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Heterologous Expression and Purification of Active Divercin V41, a Class IIa Bacteriocin Encoded by a Synthetic Gene in *Escherichia coli*

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Divercin V41, a class IIa bacteriocin with strong antilisterial activity, is produced by *Carnobacterium divergens* V41. To express a recombinant version of divercin V41, we constructed a synthetic gene that encodes the mature divercin V41 peptide and then overexpressed the gene in pET-32b by using the T7 RNA polymerase promoter in the *Escherichia coli* Origami (DE3)(pLysS) strain. The DvnRV41 peptide was expressed as a translational fusion protein with thioredoxin and accumulated in the cell cytoplasm in a soluble anti-*Listeria* active form. The fusion protein was then purified and cleaved to obtain pure, soluble, folded DvnRV41 (462 μg per 20 ml of culture). This paper describes the first design of a synthetic bacteriocin gene and the first bacteriocin expressed in the *E. coli* cytoplasm.

In the struggle for a niche and for nutrients, bacteria can produce peptides that serve as weapons to kill competing bacteria. In view of the decrease in the effectiveness of classical antibiotics due to the spread and increase of resistance mechanisms, antibacterial peptides are gaining further interest as potential antibiotics. Bacteriocins are a subgroup of antimicrobial peptides which were originally defined as proteinaceous compounds that kill closely related bacteria (59).

Although bacteriocins may be found in numerous gram-positive and gram-negative bacteria, those produced by lactic acid bacteria (LAB) have received particular attention due to their potential application in the food industry as natural preservatives (14). The inhibitory range of LAB bacteriocins is relatively narrow compared to that of their counterparts from eukaryotic cells, such as pleurocidin, which is active against both gram-negative and gram-positive bacteria (15). Among bacteriocins produced by LAB, the subclass IIa (also referred to as pediocin-like bacteriocins) is defined as a group of antilisterial, small, heat-stable, non-lanthionine-containing peptides consisting of 30 to 60 amino acids (<10 kDa) with the consensus sequence YGNGVxC in their N-terminal region (30). During the last decade, major advances have been made in highlighting the genetic and molecular basis of several class IIa bacteriocins (22).

Studies of the primary structure of class IIa bacteriocins have delineated two domains, a highly conserved hydrophilic N-terminal domain and an amphiphilic or hydrophobic C-terminal domain. Functionally, the N-terminal domain binds to target cells through electrostatic interactions (11) facilitating anchoring of the C-terminal domain to the hydrophobic core of the target cell membrane, leading to membrane leakage (44). Another relevant feature of class IIa bacteriocins is their cysteine content, and subsequently, their ability to form disulfide bridges. Pediocin PA-1/AcH (4), enterocin A (1), divercin V41 (43), sakacin G (56), and plantaricin 423 (60) are structured by two disulfide bridges, while the others possess only one disulfide bridge. Class IIa bacteriocins with two disulfide bridges are significantly more effective than those with only one (26). Moreover, the introduction of a second disulfide bridge within the C-terminal domain of sakacin P enhances the activity of recombinant sakacin P (a bacteriocin containing one disulfide bridge) 10- to 20-fold (24). The tryptophan residue at the carbonyl end is crucial for the antimicrobial activities of sakacin P and chemically synthesized mesenterocin Y105 (23, 25).

Divercin V41 is a class IIa bacteriocin produced by *Carnobacterium divergens* V41 that was isolated from fish and characterized in our laboratory (43, 47). The anti-*Listeria* activity of *C. divergens* V41 in cold smoked salmon has been investigated and is essentially due to its bacteriocin, divercin V41 (18, 49). The chromosomal *dvnV41* gene encodes a prebacteriocin of 66 amino acids whose 23-residue N-terminal extension is cleaved to yield the active mature 43-amino-acid divercin V41 of 4,509 Da with two disulfide bonds (43). Chemical modifications and enzymatic hydrolysis of divercin V41 have demonstrated that the C-terminal region (but not the N-terminal region) is necessary for antimicrobial activity and that disulfide reduction abolishes its inhibitory activity. The tryptophan residues were shown to be crucial for the antilisterial activity (6, 23).

All bacteriocins can be purified by standard methods of three, four, or more steps to obtain pure (nearly 100%) bacteriocins, but peptide recovery is generally low (about 100 μg liter of culture supernatant^{-1}) (42). The development of heterologous expression systems for bacteriocin production may offer several advantages over native systems, such as facilitating the control of bacteriocin gene expression or achieving increased production levels. The production and secretion of pediocin PA-1 was achieved in *Pediococcus pentosaceus* (12). The coproduction of pediocin PA-1 and enterocin A in *Lac-*

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TABLE 1. Strains, plasmids, and primers used for this study

Strain, plasmid, or primer	Characteristics[a] or sequence (5′→3′)	Source or reference
Strains		
C. divergens	Divercin V41 natural producer	47
Listeria innocua F	Indicator organism	DSV[b]
E. coli JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/e14$^-$ (*mcrA*) Δ(*lac pro*AB) *thi gyrA96* (*Nal*^{r}) *endA1 hsdR17* (r_K^- m_K^+) *relA1 supE44 recA1*	Stratagene
E. coli Origami (DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1 ahpC gor522*::Tn*10*(Tc^r) *TrxB*::Kan (DE3)	Novagen
Plasmids		
pPR16	Ap^r, cloning vector	21
pET-32b	Ap^r, expression vector	Novagen
pLysS	Cm^r	Novagen
pCR01	Ap^r, pPR16 with NcoI-HindIII fragment of double-stranded fragment 1	This work
pCR02	Ap^r, pCR01 with MscI-HindIII fragment of double-stranded fragment 2 insert	This work
pCR03	Ap^r, pET-32b with the NcoI-HindIII insert of pCR02	This work
Primers		
MO123	ATGCCATGGATCCGACCAAATATTACGGCAACGGTGTGTATTGCAACAGCAAAAAATGCTGG	This work
MO124	GCAAGCTTTGGCCAATGCAGCCGCTCGCCTGGCCCCAATCCACCCAGCATTTTTTGCTGTT	This work
MO125	TAGGTGGCCAGACCGTGGTTGGCGGTTGGCTGGGCGGTGCGA	This work
MO126	GTCAAGCTTAGCATTTGCCCGGAATCGCACCGCCCAGCCAAC	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.
[b] DSV, Direction des Services Vétérinaires, Nantes, France.

tococcus lactis IL-1403 has also been reported (39). The heterologous production of bacteriocins can also be achieved by exchanging leader peptides and/or dedicated ABC secretion and processing systems as well as by adding signal peptides recognized by general secretory pathways. The food-grade expression of genes encoding the secretion and processing machinery based, for example, on the inducible P*nisA* promoter (17, 31, 32) or the chloride-inducible P*gad* promoter (52, 53) could be another interesting approach for obtaining higher levels of heterologous bacteriocins (R. Kemperman, J. W. Sanders, G. Venema, and J. Kok, Abstr. Sixth Symp. Lactic Acid Bacteria, p. C80, 1999). Those heterologous systems so far developed for LAB bacteriocin production may also have some drawbacks. For example, low production levels have often been found. Consequently, *Escherichia coli* has been selected as a host for cloning a variety of genes involved in the biosynthesis of LAB bacteriocins. Three class IIa bacteriocins (piscicolin 126, mesentericin Y105, and pediocin PA-1) have been expressed in *E. coli*. These bacteriocins were secreted into the medium, but at a low production level, and they required fastidious purification processes (7, 10, 28, 40, 44, 61).

Every gene cannot be expressed efficiently in *E. coli*. This can be explained by many factors, including major differences in codon usage, the potential toxicity of the recombinant protein, structural features of the recombinant gene sequence, and the stability and translational efficiency of the mRNA (50). Synthetic DNA, using the recombinant producer organism alphabet, could be used to avoid most of these drawbacks.

Our goal was to develop a genetic tool that could easily be used to produce and purify large quantities of a pure and active bacteriocin. In this paper, we report the construction of a synthetic gene in an efficient bacterial expression system that was successfully used to obtain significant levels of highly active, soluble, and pure recombinant divercin V41 (DvnRV41). As far as we know, the present work is the first to focus on the design and expression of a synthetic gene encoding a bacteriocin and the first system developed for the production of a class IIa bacteriocin in the *E. coli* cytoplasm.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and bacteriocin activity determination. *E. coli* K-12 strain JM109 (Stratagene, Austin, Tex.) was used for standard cloning procedures, and *E. coli* K-12 strain Origami (DE3)(pLysS) (Novagen, Madison, Wis.) was used for gene expression experiments (Table 1). *E. coli* strains were grown aerobically in Luria-Bertani (LB) or Terrific-Broth medium at 37°C (51). Competent cells of *E. coli* were prepared and transformed by the transformation and storage solution procedure described by Chung et al. (13). The plasmids pPR16 (21) and pET-32b (Novagen) were used for gene construction and expression, respectively. Transformants containing pPR16 or its derivatives, pCR01 and pCR02, were selected on LB agar medium containing ampicillin (100 μg ml^{-1}) (Sigma, St. Louis, Mo.) and those containing pET-32b or its derivative pCR03 were selected on LB agar medium containing ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}) (Sigma). *C. divergens* V41 (47) (Table 1), used as a divercin V41 producer, was grown in MRS medium without Tween 80 (48) at 30°C without shaking. *Listeria innocua* F (Table 1), used as a divercin V41-sensitive indicator microorganism, was grown in Elliker medium (Biokar, Beauvais, France) at 30°C without shaking. The bacteriocin activity was determined by the agar diffusion test, performed as previously described (48). The bacteriocin activity was expressed in arbitrary units per ml (AU ml^{-1}) and was defined as the reciprocal of the lowest dilution that did not show growth inhibition of *Listeria innocua* F.

Oligonucleotide design and DNA manipulations. The DNA sequence of the synthetic divercin V41 gene (*dvnRV41*) was designed by using the *E. coli* K-12 codon usage tabulated from GenBank (45) and following the cloning strategy described in Results. The oligonucleotide primers used for this study were obtained from Invitrogen (Cergy Pontoise, France) and are listed in Table 1. Annealing was achieved with 2.5 μg of sense and antisense oligonucleotides and 2 μl of 10× annealing buffer (Clontech, San Jose, Calif.) used according to the manufacturer's instructions. The reaction mixture was incubated at 95°C for 5 min and then at 28°C (MO123 and MO124) or 42°C (MO125 and MO126) for 20 min and finally was kept on ice until used. Polymerization of the double-stranded DNA (dsDNA) fragments was obtained by using Klenow DNA polymerase according to the manufacturer's instructions. Plasmid DNAs and dsDNA fragments were isolated and purified by using Qiagen (Courtaboeuf, France) spin columns according to the manufacturer's recommendations. Restriction enzymes and Klenow DNA polymerase were obtained from New England Biolabs (Beverly, Mass.) and T4 DNA ligase was obtained from Promega (Charbonnieres, France). The nucleotide sequence of the cloned DNA located between the T7

terminator and the T7 promoter in pCR03 was determined by the dideoxynucleotide chain termination method (54) in an ABI 370 automated sequencer by use of a Taq Dye-Deoxy TM terminator cycle sequencing kit (Perkin-Elmer, Boston, Mass.) and the universal T7 terminator primer.

Expression of *dvnRV41* in *E. coli*. Overnight cultures of *E. coli* strain Origami (DE3)(pLysS) harboring the plasmid pCR03 or pET-32b were diluted to 3% (vol/vol) in Terrific-Broth medium containing ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}) and then were grown aerobically at 37°C. When the optical density at 600 nm (measured in a spectrophotometer; Biotek Instruments, Winooski, Vt.) reached 0.8, gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) to a concentration of 1 mM. The cells were grown for another 3 h, harvested by centrifugation (8,000 × *g*, 6 min, 4°C) (2K15 laboratory centrifuge; Sigma) at regular time intervals, and used for different experimental needs.

Recombinant and native divercin V41 purification procedures. The divercin V41 (DvnV41) produced by *C. divergens* V41 was purified as previously described (42). The recombinant divercin V41 (DvnRV41) was purified as follows. The cells from 20 ml of a 3-h *E. coli*/pCRO3 IPTG-induced culture were harvested by centrifugation (8,000 × *g*, 6 min, 4°C). The cell pellet was resuspended in 2 ml of binding buffer (BB) containing 10 mM imidazole (BB10; pH 7.9) (Amersham Biosciences, Freiburg, Germany). The cells were disrupted by sonication (Aerosec Industrie, Fecamp, France) in ice-cold water (225 W; five times for 2 min each) until the required visual viscosity was obtained. The separation of the cytoplasmic soluble fraction (CSF) from the cytoplasmic insoluble fraction and cell debris was performed by centrifugation (14,000 × *g*, 15 min, 4°C). The CSF was filtered (0.45-μm-pore-size filter; Sartorius, Goettingen, Germany) and then loaded directly onto a 1-ml nickel His-Trap chelating column (Amersham Biosciences). After loading, the column was successively washed with BB10, BB20 (20 mM imidazole, pH 7.9), and BB60 (60 mM imidazole, pH 7.9). The TRX-$(His)_6$-DvnRV41 fusion protein was eluted with 2 ml of BB500 (500 mM imidazole, pH 7.9). After this first immobilized metal-affinity chromatography (IMAC) purification step, the fraction containing the fusion protein was desalted against distilled water in a PD-10 column (Amersham Biosciences). The TRX-$(His)_6$-DvnRV41 fusion protein was cleaved with enterokinase EKMax according to the manufacturer's suggestion (Invitrogen). Imidazole and NaCl were added to the cleaved fusion protein mixture to final concentrations of 10 and 500 mM, respectively, and the pH was adjusted to 7.4 with 1 M HCl. The separation of DvnRV41 and TRX-$(His)_6$ from the fusion protein was achieved by a second IMAC step. The DvnRV41 was found in the flowthrough fraction and the noncleaved fusion protein and TRX-$(His)_6$ were found in the fraction eluted by BB500.

ELISA. Microtiter plates (Maxisorp; Nunc, San Diego, Calif.) were coated overnight at 37°C with 100 μl of the different protein samples diluted in 100 mM phosphate-buffered saline (PBS, pH 7.4). After this and each subsequent step, the coated microtiter wells were washed three times with PBS containing 0.05% (wt/vol) Tween 20 (Sigma) (PBS/T). Unoccupied sites in the wells were blocked by adding 250 μl of PBS/T containing 2% (wt/vol) freeze-dried low-fat milk (PBS/T/M) to each well and incubating the plates at 37°C for 1 h. Each well was filled with 100 μl of a polyclonal antiserum (anti-DvnCt-KLH) against the C terminus of divercin V41 (48) diluted 1:2,000 in PBS/T/M and then incubated at 37°C for 90 min. One hundred microliters of alkaline-phosphate-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:3,000 in PBS/T/M was added to each well, and the plates were incubated at 37°C for 1 h. Bound antibodies were detected by using 150 μl of *p*-nitrophenyl phosphate (Sigma) at 1 μg ml^{-1} in 1 M Tris-HCl (pH 9.8) per well. After 30 min of incubation at 37°C, the absorbance of each well was read at 405 nm on an automated enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek).

Protein analysis. Proteins were separated under reducing conditions by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (16.5% polyacrylamide [Sigma]) (55) or glycine-SDS-PAGE (15% polyacrylamide) (33). Proteins in Tricine-SDS-PAGE gels were stained with $AgNO_3$ (Sigma) according to the method of Blum et al. (8). Proteins in glycine-SDS-PAGE gels were stained with Coomassie blue R-250 (Sigma). An ultra-low-range marker (Sigma) was used as a molecular mass marker (26.6, 17.0, 14.2, 6.5, 3.5, and 1.1 kDa). The protein concentration was determined by using the BCA protein assay reagent (Pierce, Rockford, Ill.), with bovine serum albumin as a standard. The purity of DvnV41 and DvnRV41 was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C_{18} nucleosyl column (250 by 4.6 nm, 5-μm-diameter particles, column 300A; CIL, Sainte Foy la Grande, France). Elution was performed at 40°C with a flow rate of 0.5 ml min^{-1}, using a gradient of 0.1% trifluoroacetic acid (Sigma) (solvent A) and 0.01% trifluoroacetic acid in 90% CH_3CN (Sigma) (solvent B). After 10 min in 100% solvent A, separation was carried out with an elution gradient of solvent B ranking from 0 to 100% over a period of 55 min. Peptides were detected at 280 nm. The polypeptide purity and molecular masses were assessed with an ion-trap mass spectrometer equipped with an electrospray ionization source at atmospheric pressure (LCQ Advantage electrospray mass spectrometer; Thermo-Finnigan, San Jose, Calif.).

Immunoblot analysis. The proteins separated by Tricine-SDS-PAGE were transferred to a nitrocellulose membrane (0.2-μm pore size; Bio-Rad) at 250 mA for 55 min in a buffer containing 25 mM Tris, 0.1% (wt/vol) SDS, 192 mM glycine, and 20% (vol/vol) ethanol by using a Mini Trans-Blot cell apparatus (Bio-Rad). After transfer, the membrane was saturated at room temperature for 1 h with PBS containing 5% (wt/vol) freeze-dried low-fat milk and then washed three times with PBS/T. The membrane was incubated at room temperature for 1 h with a polyclonal antiserum (anti-DvnCt-KLH) (48) diluted 1:2,000 in PBS/T/M. After three washes with PBS/T, the membrane was incubated at room temperature for 1 h with goat anti-rabbit immunoglobulin G (heavy plus light chains)–horseradish peroxidase conjugate (Bio-Rad) diluted 1:30,000 in PBS/T/M. The membrane was washed three times with PBS/T and twice with PBS. The substrate (Super Signal West Dura extended duration substrate; Pierce) was deposited for 5 min onto the membrane. The chemiluminescence produced was revealed on Kodak X-OMAT film (Sigma) with Kodak Polymax RT solutions (Sigma).

Nucleotide sequence accession number. The nucleotide sequence of *dvnRV41*, encoding the recombinant divercin (DvnRV41), has been deposited in the GenBank database under accession number AY463965.

RESULTS

Construction of the synthetic *dvnRV41* gene. Early attempts to clone the DNA encoding DvnV41 of *C. divergens* V41 by PCR were unsuccessful due to DNA instability. To circumvent this problem, we designed and polymerized a synthetic gene specifying the mature polypeptide DvnV41. The primary structure of this open reading frame was designed to match optimally with the *E. coli* K-12 alphabet. The gene was constructed by a unidirectional ligation of two double-stranded fragments according to the strategy depicted in Fig. 1. The sequence of the synthetic gene, *dvnRV41*, its corresponding protein, DvnRV41, and a restriction map are shown in Fig. 1. The sense and antisense strands of the synthetic gene correspond to a set of four oligonucleotides (Fig. 1B) containing designed enzyme restriction targets. Sense and antisense oligonucleotides were partially annealed at a short duplex (18 bp) at their respective 3′ DNA ends and were used both as templates and as primers to generate the corresponding dsDNA fragments, which are targets of convenient endonucleases.

The polymerized dsDNA fragment 1 specifies an N-terminal fragment of the DvnV41 protein starting with an ATG codon (included in the NcoI endonuclease target) and continuing to residues G27Q28. This dipeptide is specified partially by 5 of the 6 bp of the target sequence of the endonuclease MscI. Downstream from this site, a HindIII endonuclease target was also designed. MscI was chosen because its palindromic recognition sequence can specify a dipeptide, GQ, that occurs in the amino acid sequence of the native DvnV41 protein. In addition, this endonuclease fulfills a second requirement: it has no target restriction sequence in the construction vector.

The double-stranded fragment 1 was then double digested with NcoI and HindIII and cloned into pPR16 (21) linearized with the same endonucleases. The resulting plasmid was named pCR01. The double-stranded fragment 2 was digested with MscI and HindIII and inserted into pCR01 cut with the same restriction enzymes. Owing to the DNA end compatibilities, this unidirectional cloning resulted in the construction of an open reading frame of 138 bp encoding a 46-residue

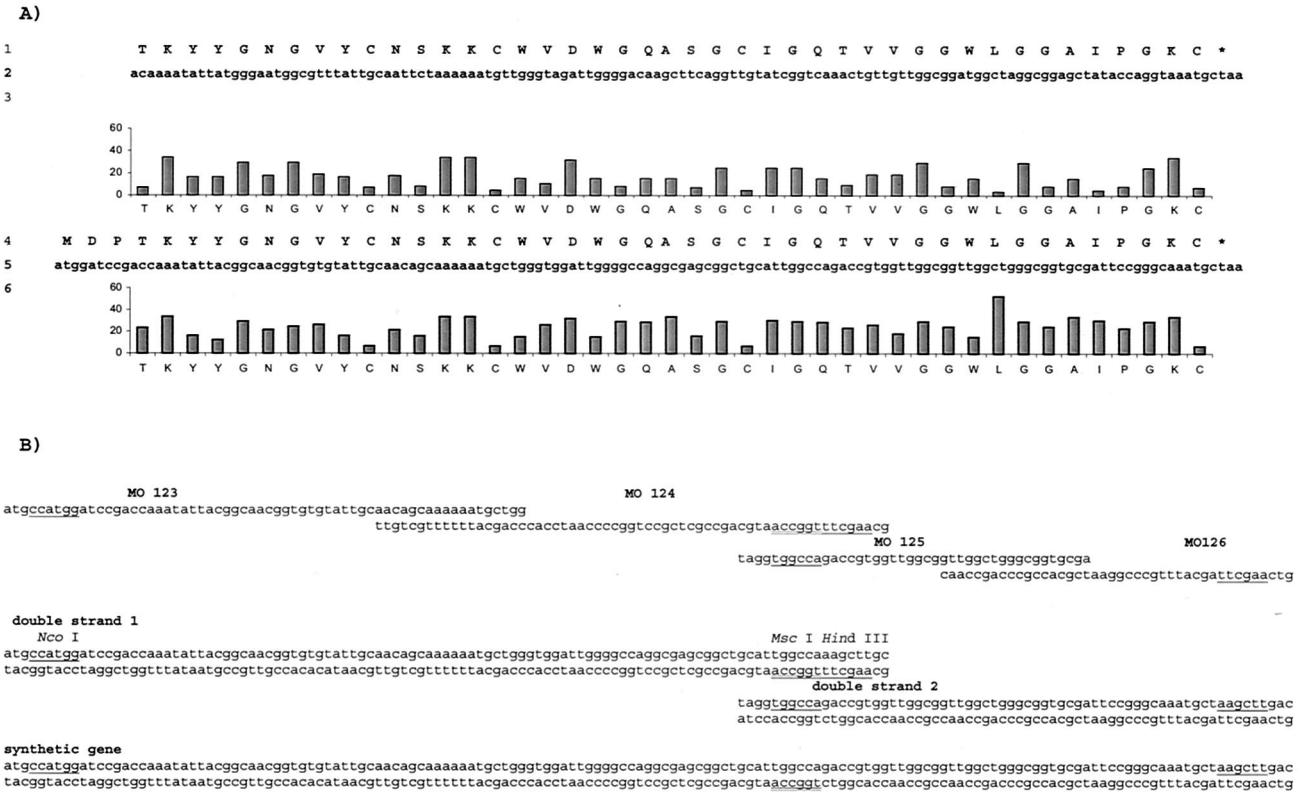

FIG. 1. Construction of optimized divercin V41 gene in *E. coli*. (A) Amino acid sequence (sections 1 and 4), DNA sequence (sections 2 and 5), and frequency in *E. coli* K-12 of each noncoding codon on the *x* axis (sections 3 and 6) of wild-type (sections 1 to 3) and recombinant (sections 4 to 6) divercin V41. (B) Design, assembly, and construction of optimized divercin V41. The sequences of the sense and antisense oligonucleotides used for this study are shown. The designed restriction sites are underlined. Start and stop codons are shown in bold.

polypeptide identical to the secreted mature DvnV41 protein, with a methionine N-terminal extension. Downstream from this ATG, an additional dipeptide, DP, was also designed to allow the separation of DvnRV41 from its fusion partner. Indeed, this peptidic bond has been shown to be very susceptible to incubation at a low pH (1.5) (34, 57). The *dvnRV41* gene was cloned into an expression vector and allowed for the expression of the fusion protein TRX-$(His)_6$-$(Asp)_4$-Lys-Ala-Met-Asp-Pro-DvnV41.

Production of active recombinant divercin V41 from the *dvnRV41* gene expressed in *E. coli*. The expression of TRX and the TRX-$(His)_6$-DvnRV41 fusion protein in *E. coli* Origami (DE3)(pLysS/pET-32b) and *E. coli* Origami (DE3)(pLysS/pCR03), respectively, was induced by the addition of IPTG as described in Materials and Methods. The induction of the T7 RNA polymerase promoter resulted in the expression of a neo-synthesized polypeptide with an apparent molecular mass that was higher in *E. coli*/pCR03 than that of the induced thioredoxin (*E. coli*/pET-32b) used as a control (Fig. 2A). The apparent molecular mass of the polypeptide expressed from pCR03 was estimated to be 20 kDa (Fig. 2A, lane 4), which was in agreement with that calculated for the TRX-$(His)_6$-DvnRV41 fusion protein (22 kDa). The soluble and insoluble cytoplasmic fractions, separated from induced *E. coli*/pCRO3 cells and analyzed by glycine-SDS-PAGE, revealed that the fusion protein accumulated essentially as soluble material in the cytoplasmic fraction of *E. coli* (data not shown). ELISA

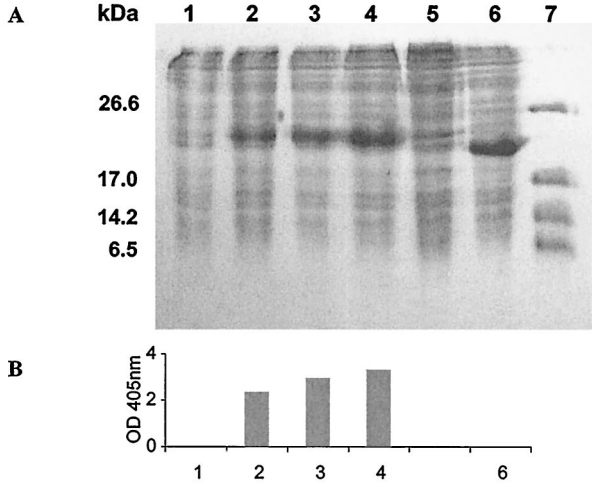

FIG. 2. Expression of fusion protein in *E. coli*. The figure shows a glycine-SDS-PAGE gel stained with Coomassie blue R-250 (A) and the results of an ELISA (B) for total cell proteins obtained from the *E. coli* Origami strain carrying plasmid pCR04 before induction (lane 1), after 1 h of induction (lane 2), after 2 h of induction (lane 3), after 3 h of induction (lane 4), and after 3 h without induction (lane 5); the *E. coli* Origami strain carrying plasmid pET-32b after 3 h of induction (lane 6); and an ultra-low-range molecular mass marker (Sigma) (lane 7).

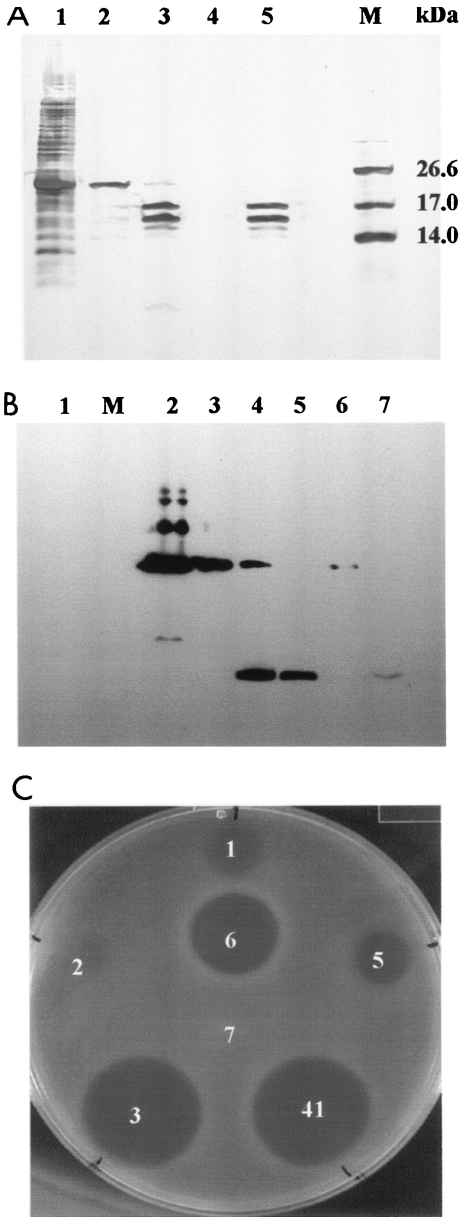

FIG. 3. Purification process by affinity chromatography of recombinant divercin V41. (A) Tricine-SDS-PAGE gel stained with $AgNO_3$ showing a CSF of *E. coli* Origami (DE3)(pLysS) carrying plasmid pCR03 after 3 h of induction (lane 1), the first desalted IMAC-immobilized eluted fraction (lane 2), enterokinase cleavage products (lane 3), purified divercin RV41 (lane 4), a second IMAC-immobilized eluted fraction (lane 5), and an ultra-low-range marker (lane M). (B) Western blot of Tricine-SDS-PAGE products of CSF of *E. coli* Origami (DE3)(pLysS) carrying plasmid pET-32b after 3 h of induction (lane 1), a CSF of *E. coli* Origami carrying plasmid pCR03 after 3 h of induction (lane 2), the first desalted IMAC-immobilized eluted fraction (lane 3), enterokinase cleavage products (lane 4), purified divercin RV41 (lane 5), a second IMAC-immobilized eluted fraction (lane 6), and the supernatant of *C. divergens* V41 (lane 7). (C) Agar diffusion test. The CSF of *E. coli* Origami (DE3)(pLysS/pCR03) after 3 h of induction (spot 1), the first desalted IMAC-immobilized eluted fraction (spot 2), enterokinase cleavage products (spot 3), purified divercin RV41 (spot 4), a second IMAC-immobilized eluted fraction (spot 5), the supernatant of *C. divergens* V41 (spot 6), and the CSF of *E. coli* Origami carrying pET-32b after 3 h of induction (spot 7) are shown.

experiments confirmed that the soluble fraction extracted from cells expressing the fusion protein TRX-$(His)_6$-DvnRV41 contained a protein that carried the epitopes recognized by polyclonal antibodies (anti-DvnCt-KLH) raised against the C-terminal domain of divercin V41 (Fig. 2B).

Western blot analyses with the same polyclonal antibodies were performed with CSFs of *E. coli*/pET-32b and *E. coli*/pCRO3 after 3 h of the induction process. No band could be detected from the *E. coli*/pET-32b CSF, showing that there was no cross-reactivity with other *E. coli* proteins (Fig. 3B, lane 1). A main band corresponding to the expected molecular mass of the TRX-$(His)_6$-DvnRV41 fusion protein was clearly detected for *E. coli*/pCRO3 (Fig. 3B, lane 2). These results confirmed that the recognized protein was the induced polypeptide TRX-$(His)_6$-DvnRV41 and also showed that it is the DvnRV41 part of the fusion protein that carries such epitopes, since no cross reaction was observed in the CSF of *E. coli* carrying plasmid pET-32b (Fig. 2B, lane 5).

Moreover, the CSF of *E. coli*/pCR03 was active against *Listeria innocua* F (Fig. 3C). The bacteriocin activity measured (100 AU ml^{-1}) was also attributed to the DvnRV41 part of the TRX-$(His)_6$-DvnRV41 fusion protein since no such inhibitory effect was observed when *Listeria innocua* F was grown in the presence of a CSF extracted from *E. coli* expressing thioredoxin alone (Table 2; Fig. 3C).

Purification of recombinant divercin V41. In order to purify the fusion protein TRX-$(His)_6$-DvnRV41, we loaded the clarified lysate of induced *E. coli*/pCRO3 onto an IMAC column. The column was successively washed with binding buffers containing imidazole from 10 to 60 mM, and then the immobilized proteins were eluted with 500 mM imidazole. The efficiency of this purification procedure was checked by Tricine-SDS-PAGE (Fig. 2B). A protein showing a similar molecular mass to that of the neo-synthesized TRX-$(His)_6$-DvnRV41 polypeptide in the *E. coli*/pCR03 CSF was only identified in the IMAC-eluted fraction. This protein cross-reacted with the anti-DvnCt-KLH antibodies (Fig. 3B, lane 3). The IMAC-immobilized eluted fraction had an antilisterial activity (1,600 AU) (Table 2; Fig. 3C) corresponding to an eightfold increase in the total activity detected in the CSF fraction of *E. coli*/pCR03. These results show that this activity belongs to the recombinant TRX-$(His)_6$-DvnRV41 fusion protein. The histidine tag located between TRX and DvnRV41 allowed the selective immobilization of the TRX-$(His)_6$-DvnRV41 fusion protein on a nickel chelation column and its specific elution by increasing the imidazole concentration.

The $(Asp)_4$-Lys sequence located upstream of the DvnRV41 sequence is the target of enterokinase. This enzymatic cleavage was used to recover the DvnRV41 protein. The TRX-$(His)_6$-DvnRV41 fusion protein was subjected to an overnight cleavage process in the presence of enterokinase. The IMAC-immobilized eluted and enterokinase-treated fractions were analyzed by Tricine-SDS-PAGE and Western blotting. Figure 3A shows that the enterokinase proteolytic products consisted of four polypeptides, of 5, 15, 17, and 20 kDa (Fig. 3A, lane 3). The apparent SDS-PAGE mobilities of the bands of 15 and 17 kDa corresponded to the two TRX forms and were not recognized in Western blots (Fig. 3B, lane 4). The band with an apparent molecular mass of 5 kDa was revealed by Western blotting and identified as DvnRV41. The weak band of 20 kDa

TABLE 2. Purification of recombinant divercin V41 from heterologous expression in *E. coli* Origami

Fraction	Volume (ml)	Protein concn ($\mu g\ ml^{-1}$)	Activity (AU ml^{-1})	Total activity[b] (AU)	Sp act (AU μg^{-1})	Fold increase in sp act	Yield (%)	Total protein (μg)
Cells	20							
CSF	2	4,015	100	200	0.0	1	100	8,031
Filtered CSF	1.3	4,015	800	1,000	0.2	8	62.50	5,019
Fusion protein[a]	2	352	800	1,600	2.3	91	8.76	704
Desalted fusion protein	3.5	351	400	1,400	1.1	46	15.28	1,227
Enterokinase cleavage products	3.8	410	102,400	389,120	249.8	10,031	19.40	1,558
Purified DvnRV41	3.8	122	102,400	389,120	842.8	33,842	5.75	462
Second IMAC-immobilized eluted fraction	2	271	800	1,600	3.0	118	6.75	542

[a] Elution fraction of the first IMAC step.
[b] DvnV41 AU per milliliter × volume (milliliters).

observed by Tricine-SDS-PAGE corresponded to the remaining uncleaved TRX-$(His)_6$-DvnRV41 fusion protein, as confirmed by its detection on a Western blot (Fig. 3B, lane 4).

The anti-*Listeria* activity of the enterokinase cleavage products was determined (Table 2). The liberation of DvnRV41 from its fusion protein resulted in a 278- and 227-fold increase in the total (389,120 AU) and specific (249.8 AU μg^{-1}) anti-*Listeria* activities, respectively (Table 2; Fig. 3C).

In order to purify the cleaved DvnRV41, we subjected the enterokinase cleavage products to a second IMAC step. The IMAC-immobilized eluted (500 mM imidazole) and flowthrough fractions were analyzed by Tricine-SDS-PAGE and Western blotting (Fig. 3A, lanes 4 and 5, and B, lanes 5 and 6). Owing to their respective His tags, the uncleaved fusion protein TRX-$(His)_6$-DvnRV41 and the cleaved TRX-$(His)_6$ protein could be immobilized fairly well on a nickel chelation resin, while DvnRV41 was collected in the flowthrough fraction. Tricine-SDS-PAGE of the second IMAC-immobilized eluted fraction revealed two bands (15 and 17 kDa) which were not recognized by antibodies and which corresponded to the two forms of TRX (Fig. 3A, lane 5, and B, lane 6). The band corresponding to the remaining uncleaved TRX-$(His)_6$-DvnRV41 fusion protein was detected on a Western blot at the expected molecular size of 20 kDa. The purified DvnRV41 protein was not detected by Tricine-SDS-PAGE of the flowthrough fraction (Fig. 3A, lane 4). However, the Western blot analysis showed a clear immunoreactivity at the same migration level for the IMAC flowthrough fraction and the supernatant of *C. divergens* V41 (DvnV41 producer strain) containing native divercin V41 (Fig. 3B, lane 7). This result confirmed that DvnRV41 was purified in the flowthrough fraction and not subjected to proteolysis.

The anti-*Listeria* activity of purified DvnRV41 (389,120 AU) was identical to the activity detected in the enterokinase-cleaved product fraction (Table 2; Fig. 3C). The second IMAC-immobilized eluted fraction was shown to exhibit an activity of 800 AU ml^{-1} (Table 2). This low level of bacteriocin activity could be due to the remaining uncleaved TRX-$(His)_6$-DvnRV41 fusion protein, which was detected by Western blotting (Fig. 3B, lane 6). However, the total activity of the second IMAC elution fraction, due to the remaining uncleaved fusion protein, represented only 0.4% of the total activity of the cleaved fusion protein fraction before the second IMAC step. In the second IMAC eluted fraction, only 0.18% (1.9 μg) of the fusion protein was not cleaved (Table 2). These results confirmed that the cleavage of the fusion protein could be optimized in order to obtain 100% cleaved protein.

To achieve the purification of DvnRV41, we subjected the protein collected in the flowthrough of the second IMAC and the native divercin V41, purified from a *C. divergens* V41 culture, to HPLC. The RP-HPLC patterns of native divercin V41 and recombinant divercin V41 were analyzed (Fig. 4). Interestingly, the purified DvnRV41 fraction displayed the same RP-HPLC pattern as purified DvnV41. However, the elution of the recombinant DvnRV41 protein was significantly delayed compared to that of native DvnV41. This is probably related to the three additional amino acid residues. This N-terminal extension (Ala-Met-Asp-Pro), added because of the synthetic gene construction requirements, led to an enhancement of the molecular mass (+414.5 Da) and of the number of negative charge residues (+1). The RP-HPLC pattern confirmed the purity of DvnRV41 (Fig. 4). The identity of the purified polypeptide was also confirmed by mass spectrometry (data not

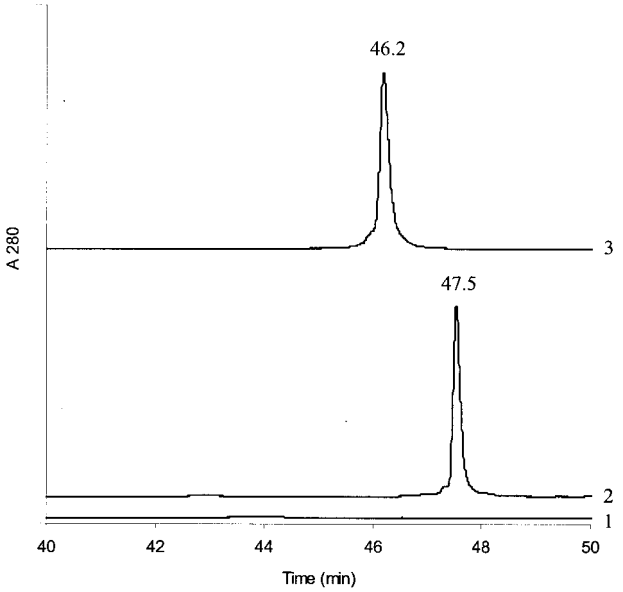

FIG. 4. RP-HPLC elution profiles (C_{18} nucleosyl) of buffer (profile 1), DvnRV41 purified after *E. coli* expression (profile 2), and DvnV41 purified from *C. divergens* V41 (profile 3). The retention time (in minutes) is given at the top of each elution peak.

shown). Indeed, mass spectrometry returned a single polypeptide mass of 4,923.2 Da, in full agreement with the expected theoretical molecular mass (4,927.6 Da). This value corresponds to the molecular mass of native DvnV41 bearing an N-terminal four-amino-acid extension (AMDP) as a result of enterokinase proteolysis and in order to fit the requirements of both the DNA polymerization strategy and the chemical cleavage procedure target. This result also confirmed that all of the cysteine residues of DvnRV41 were involved in the disulfide bond.

After 3 h of induction, the expression system developed and successfully tested in this study allowed 12.5% (wt/wt) fusion rotein to be obtained from the total cytoplasmic protein. This system is very promising with regard to the amount of DvnRV41 obtained. We purified 462 μg of DvnRV41 that led to 389,120 AU of antilisterial activity by starting with 20 ml of culture (Table 2), and we expect to obtain 23 mg of purified DvnRV41 from 1 liter of culture. Additionally, this DvnRV41 protein cross-reacts with anti-DvnCt-KLH polyclonal antibodies, has the same migration level as natural DvnV41, and is active against *Listeria innocua* F, with a specific activity of 842.8 AU μg^{-1}, despite the three amino acid residues added to its N-terminal part. As anticipated, this N-terminal extension had no repercussions on either the recombinant protein folding or its antilisterial activity.

DISCUSSION

Our strategy was to construct a synthetic gene encoding mature divercin V41 by using the *E. coli* alphabet and to express this gene in a redox environment, allowing the formation of two disulfide bonds in a cytoplasmic soluble peptide. To allow the formation of these two disulfide bonds, which are essential for the anti-*Listeria* activity (6), we used *E. coli* Origami (*TrxB gor* double mutant) to express divercin V41 as a translational fusion with thioredoxin in the pET-32b expression vector. The cytoplasm of *E. coli* is normally maintained at a low redox potential (approximately −270 mV) via the action of thioredoxin and glutathione-glutaredoxin. Genetic studies showed that no mutation in thioredoxin reductase (the *TrxB* gene product) rendered the cytoplasm more oxidizing, allowing the formation of structural disulfide bonds in the proteins expressed (16). According to Bessette et al. (5), a higher yield of oxidized proteins can be obtained in the cytoplasm of the *TRXB gor* double mutant (*gor* encodes glutathione reductase). The expression of proteins by the pET-32b expression vector in *E. coli* Origami (DE3) has been successfully achieved for plant, bacterial, and human proteins (3, 19, 20, 29, 37). However, to our knowledge, no peptides containing two disulfide bridges have been expressed in the *E. coli* Origami/pET 32b system.

In order to solve the problem of the inherent genetic instability of the *dvnV41* native gene cloned in *E. coli*, we designed a *dvnRV41* synthetic gene encoding recombinant divercin V41 (DvnRV41) to achieve both stability and an optimal heterologous expression in this host. The DNA sequence of the *dvnRV41* synthetic gene designed in this work contains a large number of substitutions that replace rarely used codons with those found frequently in *E. coli*. These codon substitutions have already been described as contributing to a higher synthetic gene expression level for a number of recombinant proteins produced in *E. coli* (9). The expression of genes encoding recombinant human DNA methylguanine transferase, interleukin-5, and apical membrane antigen 1 was improved by the replacement of *E. coli* low-usage codons in the DNA sequences of the corresponding synthetic genes (9, 19, 41). The *dvnRV41* synthetic gene was inserted into the pET-32b expression vector in frame and as a translational fusion with thioredoxin. The expression of *dvnRV41* was placed under the control of the inducible T7 promoter. Thioredoxin enabled a more soluble fusion protein and the establishment of the disulfide bonds (58). LaVallie et al. proposed that the high solubility of thioredoxin imparts to the hybrid a lower propensity to aggregate (36).

The production of several protein-TRX fusions has been previously described. The bovine enterokinase catalytic subunit was successfully produced in *E. coli* with a TRX fusion partner (63). However, this strategy involves a cleavage procedure to obtain the protein without its TRX partner. Generally, the enterokinase cleavage site is used. The aspartyl-prolyl bond has been shown to be extremely labile upon an acidic incubation (34). Given that the first residue of mature divercin V41 is a tyrosine, we genetically engineered the sequence upstream of the sequence encoding mature divercin V41 to set the four residues Ala-Met-Asp-Pro, including an Asp-Pro acid-labile bond. Sourice et al. have expressed prolamin repetitive domains in a translational fusion with TRX (57). The recombinant polypeptides were liberated by acid cleavage of the Asp-Pro dipeptide bond.

E. coli Origami (DE3) was shown to be a suitable host for the heterologous expression of a properly folded and accurately disulfide-linked extracellular protein with several cysteine residues (35). Kaomek et al. successfully expressed an antifungal chitinase from *Leucaena leucocephala* in *E. coli* Origami. The recombinant protein was active, but the authors did not directly demonstrate proper disulfide bond formation (29). Ara h 2, the major peanut allergen, was expressed in *E. coli* Origami, and all of the cysteines were oxidized, indicating that they were all involved in disulfide bridges (37). DvnRV41 possesses four oxidized cysteine residues, as shown by mass spectrometry analysis.

Guyonnet et al. and Métivier et al. obtained significant purification yields, of about 1.6 and 9.8 mg/liter of culture, respectively (26, 42). However, the heterologous expression in *E. coli* enabled a yield of about 23 mg of pure DvnRV41/liter by a short purification procedure. This yield could be increased by using a high-density culture with a regulated pH and oxygen level. Moreover, it is noteworthy that the bacteriocin and salt concentrations were 0.12 g $liter^{-1}$ and 0.5 M at pH 7.4 after the second IMAC step. These concentrations are compatible with many structure-function studies and with antimicrobial experiments using complex foodstuffs.

Bacteriocins produced by LAB have received particular attention because of their economic importance in the food industry as natural preservatives and in clinical areas as antiviral agents (62). Accordingly, attempts have been made to develop convenient bacteriocins by the use of heterologous production systems (27, 50). However, these systems have often been faced with multiple biological barriers, such as the immunity and protection of the host cell, the role of prebacteriocins, and secretion and transport within the host cell. The

production of enterocin A in *Lactococcus lactis* IL-1403 was made possible by the constitutive expression of the four-gene cassette *entAITD* under the control of the lactococcal promoter P32, but plasmid and phenotypic instability was observed (46). The coproduction of pediocin PA-1 and enterocin A in *Lactococcus lactis* IL-1403 has been reported (39), but the concentrations of pediocin PA-1 and enterocin A in the supernatant of the recombinant *Lactococcus lactis* derivative were approximately 5 and 4%, respectively, of those found in the supernatants of the wild-type bacteriocin producers *Enterococcus faecium* T136 and *Pediococcus acidilactici* 347. Chikindas et al. achieved production and secretion of pediocin PA-1 in *P. pentosaceus* PPE1.2 that had been transformed with pMC117, a plasmid containing the *ped* operon under the control of the lactococcal promoter P32 (12). The amount of pediocin PA-1 produced was up to fourfold higher than that of the natural producer *P. acidilactici* PAC1.0. *Lactococcus lactis* IL-1403 transformed with pMC117 also produced pediocin PA-1, but the yield was <1% of the production level by the *Pediococcus* parental strain. It was possible to increase the relative pediocin PA-1 production level to approximately 50% in *Lactococcus lactis* LL108 (a *Lactococcus lactis* MG1363 derivative carrying several copies of *repA* in its chromosome) by increasing the copy number of the plasmid-encoded *ped* operon. Thus, these systems for the heterologous production of LAB bacteriocins used ABC transporters or *sec*-dependent secretion systems and often had low production levels.

The fusion strategy described in the present work ensures the synthesis of a soluble cytoplasmic protein that is resistant to proteolysis, which has been responsible for the loss of recombinant proteins from *E. coli* (38). This method could allow for divercin V41 production and easy purification on a large scale and at a low cost by use of an acid cleavage procedure and IPTG replacement by lactose (2). Moreover, the fusion protein has a significant antilisterial activity that can be exploited for the screening of the large mutant library before recourse to cleavage and mature bacteriocin purification.

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