GE Healthcare, Little Chalfont, United Kingdom) was employed. The MS/MS spectra recorded for derivatized peptides were manually annotated using flexAnalysis software (v.3.3; Bruker Daltonics).

**Cloning, expression, and purification of *CmmAMP-I*.** The gene encoding *CmmAMP-I* (NC\_009480.1; 1899661-to-1900137 complement; locus tag CMM\_1675), excluding the signal sequence, was amplified by PCR from genomic DNA of *C. michiganensis* subsp. *michiganensis* NCPPB 1468, which was purified by phenol-chloroform extraction and ethanol precipitation after disruption of the cells with glass beads in a beadbeater (Biospec). The primers used were as follows: AMP-1 fw (5'-GGTGGTCA TATGGCCACGATCTCCAGCCCGAT-3'; NdeI site italicized) and AMP-1 rev (5'-GGTGGTTGCTCTCCGCGAGTTGCTGAATCCACCCA GCGAAC-3'; SapI site italicized). The PCR product was purified by standard agarose gel electrophoresis (1% gel), digested with NdeI and SapI, and ligated into NdeI-SapI-digested vector pTYB1, thus coupling the gene to an N-terminal intein tag (Impact System; New England BioLabs, Beverly, MA). After transformation of the ligation mix into chemi-

cally competent *E. coli* TOP10 cells, plasmids were isolated using a NucleoSpin plasmid miniprep kit (Macherey-Nagel, Düren, Germany) and the presence of a correct insertion was confirmed by sequencing. A correct plasmid was then transformed into chemically competent *E. coli* C3016 cells for protein expression. A positive transformant was grown in 2× TY medium containing ampicillin at 37°C. When the *A*<sub>600</sub> reached 0.5, gene expression was induced by addition of IPTG (isopropyl-β-D-1-thiogalactopyranoside) to reach a final concentration of 0.4 mM. After overnight growth at 25°C, cells collected by centrifugation were resuspended in 20 mM Tris-HCl–500 mM NaCl, pH 8.5. The cells were lysed by sonication at 33% amplitude with 30 5-s pulses (with a 5-s delay between pulses) on ice, with a Vibra cell ultrasonic processor, converter model CV33, equipped with a 3-mm probe (Sonics, Newtown, CT). The sonicated material was centrifuged at 23,700 × g for 30 min at 4°C to obtain a cell-free protein extract.

Recombinant *CmmAMP-I* was enriched with a chitin bead column, according to the manufacturer's instructions (Impact kit; New England BioLabs, Beverly, MA). As part of this procedure, the intein tag was removed while on the column by application of 50 mM DTT. The detached eluted protein was further purified by standard gel filtration chromatography using a prepacked resin column (HiLoad 16/60; GE Healthcare) operated in TN buffer (20 mM Tris [pH 8.0], 20 mM NaCl), analyzed by SDS-polyacrylamide gel electrophoresis, and stored at –20°C in TN buffer containing 50% (vol/vol) glycerol. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad), with bovine serum albumin as a standard. Immediately before use, glycerol was removed from the *CmmAMP-I* stock solutions using a centrifugal filter (Millipore) (molecular weight [MW], 3,000) for buffer exchange to TN buffer.

**Agar plate assays for screening of antimicrobial activity.** To test AMP-I activity, different strains were plated on solidified media in 100-mm-diameter petri dishes. Immediately after spreading the cells, holes punched into the agar using a cut pipet tip were filled with 25 μl of 5 μM *CmmAMP-I* in TN buffer or with TN buffer (negative control). Zones of inhibition were assessed after incubating the plates at appropriate temperatures for 2 to 3 days or as soon as a confluent layer of bacteria was visible.

A similar plate assay was used to analyze whether *C. michiganensis* subsp. *michiganensis* NCPPB 1468 and *C. michiganensis* subsp. *sepedonicus* NCPPB 2136 could inhibit each other. Supernatants were derived from 2-day-old liquid cultures of *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus* in NBY medium and sterilized by filtration using a 0.22-μm-pore-size filter. *C. michiganensis* subsp. *michiganensis* or *C. michiganensis* subsp. *sepedonicus* was plated on NBY plates, and holes punched in the plate with a cut pipet tip were filled with 50 μl of culture supernatant. The plates were incubated for 3 days at 20°C, after which the occurrence of inhibition zones was assessed.

**Growth inhibition in liquid cultures and determination of IC<sub>50</sub>.** Growth inhibition was further analyzed by microtiter plate dilution assays and quantified by determining the *CmmAMP-I* concentration required for 50% growth inhibition (IC<sub>50</sub>), essentially as described in reference 13. The indicator strain (*C. michiganensis* subsp. *sepedonicus*) was grown in liquid NBY medium until the *A*<sub>600</sub> reached 0.5 and then diluted 200 times with medium. Liquid NBY medium (50 μl) was added to each well of a 96-well microtiter plate. A 50-μl volume of a 0.5 mg/ml *CmmAMP-I* solution in TN buffer was added to the first well. Then a serial dilution (1:2) was made by stepwise transferral of 50 μl to subsequent wells. Serially diluted buffer was used as a negative control. After preparing the serial dilutions, 150 μl of the diluted indicator strain was added to each well (this yields a final AMP-I concentration of 4.8 μM in the first well). After incubation of the microtiter plate at 20°C and 100 rpm for 40 h, *A*<sub>600</sub> values were measured using a microplate reader. The IC<sub>50</sub> was estimated by calculating the protein concentration in the wells with an *A*<sub>600</sub> value equal to approximately 50% of the average *A*<sub>600</sub> value in wells without AMP-I (i.e., noninhibited growth).

To analyze whether *CmmAMP-I* kills or only inhibits *C. michiganensis* subsp. *sepedonicus*, survival of the indicator strain was analyzed. Cells were

collected from appropriate microtiter plate wells, washed with NBY medium, and grown on NBY plates for colony counting.

**Phytoxicity tests.** Potato (*Solanum tuberosum*) cultivar Bintje and tomato (*Solanum lycopersicum*) cultivar Espero were cultivated in the greenhouse under conditions of natural day light supplemented with artificial light (16-h photoperiod) at 16 to 18°C. Leaflets and fruits were harvested at the start of the experiments, whereas tubers were from cold storage. Fruits and tubers were surface disinfected using 0.5% sodium hypochlorite and rinsed in autoclaved Milli-Q water before the start of the experiment. The experiment was performed in large humid chambers (trays lined with moist filter paper under an aluminum grid and covered with plastic). Detached leaflets were placed directly on the metal grid, while tubers and fruits were placed in 150-mm-diameter petri dishes. Drops (5  $\mu$ l) of AMP-I solution (55 ng/ $\mu$ l in TN buffer) were placed onto each of 10 leaflets, 5 tuber slices, and 3 tubers of potato and 10 leaflets, 5 fruit slices, and 3 whole fruits of tomato. Controls (same numbers) were treated with double-distilled water (dH<sub>2</sub>O), TN buffer, or the nonselective contact herbicide Reglone (active ingredient dikvat-dibromid; Syngenta). The plant samples were incubated at 16°C for 7 days, leaflets under conditions of a 16-h photoperiod, and tubers and fruits in the dark.

## RESULTS

**Identification of the gene encoding *CmmAMP-I*.** Analysis of tryptic fragments obtained from *CmmAMP-I* yielded good-quality sequence information for two peptides: AVAAAAYTPYFE(I/L)YYGP NCASASR and GNDGTY(I/L)AFSSQGACVP(I/L)DSR (Fig. 1), which led to the identification of a locus called CMM\_1675 as the gene encoding the protein (NC\_009480.1; 1899661-to-1900137 complement). This gene encodes a 158-residue protein of which the first 37 amino acids (aa) are predicted to be a Sec-type signal peptide according to SignalP (14). The remaining 121-residue protein has a predicted mass of 13,012 Da, which is in good agreement which the results from SDS-PAGE (6). The theoretical pI (15) of the protein is 8.69.

BLAST searches (16) with the *CmmAMP-I* protein sequence against the translated nucleotide database yielded only two significant hits. One of these hits was a protein with unknown function from *C. michiganensis* subsp. *sepedonicus* (CMS\_0380) annotated as “putatively secreted.” This protein has the same number of residues as *CmmAMP-I* and shows 68% sequence identity (Fig. 2). The only other significant hit was to part of a postulated insertion sequence (IS) element in the related bacterium *Leifsonia xyli* subsp. *cynodontis* (previously *Clavibacter xyli* subsp. *cynodontis*), the causal agent of ratoon stunting disease in sugarcane (17). In this case, a 41% sequence identity was observed for a stretch of 105 residues.

The CMM\_1675 gene in *C. michiganensis* subsp. *michiganensis* is encoded on the reverse strand, and no obvious operon structure can be observed. CMM\_1674 encodes a putative endo-1,4-beta-xylanase A and is located as much as 966 bp upstream on the forward strand. CMM\_1676, located 108 bp downstream on the forward strand, encodes a putative protein of 162 aa with no similarity to any other proteins (meaning that this protein is absent in *C. michiganensis* subsp. *sepedonicus*).

**Expression of *CmmAMP-I*.** AMP-I was produced with an N-terminal intein tag that permitted purification with a chitin column and precise on-column removal of the tag, as described in Materials and Methods. The fusion protein was expressed in soluble form in *E. coli* C3016. After chitin affinity chromatography with on-column tag removal, the protein preparation was subjected to one-step size exclusion chromatography to obtain maximal purity. Figure 3 shows that this procedure led to the produc-

tion of reasonably pure material. The typical yield of this procedure was 0.5 mg of pure protein from 1 liter of culture of *E. coli* C3016. An agar plate assay showed that the recombinantly produced protein was active (Fig. 4).

**Antimicrobial activity.** The antimicrobial activity of purified *CmmAMP-I* was tested using plate assays with a total of 23 indicator strains: *C. michiganensis* subsp. *michiganensis* NCPPB1468 (the producer strain), *C. michiganensis* subsp. *sepedonicus* NCPPB2136, 1977-2, 1979-9, 1982-4, 1982-11, and 2006-A55, *L. plantarum* NC8, *L. sakei* Lb790, *L. innocua* 4202, *E. faecalis* V583, *S. marcescens* BJL200, *S. faecalis* NCDO581, *P. pentosaceus* LMGT2001, *E. coli* Top10, *E. coli* C3016, *B. subtilis* BAB-1, and 6 strains of *Streptomyces* spp. called 1-A, 1-B, 5-A, 5-B, 6-A, and 10-A. Using the setup and the concentrations shown in Fig. 4, no inhibitory activity was detected, except for the clear inhibitory activity on *C. michiganensis* subsp. *sepedonicus* illustrated by Fig. 4. Five of the six tested *C. michiganensis* subsp. *sepedonicus* strains were sensitive to *CmmAMP-I*, showing approximately similar halo sizes, whereas one, strain 1982-11, was not sensitive. These results show that *CmmAMP-I* has a very narrow spectrum of target organisms.

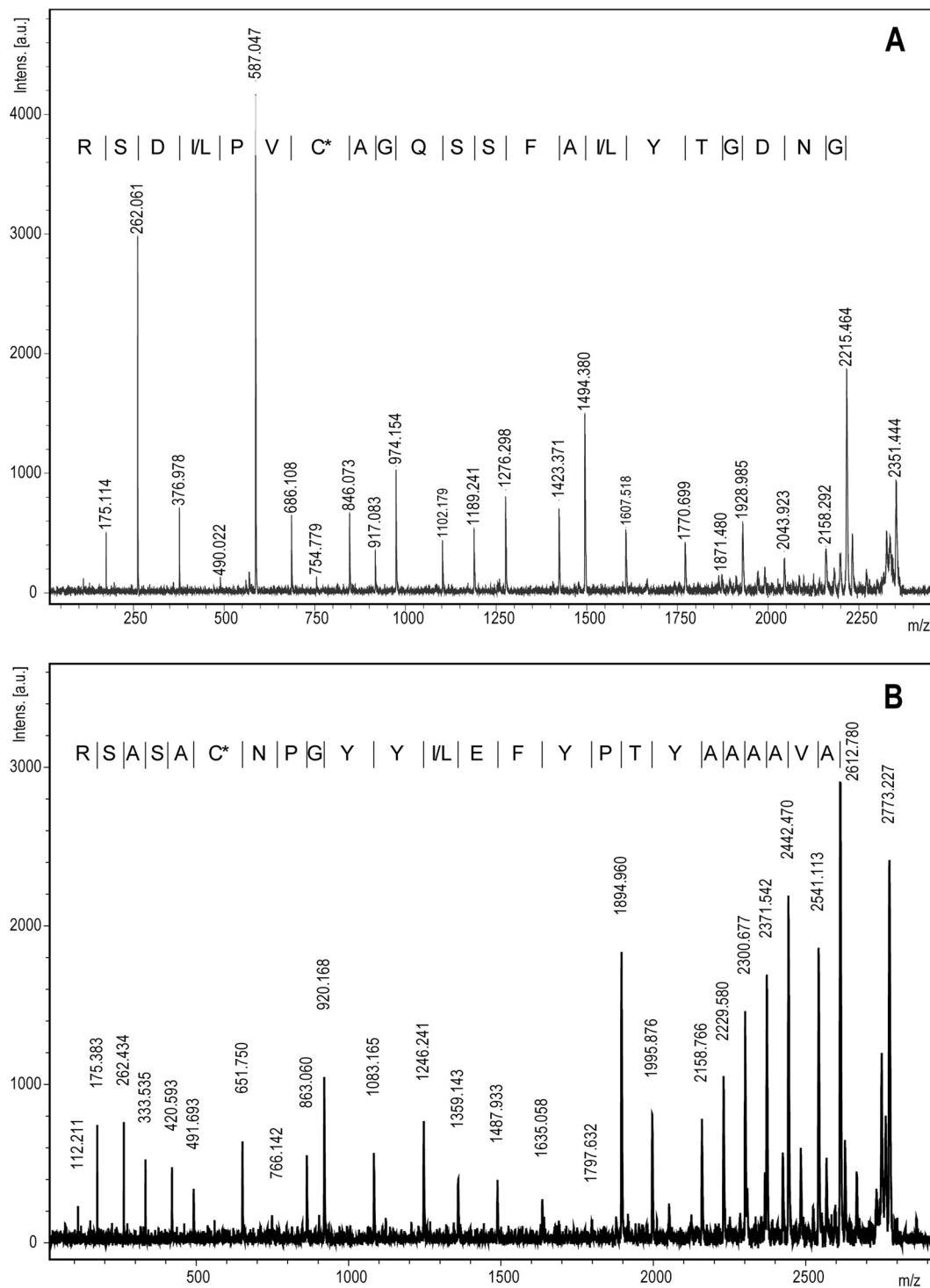
The IC<sub>50</sub> for *CmmAMP-I* acting on *C. michiganensis* subsp. *sepedonicus* in liquid culture was estimated using microtiter plate dilution assays, which consistently yielded an IC<sub>50</sub> of approximately 10 pM (i.e., *CmmAMP-I* was diluted 2<sup>19</sup> times relative to the concentration in well 1). Plating of cells recovered from the microtiter plate wells, and washed with NBY medium before plating, showed that all indicator cells were killed in wells containing *CmmAMP-I* at concentrations of approximately 40 pM and higher.

Despite several attempts, we did not manage to recombinantly produce the putative homologous AMP from *C. michiganensis* subsp. *sepedonicus* (Fig. 2). The same intein-tag-based approach that was successful for *CmmAMP-I* consistently yielded inclusion bodies under all conditions tested (various promoters, various expression temperatures). Attempts to refold the protein in the inclusion bodies failed. We also tested the possible presence of a *C. michiganensis* subsp. *michiganensis* strain inhibiting activity in the supernatant from *C. michiganensis* subsp. *sepedonicus* and did not observe any such inhibition.

**Phytoxicity.** For application in bacterial ring rot control, *CmmAMP-I* should have no toxic effects on potato plants. To test the effect of *CmmAMP-I*, 275 ng (55 ng/ $\mu$ l) of the bacteriocin was spotted on potato leaflets and tubers as well as on slices of tubers. Because of its origin in a tomato pathogen, phytotoxicity was also tested on leaflets and on whole and sliced fruits of tomatoes. No visible effects could be observed on any of the plant parts treated with *CmmAMP-I* after 1 week, while the herbicide-treated leaflet controls, in particular, the potato leaflets, showed extensive necrosis.

## DISCUSSION

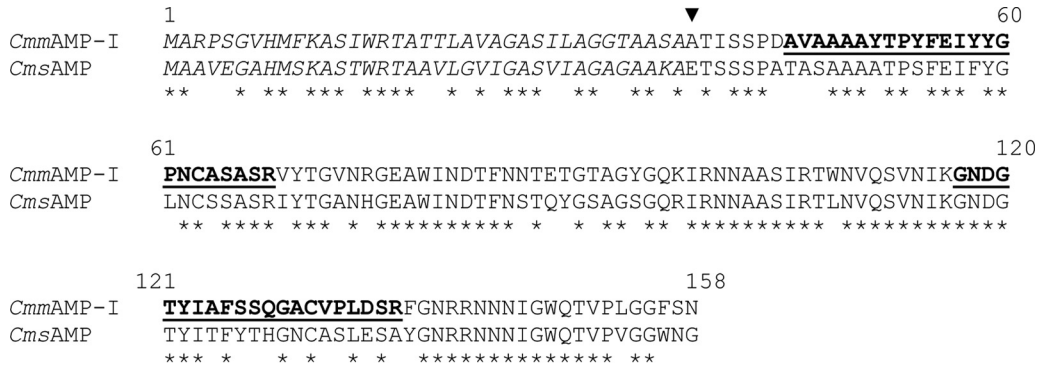
The present data show that *CmmAMP-I* is a novel type of protein bacteriocin which currently does not have any reported characterized homologues. This novel bacteriocin kills its target bacteria at concentrations in the low picomolar range, corresponding to 0.1 to 0.5 ng/ml. Thus, *CmmAMP-I* is at least as powerful as the most powerful of peptide bacteriocins (18). Most known protein bacteriocins from plant pathogens are from Gram-negative species, as these are the most dominant among plant-pathogenic bacteria. Known protein bacteriocins



**FIG 1** *De novo* sequencing of tryptic peptides from AMP-I. MALDI MS/MS spectra of the two tryptic peptides GNDGTY(I/L)AFSSQGACVP(I/L)DSR (A) and AVAAAYTPYFE(I/L)YYGPNCASASR (B). The derivatization methods used (CAF; see Materials and Methods) lead to a predominance of  $\gamma$ -ions in the MS/MS spectra, thereby significantly simplifying interpretation and annotation. C\* denotes a carbamidomethylated cysteine (see Materials and Methods). Intens. [a.u.], intensity [arbitrary units].

from the plant-pathogenic bacteria *Pseudomonas syringae*, *Pectobacterium carotovorum*, *Xylella fastidiosa*, and *Xanthomonas oryzae* are related to the high-molecular-mass (>50-kDa) colicins (19–22). Another group of high-molecular-mass protein bacteriocins

from Gram-negative plant-pathogenic bacteria comprises proteins that affect the target cell membrane by self-assembly into particles resembling the tails of bacteriophages. The best-studied of the latter bacteriocins is probably carotovoricin (23). There are



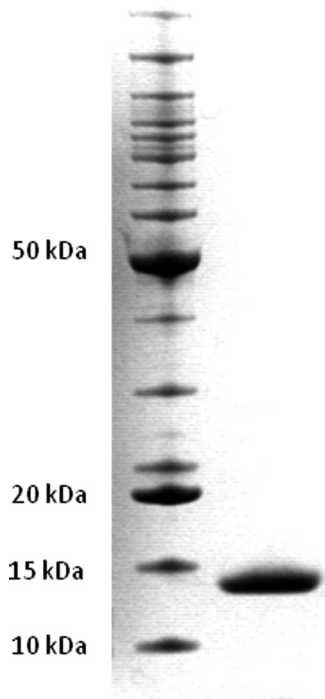
**FIG 2** Sequence alignment of AMP-I from *C. michiganensis* subsp. *michiganensis* (*CmmAMP-I*) with a homologous protein from *C. michiganensis* subsp. *sepedonicus* (*CmsAMP*). Peptide sequences that led to the identification of the AMP-I open reading frame (ORF) (see Fig. 1) are printed in bold face and underlined. According to SignalP predictions, both proteins contain a signal peptidase I cleavage site for Sec-driven secretion. The signal peptides are printed in italics, and the first amino acid of the mature secreted protein is marked with a solid triangle. The gene encoding *CmsAMP* (locus CMS0380) seems to be incorrectly annotated in the publicly available genome sequence of *C. michiganensis* subsp. *sepedonicus*. In the gene bank entry, the ORF is covering nucleotides 417755 to 418165, whereas an ORF spanning nucleotides 417689 to 418165 seems more correct. Here, the corrected ORF has been used.

several examples of plant pathogens producing other protein bacteriocins that do not resemble the “standard” types of antimicrobial proteins mentioned above and/or that have not been sufficiently characterized to allow some sort of classification (4).

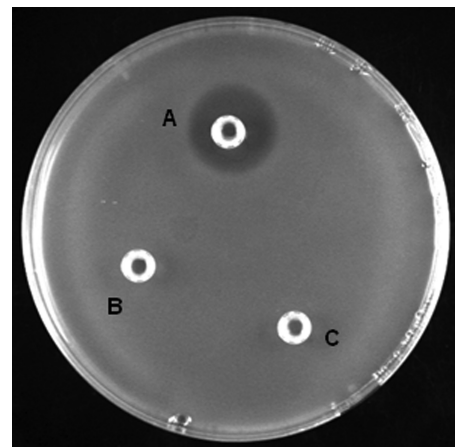
In general, very little is known about protein bacteriocins from Gram-positive bacteria and evidently even less about protein bacteriocins from Gram-positive plant pathogens. Protein bacteriocins from Gram-positive species show large variations in structure, and their activities seem to involve a wide range of mechanisms. Several protein bacteriocins from Gram-positive species are in fact cell-wall hydrolyzing (murolytic) enzymes, such

as lysostaphin (24). Others, with largely unknown mechanisms, include the 37-kDa helveticin J (25), the 17-kDa streptococcal A-M57 (26), the 21.5-kDa dysgalactin (27), and the similar 17-kDa proteins SA-M57, enterococcal V583, and corynicin JK (28). The three latter proteins have been studied in some detail, and it has been shown that the basic C-terminal regions of these otherwise acidic proteins are responsible for their killing activity. The best-studied protein bacteriocin from plant-pathogenic Gram-positive species is ipomicin, a heat-sensitive 10-kDa protein produced by the sweet-potato pathogen *Streptomyces ipomoea* (29). The antagonistic activity of ipomicin seems limited to closely related strains, i.e., primarily other strains of *S. ipomoea*. It is known that ipomicin works by lysing the target cells, but the exact mechanism remains to be elucidated (29). *CmmAMP-I* did not show any features that suggest a functional relationship with any of these known protein bacteriocins.

Interestingly, one of the two homologous sequences found in the database is that of a secreted protein of unknown function in



**FIG 3** SDS-PAGE analysis of purified recombinant *CmmAMP-I*. Right lane, purified *CmmAMP-I*; left lane, BenchMark protein ladder (Life Technologies) with 15 proteins ranging from 10 to 220 kDa.



**FIG 4** Inhibition of *C. michiganensis* subsp. *sepedonicus* by recombinant *CmmAMP-I*. An NBY plate with a confluent layer of bacterial *C. michiganensis* subsp. *sepedonicus* cells, except for a halo around the well labeled “A,” is shown. The following samples were added to the wells: A, 25  $\mu$ l of 5  $\mu$ M *CmmAMP-I* in TN buffer; B and C, 25  $\mu$ l TN buffer.

*C. michiganensis* subsp. *sepedonicus*. It is tempting to speculate that, by analogy to the function of *CmmAMP-I*, this *C. michiganensis* subsp. *sepedonicus* protein could be a selective inhibitor of *C. michiganensis* subsp. *michiganensis*. Despite quite massive attempts, as described above, we have not been able to demonstrate such an activity. Recombinant production of the protein failed, whereas we could not detect activity against *C. michiganensis* subsp. *michiganensis* in culture supernatants of *C. michiganensis* subsp. *sepedonicus*. The present results show that more work on the potential *C. michiganensis* subsp. *sepedonicus* AMP (*CmsAMP*) is warranted. It is quite likely that this protein will be produced and secreted under certain conditions and that it will have an antimicrobial activity, albeit perhaps not against *C. michiganensis* subsp. *michiganensis*.

*CmmAMP-I* has a strong antibacterial activity *in vitro*, specifically inhibiting the growth of the ring rot pathogen by killing it. Studies of phytotoxicity showed no apparent toxic effect on potato (or tomato) plants when droplets of *CmmAMP-I* were applied with concentrations approximately 5 orders of magnitude higher than the  $IC_{50}$ . Hence, *in vitro* data suggest that *CmmAMP* is a good candidate for potential management of potato ring rot. Since there currently are no direct tools available to combat potato ring rot, the commercial potential of a new, environmentally friendly combat strategy is significant. Studies on other systems (30) indicate that one could consider using the producing organism itself as a biocontrol agent. This, however, is not possible because *C. michiganensis* subsp. *michiganensis* has the status of a serious plant pathogen itself. This leaves two potential application routes for *CmmAMP-I*. One is to overproduce the bacteriocin and apply it directly to plants and seed potatoes, a strategy that has worked well for other bacterial plant diseases (31). The other is to develop transgenic potatoes that express *CmsAMP-I* and hence are likely to be resistant to *C. michiganensis* subsp. *sepedonicus*. The latter strategy is well established in potato (32).

## ACKNOWLEDGMENTS

This research was supported by two grants from The Research Council of Norway (grants 148024 and 199718). P.M. thanks the China Scholarship Council for a visiting scholarship.

We thank Vinh Hong Le for performing the phytotoxicity test.

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