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Human- and Plant-Pathogenic *Pseudomonas* Species Produce Bacteriocins Exhibiting Colicin M-Like Hydrolase Activity towards Peptidoglycan Precursors▽

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Genes encoding proteins that exhibit similarity to the C-terminal domain of *Escherichia coli* colicin M were identified in the genomes of some *Pseudomonas* species, namely, *P. aeruginosa*, *P. syringae*, and *P. fluorescens*. These genes were detected only in a restricted number of strains. In *P. aeruginosa*, for instance, the colicin M homologue gene was located within the ExoU-containing genomic island A, a large horizontally acquired genetic element and virulence determinant. Here we report the cloning of these genes from the three *Pseudomonas* species and the purification and biochemical characterization of the different colicin M homologues. All of them were shown to exhibit Mg^{2+}-dependent diphosphoric diester hydrolase activity toward the two undecaprenyl phosphate-linked peptidoglycan precursors (lipids I and II) in vitro. In all cases, the site of cleavage was localized between the undecaprenyl and pyrophospho-MurNAc moieties of these precursors. These enzymes were not active on the cytoplasmic precursor UDP-MurNAc-pentapeptide or (or only very poorly) on undecaprenyl pyrophosphate. These colicin M homologues have a narrow range of antibacterial activity. The *P. aeruginosa* protein at low concentrations was shown to inhibit growth of sensitive *P. aeruginosa* strains. These proteins thus represent a new class of bacteriocins (pyocins), the first ones reported thus far in the genus *Pseudomonas* that target peptidoglycan metabolism.

Certain *Escherichia coli* strains produce and release in the growth medium toxins designated colicins in order to kill competitors belonging to the same species or to related species (8, 28, 29). The various modes of action of colicins include formation of pores in the cytoplasmic membrane, inhibition of protein synthesis, enzymatic degradation of cellular DNA or 16S rRNA, and interference with cell envelope biosynthesis. Colicins and proteins conferring immunity to the producer are generally encoded by plasmids. Depending on the import pathway they use to enter the cells, the Tol or TonB system, the colicins have been classified in group A or B, respectively. Their lethal action occurs in three steps: binding to a specific outer membrane receptor protein, translocation through the cell envelope, and interaction with the target leading to the bactericidal effect. A specific protein domain corresponds to each of the three steps, and the different colicins display a similar three-domain structural organization (8).

Colicin M (ColM) exhibits a unique mode of action, as it is the only colicin known to interfere with peptidoglycan biosynthesis and to cause cell lysis (35). This class B colicin is internalized via the TonB translocation machinery and uses the FhuA outer membrane protein as the receptor. ColM lacks peptidoglycan-degrading activity and acts synergistically with β-lactam antibiotics, which inhibit the last polymerization step of peptidoglycan synthesis performed by penicillin-binding proteins (34, 35). Since ColM inhibited both peptidoglycan synthesis and lipopolysaccharide O-antigen synthesis, the target of colicin was assigned to a common step of the two pathways, tentatively the dephosphorylation of the undecaprenyl pyrophosphate (C_{55}-PP) lipid carrier (17, 19). However, inhibition of O-antigen synthesis was more recently shown to be an indirect consequence of the action of ColM on peptidoglycan precursors (12). In vitro and in vivo analyses showed that ColM was an enzyme that catalyzed the hydrolysis of the peptidoglycan lipid intermediates, C_{55}-PP-MurNAc-pentapeptide (lipid I) and C_{55}-PP-MurNAc(-pentapeptide)-GlcNAc (lipid II). The site of cleavage was located between the C_{55} and pyrophospho groups of these precursors. The corresponding degradation products, namely, undecaprenol (C_{55}-OH) and either 1-pyrophospho-MurNAc-pentapeptide or 1-pyrophospho-MurNAc-(-pentapeptide)-GlcNAc, that normally do not exist in *E. coli* cells, were shown to accumulate in ColM-treated cells (12). The hydrolysis of the peptidoglycan lipid precursors resulted in an arrest of the polymerization steps and subsequent loss of the cell integrity. The depletion of the pool of carrier lipid C_{55}-P that results from its progressive conversion into the "inactive" C_{55}-OH form explained why O-antigen synthesis was concomitantly blocked in ColM-treated cells.

Genes of unknown function present in the chromosome of

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence[a]
PaeO1	5′-GTGATCC**ATGG**TTATGGATCTTGGAACTACCACTATAGTC-3′ (NcoI)
PaeO2	5′-TGTTA**AGATCT**ACCAGAAATATTTACAGGGATAGCTC-3′ (BglII)
PflO1	5′-AGGA**GGATCC**ATGGAATTCGAGCTTCCAGCTACTTATGTATATC-3′ (BamHI)
PflO2	5′-GCCG**AAGCTT**CACCTATCGGGCGTAGCTAATAGGGAGCTC-3′ (HindIII)
PsyO1	5′-GGTA**GGATCC**ATGCCTATTGAGCTTCCTCCGACATACATCACCC-3′ (BamHI)
PsyO2	5′-CTGG**GAGCTC**CACTGCTCAGGCGCTACCTGTCATGCCTTTGAC-3′ (SacI)

[a] Restriction sites (in boldface type) introduced in oligonucleotide primers are indicated in parentheses. The initiation codons of the genes are underlined.

particular *Pseudomonas* species that encode proteins exhibiting some sequence similarity in their C-terminal regions with ColM were identified. These proteins were overexpressed in *E. coli*, purified to homogeneity, and biochemically characterized. Their enzymatic nature and capability to catalyze the specific degradation of peptidoglycan precursors are reported here.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strain DH5α (*supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* ϕ80 Δ*lacZ*ΔM15) (Bethesda Research Laboratories) was used as the host for propagation of plasmids, and strain C43(DE3) (Avidis) was used for the production of proteins. The *exoU*-containing *Pseudomonas aeruginosa* strains JJ692, 6077, X13273, 19660, and S54485 (20), the *Pseudomonas syringae* pv. tomato strain DC3000 (10), and the cosmid 2E5 carrying the *Pseudomonas fluorescens* ColM homologue gene (22) were kindly provided by A. Filloux (LISM-CNRS, Marseille, France), M. Langlois-Meurinne (IBP, Orsay, France), and D. Mavrodi (USDA-ARS, Pullman, WA), respectively. The plasmid vector pET2130, a pET21d (Novagen) derivative, was constructed by removal (filling-in) of the unique BglII site of pET21d and replacement of the BamHI-HindIII polylinker with that from the Qiagen pQE30 vector. Similarly, the pET2160 vector derived from pET21d by removal of the BglII site and replacement of the NcoI-HindIII polylinker with that from the Qiagen pQE60 vector. The pET2130 and pET2160 plasmids allow expression of proteins with a six-histidine tag (His_6) at the N- and C-terminal extremity, respectively. The pREP4groESL plasmid allowing overproduction of the bacterial chaperones was obtained from K. Amrein (1). Cells were grown in 2YT medium (23) at a temperature of 37°C except *P. syringae* pv. tomato (28°C). Ampicillin, kanamycin, chloramphenicol, and tetracycline were used at 100, 35, 25, and 12.5 μg/ml, respectively. Growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer.

General DNA techniques and *E. coli* cell transformation. PCR amplification of genes was performed in a Thermocycler 60 apparatus (Bio-med) using the Expand-Fidelity polymerase (Roche). DNA fragments were purified using the Wizard PCR Preps DNA purification kit (Promega). Standard procedures were used for digestion of DNA with restriction endonucleases, ligation, and agarose gel electrophoresis (32). Plasmid isolations were carried out by the alkaline lysis method (32), and *E. coli* cells were transformed with plasmid DNA by the method of Dagert and Ehrlich (11) or by electroporation.

Construction of expression plasmids. Plasmid pMLD245 allowing overproduction of the ColM homologue gene from *P. aeruginosa* was constructed as follows: PCR primers PaeO1 and PaeO2 (Table 1) were designed to incorporate NcoI and BglII sites at the 5′ and 3′ extremities of the gene, respectively. The gene was amplified from *P. aeruginosa* strain JJ692 or 6077 (20), treated with NcoI and BglII, and ligated between the same sites of the vector pET2160. The resulting plasmid, pMLD245, allowed expression of the protein with a His_6 tag (Arg-Ser-His_6 extension) at the C-terminal extremity.

For the ColM homologue from *P. fluorescens*, the gene was amplified from cosmid 2E5, which carries a chromosomal fragment from strain Q8r1-96 (22), using the Pfl1 and Pfl2 primers (Table 1). The resulting fragment was digested with BamHI and HindIII and then inserted into the same sites of the pET2130 vector. The plasmid thus generated, pMLD268, allowed expression of the protein with a Met-His_6-Gly-Ser N-terminal extension.

Plasmid pMLD261 allowing overproduction of the ColM homologue gene from *P. syringae* was similarly constructed by amplifying a fragment of the chromosome of strain DC3000 with primers PsyO1 and PsyO2 (Table 1). The fragment gene was cloned into the pET2130 vector using BamHI and SacI. The resulting plasmid, pMLD261, allowed expression of the protein with a Met-His_6-Gly-Ser N-terminal extension.

In all cases, DNA sequencing was performed to confirm that the sequence of the cloned fragments was correct.

Preparation of crude extracts and purification of ColM homologues. *E. coli* C43(DE3) cells carrying plasmid pMLD245, pMLD268, or pMLD261 were grown at 37°C in 2YT medium containing ampicillin (1-liter cultures). For production of the *P. syringae* protein, host cells also carried the pREP4groESL plasmid encoding the GroES and GroEL bacterial chaperones. When the optical density of the culture reached 0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and growth was continued overnight at 37°C (pMLD245) or 22°C (pMLD268 and pMLD261). Cells were harvested and washed with 40 ml of cold 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM β-mercaptoethanol and 200 mM NaCl (buffer A). The cell pellet was suspended in 12 ml of the same buffer, and cells were disrupted by sonication in the cold (Bioblock Vibracell sonicator; model 72412). The resulting suspension was centrifuged at 4°C for 30 min at 200,000 × *g* in a TL100 Beckman centrifuge, and the supernatant (~200 mg of proteins) was stored at −20°C.

One-step purification of the His_6-tagged proteins was performed under native conditions following the manufacturer's recommendations (Qiagen): crude soluble protein extracts obtained as described above were incubated for 1 h at 4°C with nickel-nitrilotriacetate (Ni^{2+}-NTA) agarose preequilibrated in buffer A containing 10 mM imidazole. The resin was then washed extensively with buffer A containing 20 mM and 40 mM imidazole. Proteins eluted with 100 and 200 mM imidazole were pooled, concentrated by ultrafiltration (10,000 Amicon Ultra-15 centrifugal filter; Millipore), and dialyzed overnight against 100 volumes of buffer A. The final preparations were stored at −20°C after the addition of 10% glycerol.

In order to determine the oligomerization state of these proteins, samples of the purified preparations (8 to 10 mg) were loaded on Hi-Load 16/60 Superdex 75 (Amersham Pharmacia Biotech), and elution was performed with buffer A at a flow rate of 1 ml/min. The column was calibrated with β-amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, cytochrome *c*, and vitamin B_{12}.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins was performed by the method of Laemmli and Favre (21). Protein concentration was determined by the method of Bradford (5), with bovine serum albumin as the standard, or by quantitative amino acid analysis with a Hitachi model L8800 analyzer (ScienceTec) after hydrolysis of samples for 24 h at 105°C in 6 M HCl containing 0.05% 2-mercaptoethanol.

Hydrolase activity assays. The enzymatic activity of ColM and its different homologues was tested in a reaction mixture (10 μl) containing 100 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 12 μM of ^{14}C-radiolabeled lipid II (140 Bq), and 0.2% *n*-dodecyl-β-D-maltoside. The reaction was initiated by the addition of the purified protein (from 2 ng to 10 μg in 5 μl of buffer A), and the reaction mixture was incubated for 2 h at 37°C with shaking (Thermomixer; Eppendorf). For the determination of Michaelis constants (K_m), assay conditions were as described above except that the concentration of lipid II varied from 6 to 200 μM. The reaction was stopped by heating at 100°C for 1 min and analyzed by thin-layer chromatography (TLC) on LK6D silica gel plates (Whatman) using 1-propanol–ammonium hydroxide–water (6:3:1, vol/vol/vol) as the mobile phase. The radioactive spots were visualized with a Storm 860 PhosphorImager and were located and quantified with a radioactivity scanner (model Multi-Trace-master LB285; Berthold, France). Under these conditions, the radiolabeled substrate (lipid II) and its product (1-pyrophospho-MurNAc[-pentapeptide]-GlcNAc) generated by ColM migrated with R_f values of 0.7 and 0.3, respectively. Similar assays were performed that used ^{14}C-radiolabeled lipid I or UDP-MurNAc-pentapeptide (labeling in the L-Ala residue). When ^{14}C-labeled C_{55}-PP

was tested as the substrate, a different solvent system was used for TLC analysis of reaction mixtures as described previously (12, 13).

Identification of lipid I and lipid II degradation products by HPLC. Standard assays were performed as described above, except that higher quantities of the different ColM homologues (10 to 75 μg) were used to allow a complete degradation of the radiolabeled lipid intermediates I and II (0.5 nmol). The reaction mixtures were diluted into 1 ml of 50 mM sodium phosphate buffer (pH 4.5) and applied onto a C_{18} column (Nucleosil 100C_{18} column; Alltech) (5 μm; 4.6 by 250 mm). Elution was performed at a flow rate of 0.6 ml/min, with a linear gradient of methanol (0 to 20%) in 50 mM sodium phosphate buffer (pH 4.5) that was applied between 30 and 60 min. In these conditions, the lipid I and lipid II degradation products generated by ColM (12), namely, 1-pyrophospho-MurNAc-pentapeptide and 1-pyrophospho-MurNAc(-pentapeptide)-GlcNAc, were eluted at 16 and 34 min, respectively. Different compounds generated by chemical or enzymatic treatments of lipids I and II were used as references in the high-performance liquid chromatography (HPLC) analyses. In the same conditions, the two anomers (α and β) of MurNAc-pentapeptide were eluted at 30 and 47 min, respectively, and 1-phospho-MurNAc-pentapeptide was eluted at 24 min. The α and β anomers of MurNAc(-pentapeptide)-GlcNAc obtained by mild acid hydrolysis of lipid II were eluted at 48 and 53 min, respectively.

Antibacterial spectrum of colicin M and its homologues from *Pseudomonas* species. 2YT top agar (3 ml) was inoculated (10^8 cells) with the tested strains and poured onto 2YT agar plates. Samples of the purified ColM homologues (2 μl) were spotted onto the surface of the top agar, and inhibition of growth was recorded after 24 h of incubation at 37°C.

The activity of the *P. aeruginosa* bacteriocin was also tested on 2YT liquid cultures of the *P. aeruginosa* DET08 strain. Briefly, 2YT broth (6 ml) was inoculated with an overnight culture (0.3 ml), and the bacteriocin (0, 2, 6, 20, 60, 200, and 300 μg/ml) was added at a optical density at 600 nm of 0.4.

MALDI-TOF mass spectrometry. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were recorded in the linear mode with delayed extraction on a PerSeptive Voyager-DE STR instrument (Applied Biosystems) equipped with a 337-nm laser. Buffer and glycerol were removed from the samples by using a ZipTip C_4 (Millipore) according to the manufacturer's recommendations with slight modifications. Briefly, the bacteriocins were adsorbed on ZipTip, and after the bacteriocins were washed with 0.1% trifluoroacetic acid (TFA), they were eluted with 7.5 μl of 0.1% TFA in 70% acetonitrile. Subsequently, 1 μl of matrix solution (10 mg/ml sinapinic acid in 0.1% TFA–acetonitrile [70:30, vol/vol]) was deposited on the plate, followed by 0.5 or 1 μl of concentrated proteins. After evaporation of the solvents, spectra were recorded in the positive mode at an acceleration voltage of +25 kV and an extraction delay time of 300 ns. Enolase was used as an external calibrant.

Chemicals. C_{55}-PP, C_{55}-P, and C_{55}-OH were provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, and [^{14}C]C_{55}-PP was synthesized as described earlier (13). UDP-[U-^{14}C]GlcNAc (9.85 to 11.1 GBq · mmol^{-1}) was purchased from Amersham Pharmacia Biotech, and ^{14}C-lipid II labeled in the GlcNAc moiety was prepared as previously described (12) using pure MraY and MurG transferases (3, 9). The lipid II used in this work was C_{55}-PP-MurNAc(-L-Ala–γ-D-Glu–*meso*-A_2pm–D-Ala–D-Ala)-GlcNAc, where A_2pm represents diaminopimelic acid. The ^{14}C-radiolabeled forms of UDP-MurNAc-pentapeptide and lipid I (labeling in the L-Ala residue) were synthesized as described previously (3, 12). *n*-Dodecyl-β-D-maltoside was purchased from Fluka, and Ni^{2+}-NTA agarose was from Qiagen. Antibiotics and reagents were from Sigma. Oligonucleotides and DNA sequencing were done by MWG-Biotech.

RESULTS

Identification of genes encoding colicin M homologues in the genomes of *Pseudomonas* species. Genes encoding ColM homologues were identified in certain strains of *P. aeruginosa*, *P. fluorescens*, and *P. syringae*. Interestingly, as will be discussed below, not all the strains but only particularly virulent ones carried the ColM homologue gene in their genome (20, 22, 38). Among bacteria belonging to the genus *Pseudomonas* whose genomes have been fully sequenced, only the strain *P. syringae* pv. tomato DC3000 was shown to contain this gene. The sizes of these putative proteins (289, 271, and 276 residues, respectively) and of ColM from *E. coli* (271 residues) were quite similar. An alignment of the amino acid sequences showed that homologies were mainly found in their C-terminal region, i.e., the region that is known to contain the activity/catalytic domain in ColM (26) and colicins in general (8) (Fig. 1). On the basis of the recently determined crystal structure of *E. coli* ColM (40), this domain should start around residue 140 and thus comprises approximately the second half of the protein sequence. Thirty-six to 42% identity was observed in this region, with a total of 28 invariant residues found in the four sequences (Fig. 1). No significant similarity and no invariant residue were detected in the N-terminal and central regions of these proteins, which are required in ColM for binding to the FhuA receptor and translocation through the cell envelope, respectively.

Overproduction and purification of the *Pseudomonas* colicin M homologues. The *P. aeruginosa* gene was amplified from the *P. aeruginosa* JJ692 and 6077 *exoU*-containing clinical isolates (20), and the two sequences were identical to the one found in databases. The *P. syringae* gene was amplified from the chromosome of the *P. syringae* pv. tomato strain DC3000 (7), and the *P. fluorescens* gene was amplified from a cosmid carrying a chromosomal fragment from strain Q8r1-96 (22). The three genes were cloned into pET expression vectors to generate translational fusions with sequences specifying N- or C-terminal His_6 tags. Inducible expression of the three genes was not associated with lysis of the *E. coli* host. Upon induction at 37°C, SDS-PAGE analysis of crude cell extracts showed accumulation of proteins of about 30 kDa in agreement with the calculated masses of 32.0, 29.4, and 29.6 kDa for the proteins from *P. aeruginosa*, *P. fluorescens*, and *P. syringae*, respectively (data not shown). Although the *P. aeruginosa* ColM-like protein was soluble, ColM-like proteins from *P. fluorescens* and *P. syringae* were almost exclusively found in inclusion bodies. This problem was overcome by inducing the genes at 22°C in an *E. coli* strain producing the GroESL chaperones. The three proteins recovered in soluble form were purified by affinity chromatography on Ni^{2+}-NTA agarose with an overall yield of ca. 15 to 20 mg per liter of culture. The proteins were at least 95% pure, as judged by SDS-PAGE (Fig. 2). MALDI-TOF mass spectrometry analyses revealed peaks at *m/z* 33,081, 30,533, and 30,716 for the $[M+H]^+$ ions, in agreement with the calculated values, 33,093, 30,532, and 30,720, for the *P. aeruginosa*, *P. fluorescens*, and *P. syringae* proteins, respectively (His_6 tag included). An additional peak at *m/z* 32,963 was detected in the protein preparation from *P. aeruginosa*, suggesting partial loss of the N-terminal methionine.

Gel filtration experiments showed that ColM and its *P. fluorescens* homologue eluted as monomers. A dimer and a mixture of dimer (20%) and monomer (80%) were detected for the homologues from *P. syringae* and *P. aeruginosa*, respectively. No higher-molecular-mass species or protein aggregates were observed using this technique (data not shown), confirming that these protein preparations were soluble and homogenous.

Enzymatic properties of the *Pseudomonas* colicin M homologues. The ColM homologue proteins purified from *Pseudomonas* species were tested for their capability to catalyze in vitro the degradation of the peptidoglycan lipid II intermediate, as established previously for *E. coli* ColM (12). As the peptidoglycan composition and structure of its precursors are identical in *E. coli* and *Pseudomonas* species (36), the classical A_2pm-containing lipid II synthesized using the *E. coli* enzymes was used as the substrate in these assays. It was shown, using

A

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Ecoli   ----METLTVHAPSPSTNLPSYGNGAFSLSAPHVPGAGPLLVQVVYSFFQSPNMCLQALT   56
Paeru   MVMDLGTTTIVAGQGDFSAYNYGNNGANWPAPSPAQVGYISKGQRDVAYVNGDWEKPLLA   60
Pfluo   MEFELPATYVYPDEYGTS------RSTGFTVPSPGAIVNRMVQERSAAYARGDWAPMLER   54
Psyri   MPIELPPTYITPYPEISAGGNGTYRGQDLSSGQSFPRGMQNPVATVLLLQGDLYCSPNCL   60
           :*::*:: :    ::  :::::  :::*::   *   :: :  ::: :::: :::

Ecoli   QLEDYIKKHGASN-----------PLTLQIISTNIGYFCNADRNLVLHPGISVYDAYHF-  104
Paeru   LWAQWANWGTTLYGYPSLPFPNYEFIWDIFDLPQADYNQFSLGDDRITAMNRAQNHQYP-  119
Pfluo   HLHAMHSNKPAYG-------------LDGFIMGDLKVAHGADLLLLKNPKLSTADAWNQG  101
Psyri   ATFQDQARRDSFG---------IQSKVALKTFAAADQREAEGRDLRTAYNEIATDIGRS-  110
         : :      : :              : ::   :::   ::::*:  :  :: *:
                    ▼
Ecoli   -AKPAPSQYDYRSMNMKQMSGNVTTPIVALAHYLWGNGAERSVNIANIGLKISPMKINQI  163
Paeru   -FPNNEGIPSWDRPKIDTFNGGVYTPAAAFAHYLTGKGKKMNFPIERLNIKPNVKAMPQF  178
Pfluo   IKEGSKIQNNAQLAVPTEFSGGTFTPFKAIQHWLLGNGNTASVQISRIGINPTPEKIPDL  161
Psyri   -QQINENIIKYP-PGNHVLSGGLMTPFHALAHGMFGLGAPLTFPIQNVGLNVDIRGIPDV  168
           :: :  :  :    :*G*: TP: A:*H:* G:G:  :::I ::*::: : :**:

Ecoli   KDIIKSG-VVGTFPVSTKFTHATGDYNVITGAYLGNITLKTEGTLTISANGSWTYNGVVR  222
Paeru   IGVLTSS-PMGQTTVDFNVPYATAKDSWVAGNTVGEITLRIVGILVKSTSGQWSFRGEIR  237
Pfluo   MAIINTA-RIGETTVNFNTSYYTGQDSVIPRIYLGTITLNITGKITRNTSGTVSFSGVVK  220
Psyri   MNVIQSARPVGTSSLDVNFAYDVGKDSNASWLTLGNITLRLVGTIDKNASGAWTFSGEIR  228
        : :* *: ::G:::*::*: *:**:**:: : :*G:ITL:::G::::::*G *:*:G::*

Ecoli   SYDDKYDFNASTHRGIIGESLTRLGAMFS-----GKEYQILLPGEIHIKESGKR  271
Paeru   AYDDLYDFNPSNHRTETAEGMTRLGREVGQKFKDTTPYPIGIPGAIPVNISG--  289
Pfluo   AFSDRYDANASSHRTGFDEAATTALREVG-RVANAKDYAIQINGELPISYAR--  271
Psyri   AFNDVYDANPSNHRGWLGENLTSLLSAV-----PFTSYSIEIPGSLPVTVSGN-  276
        *::D YD:N:S:HR:  :E :T:*:::*:      : Y I **G::*:  **
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B

			% identity			
	Ecoli		Paeru		Pfluo	
	N-ter	C-ter	N-ter	C-ter	N-ter	C-ter
Paeru	17	35.6				
Pfluo	15.8	36.2	20	37.1		
Psyri	12.3	37.6	13.4	42.3	20	39.6

FIG. 1. Sequence alignment of the different colicin M homologues. (A) Sequences of *E. coli* colicin M (UniProtKB/Swiss-Prot accession number P05820) (Ecoli) and its homologues from *P. aeruginosa* (UniProtKB/TrEMBL accession number Q1W548) (Paeru), *P. fluorescens* (GenBank accession number EU982300; Dmitri Mavrodi, personal communication) (Pfluo), and *P. syringae* (UniProtKB/TrEMBL accession number Q88A25) (Psyri) were aligned using the ClustalW program. Amino acids that are invariant in the four proteins are indicated by the one-letter abbreviation for the amino acid below the sequences, and amino acids that are conserved in three or two of the proteins are indicated by asterisks and colons below the sequences, respectively. Gaps introduced to maximize alignment are indicated by dashes. (B) Percentages of identity of residues in the N- and C-terminal regions (N-ter and C-ter, respectively) of the ColM homologues are indicated for pair-wise comparisons. The black triangle in panel A indicates the limit of these regions that was chosen for this comparison.

the standard ColM assay conditions, that incubation of either of these three proteins with ^{14}C-lipid II labeled in the GlcNAc moiety resulted in the conversion of the substrate into a compound migrating much slowly on TLC plates, with a R_f of 0.3 compared to 0.7 for lipid II, highlighting the enzymatic nature of the three *Pseudomonas* proteins (Fig. 3). Further HPLC analyses of these reaction mixtures (see Materials and Methods) showed that the lipid II degradation products generated by these enzymes comigrated with authentic 1-pyrophospho-MurNAc(-pentapeptide)-GlcNAc (data not shown; see below for such an analysis with lipid I). The site of cleavage, identical for all these enzymes, was thus located between the C_{55} and pyrophospho moieties of lipid II.

Under the in vitro standard assay conditions used (12 μM lipid II), the specific activities of the *P. aeruginosa*, *P. fluorescens*, and *P. syringae* enzymes were estimated at 219 ± 34, 0.017 ± 0.003, and 1.07 ± 0.22 nmol/min/mg of protein, respectively. The value previously determined for *E. coli* ColM was 0.4 nmol/min/mg (12). Assays using lipid I instead of lipid II were also performed and yielded quite similar results. The activities determined for this substrate with the *P. aeruginosa*, *P. fluorescens*, and *P. syringae* enzymes were 215 ± 29, 0.007 ± 0.001, and 1.17 ± 0.23 nmol/min/mg of protein, respectively, indicating that the presence of the GlcNAc moiety was not essential for activity of the three ColM homologues. HPLC analyses of the reaction mixtures were performed (see Materials and Methods) that confirmed that the ^{14}C-labeled lipid I degradation product was 1-pyrophospho-MurNAc-pentapeptide in all cases (Fig. 4). None of these enzymes exhibited activity on the nucleotide precursor UDP-MurNAc-pentapeptide. Although C_{55}-PP did not appear to be a substrate in the standard assay conditions, prolonged incubation (16 h) with large quantities of the *P. aeruginosa* enzyme (90 μg) finally resulted in formation of C_{55}-OH. These results showed that both the C_{55} and MurNAc-peptide moieties of the substrate were important for enzyme activity. The activity of ColM and

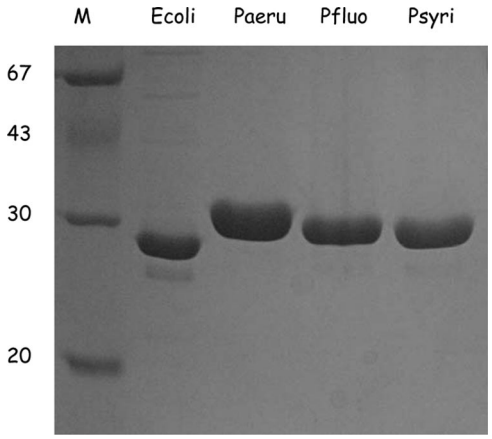

FIG. 2. Purification of ColM homologues from *Pseudomonas* species. The different ColM homologues were overproduced in His_6-tagged form and were purified by affinity chromatography on Ni^{2+}-NTA agarose. The lanes contain ColM from *E. coli* (Ecoli) (12) and purified preparations of its three homologues from *P. aeruginosa* (Paeru), *P. fluorescens* (Pfluo), and *P. syringae* (Psyri). Lane M contains molecular mass standards, namely, bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa). Staining was performed with Coomassie brilliant blue R250.

of the three homologues was dependent on the presence of Mg^{2+}, since no activity was observed when the divalent cation was omitted from the reaction mixture or when EDTA was added (data not shown).

The K_m for lipid II and k_{cat} value were determined for each enzyme (Table 2). The *P. aeruginosa* enzyme was clearly the most active one, catalyzing the hydrolysis of lipid II about 600-fold more rapidly than its *E. coli* homologue did. The K_m values of the *P. fluorescens* and *P. syringae* enzymes were four- to fivefold higher than those of the *E. coli* and *P. aeruginosa* homologues. The *P. fluorescens* enzyme exhibited the lowest activity, its catalytic efficiency (k_{cat}/K_m) being 15-fold lower than that of the *E. coli* ColM.

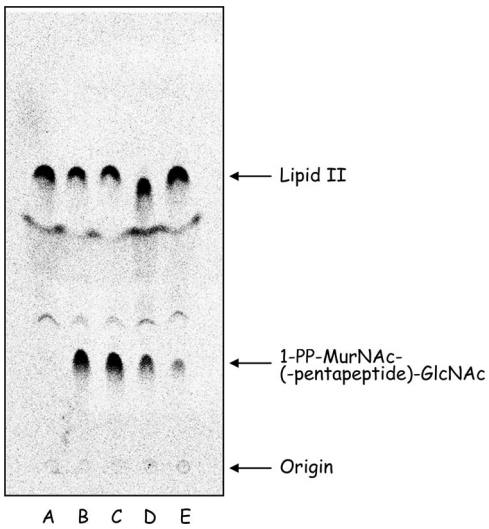

FIG. 3. In vitro degradation of the peptidoglycan lipid II intermediate by ColM-like proteins from *Pseudomonas* species. The activity of ColM and its different homologues was tested under standard assay conditions using ^{14}C-lipid II labeled in the GlcNAc moiety as the substrate. Conditions that result in partial (~20 to 50%) conversion of lipid II into 1-pyrophospho-MurNAc(-pentapeptide)-GlcNAc product were used. The radiolabeled substrate and product were separated by TLC (R_f values were 0.7 and 0.3, respectively,) and spots were visualized with the PhosphorImager. Lane A, lipid II incubated in the absence of ColM; lanes B to E, lipid II incubated in the presence of *E. coli* ColM (0.4 μg) or one of the ColM homologues from *P. aeruginosa* (2 ng), *P. fluorescens* (28 μg), and *P. syringae* (0.1 μg), respectively.

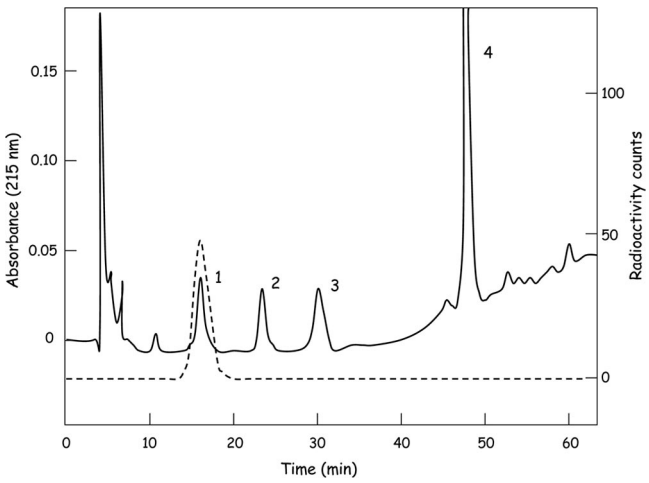

FIG. 4. Identification of lipid I degradation product by HPLC. L-[^{14}C]alanine-labeled lipid I was digested to completion by colicin M and the three homologues from *Pseudomonas* species, and the reaction mixtures were analyzed by HPLC as described in Materials and Methods. The solid line (detection at 215 nm) depicts analysis of a standard mixture of 1-pyrophospho-MurNAc-pentapeptide (peak 1), 1-phospho-MurNAc-pentapeptide (peak 2), and MurNAc-pentapeptide (peaks 3 and 4; anomeric forms β and α, respectively). The broken line (detection of radioactivity) depicts analysis of the reaction mixture following incubation of ^{14}C-labeled lipid I with the *P. aeruginosa* enzyme, showing the formation of a single radiolabeled product comigrating with authentic 1-pyrophospho-MurNAc-pentapeptide. The same results were obtained with ColM and the *P. fluorescens* and *P. syringae* homologue proteins, and no radiolabeled product was observed in the absence of enzyme (data not shown).

Antibacterial spectra of colicin M homologues. The activity of the three purified enzymes from *Pseudomonas* spp. and ColM from *E. coli* was tested on a collection of strains of *P. aeruginosa* ($n = 14$), *P. fluorescens* ($n = 4$), and *P. syringae* ($n = 24$) (Table 3), as well as on classical laboratory strains of *E. coli*. Among the *Pseudomonas* spp., only two strains of *P. aeruginosa* were found to be inhibited: strain NCK007, which was inhibited by the enzyme from *P. aeruginosa*, and strain DET08, which was inhibited by the enzymes from *P. aeruginosa* and *P. fluorescens* (Fig. 5A and data not shown). ColM did not inhibit any of the 42 *Pseudomonas* strains and was active on all

TABLE 2. Kinetic parameters of *E. coli* ColM and homologues from *Pseudomonas* species

ColM homologue	K_m (μM) for lipid II	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$) ($10^3$)
E. coli	44 ± 4	0.055 ± 0.002	1.2 ± 0.1
P. aeruginosa	42 ± 6	32 ± 1	760 ± 110
P. fluorescens	150 ± 20	0.012 ± 0.001	0.080 ± 0.013
P. syringae	200 ± 30	0.52 ± 0.06	2.6 ± 0.5

TABLE 3. Spectrum of activity of the purified bacteriocins against *Pseudomonas* strains

Species[a]	No. of strains tested	No. of strains susceptible to the indicated bacteriocin			
		ColM from *E. coli*	*Pseudomonas* ColM homologue from:		
			P. aeruginosa	*P. fluorescens*	*P. syringae*
P. aeruginosa	14	0	2[b]	1[b]	0
P. fluorescens	4	0	0	0	0
P. syringae	24	0	0	0	0

[a] The strain collection tested comprised strains from three *Pseudomonas* spp.: *P. aeruginosa* strain ATCC 1045 and clinical isolates NCK001, -002, -003, -004, -005, -006, -007, -008, and -009 (Hôpital Necker); clinical isolates CLE08 and DET08 (Hôpital Européen Georges Pompidou); PA14 (CHU de Besançon); and PAO1 (LRMA); *P. fluorescens* CFBP strains 2392, 5755, 5756, and 5759 (Collection Française de Bactéries Phytopathogènes [CFBP], INRA); and *P. syringae* CFBP strains 1067, 1573, 1620, 1634, 1657, 1674, 1908, 2104, 2212, 2215, 2216, 2346, 2898, 2899, 3205, 3226, 3228, 8486, 10971, 11005, 11007, 11033, 11040, and 11056.

[b] Strain NCK007 was inhibited by the enzyme from *P. aeruginosa*, and strain DET08 was inhibited by the enzymes from *P. aeruginosa* and *P. fluorescens*.

classical *E. coli* laboratory strains tested (DH5α, JM83, and BW25113) except those (C600, BL21, C43…) that do not express a functional FhuA receptor. Inhibition of *P. aeruginosa* strain DET08 by the enzyme of *P. aeruginosa* was further tested in liquid medium (Fig. 5B). The enzyme inhibited growth (bacteriostatic effect) at a low concentration (6 μg/ml) but in contrast to what is observed with ColM on sensitive *E. coli* cells, no bacteriolytic effect was observed up to the highest enzyme concentration tested (300 μg/ml). It is well-known that antibiotics that inhibit peptidoglycan differ greatly in their capacity to cause lysis of different bacteria. The difference in the capacity of the ColM orthologues to lyse their respective *E. coli* and *Pseudomonas* target cells could likely be due to differences in the regulation of the autolysin complements in these bacterial species.

DISCUSSION

ColM is one of the smallest colicins known thus far (8). It was earlier established that this group B (TonB-dependent) colicin interfered with both peptidoglycan and lipopolysaccharide O-antigen biosynthesis pathways (17, 19, 35), but its precise mode of action was elucidated only recently (12). ColM exerts its cytotoxic effect through enzymatic degradation of undecaprenyl-phosphate-linked peptidoglycan intermediates (lipids I and II). It is the only known colicin that targets peptidoglycan and the only one whose bacteriolytic effect can be controlled by the osmolarity of the growth medium (6). It should also be noted that unlike the other enzymatic colicins, ColM is not released in complex with its immunity protein (ImM, also named Cmi) (8). The ImM protein is located in the periplasm and anchored to the outer side of the cytoplasmic membrane (15, 24), i.e., in the region where ColM exerts its cytotoxic activity. *E. coli* and related species are not the only bacteria to produce bacteriocins to kill neighboring bacteria. Toxins with similar domain organizations and infection characteristics have been described in other species (8). Interestingly, a pesticin of *Yersinia pestis* was earlier described that shared with ColM the capability to convert sensitive cells into osmotically stable spheroplasts (16) and to have its immunity protein located in the periplasm (27). This pesticin contained a typical TonB box (27) and was thus assigned to the group B colicins (14). Evidence that it displayed a muramidase activity and thus exerted its bacteriolytic effect through the specific degradation of the peptidoglycan polymer was then provided (37).

The pyocins characterized in this work constitute a novel class of bacteriocins interfering with peptidoglycan metabolism. The purification and biochemical characterization of these ColM homologues from *P. aeruginosa*, *P. fluorescens*, and *P. syringae* showed that they all display similar enzymatic activities of degradation of the peptidoglycan lipid intermediates in vitro. High-level expression of these ColM orthologues in *E. coli* cells was not toxic and did not result in cell lysis. It confirmed the previous observation that ColM exhibits its bactericidal properties only when entering the cell from outside (18). ColM could be effectively expressed in the absence of immunity protein ImM if the producing strain does not express a functional outer membrane receptor FhuA (12, 18). There is clearly no need for protection against the intracellular ColM. The lipid intermediates I and II, the ColM targets, are synthesized by the MraY and MurG enzymes on the inner side of the cytoplasmic membrane and are subsequently translocated to the outer side of the membrane where the polymerization steps occur (4). The absence of toxic effects therefore implies that the lipids located on the cytoplasm side of the membrane are not accessible to ColM-like proteins. They might be protected within the active sites of MraY and MurG and rapidly transferred to the outer leaflet of the membrane. The immunity protein ImM, which is essential in ColM producers expressing the FhuA receptor, is known to be attached to the inner membrane (N-terminal hydrophobic sequence from amino acids 1 to 23) and to be exposed toward the periplasmic space (24). Thus, the localization of the immunity protein also suggests that protection of the lipids is not needed in the cytoplasm.

The genes encoding this specific class of pyocins were detected in the chromosome from only a limited number of *Pseudomonas* strains. Interestingly, these particular strains

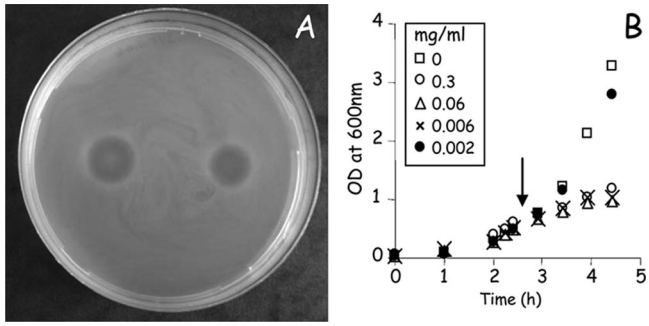

FIG. 5. Effect of the ColM homologue from *Pseudomonas aeruginosa* on the growth of *P. aeruginosa* DET08. (A) Two microliters of the purified bacteriocin (4 μg) were loaded in duplicate at the surface of a 2YT agar plate that was previously inoculated with the indicator strain *P. aeruginosa* DET08. Diffusion of the enzyme inhibited growth, resulting in darker zones. (B) *P. aeruginosa* DET08 was grown at 37°C in 2YT medium, and the purified bacteriocin was added at various concentrations (mg/ml) as indicated by the arrow. OD, optical density.

were often described to exhibit some advantages in terms of growth or virulence properties over their congeners. In *P. aeruginosa*, this gene was found within an 80-kb genomic island (ExoU island A) that carries the *exoU* gene coding for a potent cytotoxin with phospholipase A2 activity (20, 33). The genome of *P. aeruginosa* is known to be highly conserved, and remarkably, a core set of genes encoding nearly all known virulence factors was shown to be maintained in all clinical and environmental isolates (39). The ExoU island, however, was present only in particular strains and was located in a highly polymorphic region of the chromosome where insertion and rearrangement of genetic material frequently occurs (20). It was suggested that the acquisition of this virulence determinant through horizontal gene transfer may enhance colonization and survival in different host environments. Within this island, the open reading frame EXA13 was annotated as a hypothetical protein with homology to the C terminus of colicin M (20). We demonstrated in the present work that this gene is functional and encodes a protein with biological activity. The advantage given to *P. aeruginosa* strains by the expression of this particular gene remains to be established. The library of transposon insertions in the ExoU island A that was generated by Kulasekara et al. (20) could be useful for such a functional analysis.

A gene for this type of bacteriocin was also detected in *P. fluorescens*, but here again only in a limited number of strains. A genomic subtraction method was used by Mavrodi and coworkers for exploring structural differences between the genomes of closely related fluorescent pseudomonads (22). The aim of this study was to identify the genes contributing to the exceptional rhizosphere competence of D-genotype strains that colonize roots and suppress soilborne diseases much more efficiently than others. DNA sequences present in the superior root colonizer *P. fluorescens* Q8r1-96 but not in the less competent strain Q2-87 were cloned and sequenced, and one of the 32 Q8r1-96-specific fragments thus identified was shown to exhibit sequence similarity with *E. coli* ColM (22). The product of this gene has now been purified, and its enzymatic activity of degradation of peptidoglycan precursors is demonstrated here. Its expression could potentially be beneficial and have an ecological role, as proposed for bacteriocins in general (30). As suggested by Mavrodi et al. (22), it may function in intraspecific interactions or the competitiveness of the Q8r1-96 strain in the rhizosphere. Interestingly, an analysis of the distribution of the aforementioned 32 subtracted fragments in a large series of *P. fluorescens* strains of different genotypes showed that the gene encoding this bacteriocin was particularly well distributed in strains exhibiting superior rhizosphere competence compared to the less efficient A-, B-, E-, and L-genotype strains.

The third ColM homologue gene was cloned from the genome of the bacterial pathogen *P. syringae* pv. tomato strain DC3000 (7) that in nature causes bacterial speck disease on tomato and can also infect *Arabidopsis* and *Brassica* species (41). This gene was not found in the *P. syringae* pv. syringae 728a and pv. phaseolicola 1448a strains whose genomes have been sequenced. *P. syringae* pv. tomato DC3000 has emerged as an important model organism for molecular studies of plant-pathogen interactions. A search for genes important for colonization, growth in planta, and disease production of strain DC3000 in *Arabidopsis thaliana* yielded genes for lytic murein transglycosylase and muropeptide transporter activities, indicating that functional peptidoglycan recycling is important for the virulence of plant pathogens (2). The physiological role of the bacteriocin identified in this work, which targets peptidoglycan metabolism, remains to be elucidated. As is the case for the other bacteriocins described in this paper, it can be speculated that increased degradation of the peptidoglycan polymer and the release of its fragments in the external medium that may occur in strains producing these pyocins could contribute in some way to the pathogenicity of these strains in their hosts. However, given the available data, the observed correlation between the expression of these ColM-like proteins and the virulence level of the *Pseudomonas* species could just be a coincidence. These bacteriocins may simply serve as anticompetitors, enabling the invasion of the bacteriocin-producing strain into microbial communities.

Colicins and other bacteriocins are known to exhibit a narrow range of killing activity because they parasitize receptors and translocation machineries that are specific of the bacterial species. They were therefore expected to play a role restricted to mediating within-species dynamics. In fact, it was shown that the killing breadth of the bacteriocins was not only limited within the species of the producer strain but could even specifically also target isolates of a different species (31). ColM and the three *Pseudomonas* proteins we have analyzed clearly have a narrow range of antibacterial activity. Although the *P. aeruginosa* enzyme was much more active in vitro (catalytically) than the *E. coli* ColM, it did not exhibit any effect on the growth of the various *E. coli* strains tested. This suggests that the *P. aeruginosa* protein could not parasitize the *E. coli* receptor and translocation machinery used by ColM. This protein was shown, however, to have a bacteriostatic effect at low concentrations on the growth of some *P. aeruginosa* strains. Similarly, ColM did not show any activity on the different strains of *Pseudomonas* species tested, but it was readily active on all classical *E. coli* strains tested, except those that do not express functional FhuA receptor or TonB translocation proteins. The finding that only a limited number of *P. aeruginosa* strains were sensitive to the *P. aeruginosa* bacteriocin was somewhat surprising, but whether *P. aeruginosa* species are the natural targets of this bacteriocin remains to be demonstrated.

The present discovery of ColM-like proteins produced by some *Pseudomonas* species now opens the way to detailed investigations on how these bacteriocins are released in the external medium, how they are imported into cells, and by which mechanisms the bacteriocin-producing strains resist them. Concerning the immunity mechanism, an analysis of the genomes of bacteriocin-producing strains revealed that putative immunity genes were located in the vicinity of the genes coding for these ColM orthologues. The sizes of the deduced products of these genes (140 to 150 amino acid residues) and ImM protein (117 residues) (25) are similar and display 15 to 25% identity in pair-wise alignments, although invariant residues were not detected (data not shown). These proteins could therefore be related and potentially exert a similar immunity function.

To our knowledge, only two other putative proteins from *Burkholderia* species (UniProtKB/TrEMBL accession numbers B1YRT6 and Q0BIYg) (40) were found in databases that exhibit some similarity with ColM and thus likely belong to the

same protein family. The recent elucidation of the mode of action of ColM and the detection of homologous genes in the genomes of pathogenic species constitute a favorable context for the development of a detailed and multidisciplinary study of this family of enzymatic colicins targeting the peptidoglycan metabolism. The catalytic mechanism of these enzymes should now be elucidated, as well as the mapping of their active site and the role of conserved amino acid residues. Furthermore, the fact that the targets of these enzymes (lipids I and II) are found in all types of bacteria and are specific for bacteria clearly opens the way toward a potential exploitation of these enzymes as broad-spectrum antibacterial agents.

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