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# Characterization of a two-gene operon *epeRA* involved in multidrug resistance in *Streptomyces clavuligerus*

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**Abstract**

Two genes, *epeR* and *epeA*, are located downstream of *argH* in the *Streptomyces clavuligerus* genome. EpeR belongs to the TetR family of transcriptional regulators. It is homologous to PqrA of *Streptomyces coelicolor* (74.3% identity) and to NfxB of *Pseudomonas aeruginosa* (30.9% identity). EpeA encodes a protein with 14 transmembrane spanning domains (TMS) of the major facilitator superfamily. It shares 68.9% identity to PqrB of *S. coelicolor* and 46.5% identity to LfrA, conferring resistance to fluoroquinolones in *Mycobacterium smegmatis*. Disruption of *epeR* results in a *S. clavuligerus epeR*::*aph* mutant which shows increased resistance to ethidium bromide and proflavine (16- and 32-fold higher than the wild type). Taking into consideration the sensitivity to drugs of different transformants carrying functional copies of either *epeR* or *epeA*, it might be concluded that both genes appear to be co-transcribed, with *epeR* encoding a regulatory protein which controls the expression of *epeA*.


*Keywords: epeRA*; *Streptomyces clavuligerus*; Two-gene operon; Multidrug resistance

## 1. Introduction

*Streptomyces* are Gram-positive filamentous bacteria well known for producing numerous secondary metabolites, including many clinically important antibiotics. Antibiotic biosynthesis is coordinated with morphological development leading to aerial mycelium and spore formation [7]. In each producing strain, one or more genes for resistance to their own antibiotic are often clustered with antibiotic biosynthesis genes [37]. Self-defense mechanisms include drug binding or inactivation, target alteration and reduction of intracellular concentration by active transport [12]. It has been suggested that these genes might be at the origin of resistance determinants found in pathogenic bacteria [11,13,60].

*Streptomyces clavuligerus* produces the β-lactam antibiotic cephamycin C and the β-lactamase inhibitor clavulanic acid [25,33,34]. A gene for a β-lactamase is present in the cephamycin cluster, encoding an enzyme involved in the extracellular hydrolysis of active β-lactam intermediates of cephamycin biosynthesis [43]. Genes encoding a β-lactamase inhibitory protein (Blp), two penicillin-binding proteins (PBP) and a transmembrane protein (CmcT) of the major facilitator superfamily (MFS) to export cephamycin are also present in the cephamycin–clavulanic acid supercluster [33,43].

Additionally, the presence of multiple drug transporter proteins in membranes associated with active efflux and resistance to structurally different toxic substances not produced by the strain has been observed in *Streptomyces* [5,15] and other Gram-positive bacteria, such as *Staphylococcus* [17] and *Mycobacterium* [59]. When the sequence of known genomes is considered, soil microorganisms are found to encode a number of multidrug resistance genes (MDR), well above the average encoded by other microorganisms [42]. These genes might protect the cell from antibiotics produced by different *Streptomyces* species or from toxic compounds present in soil [32]. In *Streptomyces*, two MDR systems have been well characterized: (i) the *ptr* gene of *Streptomyces pristinaespiralis*, encoding a

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MFS protein which confers resistance to rifampicin and to the synergistically acting antibiotics produced by the strain, pristinamycins I and II [5]; and (ii) a silent ABC transporter system, named *msr*, isolated from the streptothricin producer *Streptomyces rochei* F20. This gene, when expressed in *Streptomyces lividans*, raises multiresistance to oleandomycin, erythromycin, spiramycin, doxorubicin and tetracycline [15]. More recently, three MDR systems have been isolated from *S. lividans* mutants selected by means of ethidium bromide resistance [29].

Therefore, it was of interest to study MDR systems in *S. clavuligerus* to establish their role in antibiotic resistance and/or exportation. In this article we present an operon encoding a regulatory protein which controls the expression of a transmembrane protein involved in ethidium bromide, proflavine and other drug resistance in the clavulanic acid producer *S. clavuligerus*.

## 2. Materials and methods

### 2.1. Media and culture conditions

The bacterial strains used in this work are listed in Table 1. ME medium [51] was used to obtain spores of *S. clavuligerus* strains. *S. clavuligerus* ATCC27064 and derived strains were grown in 500 ml triple-baffled flasks containing 100 ml of TSB medium (trypticase soy broth). The cultures were incubated at 28 °C and 220 rpm until the $OD_{600\,nm}$ was 7. Aliquots (1 ml) from these cultures were mixed with glycerol to a final 20% concentration, stored at −70 °C and used as inoculants. Liquid cultures of *S. clavuligerus* transformants were supplemented with kanamycin (50 μg/ml) or thiostrepton (5 μg/ml) when appropriate, except when the strains were used for drug resistance tests (see below). TSA plates (TSB with 2% agar) were used for drug resistance assays.

### 2.2. DNA procedures and sequence analysis

DNA manipulations, restriction endonuclease digestions, ligations and *Escherichia coli* or *S. clavuligerus* transformation, were performed according to standard procedures [16,27,50]. Fragments to be sequenced were subcloned in pBluescript I KS(+) and pBluescript II SK(+) and exonuclease mediated deletions were generated with an Erase-a-Base kit (Promega). The resulting clones were sequenced on both strands using an ALF-DNA sequencer (Pharmacia). Sequence analysis was performed using the DNASTAR package and the following internet resources: (i) the Neural Network Method for Promoter Predictions developed by Martin Reese, the Berkeley Drosophila Genome Project website (http://www.fruitfly.org/seq_tools/promoter.html); (ii) the TopPred 2 program for Topology Prediction of Membrane Proteins, Department of Biochemistry, Stockholm University (http://www.biokemi.su.se/~server/toppred2); (iii) protein motif searches were performed at the Bioinformatics Group website of the Swiss Institute for Experimental Cancer Research (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) and at the Conserved Domain Database using RPS-Blast (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). DNA sequences were screened for putative binding sites using the Patser algorithm [21], in the web resource Regulatory Sequence Analysis Tools [61]. The logo and the information content value, $R_i$, were calculated using the Bipad web server [9]. $R_{sequence}$ values were calculated with encode and rseq programs [53]. Sequence information of the downstream region of the arginine cluster, including the *epeRA* genes, has been deposited under EMBL Nucleotide Sequence database accession number AJ302083.

Table 1
Bacterial strains and plasmids used in this work

| Strains or plasmids | Description | Reference or source |
|---|---|---|
| *Streptomyces clavuligerus* ATCC27064 | Producer of cephamicin C and clavulanic acid | Higgens and Kastner, 1971 [22] |
| *E. coli* DH5α | Used as host in transformation | Hanahan, 1983 [20] |
| *E. coli* XL1-Blue | Used as host in transformation | Bullock et al., 1987 [6] |
| *E. coli* WK6 *mutS* | Used to obtain ssDNA | Kramer et al., 1984 [28] |
| *E. coli* ET12567 | Used to obtain unmethylated DNA to transform *S. clavuligerus* | MacNeil et al., 1992 [36] |
| pBluescript II SK(+)/pBluescript I KS(+) | *E. coli* vectors | Stratagene |
| pIJ699 | *E. coli–Streptomyces* vector | Kieser and Melton, 1988 [26] |
| PULVK99 | *E. coli–Streptomyces* vector | Chary et al., 1997 [8] |
| pTC192 | *E. coli* vector | De la Fuente et al., 1994 [14] |
| pTC192-Km | It contains a 1.3 kb *Hind*III-blunt ended fragment with the *aphII* gene in pTC192 | This work |
| pULAR10/pULAR11 | They contain, in pBluescript vectors, a 6.1-kb DNA fragment with *argDRGH, epeR* and part of *epeA* | Rodriguez-Garcia et al., 1995 [47] |
| pULAR699-*aph* | Shuttle vector carrying *epeR*::*aph* | This work |
| pUL69*epeA*1 | a pIJ699 derivative with a 1.9-kb DNA fragment carrying *epeA* downstream of the *pcbAB* promoter of *N. lactamdurans* | This work |
| pUL69*epeA*2 | the same as pUL69*epeA*1 with the promoter in the opposite orientation | |
| pULSKapa5A/B | pBluescript II SK(+) carrying in both orientations a 5.2-kb *Apa*I DNA fragment from *S. clavuligerus epeR*::*aph* | This work |
| pUL99*epeR* | pULVK99 containing *epeR* in a 2-kb *Xba*I-*Nco*I fragment | This work |
| pUL99*epeR*p | pULVK99 carrying in a 0.4-kb fragment the 5′ end of *epeR* | This work |

### 2.3. Gene disruption

Disruption of *epeR* was achieved using standard *Streptomyces* procedures [2]. The vector pULAR699-*aph* carrying the *aphII* gene inserted in the *Sal*I site of *epeR* was used for the disruption.

### 2.4. Southern hybridization analysis

Genomic DNA was isolated as described by Kieser et al. [27], digested with suitable restriction enzymes and probed using standard methods with a radiolabeled 0.9-kb *Sac*II *epeRA* fragment or the *aphII* cassette.

### 2.5. Drug-resistance determination

In experiments to test drug resistances, *S. clavuligerus* transformants were grown as indicated above in the presence of thioestrepton (25 μg/ml) for plasmid maintenance. After 24 h of growth, cells were collected by centrifugation and resuspended in 0.5 vol. of TSB. Petri dishes of five centimeter diameter containing 5 ml of TSA medium supplemented with the drug to be assayed (and the antibiotic for selection when required), were prepared and seeded immediately by spreading 8 μl of a fresh mycelium suspension obtained from a 24 h TSB culture concentrated twofold in TSB medium. The minimal inhibitory concentration (MIC) was determined as the concentration inhibiting growth after incubation for 3 days at 28 °C in a water-saturated atmosphere to prevent plates from drying. Experiments were performed in triplicate.

### 2.6. Antimicrobials

The following antimicrobials were used in this work: cetrimide and benzalkonium chloride were from Acofarma (Tarrasa, Spain), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), proflavine and ethidium bromide were purchased from Sigma Ch. Co. (St. Louis, MI, USA), hexamidine was acquired from Rhône-Poulenc Rorer (Alcorcón, Spain), acridine orange and crystal violet were obtained from Merck, Sharp and Dohme (Madrid), chlorhexidine from Guinama Laboratories (Valencia, Spain), pefloxacin (Azuben®) from Lasa Laboratories (Sant Feliu de Llobregat, Spain) and ciprofloxacin (Baycip®) from Bayer (Barcelona, Spain).

## 3. Results

### 3.1. Analysis of the DNA region located downstream of argH: Disruption of ORF1 does not prevent arginine utilization

Downstream of the arginine biosynthesis gene cluster *argCJBDRGH* [46], in plasmid pULAR10 (Fig. 1), a complete 555 bp open reading frame (ORF1) and 586 bp of an incomplete ORF (ORF2) were detected. The genetic loci and initial sequence data of these ORFs suggested that these genes might be involved in arginine transport. To test this hypothesis experiments were performed to disrupt ORF1. Plasmid

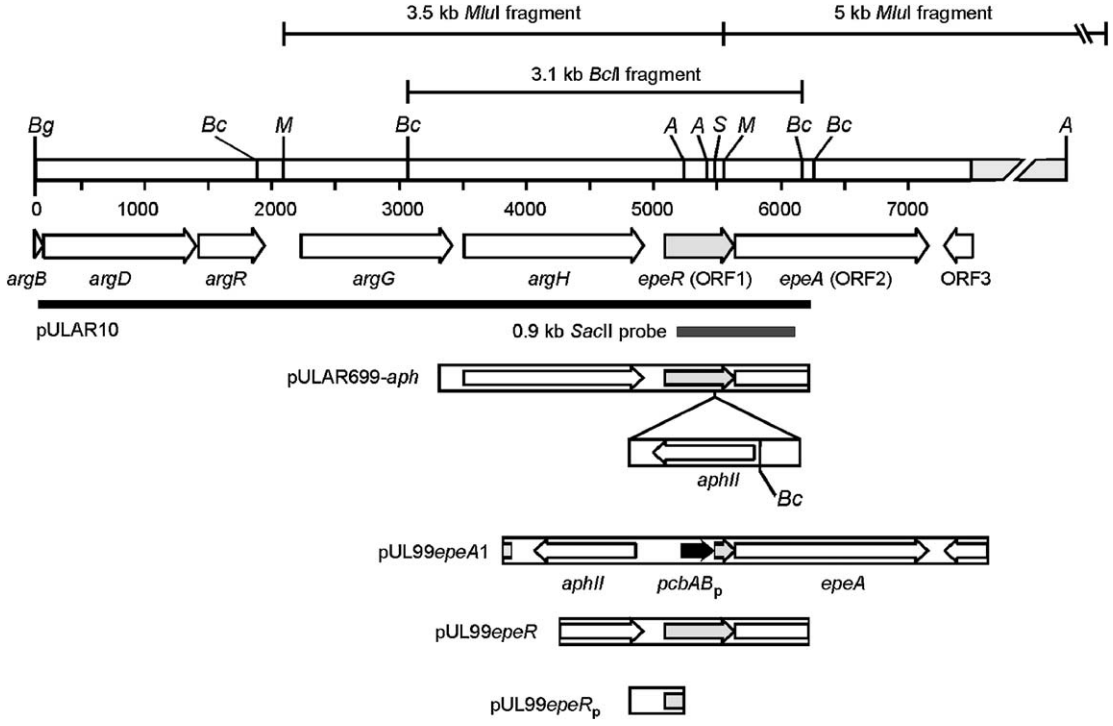


Fig. 1. Physical map of the *S. clavuligerus* DNA region studied in this work. The shaded bar at the right end represents the partially sequenced *Apa*I fragment. Restriction sites: *Apa*I, A; *Bgl*II, Bg; *Bcl*I, Bc; *Mlu*I, M; *SalI*, *S* (only shown the *SalI* site where the *aphII* cassette is inserted). ORF1 and ORF2 correspond to the *epeR* and *epeA* genes, respectively. The extension of the pULAR10 fragment in which *epeR* and *epeA* were initially found is indicated by a thick bar. The gray rectangle bellow represents the *Sac*II probe used in Southern hybridization in Fig. 2. The *Mlu*I and *Bcl*I fragments of the wild-type strain that were detected in Southern hybridization are also indicated.

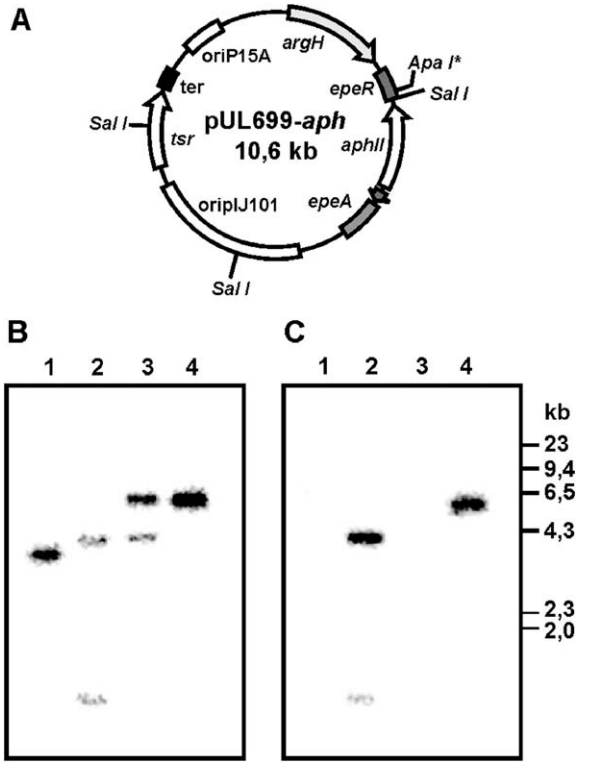


Fig. 2. (A) Plasmid pULAR699-*aph* used to disrupt *epeR*. (B, C) Hybridization of total DNA of *S. clavuligerus* (1, 3) and *S. clavuligerus epeR*::*aph* (2, 4) digested with *Bcl*I (1, 2) or *Mlu*I (3, 4) with a 0.9 kb *Sac*II probe overlapping *epeR* and *epeA* (B) and a probe containing the whole *aphII* gene (C). Expected fragments for the *Bcl*I digestion were: one fragment of 3.1 kb for the wild type, and two fragments of 1.1 and 3.4 kb for the disrupted strain, because of the presence of a *Bcl*I site in the *aphII* cassette (Fig. 1). For *Mlu*I digestion of wild-type total DNA, a fragment of 3.5 kb was expected, plus a fragment of unknown size that turned out to be 5-kb of length. The disrupted strain showed this 5-kb fragment as well, and another fragment of 4.9 kb, composed of the wild-type 3.5-kb fragment plus 1.4 kb from the *aphII* cassette.

pULAR699-*aph*, a pIJ699-derived plasmid containing the *aphII* cassette inserted in the *Sal*I site of ORF1 (Fig. 1), was transformed in *S. clavuligerus* and, after two steps of sporulation in antibiotic-free medium, about 1% of the clones were found to be thioestrepton-sensitive and kanamycin-resistant. DNA from the putative disrupted clones was digested with *Bcl*I and *Mlu*I and hybridized with: (i) a 0.9 *Sac*II kb probe overlapping ORF1 and ORF2, and (ii) a probe containing the whole *aphII* gene (Fig. 2). Results showed that only the *thio*$^{S}$ *kan*$^{R}$ strains hybridize with the *aphII* probe (Fig. 2C, lanes 2 and 4). Additionally, the size pattern of fragments hybridizing with the ORF1–ORF2 probe were consistent with the gene organization shown in Fig. 1 for *S. clavuligerus* and *S. clavuligerus* ORF1::*aph*.

However, *S. clavuligerus ORF1*::*aph* was still able to grow on arginine as sole nitrogen source. Therefore the sequence of the second ORF2 was completed in order to have a clear idea of the function of ORF1 and ORF2. The complete ORF2 was cloned from a *S. clavuligerus* ORF1 disruptant, by marker rescue. DNA from *S. clavuligerus ORF1*::*aph* was digested with *Apa*I, a restriction enzyme with no target sequence in the 5 kb around ORF1. The resulting fragments were ligated into pBluescript I KS(+) and transformants carrying the *aphII* marker were selected with kanamycin. Two plasmids, pULSKapa5A and pULSKapa5B, were found to contain a 5.2-kb *Apa*I DNA fragment carrying the *aphII* gene upstream of ORF2, the complete ORF2 and 2.1-kb fragment downstream of ORF2.

### 3.2. Characteristics of the genes located downstream of argH

ORF1 (nt 155–709 of the sequence) is found 151 nt downstream of *argH*. The gene is 555 nt in length and has a C + G content of 73%. The neural network method predicts the presence of a *Streptomyces–E. coli* promoter, characteristic of primary metabolism genes, upstream of ORF1, with −35 and −10 sequences TTAATG and TGCAGT, respectively. No obvious Shine–Dalgarno sequence is present upstream of the ATG start codon. The ATG codon of ORF2 (nt 706–2244) overlaps the TGA stop codon of ORF1. A Shine–Dalgarno sequence GGAG is present at nucleotides 693–696 preceding the ATG start codon. Additionally, upstream of the ATG start codon of ORF2, internal to ORF1, there is an inverted repeat sequence (nt 660–668 and 673–681), predicted to form a hairpin structure in the mRNA, with a calculated free energy of −17.2 kcal/mol. ORF2 has 1539 nt and a C + G content of 72.6%. A second potential hairpin structure was identified downstream of ORF2 (nt 2291–2299 and 2305–2313), with a free energy of −26.6 kcal/mol. This may function as a transcription terminator since the following ORF (ORF3) is in the complementary DNA strand. They will be named hereafter *epeR* and *epeA*, respectively (for *ethidium proflavine efflux*) due to the function identified for ORF1 and ORF2 (see below).

Comparison of the *epeRA* sequence of *S. clavuligerus* with *Streptomyces coelicolor* cosmid SCL24 (AL157956) shows that homologous genes for *epeRA*, encoding proteins PqrA (SCL24.04C) and PqrB (SCL24.03C), are located downstream of *argH* in the *S. coelicolor* genome, but separated by an additional ORF (SCL24.05C). Therefore, the organization *argH*-[SCL24.05C]-[SCL24.04(*epeR*)]-[SCL24.03(*epeA*)] is very similar in both *Streptomyces* strains, whereas the organization of the *epeRA* region in the genome of both *S. coelicolor* and *S. avermitilis* is identical.

### 3.3. Characteristics of the proteins encoded by epeR and epeA

The protein encoded by *epeA* has 512 amino acids and a Mr of 52111. This protein has 74.3% identity at the amino acid level to the protein encoded by *pqrB* (SCL24.03C) of *S. coelicolor*, which has been characterized as conferring methyl viologen resistance [10]. EpeA shares 74.6% identity with ORFA of *S. cinnamoneus*—a protein with unknown function, 46.5% with LfrA [52], a protein which confers resistance to fluoroquinolone upon *Mycobacterium smegmatis*, and 35.7% with each QacB and QacA of *Staphylococcus aureus*, proteins involved in multidrug resistance (Fig. 3). Many other proteins (not shown) involved in antibiotic export, or antiporter proteins involved in drug resistance, show amino acid identity above 35% with EpeA.

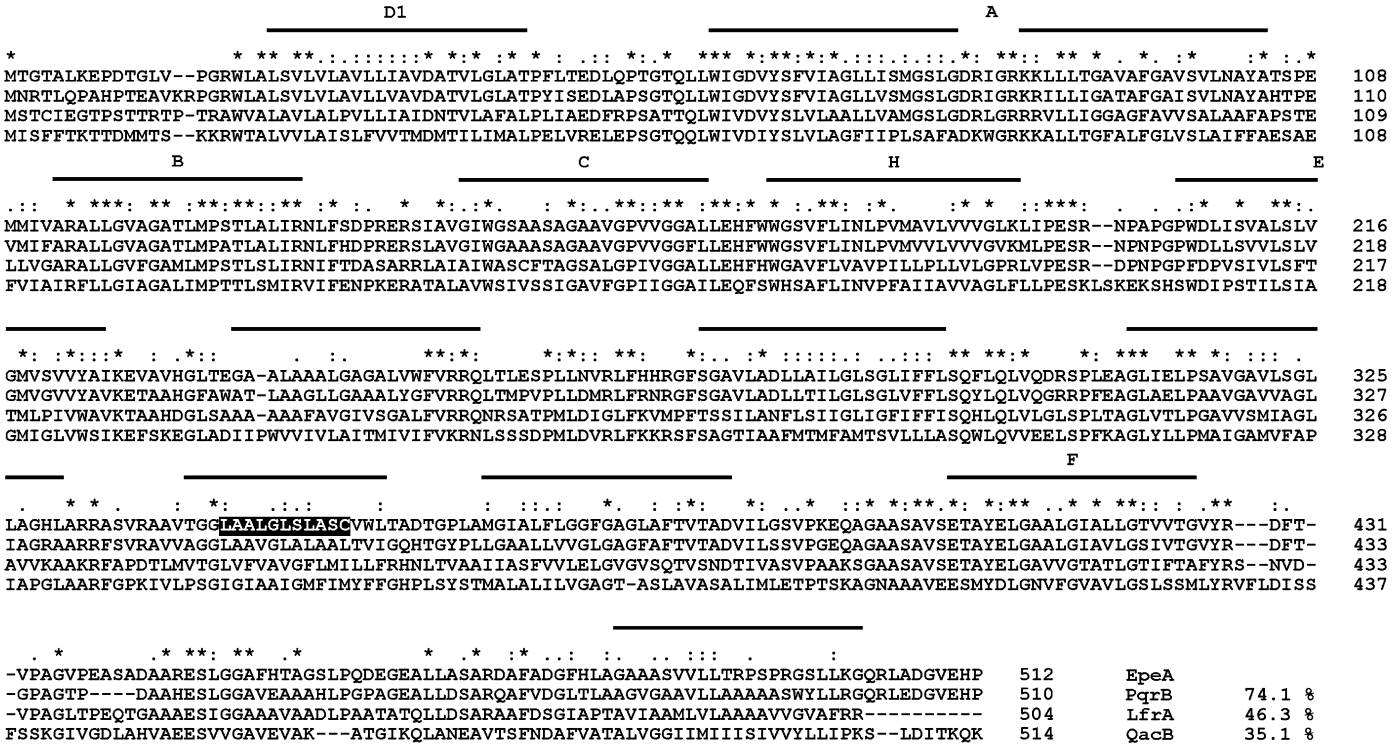


Fig. 3. Amino acid sequence alignment of EpeA from *S. clavuligerus*, PqrB from *S. coelicolor*, LfrA from *M. smegmatis* (Q50392) and QacB from *S. aureus* (P23215). Identical amino acids are indicated by asterisks. The 14-TMS transmembrane domains are indicated by thick bars. Motifs D1, A, B, C, H, E and F correspond to highly conserved sequences present in members of the 14-TMS family [41]. A sequence characterized as a lipid attachment site in lipoproteins (LAALGLSLASC, Prosite entry PS00013) present in transmembrane domain TMS11 of EpeA is outlined in black.

EpeA possesses the characteristic 14 transmembrane segments of the 14-TMS subfamily of MFS proteins identified by the TopPred 2 Program (see Fig. 3). A sequence characterized as a lipid attachment site in lipoproteins (LAALGLSLASC, Prosite entry PS00013) is present in transmembrane domain TMS11 of EpeA. Acylation of proteins has been reported as a mechanism to increase affinity to biological membranes [58], but whether this acylation site has any function in EpeA remains to be established.

Gene *epeR* encodes a protein with 184 amino acids and a deduced Mr of 20032. The amino acid sequence of EpeR showed homology to PqrA, the cognate regulator of *pqrB* in *S. coelicolor* (68.9% identity in 180 amino acids) and 30.9% identity to the NfxB regulator of *Pseudomonas*, associated with quinolone resistance (Fig. 4). Amino acid residues 22–52 of EpeR, ATMDEVAKAAGIGRATLHRHFAGRDALVRAL, match the Pfam motif 00440 from bacterial regulatory proteins of the TetR family.

Orthologous genes of *epeRA* of *S. clavuligerus* and *pqrAB* of *S. coelicolor* can be found in the *Streptomyces avermitilis* chromosome, genes SAV6781–SAV6782 [39], and in the streptomycin gene cluster of *Streptomyces griseus* (accession number AJ862840), genes SG7F10.61–SG7F10.62.

### *3.4. Functions of* epeR *and* epeA*: The EpeR protein controls sensitivity to some intercalating agents*

To test the effect of the proteins encoded by *epeR* and *epeA* on resistance to antiseptics, disinfectants and antibiotics, several plasmids were constructed (Fig. 1) so as to obtain strains with multiple copies of *epeA* (pUL69*epeA1*, *A*2) or *epeR* (pUL99*epeR*), as well as a strain with the putative promoter region of *epeR* in multicopy (pUL99*epeR*p). The plasmids were transformed in *S. clavuligerus* and in the disrupted strain *S. clavuligerus epeR*::*aph*, and then tested for sensitivity to several drugs (Table 2).

All strains tested (Table 2) displayed identical sensitivity to the intercalating agents crystal violet (MIC 64 μg/ml) and acridine orange (MIC 10 μg/ml), to the ammonium quaternary agents cetrimide (MIC 256 μg/ml) and benzalkonium hydrochloride (MIC 512 μg/ml), to the biguanidine agent chlorhexidine (MIC 64 μg/ml), and to the protonophore CCCP (MIC 16 μM).

A clear difference in drug sensitivity to several drugs was found in *S. clavuligerus epeR*::*aph* with respect to the wild-type strain. The MICs for the fluoroquinolone ciprofloxacin and for the diamidine agent hexamidine increased only twofold, but consistently among culture replicates. However, the greatest difference was found for the DNA intercalating agents ethidium bromide and proflavine, MIC values of which increased 16- and 32-fold, respectively. The lower sensitivity to intercalating agents of *S. clavuligerus epeR*::*aph* appears to be related to the absence of EpeR formation, a putative repressor protein. EpeR might control *epeA* expression from an operator within the EpeR-encoding sequence. Alternatively, *epeR* and *epeA*, might be co-transcribed and the control might be exerted on the *epeR* promoter.

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      :: * *:::::: *. :. :. **:.*:*.***:.:***** *.* ** **: **
------MTIDRERVLRAAAALLARKSTATMDEVAKAAGIGRATLHRHFAG-RDALVRALE    53
MGYCCLMAVDREQVLRSAATLLTRKSTATMDEVARAAGLSRATLHRHFAG-RDALVRALE    59
---MTLISHD-ERLIKALAVAIVDRPRATLKELAEAAGVSKATLHR-FCGTRDNLVQMLE    55

. *    :  :  .  :.. . :*::**: .  .   ** **  :   :::: .  .  *
EFGLRELEAAVEEARPDEGSAPEALRRLIGRTAPASPLLGFLITEN--QLFEGEGVNEGW   111
SLGIAECEAALAAARTDEGPAADAVRRLVREMEPSAALLAFLYTEN--QLFEGEEQNEGW   117
DHGETVLNQIIQACDLEHAEPLEALQRLIKEHLTHRELLVFLVFQYRPDFLDPHGEGARW   115

         :* ***:.* **:*:*.. :** :  *: . . *   **.* .:  : : :
ARIDAVCTTLFRRGQEEGHFRVDLTAVWLTEALYGLLSAGAWAVQAGRVAPKDYQYMIAE   171
ARIDTGLTELFRRGQESGEFRIDLTPAWLTEALYGLLASGAWAVTEGRVARNDFTHMIVE   177
QSYLEALDEFFLRGQQKGVFRIDITAAVFTELFITLVYGMVDAERRGRAASSNSAHTLEQ   175

:.* *. .
LVLGGCVRRSVEA   184       EpeR
LLLGGALRREEP-   189       PqrA      68.9 %
MFLHGASNPARS-   187       NfxB      30.9 %
```

Fig. 4. Amino acid sequence alignment of EpeR from *S. clavuligerus*, PqrA from *S. coelicolor*, and NfxB from *P. aeruginosa* (P32265). Identical amino acids are indicated by asterisks. Amino acid residues 22–52 of EpeR, matching the Pfam motif 00440 from bacterial regulatory proteins of the TetR family, are outlined in black.

Table 2
Susceptibilities of *S. clavuligerus* transformants to several intercalant drugs and fluoroquinolones

| Strain | Minimal inhibitory concentration (μg/ml) | | | | |
|---|---|---|---|---|---|
| | Ethidium bromide | Proflavine | Hexamidine | Ciprofloxacin | Pefloxacin |
| *S. clavuligerus* 27064 | 32 | 16 | 100 | 64 | 64 |
| *S. clavuligerus epe*R::*aph* | 512 | 512 | 200 | 128 | 64 |
| *S. clavuligerus epe*R::*aph* | 512 | 512 | 200 | 128 | 64 |
| *S. clavuligerus epe*R::*aph* [pUL99*epe*R] | 32 | 16 | 100 | 64 | 64 |
| *S. clavuligerus epe*R::*aph* [pUL69*epe*A1] | 512 | 512 | 200 | 128 | 64 |
| *S. clavuligerus* [pULVK99] | 32 | 16 | 100 | 64 | 64 |
| *S. clavuligerus* [pUL99*epe*R] | 32 | 16 | 100 | 64 | 64 |
| *S. clavuligerus* [pUL69*epe*A1] | 32 | 16 | 100 | 64 | 64 |
| *S. clavuligerus* [pULVK99] | 32 | 16 | 100 | 64 | 64 |
| *S. clavuligerus* [pUL99*epe*Rp] | 32 | 16 | 100 | 16 | 16 |

When the *S. clavuligerus epeR*-disrupted strain was transformed with plasmid pUL99*epeR*, the sensitivity to drugs returned to wild-type levels, confirming that EpeR acts as a diffusible repressor of *epeA* expression. No differences were found by overexpressing *epeR* in the wild-type strain, in which the concentration of EpeR molecules is probably high enough to repress transcription from the promoter controlled by this protein.

The possible effect of EpeR on EpeA formation, and therefore on drug sensitivity, was tested in a transformant carrying *epeA* (pUL69*epeA*1) under the strong *N. lactamdurans pcbAB* promoter ($pbcAB_p$), which is known to be active in *Streptomyces* [30]. A transformant with $pbcAB_p$ in the opposite orientation was used as a control strain (pUL69*epeA*2). In both plasmids $pbcAB_p$ was placed upstream of the sequence present at the 3′ end of *epeR* which is predicted to form a stem loop structure. No differences in drug sensitivity were found between the two transformants carrying plasmids or between the untransformed strains (Table 2).

In order to test whether the amplification of the *epeR* promoter can titrate out the repressor protein, the MIC for each drug used in this work was tested in *S. clavuligerus* [pUL99$epeR_p$], which contains the *epeR* promoter region in multiple copies. No differences were found for the intercalating drugs; however, the MIC's for the quinolones pefloxacin and ciprofloxacin were reduced in the order of 4-fold, indicating an interaction of the promoter of *epeR* with another possible repressor.

### 3.5. Bioinformatics identification of the EpeR operator

Two inverted repeats (IR) of 18 nt, IR1 and IR2, have been identified in the *epeR* homologous to *S. coelicolor*, *pqrA* [10]. Homologous sequences can be found in the *epeR* promoter region. The four IR sequences revealed some similarity with the NfxB operator sequences. However, the putative PqrA-EpeR-NfxB operators are best described as inverted repeats of 20 nt in length. Both inverted repeats were consecutive and overlapped with the nucleotides from −38 to +3 (relative to the initiation codon) in both genes.

Bioinformatics searches using the matrix formed from the 8 sequences of *S. coelicolor pqrA* and *S. clavuligerus epeR* (the reverse complement of each IR is included) were performed over several microbial genomes and data base entries. We found IR in the promoter regions of the *epeR* homologues of *S. avermitilis* (SAV6781) and *S. griseus* (SG7F10.61). Both genes showed two IRs overlapping the codon start, from −38 to +3 (relative to the codon start indicated by homology with

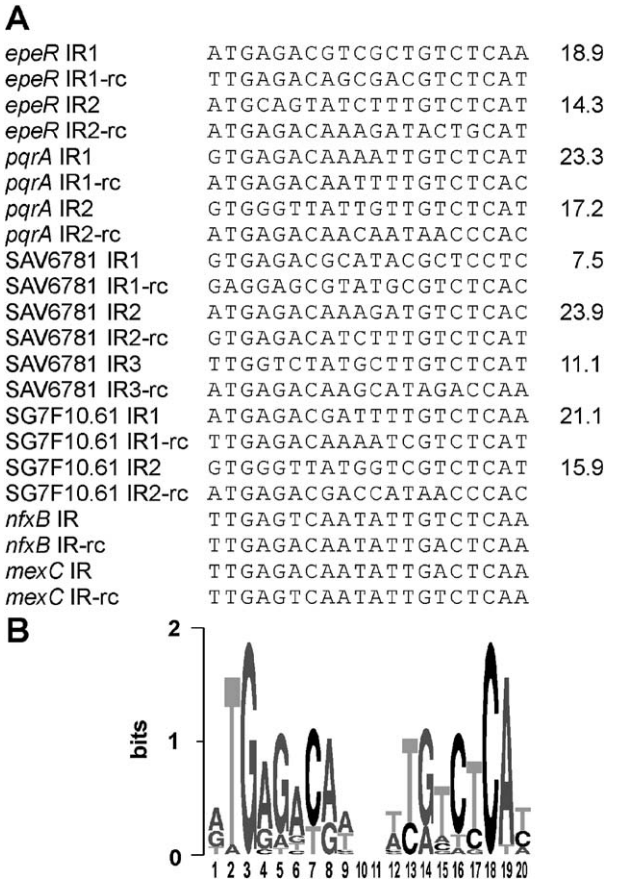


Fig. 5. (A) Nucleotide sequence alignment comprising the putative EpeR operator and related sequences. The two inverted repeat sequences (IR1 and IR2) of *epeR* were firstly aligned with the homologous inverted repeats IR1 and IR2 of *pqrA*, then with the newly found IR in the orthologous genes of *S. avermitilis* and *S. griseus*, and finally with the binding sites of NfxB in the *nfxB* and *mexC* genes of *P. aeruginosa*. Since these sequences constitute asymmetric motifs, each reverse complement (rc) sequence is included. The $R_i$ value in bits of each IR is also shown (calculated from the alignment). (B) Logo representing the binding site of the EpeR-orthologous proteins, deduced from the above alignment.

*pqrA*) as occurs in *S. clavuligerus* and *S. coelicolor*. Moreover, SAV6781 showed a third upstream IR, located from −58 to −39. The alignment of the IR identified is shown in Fig. 5A. A matrix was made and employed to screen several microbial genomes using the 18 sequences (the 9 inverted repeats and their reverse complements). No target sites, other than the previous identified, were found in the genomes of *S. coelicolor* and *S. avermitilis* (threshold estimation as adjusted information content), indicating that the operator is unique, and the only gene regulated is the multidrug pump. Other related microbial genomes, like *Corynebacterium glutamicum, Mycobacterium tuberculosis, Nocardia farcinica*, and *Bifidobacterium longum*, failed to contain target sequences. Meanwhile, *Pseudomonas aeruginosa* clearly showed the *mexC* and *nfxB* operators.

## 4. Discussion

Most multiple drug (MDR) transporter proteins involved in resistance belong to the ATP binding cassette family (ABC), the major facilitator superfamily (MFS), the resistance/nodulation (RND) family or the small multidrug resistance family (SMR) [31,45,48,49,62].

Expression and regulation of MDR proteins have not yet been completely elucidated but specific trans-acting regulatory proteins have been reported. These regulatory proteins are repressors such as in the EmrR system of *E. coli* [35] or in the QacA system of *S. aureus*, where the formation of the efflux pump involved in quinolone resistance is negatively regulated by QacR, a protein encoded by a divergently transcribed gene [17]. However in other systems, such as in the BmrR system of *Bacillus subtilis* [1], they act as activator proteins. The induction of these transport systems is usually mediated by toxic compounds that are transported from the cell by the transmembrane protein involved in the system. Additionally, global regulatory proteins and two-component systems controlling the transcription of genes for the transmembrane pump have been described [18,19,24].

After the *S. coelicolor* genome sequence project, up to 9 genes were annotated as having a putative multidrug export function [4]. The close species *S. lividans* contains at least four multidrug systems, three of them already identified [29]. These genes encode two MFS proteins (*ebrB* and *ebrC*), and one SMR transporter (*ebrA*). The *S. avermitilis* genome sequence also revealed 9 putative multidrug transporters, including 6 MFS, 2 SMR, and one RND member [39]. The soil environment of *Streptomyces* is potentially rich in toxic compounds that explain the numerous multidrug systems found in this genus, as occurs in other soil-dwelling microorganisms. However, these systems appear to be inactive in laboratory cultures, probably due to the lack of proper inducers [29].

EpeA of *S. clavuligerus*, containing 14-TMS motifs, is a proton motif force (PMF)-dependent antiporter of the DHA2 subfamily [48]. Its orthologous gene in *S. coelicolor* is *pqrB*, which has been related only to methyl viologen (paraquat) resistance [10]. This work has proved the involvement of the *epeRA* system in the export of ethidium bromide, proflavine, hexamidine, and ciprofloxacin. Therefore, EpeA, and very probably PqrB, can be included in the list of multidrug pumps.

According to its amino acid sequence, EpeR is a transcriptional regulator. The lack of clear promoter sequences upstream of *epeA* and the putative strong termination hairpins found downstream of *argH* and *epeA* suggest that *epeRA* is expressed as a transcriptional unit and the proteins formed are translationally coupled, as is usual in genes with overlapping TGA-stop and ATG-start codons [40]. The orthologous gene of *epeR* in *S. coelicolor* is *pqrA*, a repressor that is autoregulated, as shown in electrophoretic mobility shift assays [10]. Both genes, *pqrA* and *pqrB*, also appear to be transcriptionally coupled, since the codon stop of *pqrA* overlaps the codon start of *pqrB*. The disruption *epeR* produces a resistance phenotype in *S. clavuligerus*, and the *S. coelicolor* null mutant of *pqrA* is resistant to methyl viologen. The *epeR* gene complements disruption of the *epeR* genomic copy *in trans* and restores the MIC level of the complemented strain to wild-type levels, confirming that it encodes a diffusible regulatory protein. However, as shown in Table 2, increasing the copy number of *epeR* in either

*S. clavuligerus* or the *epeR*-disrupted strain does not result in greater sensitivity to ethidium bromide or proflavine, indicating either that *epeR* is autoregulated or that the chromosomal copy of *epeR* is enough to block the expression of *epeA*. A similar situation occurs in NfxB of *Pseudomonas* [38], which controls both transcription of the *mexC-mexD-oprJ* operon and its own transcription [44,56] or with ErmR of *E. coli* [35]. A mutation in the coding sequence of the *pqrA* repressor gene has been described as being the cause of methyl viologen resistance. The mutation results in an amino acid residue change near the helix-turn-helix motif, and causes the loss of DNA binding capability. The mutation is dominant and explains the resistance phenotype because of the derepression of *pqrB* [10]. The increase in MIC to ethidium bromide and proflavine of *S. clavuligerus epeR*::*aph* may be explained by expression of *epeA* from a putative derepressed promoter located in the *epeR* coding sequence. However, this is unlikely because of the lack of putative binding sites in the *epeR* coding sequence. The most plausible explanation is the deregulation of the *epeR* promoter, which may read through the *aphII* gene (lacking sequences for transcription termination) into the *epeA* gene. A similar arrangement occurs with the *lmrAB* operon of *B. subtilis*, in which the first gene is the repressor of the *lmr* promoter and the second gene is the multidrug transporter. Multidrug-resistant mutants showed increased transcription of both *lmrA* and *lmrB* genes [63]. In the *S. clavuligerus epeRA* system it is possible that the stem loop sequence found at the 3′ end of *epeR*, also present in all the plasmid constructs carrying *epeA*, modulates the expression of *epeA*. It has been indicated that bacterial MDR pumps need to be expressed at a substantially higher level than the wild type to obtain significant efflux of antimicrobial compounds [18]. The *S. clavuligerus* [pUL69*epe*A1] strain failed to show increased resistance, probably due to the lack of enough promoter activity or because of the presence of the stem loop downstream of the *pcbAB* promoter.

An additionally interesting point is the modification of MICs for the hydrophobic fluoroquinolones, pefloxacin and ciprofloxacin, due to the presence of the *epeR* promoter sequence in multiple copies. Amplification of the *epeR* promoter region might result in the sequestering of a protein required for the expression of genes for hydrophobic quinolone resistance. This protein might act as a global activator on both the *epeRA* system and a system conferring quinolone resistance. A different pattern of regulation has been found in *B. subtilis*. In this organism a positively-autoregulated global regulatory protein, named Mta [3], activates two different transport systems, *bmr* and *blt*. These two systems are, in turn, activated by specific transcriptional activators, BltR and BmrR, the latter being induced by tetraphenylphosphonium and rhodamine.

The *epeRA* genes are contiguous in four different *Streptomyces* strains and are located downstream of arginine biosynthetic genes. However, the organization of the surrounding genes is not exactly the same. In *S. clavuligerus* the *epeRA* operon is located immediately downstream of the arginine biosynthesis *argGH* genes, and is followed by a putative acyltransferase. The same organization occurs in *S. avermitilis* and in *S. griseus*, but between *argH* and their *epeR* homologues there is an oxidoreductase in *S. avermitilis*, and an oxidoreductase and a helicase, in *S. griseus*. In *S. coelicolor* the organization is similar except that the *argG* is absent. The partial sequence of the *epeA* homologue of *S. cinnamoneus* [57] does not allow us to identify its upstream gene, but there is a lipase instead of an acyltransferase downstream.

The identified binding sites of the EpeR orthologues are located in the same position, blocking the operon promoter. It is very plausible that the inverted repeats are bound by one dimer and that the tandem arrangement enables cooperative binding [23,55]. The bioinformatic search of the *Streptomyces* available genomes failed to reveal binding sites other that those found in the orthologous operons. We aligned the inverted repeats and—because the site is symmetric [53]—its reverse complement sequences, to calculate the individual information content, or $R_i$ value [54], for each binding site (Fig. 5A). The logo that represents the binding site, is also shown (Fig. 5B). The information needed to locate a unique symmetric binding site is 23.05 bits for the *S. coelicolor* chromosome and 23.21 bits for the slightly larger *S. avermitilis* chromosome, according to the $R_{frequency}$ formulae [53]. The calculated $R_{sequence}$ values of the inverted repeat sequences of each species range from 10.24 bits (*S. avermitilis*) to 15.0 bits (*S. coelicolor*). The $R_{sequence}$ [53] for the whole binding site is the sum of the $R_{sequence}$ values of the inverted repeats that form the binding site, i.e., 30.7 bits for *S. avermitilis* and 30 bits for *S. coelicolor*. These values exceed considerably the information needed to locate a unique binding site. This theoretical prediction reinforces the idea that EpeR and its orthologues exclusively regulate their own operon.

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