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Identification and modeling of a novel chloramphenicol resistance protein detected by functional metagenomics in a wetland of Lerma, Mexico

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Summary. The exploration of novel antibiotic resistance determinants in a particular environment may be limited because of the presence of uncultured microorganisms. In this work, a culture-independent approach based on functional metagenomics was applied to search for chloramphenicol resistance genes in agro-industrial wastewater in Lerma de Villada, Mexico. To this end, a metagenomic library was generated in *Escherichia coli* DH10B containing DNA isolated from environmental samples of the residual arsenic-enriched (10 mg/ml) effluent. One resistant clone was detected in this library and further analyzed. An open reading frame similar to a multidrug resistance protein from *Aeromonas salmonicida* and responsible for chloramphenicol resistance was identified, sequenced, and found to encode a member of the major facilitator superfamily (MFS). Our results also showed that the expression of this gene restored streptomycin sensitivity in *E. coli* DH10B cells. To gain further insight into the phenotype of this MFS family member, we developed a model of the membrane protein multiporter that, in addition, may serve as a template for developing new antibiotics. [**Int Microbiol** 2013; 16(2):103-111]

Keywords: *Escherichia coli* · chloramphenicol · functional metagenomics · major facilitator superfamily · homology models · membrane proteins · arsenic

Introduction

The emergence of strains resistant to antibiotics and thus to significant public health problems in many countries is mainly related to the improper management, use, and distribution of these drugs [22,40]. In Mexico, antibiotic resistance is of particular concern because of the lack of national policies regulating the use of antibiotics [3]. An understanding of the mechanisms by which uncultured and cultured microorganisms develop antibiotic resistance in particular environments is of utmost importance [4] to improve current therapeutic strategies and design alternative agents. Metagenomics is a useful approach to characterize emerging pathogens [20]. It also sheds light on the persistence and dissemination of the genetic mechanisms underlying antibiotic resistance [19]. Metagenomics techniques are best suited for DNA analysis *in situ*, since DNA can be extracted directly from the environment and subsequently cloned into commercial vectors. One of the most significant advantages of using functional metagenomics to study antibiotic resistance is that specific phenotypes can be isolated and thousands of clones can be analyzed in a single screening [25]. At the same time, the analysis allows the study of genes involved in resistance whose function

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may not be obvious, by the extrapolation routine included in the annotation process [19]. Furthermore, the use of new methods to isolate DNA from different substrates has enabled the analysis of larger fractions of microorganisms, both culturable and non-culturable [33].

Chloramphenicol is widely used in the treatment of acute diarrhea [21], even though it is now less frequently prescribed in developed countries. Although simple storage conditions and accessibility make this antibiotic attractive, its overuse has promoted the appearance of resistant strains [11]. In studies of chloramphenicol resistance, novel chloramphenicol resistance genes have been retrieved from diverse environmental samples by using functional metagenomics approaches. These genes, which encode proteins similar to the efflux pumps of the major facilitator superfamily (MFS) have been isolated from Alaskan soil samples [19]. MFS proteins are classified into 17 families, and are ubiquitous among all forms of life [28]. Another class of newly identified genes includes those encoding drug resistance transporters of the Bcr/CflA family, isolated from agricultural soil samples [41]. In this work, functional screening of a metagenomic library prepared from an arsenic-enriched water sample obtained from a wetland near to Lerma de Villada, Mexico, led to the retrieval of a gene conferring chloramphenicol resistance. Sequence analysis of the resistant clone revealed the presence of a complete open reading frame (ORF) highly similar to an MFS protein from an environmental clone of *Aeromonas salmonicida*. The resistance of this clone to other antibiotics was assessed. Finally, a model of the retrieved MFS protein was developed, which yielded new insights into the molecular mechanisms leading to the resistance phenotype.

Materials and methods

Sample collection and metal determination. The water sample used in this study was recovered from an irrigation canal in Lerma de Villada, State of Mexico, Mexico, in January 2013. This canal crosses crop fields and leads to one of the wetlands that constitute the ecosystem of the swamps of Lerma (19° 17′ 28″ N 99° 30′ 08″ W; altitude 2577). Lerma de Villada is a municipality located east of Toluca and 54 km west of Mexico City (Fig. 1).

To obtain a representative profile of the microbial diversity from this area, water samples of 50 ml were obtained from 20 different points of the canal. These samples were homogenized and stored at –20 °C until needed.

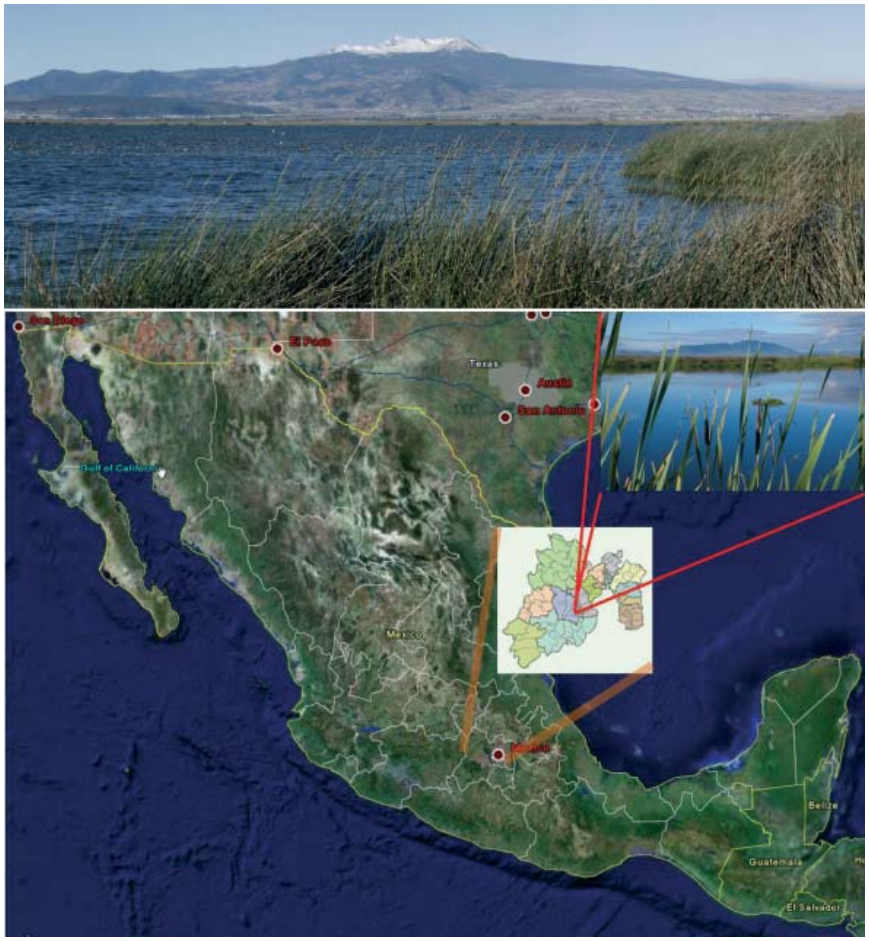

Fig. 1. Ciénegas (swamps) of the Lerma River, located about 2 km from the municipality of Lerma de Villada, State of Mexico, Mexico. This ecosystem stretches along a line of 8 km in the south direction, mixing cultivation areas with protected areas. Wetlands are usually shallow, but there are some areas that have deeper water. Emergent aquatic vegetation is abundant, mainly common bulrush (*Typha latifolia*) and giant bulrush (*Schoenoplectus californicus*).

The concentrations of metal ions present in the sample were determined by using total reflection X-ray fluorescence (TXRF), an X-ray spectrometry method that derives from the classic technique of X-ray fluorescence for the dispersal of energy (EDXRF) [18].

Bacterial strains, media, and culture conditions. *Escherichia coli* DH10B was routinely grown in Luria-Bertani (LB) medium at 37 °C. The growth medium for transformed *E. coli* strains was supplemented with 50 μg ampicillin (Ap)/ml in order to maintain the pBluescript SKII(+) plasmid (pSKII+).

Library construction and amplification. A metagenomic library was generated using DNA isolated from the above-described water sample recovered in Lerma de Villada. Biomass was collected by filtration of the sample through a 0.22-μm-pore-size membrane filter (Nalgene, Rochester, NY, USA). Metagenomic DNA was isolated from the water sample by using the BIO101 FastDNA spin kit for soil (Qbiogene, Carslbad, CA, USA) according to the manufacturer's recommendations, with no further treatment. Approximately 12.5 μg of DNA per liter of water sample was obtained. The metagenomic DNA was partially digested using Sau3AI. The resulting fragments, ranging from 1 to 4 kb, were collected directly from a 0.8 % low-melting-point agarose gel with the QIA quick gel extraction kit (Qiagen, Hilden, Germany) and ligated into the dephosphorylated and BamHI-digested pSKII+ vector, at a molar ratio of 1:1, using T4 DNA ligase (Roche, Mannheim, Germany). The ligation mixtures were incubated overnight at 16 °C and used to transform *E. coli* DH10B cells (Invitrogen, Carlsbad, CA, USA) by electroporation, using a Micropulser (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. The plasmids of 16 random clones were isolated and digested using *Xba*I and *Xho*I (Roche) to determine the average insert size of the library. To increase the number of recombinant clones, the library was amplified as previously described [13]. Briefly, transformed cells were grown on LB agar plates containing Ap and incubated at 37 °C for 24 h. Cells from each plate were mixed with 3.5 ml LB and 10 % (wt/vol) glycerol, pooled in a flask with cells from the same library, mixed again, and stored at –80 °C.

Screening for chloramphenicol resistance genes and sequence analysis. To screen for genes conferring chloramphenicol resistance, aliquots of approximately 1.4×10^5 bacteria/ml from the amplified library were plated onto LB-Ap agar plates containing a final concentration of 5 μg chloramphenicol/ml and incubated at 37 ºC for 72 h. Plasmid DNA was isolated from individual resistant clones and used to again transform *E. coli* DH10B cells to confirm that the plasmid was responsible for the resistance phenotype. Plasmid DNA isolated from a resistant, retransformed clone was sequenced on both strands by primer walking using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA) and an ABI PRISM 377 sequencer (Perkin-Elmer) according to the manufacturer's instructions.

Sequences were analyzed with the Editseq, Megalign, and Seqman programs from the DNAStar package. Putative ORFs were identified using ORF Finder, available at the NCBI website [http://www.ncbi.nlm.nih.gov/gorf/gorf.html]. Sequences with significant matches were further analyzed with rpsBlast, and their putative functions were annotated based on similarities to sequences in the COG (Clusters of Orthologous Groups) and Pfam (Protein Families) databases.

Antibiotic susceptibility test and minimal inhibitory concentration determination. A disc diffusion test was used to assess the antibiotic susceptibility of clone pLM2, by comparing pLM2-transformed cells with the host strain *E. coli* DH10B transformed with pBluescriptSKII+. Bacterial cultures grown overnight were adjusted to an OD_{600} of 0.5 and 200 μl of each culture was plated on 90-mm Petri dishes containing LB agar. Filter discs (6 mm diameter) were placed on the LB agar plates and impregnated with 10 μl of the following antibiotics solutions: tetracycline (50 μg), kanamycin (500 μg), streptomycin (2500 μg), gentamicin (100 μg), spectinomycin (500 μg), trimethoprim (500 μg), chloramphenicol (50 μg), and nalidixic acid (100 μg). The plates were incubated at 37ºC for 24 h. Antibiotic susceptibility was assessed by measuring the diameters of inhibition zones around the filter discs. All tests were carried out in triplicate.

Minimal inhibitory concentration (MIC), defined as the lowest concentration of chloramphenicol that prevented the growth of pLM2, was determined in LB liquid medium in cultures incubated at 37 °C overnight with agitation at 150 rpm.

Bioinformatic screening. Alignments were calculated by the BLAST engine for both nucleotide and amino acid sequences (http://blast.ncbi.nlm.nih.gov). The Pfam database server [http://blast.ncbi.nlm.nih.gov/Blast.cgi] was used to identify the protein family related to the new query; i.e., the major facilitator superfamily (MFS) matched with the amino acid sequence.

Protein transmembrane (TM) domains were identified based on hydropathy properties along the amino acid sequence, on the octanol as well as the translocon scales [37]. The protein model was generated using the standalone version of the Modeller 9.11 package [35]. Multiple templates and sequence alignments were performed to generate the atomic coordinates for the N-term and C-term domains. As described below, a protein model was developed by binding these two domains.

Protein modeling. Our approach to generate the protein model was based on a structural analysis of MFS proteins [45]. The domains identified by consensus in these proteins were the N-term and the C-term, each consisting of two symmetric TM bundles of three helical repeats, for a total of 12 TM helices. For the C-term domain, the PDB:2CFQ LacY structure, corresponding to a lactose permease without substrate [26], was used as template. Identity determined after alignment of the amino acid sequences was 20 %. Alignment of the N-term domain was improved by using the *E. coli* multidrug transporter PDB:2GFP [46] as template. By aligning only a fragment of the query sequence, from Leu9 to Leu149, we obtained an identity of 37 %. The fragment included the first three helical repeats of the N-term domain (TM1–TM3). Protein coordinates were generated for the C-term and N-term separately using the corresponding alignments; both structural fragments were fitted on the LacY template, which had been previously aligned along the bilayer normal. To complete the protein structure, we designed the following procedure: (i) the first repeat of the N-term, helices TM1–TM3, was inverted by rotating it 180° along an axis parallel to the bilayer normal; (ii) the first repeat was fitted on the second repeat (helices TM4–TM6) of the N-term domain; (iii) the coordinates for the TM4–TM6 backbone atoms were defined by the fitted fragment; the symmetry of N-term model was accordingly satisfied [45]; and (iv) the backbone atoms of the C-term domain (including helices TM7–TM12) showed no distortions from the helical fold and were not further adjusted. By means of the PSFGEN plugin of the VMD 1.9 package [15], the coordinates for the missing atoms were generated using the internal coordinates defined in the topology file of the CHARMM22 parameter set [23].

Molecular dynamics. The protein model was relaxed from the initial configuration to correct any geometry distortion attributable to the initial model setting. The NAMD 2.8 [30] program was used to minimize the potential energy for the protein, in vacuum, based on the all-atom CHARMM22 force field parameter set [23]. The first stage consisted of 8000 steps of energy minimization using the conjugate-gradient; in a second stage, 10,000 time steps of molecular dynamics simulation in the NVT ensemble were carried out according to the following protocol: All backbone atoms were fixed in the relaxation process to preserve protein folding. For the side-chain atoms, bonding and non-bonding interactions were calculated to evaluate the

intermolecular forces [23]. Motion equations were solved using a time step of 1 fs, and electrostatic interactions were calculated using the smooth particle mesh Ewald [9]. All bonds involving hydrogen atoms were constrained using the SHAKE [34] and SATTLE algorithms [27]. Temperature was controlled by means of Langevin dynamics [30]. Analysis scripts and graphics were prepared with the VMD 1.9 package [17].

Nucleotide sequence accession number. The nucleotide sequence obtained for the plasmid insert of the pLM2 sequence has been deposited in the GenBank database under the accession number KF169941.

Results

Identification of a chloramphenicol resistant clone. Microbial DNA from a water sample enriched in arsenic (10 mg/l) was isolated and used to construct a metagenomic library in *E. coli*. A total of 200,000 recombinant clones, with an average insert size of 2.5 kb, were obtained and screened for recombinant clones allowing *E. coli* to grow in the presence of chloramphenicol. One resistant clone (pLM2) was thus identified and further analyzed. Sequence analysis revealed that pLM2 harbored a 1859-bp insert with a G+C content of 60.89 %, one complete (*orf2*), and two incomplete heterologous ORFs (*orf1* and *orf3*), as shown in Fig. 2. These ORFs had high similarity to sequenced genes from the *Aeromonas* genus (Table 1) and displayed the same arrangement observed in *A. salmonicida*.

Among the three ORFs, *orf2* encoded an amino acid sequence almost identical (99 % amino acid identity) to a putative multidrug resistance protein of the Bcr/CflA subfamily from the fish pathogen *A. salmonicida* [31]. This ORF also shared homology with EmrD-3 (57 % amino acid identity), a multidrug efflux pump of the Bcr/CflA subfamily identified in *Vibrio cholerae* O395, which is resistant to several antimicrobials tested including chloramphenicol [36]. The other two ORFs, *orf1* and *orf3*, encoded amino acid sequences similar to an oxidoreductase (98 % amino acid identity) and a HAM1 protein (96 % amino acid identity), respectively. The slightly lower similarities between these ORFs and the sequenced genes of several *Aeromonas* species suggested the environmental origin of this clone.

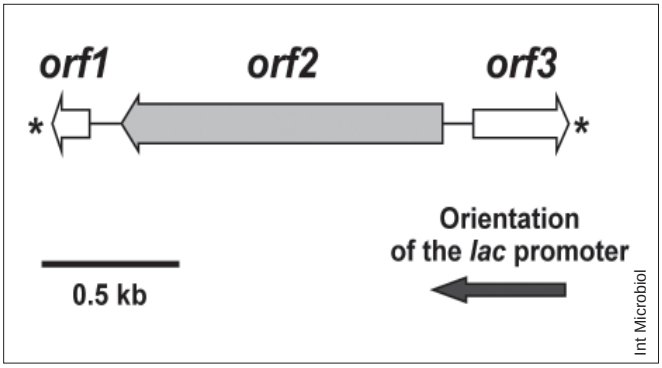

Fig. 2. Schematic organization of the ORFs identified in pLM2. The arrows indicate the locations of the ORFs in the plasmid and the direction of transcription. The ORF similar to the gene encoding a multidrug resistance protein involved in chloramphenicol resistance is indicated in gray. Asterisks indicate incomplete ORFs.

MIC determination and susceptibility test for pLM2. The strain carrying pLM2 was selected at 5 μg chloramphenicol/ml. As determined from its MIC, this strain was unable to survive in LB medium containing up to 25 μg chloramphenicol/ml. To better characterize the resistance profile of pLM2 in the presence of other antimicrobials, considering its similarity to a multidrug resistance protein, the respective strain was subjected to an antibiotic resistance susceptibility test using a wide range of antimicrobials representing different antibiotic classes, including tetracycline, aminoglycosides (kanamycin, gentamicin and streptomycin), phenicols (chloramphenicol), dihydrofolate reductase inhibitors (trimethoprim) and quinolones (nalidixic acid). Inhibition haloes of the pLM2-bearing strain were compared with those of the host strain *E. coli* DH10B transformed with pBluescriptSKII+ (DH10B-pSK) (Fig. 3A). As expected, the resistance of the pLM2 strain to chloramphenicol was higher, as shown by an

Table 1. Sequence similarities of the ORFs identified in pLM2

ORF	Length (aa)	Closest similar protein	Organism	Amino acid		Nucleotide	
				E value	Identity (%)	E value	Identity (%)
orf1	45	Oxidoreductase	*Aeromonas salmonicida*	4E-21	98	4E-60	99
orf2	387	Multidrug resistance protein	*A. salmonicida*	0.0	99	0.0	98
orf3	115	HAM1 protein	*A. salmonicida*	2E-72	96	1E-169	98

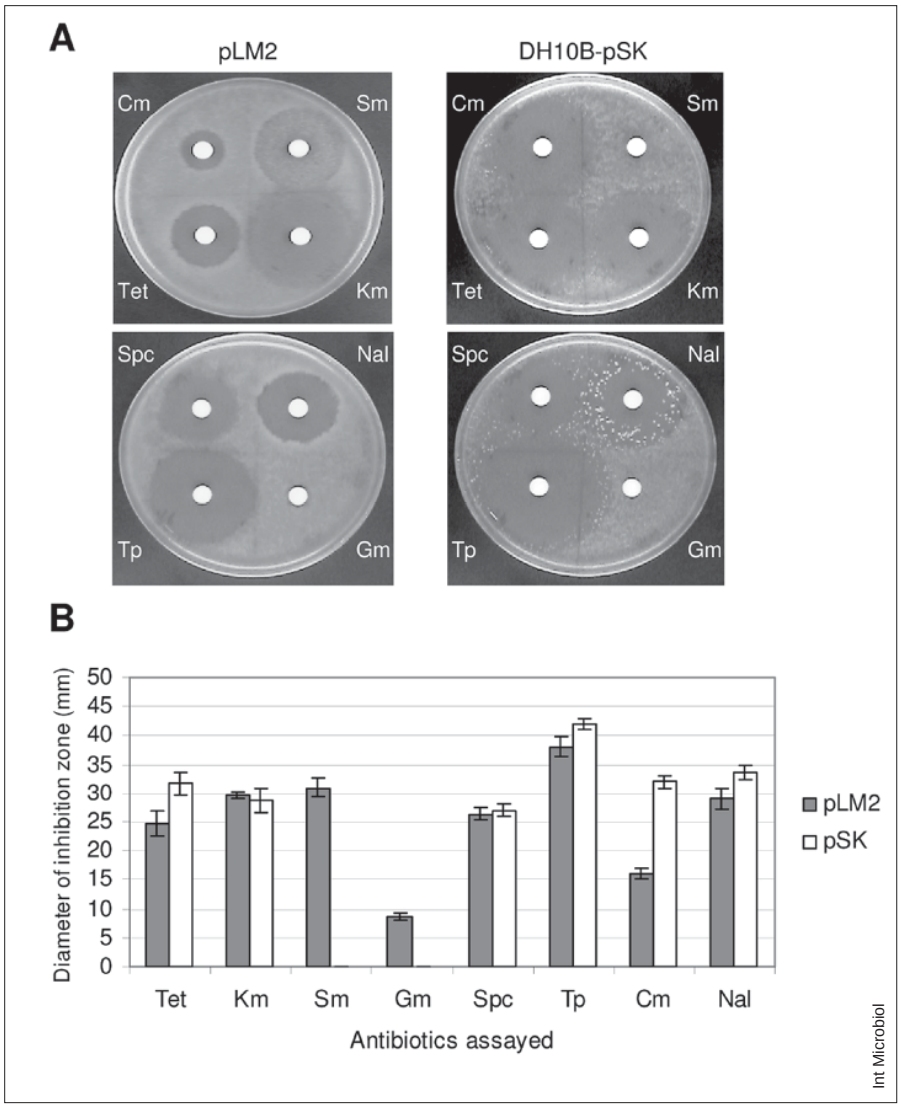

Fig. 3. (**A**) Disc diffusion test for evaluating the antibiotic susceptibility of pLM2 and *Escherichia coli* DH10B cells with pBluescriptpSKII+ (DH10B-pSK). (**B**) Size of the inhibition zone formed around diffusion discs impregnated with different antibiotics. Tet, tetracycline; Km, kanamycin; Sm, streptomycin; Gm, gentamicin; Spc, spectinomycin; Tp, trimethoprim; Cm, chloramphenicol; Nal, nalidixic acid. Values are the averages of three independent tests. Error bars indicate standard deviation.

inhibition halo smaller than that of the host strain (16 ± 1 and 32 ±1 mm, respectively), as shown in Fig. 3B. By contrast, a much larger inhibition halo was obtained with pLM2 than with DH10B-pSK in the presence of either streptomycin (31 ± 1.7 and 0 mm, respectively) or gentamicin (8.67 ± 0.58 and 0 mm, respectively). Thus, the pLM2 clone was more sensitive to these two antibiotics than to chloramphenicol. With the remaining antibiotics, the resistance profiles were similar, with the pLM2 strain showing slightly higher resistances to tetracycline, trimethoprim, and nalidixic acid.

Chloramphenicol resistance protein model. To gain further insight into the phenotype conferred by pLM2, we developed a model for the chloramphenicol resistance protein encoded by *orf2*. According to a hydropathy analysis, TM helical domains were predicted for the following fragments: TM1 Pro2-Leu22, TM2 Gly41-Ala61, TM3 Val74-Ala96, TM4 Tyr130-Ala151, TM5 Phe159-Met177, TM6 Phe206-Ala227, TM7 Tyr244-Arg266, TM8 Arg270-Val290, TM9 Val303-Ala323, TM10 Ala333-Met355, and TM11 Leu362-Leu384 (Figs. 4, 5).

The translocon hydropathy scale takes into account the bilayer partitioning of TM helices recognized by the translocon machinery, hence it is more efficient in identifying helical folding than the portioning of short peptides in n-octanol [37,44]. The hydropathy analysis identified up to 11 putative helical domains. The query sequence, however, did not yield any TM sequences already deposited in the database. Thus, this sequence displayed novel hydropathic properties. In relation to the structural data available for members of the MFS superfamily, the hydropathic analysis was consistent with that of the helical bundles of other MFS members, which fold in a bundle comprising12 TM helices. The two halves of the struc-

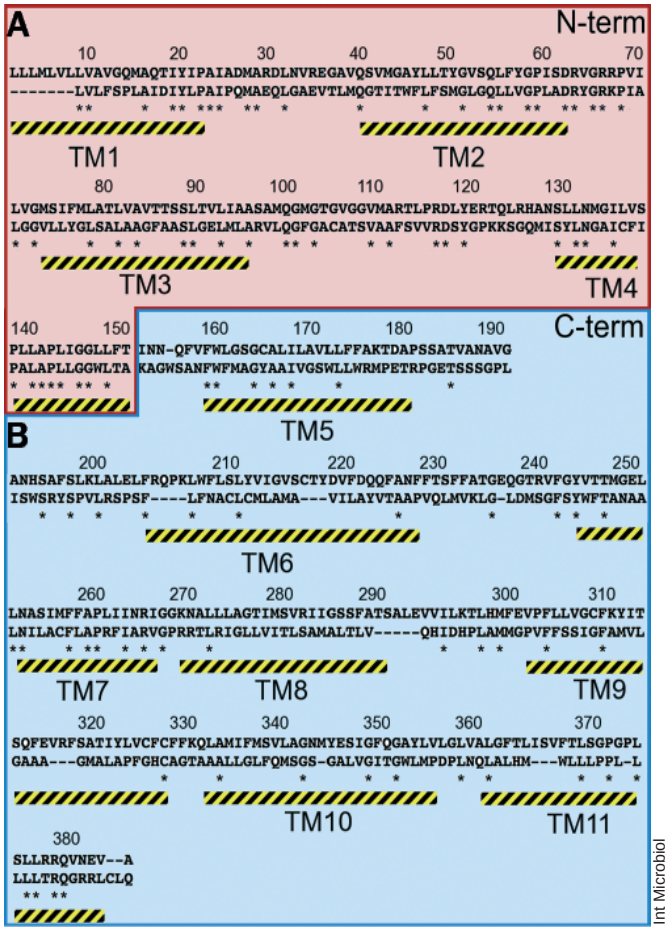

Fig. 4. Sequences alignment used to generate the chloramphenicol resistance protein model. Numbering corresponds to the novel membrane protein sequence. (**A**) Alignment for the N-term domain using the *Escherichia coli* multidrug transporter (upper sequence) as template. (**B**) Alignment for the C-term domain using the *E. coli* lactose permease LacY (upper sequence) as template. The overall sequence identity was 25 %. Helical domains along the amino acid sequence are indicated by the bars beneath the protein sequences. Eleven helical domains were predicted (TM1–TM11).

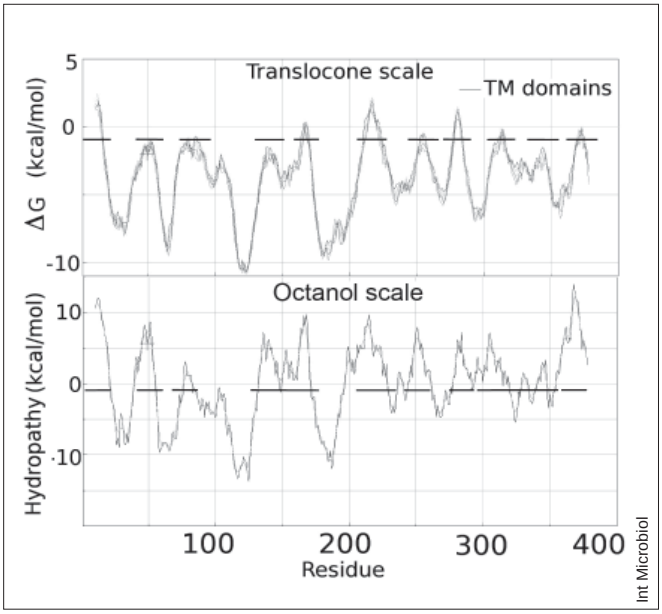

Fig. 5. Hydropathy analysis of the novel chloramphenicol resistance protein. Putative TM helical domains are identified along the amino acid sequence. Helical folds detected by the translocon machinery define the translocon scale, and the partitioning of short peptides in n-octanol defines the n-octanol scale.

ture correspond to the N-term and C-term domains, with six TM bundles each. The structural features revealed by the chloramphenicol resistance protein model were similar to those of the MFS members (Fig. 6). Table 2 summarizes the main structural motifs of the resolved structures of the MFS members and of those obtained in the MFS model.

Discussion

In this study, a functional metagenomics approach was used to search for novel chloramphenicol resistance genes. Because of the large fraction of uncultured microorganisms that may thrive in a particular environment, culture-independent methods based on metagenomics techniques have proven to be advantageous in the identification and sequencing of antibiotic resistance genes from diverse environments [2,10,29,41], in addition to providing useful tools for identifying genes and proteins in situ [8]. The water sample used to construct the metagenomic library contained higher concentrations of arsenic than of the other heavy metals detected. Arsenic enrichment likely reflected poor manure management by the surrounding farms close to the irrigation canal. Moreover, it might be indicative of the co-occurrence of antibiotic resistance genes in this particular environment. In fact, a correlation between high concentrations of heavy metals, including arsenic, and the abundance of antibiotic resistance determinants had been previously reported [48].

By screening for chloramphenicol resistance genes, we identified one positive clone, denoted as pLM2. Further analysis of pLM2 revealed a complete ORF that encoded an amino acid sequence identical to a multidrug resistance protein of *A. salmonicida*. This putative protein was subsequently determined to be a member of the MFS. The amino acid and nucleotide sequences of the other two ORFs of pLM2 differed slightly from the sequences of *A. salmonicida*, in which the same gene arrangement is found. This result suggested that

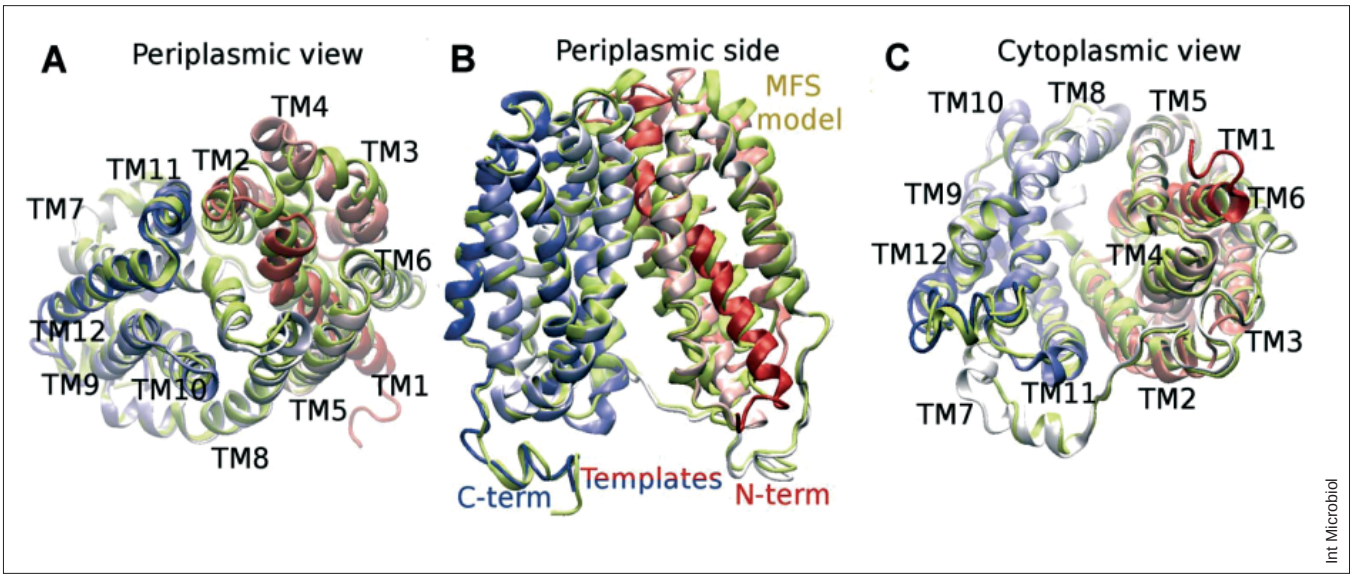

Fig. 6. Three-dimensional overlay between the chloramphenicol resistance protein model (yellow ribbons) and the LacY template. The N-term and C-term of LacY are depicted as ribbons using red-pink-white and white-light blue-blue gradients, respectively. (**A**) Periplasmic view of the membrane according to the protein model and the templates. The N-term consists of helical repeats TM1–TM3 and TM4–TM6, and the C-term TM7–TM9 and TM10–TM12. (**B**) Side view of the 12 TM segments. (**C**) Cytoplasmic view of the N-term and C-term domains.

pLM2 was retrieved from an environmental clone of this species, in agreement with the high intra-specific diversity found within other species of *Aeromonas* [1].

Previous studies described a relationship between members of the MFS and multiple antibiotic resistances [19,24,43]. MFS proteins are involved not only in the assimilation of different nutrients but also in the excretion of potentially toxic compounds in the cell [32]. Sequence analysis has shown that pLM2 shares homology with members of the Bcr/CflA MFS subfamily, a group of antiporters with known chloramphenicol resistance, such as EmrD-3 in *V. cholerae* [36]. Note that a great variety of proteins confer resistance to chloramphenicol, such as the Mdr multidrug transporters [6] and the florfenicol exporter, fexA [17]. Thus, nowadays, the clinical use

Table 2. Motifs verified in the proposed MFS model

Structural motiff	MFS Protein	Function	Reference
TM1, TM4, TM7, and TM10 form the central core domain	LacY, GlpT, EmrD, FucP, PepTso, PepTst, XylE	Residues essential for substrate coordination and co-transport coupling. TM domains involved in interactions between N and C domains of the transporter	[45]
TM2, TM5, TM8, and TM11 form shield the central core domain	LacY, GlpT, EmrD, FucP, PepTso, PepTst, XylE	Mediate inter-domain interactions. Also involved in substrate binding and co-transport	[47]
Short cytoplasmic loops at the TM2 and TM3, and TM8 and TM9 junctions	LacY, GlpT, EmrD, FucP, PepTso, PepTst, XylE	Restrain to the relative motions of the connected TM domains on the cytoplasmic side	[45]
Single substrate-binding cavity enclosed by the N and C domains located halfway into the membrane	LacY, GlpT, EmrD, FucP, PepTso, PepTst, XylE	Gating of molecules to the periplasmic side	[16,39]

LacY: Lactose proton symporter; *GlpT*: glycerol 3 phosphate:Pi antiporter; *EmrD*: multidrug transporter; *FucP*: L-fucose proton symporter; $PepT_{50}$: peptide proton symporter; $PepT_{st}$: peptide proton symporter; *XylE*: D-xylose proton symporter.

of many antibiotics may be compromised by a wide range of bacterial drug efflux pumps [12]. In this work we also found that pLM2 restored the streptomycin sensitivity of *E. coli* DH10B cells, which because of a rpsL mutation display streptomycin resistance. Although the precise mechanism underlying the sensitivity phenotype remains to be elucidated, the overproduction of MFS proteins may facilitate the uptake of antibiotics and thus increase their intracellular concentration to one that is effective [7].

A model for the multiporter membrane protein expressed by *A. salmonicida* and responsible for chloramphenicol resistance was developed to allow us to gain insights into its mode of action. This closer look into the structure and possible mechanisms of action of MFS proteins provided evidence of their importance with respect to multiple processes that influence the physiology of bacteria in their different environments [28].

The proposed MFS structure, based on the information deposited in databases and previously resolved structures [14,16,39,42,47], sheds light on non-specific transport mechanism and can be used as an initial configuration to generate a set of protein configurations in a lipid bilayer membrane in the presence of explicit solvent molecules, or as template to host antibiotic molecules in the substrate binding site. Structural data as well as molecular dynamics studies suggested that the large scale motions for the inward/outward gating of the *A. salmonicida* multiporter are triggered by the protonation of Glu135 in the fucose co-transporter [38] and the deprotonation of Glu325 in the galactopyranoside symporter [5]. The absence of acidic residues in key positions of the chloramphenicol resistance multiporter described herein may therefore point to an alternative triggering mechanism of inward/outward symport gating in MFS membrane proteins. Future work building on these findings will include characterization of the mechanism by which MFS proteins expel chloramphenicol. Elucidation of this mechanism could guide the development of new antibiotics that are less likely to generate bacterial resistance.

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Competing interests. None declared.

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