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A Silent ABC Transporter Isolated from *Streptomyces rochei* F20 Induces Multidrug Resistance

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In the search for heterologous activators for actinorhodin production in *Streptomyces lividans*, 3.4 kb of DNA from *Streptomyces rochei* F20 (a streptothricin producer) were characterized. Subcloning experiments showed that the minimal DNA fragment required for activation was 0.4 kb in size. The activation is mediated by increasing the levels of transcription of the *actII-ORF4* gene. Sequencing of the minimal activating fragment did not reveal any clues about its mechanism; nevertheless, it was shown to overlap the 3′ end of two convergent genes, one of whose translated products (ORF2) strongly resembles that of other genes belonging to the ABC transporter superfamily. Computer-assisted analysis of the 3.4-kb DNA sequence showed the 3′ terminus of an open reading frame (ORF), i.e., ORFA, and three complete ORFs (ORF1, ORF2, and ORFB). Searches in the databases with their respective gene products revealed similarities for ORF1 and ORF2 with ATP-binding proteins and transmembrane proteins, respectively, which are found in members of the ABC transporter superfamily. No similarities for ORFA and ORFB were found in the databases. Insertional inactivation of ORF1 and ORF2, their transcription analysis, and their cloning in heterologous hosts suggested that these genes were not expressed under our experimental conditions; however, cloning of ORF1 and ORF2 together (but not separately) under the control of an expressing promoter induced resistance to several chemically different drugs: oleandomycin, erythromycin, spiramycin, doxorubicin, and tetracycline. Thus, this genetic system, named *msr*, is a new bacterial multidrug ABC transporter.

The transport of molecules through cellular membranes is essential for living cells and can involve a significant part of the cell's genetic information. Most of the systems involved in this process are classified into a small number of families according to their sequences, the molecular arrangement of their individual components, and their molecular mechanisms. Of these, the so-called ABC transporter superfamily forms one of the largest and most diverse groups (2, 8, 18). Members of this superfamily are found in both prokaryotes and eukaryotes. They normally have some specificity for their substrates despite the wide diversity of compounds transported by each: peptides, amino acids, sugars, ions, antibiotics, toxins, heavy metals, etc. (2, 8, 18). Proteins of this superfamily usually have four domains: two hydrophilic domains facing the cytoplasmic side which are involved in ATP binding and hydrolysis and two hydrophobic domains (each with six membrane-spanning segments) involved in forming the structure needed for the substrates to be transported across the membrane. The four domains may be organized either in a multifunctional polypeptide or in separate proteins. One of the most relevant features of these transporters is the coupling of the energy from ATP hydrolysis for pumping their substrates across the cellular membrane against a concentration gradient. The importance and awareness of the ABC transporters has notably increased over the last few years since some of their members were implicated in single resistance or multiresistance to antibiotics in pathogenic bacteria and in lactic bacteria, as well as in multidrug resistance in tumors (2, 8, 18, 26, 44).

Here we describe the cloning and characterization from *Streptomyces rochei* F20, a producer of streptothricin (13), of a new member of the ABC transporter superfamily. This new transport system is highly similar to those conferring specific resistance to the anticancer drug doxorubicin and the macrolide oleandomycin and can, in some conditions, induce resistance to both drugs in addition to erythromycin, spiramycin, and tetracycline. Thus, this ABC transporter behaves as a multidrug resistance system, and we named it *msr* (multiresistance from *S. rochei*).

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used here were JM101 and JM110 (49). The *Streptomyces lividans* 66 strain was TK21 (*str-6* SLP2$^-$ SLP3$^-$) (20). The *Streptomyces coelicolor* strains were J1501 (20) and the *actII* ORF4 mutants B43, B58 (37), and JF1 (9).

Plasmids and bacteriophages. The *E. coli* plasmids used were pUC19 (49), pIJ2925, and pIJ2921 (21). *E. coli* M13 derivative phages mp18 and mp19 (49) were used for DNA sequencing. The *E. coli* λ phage-derived vector EMBL4 (14) was used to prepare a chromosomal library. The high-copy-number *Streptomyces* plasmid vectors used were pIJ486, pIJ487 (47), and pIJ702 (22). For promoter activity assays in *Streptomyces* strains, plasmid pIJ4083 (4) was used. The *Streptomyces* phage vector was the ϕC31 derivative KC515 (33).

Media, culture conditions, and microbiological procedures. *Streptomyces* manipulations were done as described previously (20). For streptothricin production by *S. rochei* F20, liquid FM medium was used (yeast extract 1%, Bacto Tryptone 1%, 25 mM TES buffer [pH 7.5]), at 30°C with good aeration. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth (24). The streptothricin production assays from liquid medium were made as previously described (13).

Conventional disk assay for antibiotic sensitivities was performed with DNA agar plates overlaid with 3 ml of SNA (20) containing 4×10^6 viable spores of the strains to be tested; the following quantities (given in micrograms) of several antibiotics and chemicals were applied on the paper disks: doxorubicin, 40; oleandomycin, 40; erythromycin, 30; tetracycline, 40; spiramycin, 150; strepto-

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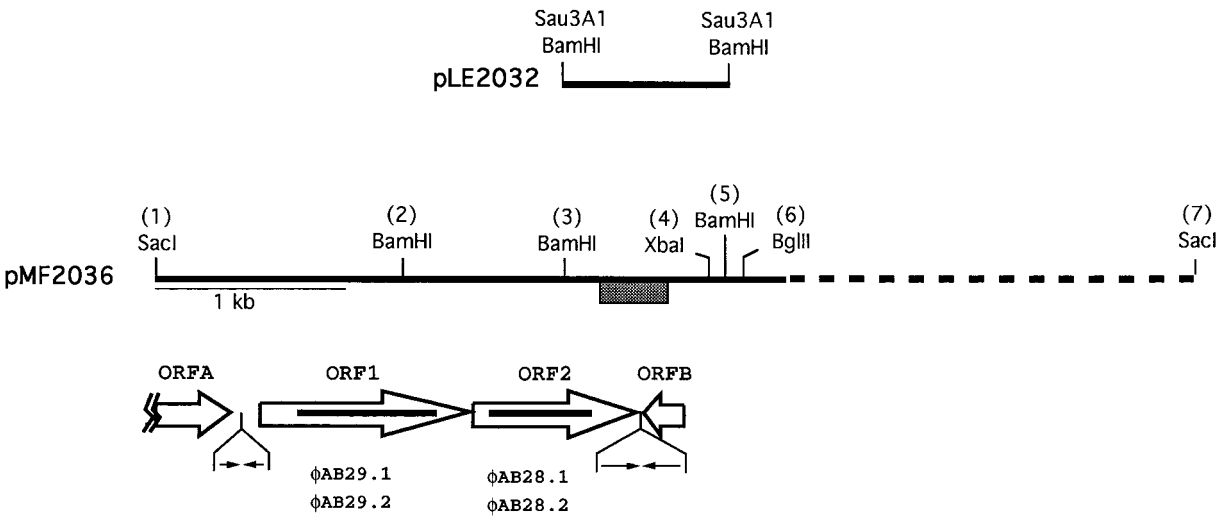

FIG. 1. Restriction map of the *S. rochei* F20 chromosome flanking the activation fragment. Organization of the ORFs adjacent to the activator fragment as deduced by DNA sequencing is shown. The solid black bar corresponds to sequenced DNA; the dotted black bar corresponds to nonsequenced DNA. The shaded box represents the activator fragment. Stem-loop structures are indicated by thin arrows. ORFs are indicated by large open arrows with thick bars inside corresponding to the fragments used for gene disruptions with the ϕC31-derived KC515 vector.

thricin, 30; streptomycin, 30; tobramycin, 10; novobiocin, 30; chloramphenicol, 30; phenol, 0.08; pentachlorophenol, 0.08; tetrachlorohydroquinone, 0.7; and ethidium bromide, 2.

Gradient plates of antibiotics were made according to the method of Szybalski and Bryson (42) on solid LB media. Strains were plated immediately after the plates were prepared.

For catechol assays, R5 as solid medium and YEME as liquid medium were used.

Nucleic acid manipulations. Isolation, cloning, and manipulation of nucleic acids were done as described for *Streptomyces* sp. (20) and for *E. coli* (24). For high-resolution S1 mapping, the method of Murray (25) was used. For RNA extraction from *S. lividans* and *S. coelicolor*, pregerminated spores were used to inoculate SY liquid medium (27), and mycelia were harvested after 48 h. RNA from *S. rochei* F20 was extracted from mycelia grown in FM (13) and harvested at several times of the cell cycle.

Construction of a chromosomal library. For constructing a chromosomal library of *S. rochei* F20 we used phage λ-EMBL4 as a vector and *E. coli* XL1-blue MRA as a host; both were from Stratagene (catalog number 242201). Then 100 μg of *S. rochei* F20 chromosomal DNA was partially digested with *Sau*3AI and fractionated on a 10 to 40% sucrose gradient (24). Next, 5 μg of the fraction containing DNA fragments of approximately 20 kb was ligated to 750 ng of vector previously digested with *Bam*HI and *Sal*I. Packaging and infection were performed according to the manufacturer's recommendations.

DNA sequencing. DNA sequencing was carried out by the dideoxy-chain termination method (38). We used the 7-deaza-dGTP reagent kit from United States Biochemical Corp. (catalog number 70750) according to the manufacturer's recommendations.

Computer analysis of sequences. The DNA sequence was analyzed by using the software programs of the University of Wisconsin Genetics Computer Group (version 8.0-AXP) (5); analysis for open reading frames (ORFs) used CODON-PREFERENCE with a codon usage table made from 100 *Streptomyces* genes (48). Comparisons of sequences were made against the EMBL Nucleic Acid Database and the Swissprot Data Base (both of them updated daily) by using FASTA, TFASTA, MOTIF, and BESTFIT. Protein alignments were made by using PILEUP and PRETTYBOX (32). Protein secondary structures were predicted with PEPTIDESTRUCTURE and PLOTSTRUCTURE. DNA secondary structure predictions were made with STEMLOOP.

Gene disruption. For gene disruption of *S. rochei* F20 we used insert-directed recombination of ϕC31 derivatives as described previously (3). In all cases, the chromosomal arrangements of lysogens were confirmed by Southern blot analysis.

Engineering of *msr* ORFs. The promoter region of *msr* was replaced by the 444-bp *Mbo*II fragment containing the well-known actinorhodin polyketide synthase (PKS) promoter (12, 29); the resulting construct, named pMF1138.1 (see Fig. 4), thus carries *msr-ORF1* and *msr-ORF2* (nucleotides 464 to 2912) under the control of a heterologous promoter. No fusion proteins between the N terminus of ActI-ORF1 with Msr-ORF1 could have occurred because of an in-frame TGA codon upstream of ORF1. Both ORFs *msr ORF1* and *msr ORF2* would be translated by using their own ribosome binding site (RBS).

For a functional characterization of the cloned genes, several constructs were made (see Fig. 4). Thus, the entire ORFB (from the *Bst*XI site at position 2532 to the right end) was removed from pMF1138.1, in several cloning steps, by replacement with the 155-bp *Hin*dIII/*Sph*I fragment from the Ω fragment (30), leaving translation and transcription stop signals immediately downstream of ORF2. This construction was named pMF1147. To prevent ORF2 expression, the following recombinant plasmids were made based on pMF1138.1 (see Fig. 4): pMF1139, in which the region extending from nucleotide 1787 until the right end of the cloned fragment was removed, leaving the whole ORF1 and 137 bp of ORF2; pMF1145, in which the integron Ω was inserted into the *Nru*I site of ORF2, introducing transcription and translation stop signals; and pMF1146, a derivative of pMF1145 in which a *Hin*dIII deletion removes the transcription stop signals, leaving the translation stop codons in all frames within ORF2. Likewise, to avoid the ORF1 expression, several pMF1138.1 derivatives were made (see Fig. 4): pMF1140, in which most of ORF1 is deleted (up to the *Nar*I site, nucleotide 1577), leaving the last 75 bp of ORF1 and the complete ORF2 including its own RBS; pMF1143, in which an *Sma*I fragment containing the Ω fragment was inserted into the *Xmn*I site of ORF1, introducing transcription and translation stop signals; and finally pMF1144, in which an *Hin*dIII fragment was deleted from pMF1143, leaving translation stop codons in all frames within ORF1.

DNA accession number. The DNA sequence reported here was submitted to EMBL-GenBank and was given accession number Y15759.

RESULTS

Cloning and analysis of a DNA fragment inducing overproduction of actinorhodin in *S. lividans*. In order to gain further insight into the signals leading to activation of antibiotic biosynthetic genes, we used the ability of *S. lividans* to be converted to a blue phenotype when extra copies of a regulatory element are introduced by transformation. We used *S. rochei* F20 as a source of DNA since this strain is not an actinorhodin producer. Thus, any heterologous activation might well be due to general regulatory signals for secondary metabolism.

A library of *S. rochei* F20 chromosomal fragments was prepared in the pIJ702 vector, and protoplasts of *S. lividans* TK21 were transformed with the ligation mixture, selecting thiostrepton resistance. Plasmid DNA from one blue transformant (named pLE2032) was isolated and characterized.

pLE2032 was found to contain a 0.8-kb DNA fragment (Fig. 1). By subcloning the original fragment, the minimal DNA region which was able to induce actinorhodin production was determined to be a 0.4-kb *Hin*cII fragment. This fragment was cloned in pIJ2925, then rescued as an *Eco*RI/*Hin*dIII fragment

and cloned in the vectors pIJ486 and pIJ487 to yield plasmids pMF1141 and pMF1142, respectively. These recombinants differ only in insert orientation and were found to induce the same blue phenotype in *S. lividans*. Southern blot analysis confirmed that the cloned DNA represented an intact chromosomal fragment in *S. rochei* F20, while no hybridization was observed with DNA from the host strain.

The DNA sequence of the *Hin*cII 0.4-kb fragment was determined and revealed to carry the C termini of two putative, convergently arranged ORFs of 153 and 210 bp. Comparison of the translated products with the databases showed no significant similarities for one of them, while the 50 amino acid residues of the other demonstrated close similarity to the integral membrane proteins related to the ABC transporter superfamily, particularly to that involved in the transport of doxorubicin (17).

Actinorhodin induction in *S. lividans* is due to increased levels of transcription of the positive pathway-specific regulatory gene, *actII-ORF4*. To analyze the possible mechanism involved in the activation of actinorhodin by the cloned fragment, the plasmid pMF1141 was introduced into *actII-ORF4* mutants B43, B58, and JF1. No complementation was observed, indicating that induction strictly requires the participation of *actII-ORF4*, the specific regulator for actinorhodin biosynthesis.

The effect of the activator fragment on *actII-ORF4* transcription was examined in *S. lividans* TK21 strain by high-resolution S1 mapping. The probe was a 634-bp fragment (nucleotides 4825 to 5458) labeled on the 5′ of the *Xho*I site at position 5458, extending therefore 354 nucleotides from the *actII-ORF4* start codon into the coding region (11). Hybridization was done with total RNA from *S. lividans* TK21 containing the vector pIJ486 or plasmid pMF1141 and from *S. coelicolor* J1501. G+A and T+C Maxam and Gilbert sequencing reactions of the probe were run in parallel. A single S1-resistant hybridizing band was observed in *S. lividans* TK21(pMF1141) and in *S. coelicolor*, suggesting a unique transcription start point located at the C residue at position 5073 (Fig. 2).

From Fig. 2 it can be deduced that: (i) there is a unique transcription start point for *actII-ORF4* in *S. lividans* as in *S. coelicolor*; (ii) this transcription start point is the same in both species; (iii) although no actinorhodin is detected in *S. lividans*, a basal transcription of *actII-ORF4* is shown by this strain; and (iv) the activation of actinorhodin production in *S. lividans* mediated by the 0.4-kb fragment from *S. rochei* F20 correlates with increased levels of transcription of *actII-ORF4*.

Subcloning and analysis of the region adjacent to the activator fragment. The DNA sequence of the activator fragment revealed that it contains part of a putative ABC transporter. To analyze if this overlapping arrangement involved a putative relationship between activation of antibiotic biosynthesis and a transport event, the adjacent region was isolated and characterized from a chromosomal library by using as a probe the 0.4-kb *Hin*cII DNA fragment from pMF1141. From a positive lambda clone, a 5.5-kb *Sac*I fragment (overlapping the probe) was subcloned, yielding the plasmid pMF2036. Sequencing and computer-assisted analysis of 3.4 kb, covering the region coding for the putative ABC transporter, identified four putative ORFs (Fig. 1), which were named ORFA, ORF1, ORF2, and ORFB; the first three ORFs would be transcribed from left to right, whereas ORFB runs convergently. Thus, the activator fragment overlaps the 3′ termini of ORF2 and ORFB. The most relevant features of this region, deduced from its DNA sequence, are summarized in Table 1.

The translation start point for each ORF was tentatively assigned according to several criteria: (i) the overall distribution of G+C content in the third position of the codons (1); (ii) the codon usage within the putative coding sequences (48); (iii) the observed similarities between the putative ORF products with those of others in the databases; and (iv) the presence of a canonical RBS at a suitable distance from the putative translation start codon (ORF1, ORF2, and ORFB showed suitable RBS sequences for their putative translation start codon positions). The putative ORF1 stop codon overlaps with the ORF2 start codon (ATGA), suggesting translational coupling, as in many other *Streptomyces* genes (19).

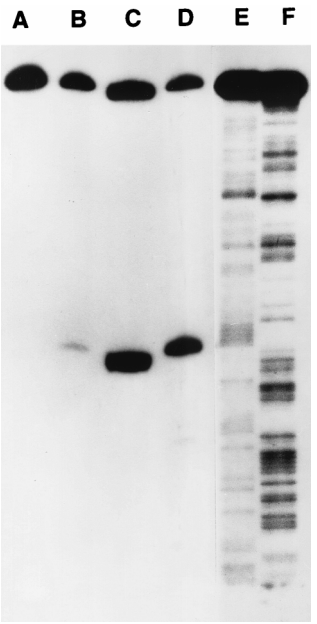

FIG. 2. High-resolution S1 mapping and transcription of the *actII-ORF4* gene. Lanes: A, *E. coli* tRNA; B, total RNA from *S. lividans* TK21(pIJ486); C, total RNA from *S. lividans* TK21(pMF1141); D, *S. coelicolor* J1501 total RNA; E and F, G+A and C+T Maxam and Gilbert sequencing reactions of the labeled probe, respectively.

Two stem-loop structures were observed at nucleotides 433 to 476 and nucleotides 2499 to 2575 (Fig. 1). These structures could act as rho-independent transcription terminators for ORFA and for ORF2+ORFB, respectively.

Deduced functions of the sequenced genes. Comparison of the deduced gene products of the truncated ORFA and ORFB with the databases showed no significant similarities and thus there are no clues about their possible roles. The ORF1 product showed strong resemblance with ATP-binding proteins belonging to the ABC transporter superfamily (18), particularly with several from *Streptomyces* species involved in the efflux of antibiotics, such as DrrA (for doxorubicin) (17), OleB and OleC-ORF4 (for oleandomycin) (28, 34), TlrC (for tylosin) (36), CarA (for carbomycin) (6) and SrmB (for spiramycin) (40), and from other genera, such as NodI from *Bradyrhizobium japonicum*, which presumably exports a lipooligosaccharide (45), and MsrA (for erythromycin) (35). Similarities varied from 50.8 to 81.6%, with identities from 25.3 to 66.6%, the highest values being found with DrrA from *S. peucetius*.

As for the ABC transporters, the typical consensus residues, known as "the signature of the family," are also present in ORF1 (residues 138 to 149), including the so-called loop 3, rich in glycine, which is postulated to interact with the phosphate group of the nucleotide (39, 46). Interestingly, a single Walker A and B motif is present in ORF1, as in DrrA, OleC, and NodI, whereas in MsrA, TlrC, CarA, SrmB, and OleB it is duplicated. The alignments of these domains are shown in Fig.

TABLE 1. Relevant features of the 3.4-kb multidrug-resistance-inducing region deduced from its DNA sequence[a]

ORF	RBS	Start/stop codons[b]	Amino acids (M_r)	Predicted function
ORFA		Truncated/388 TGA	128 (14,383)	Unknown
ORF1	^{548}GAGGGAA^{554}	ATG 561/1652 TGA	364 (37,684)	ATP binding and hydrolysis
ORF2	^{1633}AGAAGGAGA^{1641}	ATG 1649/2503 TGA	284 (31,211)	Transmembrane protein for transport
ORFB	^{2816}AAGGG^{2812}	GTG 2789/2580 TGA	69 (7,505)	Unknown

[a] RBS entry indicates significant similarity between the region upstream of the putative start codon and the 16S rRNA sequence (1).
[b] Numbers show the first and last nucleotide of the start and stop codons, respectively.

3. Based on these similarities, we postulated ATP-binding and hydrolysis activities for ORF1.

The ORF2 product showed significant similarities with the transmembrane proteins described for members of the ABC transporter superfamily (similarities vary from 72 to 44.7%, and identities vary from 48.7 to 17.4%), with the typical signature of the ABC-2 subgroup of this superfamily (31). Members of this subfamily would include: DrrB for the export of doxorubicin by *S. peucetius* (17) and those for export of carbohydrates such as NodJ from *Rhizobium* (7) and *Bradyrhizobium* (16) species, KpsM from *E. coli* (41), BexB from *Haemophilus influenzae* (23), and CtrC from *Neisseria meningitidis* (15). In addition to these proteins, ORF2 shows a remarkable similarity to OleC for export of oleandomycin by *S. antibioticus* (28) and with MtrB for mithramycin export by *Streptomyces argillaceus* (10). As in ORF1, ORF2 shows the greatest resemblance to the Drr system (DrrB; similarity, 72.4%; identity, 48.7%). The ORF2 protein is approximately 30 kDa in size and, like other members of this family, has the typical hydrophobicity profile, with six putative α-helical membrane-spanning segments and the N and C termini facing the cytoplasmic side.

These similarities to other well-known gene products suggested that ORF1 and ORF2 would presumptively constitute a two-component transport system.

Functional characterization of ORF1 and ORF2. The functional characterization of *msr* was attempted in the original strain. Thus, recombinant phages containing DNA fragments internal to ORF1 and ORF2 were constructed and used to disrupt ORF1 and ORF2 by insertional inactivation (3) (Fig. 1).

To disrupt ORF1, an internal *Bal*I fragment (nucleotides 715 to 1432) was first cloned in the *Hinc*II site of pIJ2925; the fragment was rescued with *Bgl*II and ligated to the phage KC515, which had been previously digested with *Bgl*II. Recombinant phages φAB29.1 and φAB29.2, carrying the insert in alternative orientations, were obtained. ORF2 disruptions were made in the same manner by using phages φAB28.1 and φAB28.2 carrying the 507-bp *Bss*HII/*Dde*I fragment (nucleotides 1790 to 2297, previously blunt ended with Klenow enzyme) in either orientation. Lysogens of *S. rochei* F20 were generated with the four recombinant phages as previously described (13). No phenotypic differences between the disruptants and the wild type were observed and, therefore, no meaningful information about the putative roles of these ORFs was obtained.

The expression of these genes was next explored at two levels: first, transcription was analyzed by high-resolution S1 mapping in the parental strain; second, the expression of their putative promoter region in a promoter probe plasmid was tested in a heterologous host (*S. lividans*) because *S. rochei* F20 could not be transformed with plasmid DNA by standard procedures (13). For the former analysis, we determined the transcription start point of ORF1 and ORF2 and the transcription termination point of ORF2. After hybridization with total RNA, no S1 protected fragments were obtained, strongly suggesting that the genes were not transcribed under our assay conditions. To analyze the expression of the putative promoter region in *S. lividans*, the 247-bp *Bal*I fragment (nucleotides 330 to 576, which includes the last 59 bp of ORFA and the first 16 bp of ORF1) was cloned in the promoter probe vector pIJ4083, upstream of the *xylE* reporter gene (50). No chromogenic reaction with catechol was seen either in liquid medium or solid medium or on the plates containing a gradient of the agents which were previously tested as putative inducers (see above). These results are in good agreement with the previous finding that no expression of these genes occurred and did not allow the assignment of a function for the cloned genes.

Since the apparent absence of expression of ORF1 and ORF2 might be due to a silent or inducible promoter, which would not be expressed under our experimental assay conditions, the cloned genes were engineered in order to analyze their expression in a heterologous host: (i) the *Sac*I/*Xba*I fragment (sites 1 to 4 [Fig. 1], nucleotides 1 to 2907) was cloned in the high-copy-number plasmid pIJ486 to generate pMF1128; (ii) the promoter region for ORF1 and ORF2 was replaced by the well-known promoter that controls the expression of the

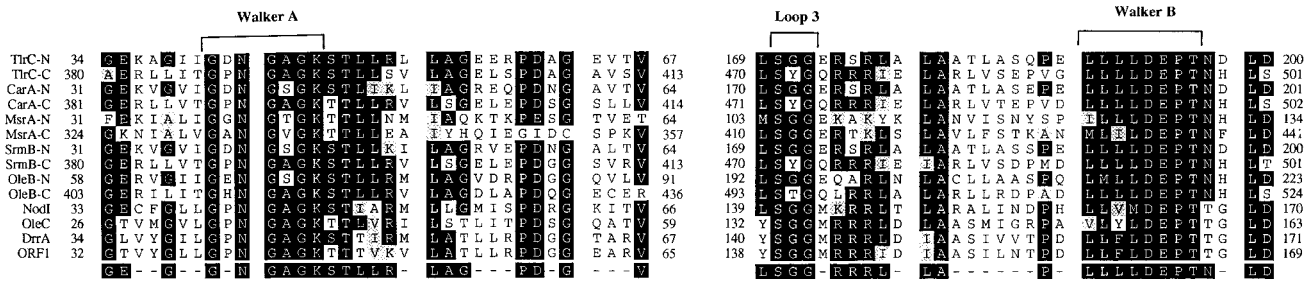

FIG. 3. Alignment of the ORF1 gene product domains with homologous domains from different ATP-binding proteins. The amino acid stretches show the Walker A, Walker B, and loop 3 domains from the following proteins (accession numbers): TlrC, tylosin resistance (M57437) (36); CarA, carbomycin resistance (M80346) (6); MsrA, macrolide resistance (X52085) (35); SrmB, spiramycin resistance (X63451) (15a); OleB, oleandomycin resistance (L36601) (28); NodI, oligosaccharide-derived transport (J03685) (16); OleCORF4, oleandomycin resistance (L06249) (34); and DrrA, doxorubicin resistance (M73758) (17). Duplicated domains (see the text) are shown and identified as "N" (N Terminus) or "C" (C terminus). Plurality = 8.

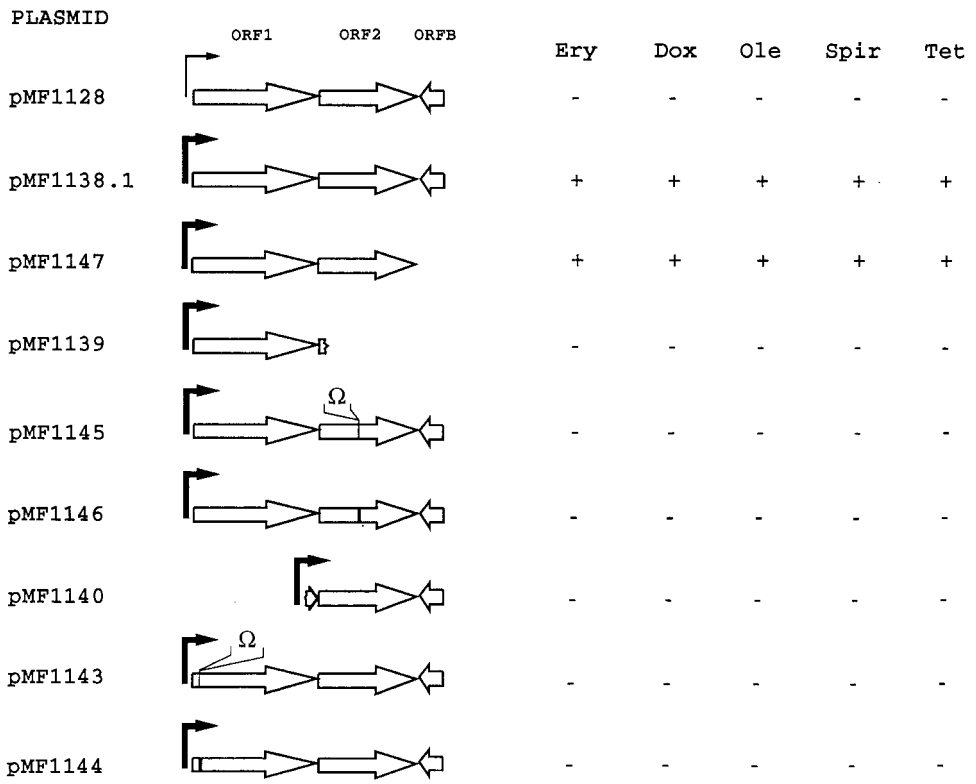

FIG. 4. Subcloning of *msr* ORFs to elucidate the genes required for the multiresistance-inducing phenotype. The thin bent arrow shows the *msr* promoter. Thick bent arrows show the actinorhodin PKS promoter replacing the *msr* promoter. Open arrows show the ORFs in the corresponding constructions. Small vertical thick lines inside the ORFs represent translation stop signals introduced "in vitro" within the recombinant genes. Ω, Ω integron from pHP45Ω; Ery, erythromycin; Dox, doxorubicin; Ole, oleandomycin; Spir, spiramycin; Tet, tetracycline; −, no resistance; +, resistance.

PKS for actinorhodin biosynthesis from *S. coelicolor* (12, 29), yielding plasmid pMF1138.1; and (iii) pMF1138.1 was manipulated in order to allow selective expression of ORF1 or ORF2 under the control of the PKS promoter (see Materials and Methods and Fig. 4). The resulting plasmids were used to transform *S. lividans* TK21. The recombinant strains were tested by the disk assay method for resistance to the following drugs and chemical agents: doxorubicin, oleandomycin, erythromycin, tetracycline, spiramycin, streptothricin, tobramycin, novobiocin, streptomycin, chloramphenicol, phenol, pentachlorophenol, tetrachlorohydroquinone, and ethidium bromide. Only strains containing ORF1 and ORF2 simultaneously under the control of the PKS promoter showed a multiresistance phenotype to erythromycin, spiramycin, oleandomycin, doxorubicin, and tetracycline. As shown in Fig. 4, removing ORFB from pMF1138.1 (pMF1147) still conferred resistance to the same drugs, excluding a possible role for ORFB in the multiresistance phenotype.

Further characterization of this multiresistance phenotype was carried out in order to quantify the level of the induced multiresistance. To do this, the recombinant plasmids were introduced into *S. lividans* or *S. coelicolor* containing (on a compatible SCP2 derivative plasmid) extra copies of the positive regulator of the *act* PKS promoter (the *actII-ORF4* gene) to overexpress the PKS promoter. The resulting phenotype was analyzed on antibiotic gradient plates, and the results are summarized in Table 2.

From these results, we can conclude that both ORF1 and ORF2 are simultaneously required for an efficient induction of the multidrug resistance phenotype.

DISCUSSION

A new multiresistance determinant has been identified in *Streptomyces rochei* by isolating an overlapping *trans*-acting transcriptional activator for actinorhodin biosynthetic genes in *S. lividans*. It was shown that this activation is mediated by increasing the transcription of the pathway-specific positive regulator of the *act* genes, *actII-ORF4*. Although we have no clue about the precise mechanism, this could involve either the neutralization of a putative repressor (by direct binding or by an antisense mechanism) or be the response to a putative stress induced by the high copy number of this DNA. In any case, there is not enough experimental information to establish a correlation between this activator and the process which leads

TABLE 2. Determination of MICs for several antibiotics as deduced from antibiotic gradient plates[a]

Plasmid	MIC (μg/ml) of:				
	Erythromycin	Doxorubicin	Oleandomycin	Spiramycin	Tetracycline
pIJ487	10	5	25	300	20
pMF1128	10	5	25	300	20
pMF1138.1 *actII-ORF4*	>250	>100	>250	>2,000	>75
pMF1139 *actII-ORF4*	10	5	25	300	20
pMF1140 *actII-ORF4*	10	5	25	300	20

[a] The multiresistance phenotype was tested on *S. lividans* TK21 carrying the engineered *msr* ORFs (see the text).

to the multiresistance phenotype. This will be the subject of future studies.

Interestingly, in our studies of the activator fragment we have identified a new member of the ABC transporter superfamily. This transporter, which is flanked by stem-loop structures, shows a high degree of similarity to the other members of the family, particularly with those involved in the resistance to the antitumor drug doxorubicin in the producer organism, *S. peucetius*. Based on the sequence similarities, we believe that this new transporter would be structurally formed by two homodimeric constituents as in many other bacterial ABC transporters: two Msr-ORF1 proteins facing the cytoplasmatic side (presumptively involved in ATP binding and hydrolysis) and two Msr-ORF2 molecules included in the cell membrane (presumptively forming the structure needed for extrusion of the corresponding substrate).

The failure to detect expression of *msr*, which has been demonstrated by gene disruption and by expression analysis both in the original strain and in heterologous hosts, suggests the existence of an inducer that might activate its promoter. Attempts to identify these putative inducers failed. Moreover, even those chemicals which were shown to be substrates for the engineered functional genes did not behave as inducers. Further experimental analysis will shed light on the existence and nature of the putative inducer of this new ABC transporter. Thus, the in vivo role of *msr* in *S. rochei* F20 is unclear: it could be involved in self-resistance to any bioactive metabolite produced by the strain (which would imply either an alternative resistance mechanism or the lack of production of such a metabolite in the disruptant strains); it could induce multiresistance as a selective advantage against biocide agents; or perhaps removing toxic chemicals from the cell could be the biochemical function of this system.

Despite the large diversity of substrates for ABC transporters described in the literature, the specificity of each system is relatively high and only a few members belonging to the ABC transporter superfamily induce multidrug resistance. All of them are of eukaryotic origin, such as the P glycoproteins, and only one characterized system is of bacterial origin (44).

From the experimental information presented in this paper, it is clear that *msr-ORF1* and *msr-ORF2* are simultaneously required for the multidrug resistance phenotype (Table 2). Neither *msr-ORF1* nor *msr-ORF2* can be replaced by any putative homologous gene in the host strain unless, if present, it is not expressed. This raises interesting questions: how widespread are *msr* or its analogs among actinomycetes, and how widespread are silent multidrug resistance systems such as the one described in this work?

Studies on efflux pumps such as the one described here are relevant because in the last few years it has been shown that pumping activities are involved in a large number of multiresistance phenomena by pathogenic bacteria and tumor cells. Particularly, tumor cells use ATP hydrolysis mediated by members of the ABC transporter superfamily to obtain the energy to efflux the chemotherapeutic agents. In addition, the unique bacterial multiresistance pump belonging to the ABC transporters is able to complement the human multidrug resistance P-glycoprotein gene (43), supporting the clinical and academic value of studying these mechanisms of transport.

As far as we know, the system described here is the first reported ABC transporter from a *Streptomyces* sp. conferring multidrug resistance. This system may be an important tool in the search for new drugs where resistance genes, with relaxed requirements for substrates, are needed. Likewise, it will offer a useful model for further studies on multidrug resistance mechanisms from either bacterial or eukaryotic cells.

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