The Structure, Function, and Origin of the Microcin H47 ATP-Binding Cassette Exporter Indicate Its Relatedness to That of Colicin V

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Microcin H47, a gene-encoded peptide antibiotic produced by a natural *Escherichia coli* strain, was shown to be secreted by a three-component ATP-binding cassette exporter which was revealed to be strongly related to that of colicin V. The results of sequence and gene fusion analyses, as well as heterologous complementation assays, are presented.

For gram-negative bacteria, several three-component ATP-binding cassette (ABC) exporters dedicated to protein secretion have been described. They are composed of an ABC transporter protein, a second component of the membrane fusion protein (MFP) family, and an outer membrane protein (3, 4, 21). There is a single peptide described to be secreted by a three-component ABC apparatus: *Escherichia coli* colicin V (ColV), an antibiotic of the microcin family. Its exporter comprises the ABC protein CvaB, the MFP CvaA, and the outer membrane protein TolC (6). The ColV ABC transporter contains a proteolytic domain, and consistent with this, the ColV precursor bears a double glycine leader peptide which is processed during export (8, 9).

In this work, results are presented on the mode of secre-

tion of microcin H47 (MccH47), an *E. coli* antibiotic peptide. Genes for its synthesis, immunity, and secretion are clustered in a 10-kb DNA segment (Fig. 1A) (5, 11, 16, 17). The secretion function was assigned to the products of two genes, *mchE* and *mchF*. In addition, *tolC* mutants were shown to produce reduced amounts of microcin. It has been proposed that MccH47 is secreted by an ABC exporter, constituted by MchF, MchE, and TolC (5).

A DNA segment containing the *mchE* and *mchF* genes was sequenced, partly in our laboratory (18) and partly in the DNA Sequencing Core Laboratory Service of the University of Florida. Two open reading frames were found in the positions expected for these genes (Fig. 1B) (5). Protein homology analysis of the deduced amino acid sequences for MchE and MchF

FIG. 1. (A) DNA region containing the MccH47 genetic system. The physical map and the *mch* genes, with their extension and direction of transcription, are shown. The genes *mchABCD* are involved in MccH47 synthesis, *mchI* codes for the immunity peptide, and *mchX* appears to be related to regulatory functions. *mchE* and *mchF* are microcin secretion genes. (B) Enlargement of the 4,197-bp sequenced DNA region containing *mchEF*. The physical map, as well as the extension and direction of transcription of the *mch* genes, is shown. B, *Bam*HI; E, *Eco*RI; H, *Hind*III.

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A

MchE: MFRQDALENRKMKWQGRAILLPGIPLWLIMLGSIVFITAFLMFIIVGTYSRRVNVSGEVT 60
CvaA: MFRQDALENRKMKWQGRAILLPGIPLWLIMLGSIVFITAFLMFIIVGTYSRRVNVSGEVT 60

MchE: TWPRAVNIYSGVQGFVVRQFVHEGQLIKKGDVPYLLIDISKSTRSGIVTDNHRDIENTQLV 120
CvaA: TWPRAVNIYSGVQGFVVRQFVHEGQLIKKGDVPYLLIDISKSTRNGIVTDNHRDIENTQLV 120

MchE: RVDNIISRLEESKKITLDTLEKQRLQYTDAFRRSSDIIQRAEEGIKIMKNNMENYRNYQA 180
CvaA: RVDNIISRLEESKKITLDTLEKQRLQYTDAFRRSSDIIQRAEEGIKIMKNNMENYRYQS 180

MchE: KGLINKDQLTNQVALYYQQQNNLLSLSGQNEQNALQITTLESQIQTAADFDNRIYQMEL 240
CvaA: KGLINKDQLTNQVALYYQQQNNLLSLSGQNEQNALQITTLESQIQTAADFDNRIYQMEL 240

MchE: QRYELQKELVNTDVEGEIIIRALTDGKVDLSLVTVGQMVNTGDSLLQVIPENIENYLLIL 300
CvaA: QRLELQKELVNTDVEGEIIIRALSDGKVDLSLVTVGQMVNTGDSLLQVIPENIENYLLIL 300

MchE: WVPNDVPIYISAGDKVNIRYEAFFAEKFGQFSATVKTISRTPASTQEMLTYKGAPQNTPG 360
CvaA: WVPNDVPIYISAGDKVNIRYEAFFSEKFGQFSATVKTISRTPASTQEMLTYKGAPQNTPG 360

MchE: ASVPWYKVIAMPEKQIIRYDEKYLPLENGMKAESTLFLEKRRIYQWMLSPFYDMKHSATG 420
CvaA: ASVPWYKVIATPEKQIIRYDEKYLPLENGMKAESTLFLEKRRIYQWMLSPFYDMKHSATG 420

MchE: PLND 424
CvaA: PLND 424

B

MchF: MTNGSFRQIINOLDNRWRRRVPVIHQETETAECGLACLAMICGHFGKNIDLISLRRKENLS 60
CvaB: MTNRNFRQIINLDRWRRRVPVIHQETETAECGLACLAMICGHFGKNIDLISLRRKENLS 60

MchF: ARGANLAGINGIAEQGMVTRALSLELDELGALKMPCILHWDHSHFVVLVSVKRNRVYLH 120
CvaB: ARGATLAGINGIAEQGMATRALSLELDELAVLKTPCILHWDHSHFVVLVSVKRNRVYLH 120

MchF: DPARGRYLGREEMSRYFTGIALEVWPGSEFLAETQIRISLRSLINSIYGIKRTLAKIF 180
CvaB: DPARGIRYISREEMSRYFTGIALEVWPGSEFOSETQIRISLRSLINSIYGIKRTLAKIF 180

MchF: CLSVVIEAINLMPVGTQLVMDHAIPAGDRGLLTLSAGLMFFILLRAAVSMLRAWSSLV 240
CvaB: CLSVVIEAINLMPVGTQLVMDHAIPAGDRGLLTLSAALMFFILLKAATSLRAWSSLV 240

MchF: MSTLINIQWQSGLFNHLRLPLAFFERRKLGDIQSRFGSLDTRLATFTTCVVGAIMDSIM 300
CvaB: MSTLINVQWQSGLFDHLRLPLAFFERRKLGDIQSRFDSLDTRLATFTTSVIGFIMDSIM 300

MchF: VVGVEVMMLLYGGYLTWIVLGFTMVYVLIRLVITYGYRQISEETLVRGARASSYFMESLY 360
CvaB: VVGVEVMMLLYGGYLTWIVLCFTTIYIFIRLVITYGNRYQISEECLVREARASSYFMETLY 360

MchF: GIATVKIQGMAGIRGTHWLNKIDAINSGIKLTRMDLLFGGINTFVAACDQVAILWLGAS 420
CvaB: GIATVKIQGMVIRGAHWLNKIDAINSGIKLTRMDLLFGGINTFVTACDQVAILWLGAS 420

MchF: LVIDNQMTIGMFVAFSSFRGQFSERVASLTSFLLQLRIMSLHNERIADIALHEKEKKPE 480
CvaB: LVIDNQMTIGMFVAFSSFRGQFSERVASLTSFLLQLRIMSLHNERIADIALHEKEKKPE 480

MchF: IEIVADMSPVSLETNQLSYRYDSQSAQVFSGLNLSVAPGESVAITGASGAGKTTLMKVLC 540
CvaB: IEIVADMCPVSLETNQLSYRYDSQSAPIFSALSLVAPGESVAITGASGAGKTTLMKVLC 540

MchF: GLFEPDSGKVLVNGTDIROGGINNYHRMIACVMQDDRLFSGSIRENICGFAEETDDEWMT 600
CvaB: GLFEPDSGRVLINGTDIROGGINNYHRMIACVMQDDRLFSGSIRENICGFAEEMDDEWMT 600

MchF: ECARASHIHDVIMPMGYETLIGELGEGLSGGQKQRIFIARALYRKPGILFMDEATSSL 660
CvaB: ECARASHIHDVIMPMGYETLIGELGEGLSGGQKQRIFIARALYRKPGILFMDEATSSAL 660

MchF: DTESERFVNAIAKMNITRVIIAHRETTLRVDRISI 698
CvaB: DSESEHFVNVAIKMNITRVIIAHRETTLRVDRISI 698

FIG. 2. Amino acid sequence alignments using the program LALIGN (10). The numbers on the right refer to amino acid positions; nonconserved residues are shaded. (A) Alignment of the entire MchE and CvaA sequences. The first residue of MchE* and CvaA* is boxed. (B) Alignment of the entire MchF and CvaB sequences.

TABLE 1. Reporter enzymatic activities in strains with *TnphoA* or *Tnlac* insertions in *mchE*

Fusion or insertion ^a	β-Galactosidase units ^b		Alkaline phosphatase units		Ratio of enzyme units (SP/LP)
	LP	SP	LP	SP	
<i>mchE54-phoA</i>			131.1	504.4	3.85
<i>mchE158-phoA</i>			49.3	355.3	7.21
<i>mchE162-phoA</i>			59.2	324.1	5.47
<i>mchE253-phoA</i>			46.1	298.8	6.48
<i>mchE343-phoA</i>			28.1	164.2	5.84
<i>mchE367-lacZ</i>	345.1	611.3			2.15
<i>mchE407-lacZ</i>	356.4	765.8			1.77
pEX100::Tnlac 7.1	81.0	126.4			1.56

^a CC118 cells ($\Delta lacX74 \Delta phoA20$) (12), carrying pMVD14 derivatives with gene fusions or carrying pEX100 with a *Tnlac* insertion, were analyzed. The fusions were named according to the *mchE* codon involved in the junction.

^b LP, logarithmic phase (optical density at 600 nm of 0.3 to 0.4); SP, stationary phase (optical density at 600 nm of 2.2 to 2.7).

revealed 98 and 89% identity with CvaA and CvaB, respectively, indicating that MchF is an ABC protein and MchE is an MFP (Fig. 2). These results are consistent with the identification of a double glycine leader peptide located in the 15 N-terminal residues of the MccH47 precursor, which would be processed concomitantly with export (9, 17). The alignment of MchE and CvaA included the methionine where a second in-frame shorter protein, CvaA*, and a putative MchE* homologue begin (Fig. 1B and 2A) (6). In fact, two proteins had previously been detected in polyacrylamide gel electrophoresis systems as products of *mchE* (5).

The *mchE* and *mchF* genes are expressed in the same direction and present a small overlap, identical to that found between *cvaA* and *cvaB*, indicating that they are arranged in an operon (Fig. 1B) (6). A previously isolated insertion on *mchE*, *Tnlac 7.1*, conferring β-galactosidase activity and thus presumed to generate a gene fusion, led us to infer that *mchEF* were expressed from the right to the left (Table 1) (5). Since the opposite direction was now confirmed, the *Tnlac 7.1* insertion site was sequenced, showing that no fusion existed: *Tnlac* mapped in *mchE*, with *lacZ* oriented from right to left.

New mutagenesis experiments with *TnphoA* and *Tnlac* were performed to analyze *mchE* expression. Strains harboring pMVD14, a pACYC184 derivative plasmid carrying the *EcoRI-EcoRI* fragment that contains *mchE*, were mutagenized as described previously (7, 12). Clones bearing *mchE-phoA* and *mchE-lacZ* active gene fusions were isolated, and their respective enzymatic activities when grown in Luria-Bertani medium were measured (Table 1) (2, 13). These activities increased from logarithmic to stationary phase, which could be indicative of a growth phase regulation of *mchE* expression. The junction sites in the *mchE-phoA* fusions were distributed between codons 54 and 343, a result that indicates a periplasmic MchE segment, in agreement with previously reported data on *cvaA-phoA* fusions. In both cases, no active fusions with PhoA were isolated at the C-terminal portion of MchE or CvaA (19). On the contrary, two active *mchE-lacZ* fusions were located near the end of *mchE*, indicating that their products would reside in the cytoplasm. We propose that these fusions encode two types of hybrid proteins, MchE-LacZ and MchE*-LacZ, and that the latter is responsible for the detected enzymatic activity. MchE*

lacks predictable transmembrane segments and thus would be a cytoplasmic protein.

In view of the strong similarities between MchEF and CvaAB, DNA homologies were searched for using the program CLUSTAL W (20). A 92.1% identity was found along the entire coding sequences of *mchEF* and *cvaAB* and ceased abruptly in noncoding DNA. Upstream of *mchE* no counterpart of the Fur box, which is responsible for *cvaAB* iron regulation, was found (1). When analyzed with the program FASTA (14), sequences downstream of *mchF*, i.e., beyond the limits of the MccH47 genetic system, exhibited 53.4% identity with *cvi* and *cvaC*, the ColV immunity and activity genes, respectively. In fact, two small open reading frames reminiscent of these ColV genes were found (data not shown). However, no antibacterial or immunity function is encoded by these sequences, as saturation mutageneses of the MccH47 system and its surroundings revealed (5, 11). This finding suggests the ancestral occurrence of a ColV genetic system that integrated next to the *mch* sequences; the determinants for its export apparatus became dedicated to MccH47 secretion, while the ColV activity and immunity genes lost their function.

A heterologous complementation analysis for MccH47 secretion by the ColV exporter was performed. For this purpose, a plasmid carrying the ColV genetic system, pUY270, was constructed by cloning a 7-kb *HindIII-BglII* fragment from pColV-K270 (15) into *HindIII/BamHI*-digested pUC13. Six RYC1000 derivative strains, each bearing plasmid pEX100 (containing the MccH47 system) with a different insertion mutation in *mchE* or *mchF*, were used. These strains produce

FIG. 3. Heterologous complementation analysis for MccH47 secretion. MccH47 production was assayed on a lawn of the MccH47-sensitive ColV-immune indicator strain PAP222 (15). Six different RYC1000 derivative strains were stabbed, all carrying pUY270 and the following pEX100 derivative plasmids: pEX100 *mchF*::MudI1681 30.9 (stab 1), pEX100 *mchF*::MudI1681 40.1 (stab 2), pEX100 *mchE*::MudI1681 30.4 (stab 3), pEX100 *mchE*::MudI1681 30.5 (stab 4), pEX100 *mchE*::MudI1681 80.9 (stab 5), and pEX100 *mchE*::Tnlac 7.1 (stab 6). RYC1000(pUY270) (stab 7) and RYC1000(pEX100) (stab 8), ColV- and MccH47-producing strains, respectively, were also stabbed onto the lawn. The plates were incubated at 37°C for 16 h to detect inhibition halos.

MccH47 but are unable to secrete it; therefore, they do not give rise to growth inhibition halos (5). pUY270 was used to transform these strains. In a halo assay performed on minimal M63 glucose medium (13, 17), the transformant clones were found to efficiently produce extracellular MccH47 (Fig. 3). In parallel, they were assayed on a lawn of an MccH47-immune and ColV-sensitive strain, confirming that they also produced ColV (data not shown). In sum, all the experimental clones produced and secreted both antibiotics, clearly revealing that the ColV exporter is competent for recognizing and exporting MccH47 to the extracellular medium in substantial amounts.

We can conclude that MccH47 is secreted by an ABC export apparatus. The ABC and the second component proteins are encoded by genes belonging to the MccH47 genetic system, while the outer membrane protein would be encoded by the unlinked *tolC* gene. The MccH47 and ColV exporters were found to be strongly related from the structural and functional points of view. Moreover, DNA sequence homologies revealed that the MccH47 exporter genes most probably derived from those of ColV.

Nucleotide sequence accession number. The sequence of the 4,197-bp *EcoRI*-*Bam*HI DNA segment containing the *mchE* and *mchF* genes has been deposited in the EMBL database under accession number AJ278866.

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