A multidrug efflux system is involved in colony growth in *Streptomyces lividans*

Li-Fong Lee,†‡ Yueh-Jung Chen,† Ralph Kirby,† Chi Chen† and Carton W. Chen†

†Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, University System of Taiwan, Shih-Pai, Taipei, Taiwan
‡Department of Biological Science and Technology, Chung Hwa College of Medical Technology, Jen-Te Hsiang, Tainan Hsien, Taiwan

Multidrug resistance (MDR) genes are abundant in *Streptomyces* genomes, and yet these bacteria are generally drug sensitive under routine laboratory conditions, indicating low or no expression of these genes. Drug-resistant mutations have been isolated that lie in regulatory genes adjacent to the MDR genes, suggesting that resistance arises by derepression. This study identified a divergently oriented pair consisting of a TetR-family regulator (ebrS) and a major facilitator-family MDR pump (ebrC) gene in *Streptomyces lividans*, which is widely conserved in *Streptomyces* species. EbrS represses transcription of ebrC as well as its own transcription. Deletion of ebrS causes overexpression of ebrC, resulting in elevated resistance to many drugs. The ebrS and ebrC promoters were used in a reporter system to test inducibility by various chemicals. Among the 15 compounds (including five EbrC target drugs) tested, none induced ebrC transcription. On the other hand, the ebrS promoter was induced by rifampicin and high concentrations of calcium and magnesium. Deletion of ebrS-ebrC did not change rifampicin sensitivity, indicating that the EbrC pump is not involved in rifampicin efflux. Moreover, deletion of ebrC caused retardation of colony growth on selected media, and the defect could be suppressed by supplementation with high concentrations of Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\) or K\(^+\). Based on these results, it is proposed that the primary biological role of most MDR systems in *Streptomyces* species is not removal of extrinsic drugs, but rather export of specific toxic compounds endogenously synthesized during growth.

INTRODUCTION

‘Multidrug resistance’ (MDR) genes are abundant in bacteria; they encode membrane-bound pumps that remove a wide variety of structurally unrelated, lipophilic cationic drugs from the cells. These pumps are actively driven by ATP hydrolysis or by the proton-motive force of the transmembrane proton gradient. Those pumps that are subject to regulatory controls typically belong to the second group and fall into two superfamilies – the major facilitator superfamily (MFS) and the resistance, nodulation and cell division (RND) superfamily (Paulsen *et al.*, 1996; Putman *et al.*, 2000).

Gram-positive bacteria of the genus *Streptomyces* are abundant in soil, and must interact extensively with various terrestrial environments. It is thus not surprising that they contain a large number of transporter proteins. For example, there are over 700 predicted transporter genes (about 8% of all genes) in the *Streptomyces coelicolor* chromosome (TransportDB, a database for predicted membrane transport protein; http://www.membranetransport.org). At least 100 of the *S. coelicolor* transporters show strong sequence similarity to MDR pumps of various families. Surprisingly, in spite of the large number of MDR pump genes, *Streptomyces* species are intrinsically sensitive to most of the drugs tested (Lee *et al.*, 1996, 2003). Resistant mutants that arise spontaneously exhibit elevated efflux of the drugs (Lee *et al.*, 1996).

Two classes of spontaneous ethidium bromide (EB)-resistant (ebr) mutants have been previously isolated from *Streptomyces lividans* (Lee *et al.*, 1996). Class I mutants (four members) are resistant to high concentrations of EB (>15 \(\mu\)M), while a class II mutant (one member, YJ18) is resistant to only 5 \(\mu\)M EB. The two classes of mutants were mapped at different chromosomal loci and exhibited...
distinct spectra of cross-resistance to other drugs (Lee et al., 2003).

Three EB resistance genes, designated ebrA, ebrB and ebrC, were cloned from these mutants based on their ability to confer elevated resistance to EB on a multi-copy plasmid (Lee et al., 2003). Their deduced protein products appear to be transmembrane proteins that resemble other bacterial MDR proteins. EbrA belongs to the small (or staphylococcal) MDR (Smr) family, while EbrB and EbrC belong to the drug resistance translocase family within the MFS. A putative regulatory gene (ebrR) was found downstream of ebrA, and another (ebrS) upstream of ebrC in the opposite orientation. Both regulatory genes encode transcription regulatory proteins of the TetR/AcrR family with a putative helix-turn-helix DNA-binding motif, like all other known repressors in this family (Grkovic et al., 2002).

When deletion mutants for each of these genes were analysed, it was found that deletion of ebrA or ebrB did not affect the EB resistance phenotype; hence these genes are not involved in the EB resistance of the ebr mutants (Lee et al., 2003). On the other hand, ebrC appears to be involved in the resistance shown by the class II ebr mutant YJ18, because a disruption of ebrC in YJ18 resulted in the loss of the EB resistance phenotype. YJ18 was found to contain a frame-shift mutation in the regulatory gene ebrS that accompanies ebrC. Lee et al. (2003) proposed that this mutation caused derepression of ebrC and thus elevated resistance to EB (and other drugs). ebrC and ebrS are separated by 65 bp and this intergenic region has a relatively high A+T content (43 mol%), characteristic of Streptomyces promoters.

The ebrC-ebrS gene pair was physically mapped at about the ‘6 o’clock’ position of the S. lividans chromosome (Lee et al., 2003), agreeing with the genetic map location of the ebrS-18 mutation in Y118 (Lee et al., 1996). Orthologues of the ebrC-ebrS gene pair (>90% identity) are present in the same synteny at similar positions on the sequenced chromosomes of S. coelicolor (SCO5516-7) and Streptomyces avermitilis (SAV2727-8). Moreover, ebrC homologues appear to be widespread among Streptomyces species based on a hybridization survey (Lee et al., 2003). All these findings suggest that the ebrC-ebrS pair may play an important biological role.

Many homologues of EbrC with lower degrees of amino acid sequence identity (36–45%) are also present among uncharacterized proteins (e.g. PqrB) in S. coelicolor (Cho et al., 2003) and S. avermitilis, and among known resistance pumps in other actinomycetes, such as SgcB of Streptomyces globisporus (Liu & Shen, 2000), LfrA of Mycobacterium smegmatis (Takiff et al., 1996), RifP of Amycolatopsis mediterranei (August et al., 1998) and VarS of Streptomyces virginiae (Lee et al., 1999).

It is apparent that these MDR systems do not normally confer MDR on their Streptomyces hosts and only derepression of the pump genes achieves elevated resistance. So, what are the biological roles of so many homologues of multidrug efflux pumps in the Streptomyces genomes? In an attempt to shed light on this question, we investigated the genetic control of the ebrS-ebrC regulator-pump pair. We demonstrate here the repressor role of ebrS in regulation of transcription of ebrC and of itself. Several compounds, including rifampicin and CaCl₂, were found to induce expression of ebrS. Most notably, deletion of ebrC resulted in retardation of colony growth on certain solid media, and the defect may be suppressed by supplementation of a number of different metal cations. We propose that, under these experimental conditions, the EbrC pump is involved in colony growth through removal of intrinsically produced toxic compounds, and that other MDR systems may also play a similar physiological role in Streptomyces.

**METHODS**

**Bacterial strains, plasmids and general methods.** The bacterial strains and plasmids used in this study are listed in Table 1. Microbiological and genetic manipulations in *Escherichia coli* and *Streptomyces* were according to Kieser et al. (2000). Five solid media were used for culturing *Streptomyces* strains: NE (per litre: 10 g glucose, 2 g yeast extract, 2 g Casamino acids, 1 g beef extract, 15 g agar, pH 7.0) (Thiara & Cundliffe, 1995), PYM (per litre: 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g glucose, 20 g agar), LB, R5 (Kieser et al., 2000) and HAUCM (Qin et al., 1994).

**Dot-blot hybridization.** Spores of TK64 or Y118 were inoculated in 200 ml TSB broth to give an OD₆₅₀ of about 0.05, and shaken at 30°C. Mycelium was collected at different growth phases from early exponential to stationary phases. RNA was isolated by the methods described by Kieser et al. (2000). RNA samples (20–40 μg) were applied to a nylon membrane. Hybridization was performed at 45°C in a buffer containing 5× SSPE, 5× Denhardt’s solution, 0.5% (w/v) SDS, 50% (v/v) formamide and 0.1 mg denatured salmon sperm DNA ml⁻¹. The following probes were labelled with [α-³²P]dCTP using a random priming kit (Amersham): (i) ebrC: nt 1023–1782; (ii) ebrS: nt 155–774 (GenBank accession no. AY043331); and (iii) 16S rRNA: nt 1–632 (GenBank accession no. X63847).

**S1 mapping.** Primer PS6 (CTTCTCCAGCAGCGAGCG, in ebrS) and PS7 (AGCATTAGCGCGCGAG, in ebrC) were used to prepare for PCR a 0.2 kb fragment spanning the promoter region, which was used as template for S1 mapping according to Kieser et al. (2000). To map the transcription start site of ebrC, PS7 was labelled at the 5’ end with [α-³²P]ATP using polynucleotide kinase. The same primer was used for sequence determination in parallel.

**Gene disruption.** The suicide plasmid pLUS944 was digested with Asel and BglII. The linearized DNA was used to transform protoplasts of TK64 and Y118 according to Oh & Chater (1997). After overnight incubation at 30°C, thioestrepton-resistant transformants were selected by overlaying the agar plates with 200 μg thioestrepton ml⁻¹.

**Induction of ebrC and ebrS promoters.** The basic design of Salah-Bey (1995) was adopted. About 1.5×10⁵ spores were spread on a 9 cm Petri dish containing 25 ml NE medium supplemented with 30 μg kanamycin ml⁻¹. Paper discs (8 mm, Toyo Roshi) loaded with various test compounds in a volume of less than 20 μl were placed on the medium, and the plates were incubated at 30°C.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. lividans 1326</td>
<td>Wild-type, SLP2+ SLP3+</td>
<td>Hopwood et al. (1983)</td>
</tr>
<tr>
<td>S. lividans TK64</td>
<td>pro-2 str-6 SLP2- SLP3-</td>
<td>Hopwood et al. (1983)</td>
</tr>
<tr>
<td>S. lividans Y18</td>
<td>TK64 containing ebrS-18 mutation</td>
<td>Lee et al. (1996)</td>
</tr>
<tr>
<td>S. lividans Y18-8</td>
<td>Y18 containing Δ(ebrS-ebrC)::tsr mutation</td>
<td>This study</td>
</tr>
<tr>
<td>S. lividans LF13</td>
<td>TK64 containing Δ(ebrS-ebrC)::tsr mutation</td>
<td>This study</td>
</tr>
<tr>
<td>S. lividans LF16</td>
<td>TK64 containing ΔebrS::tsr mutation</td>
<td>This study</td>
</tr>
<tr>
<td>S. lividans LF18</td>
<td>TK64 containing ΔebrS::tsr mutation</td>
<td>This study</td>
</tr>
<tr>
<td>S. lividans LF24</td>
<td>TK64 containing Δ(ebrS-ebrC)::tsr mutation</td>
<td>This study</td>
</tr>
<tr>
<td>S. coelicolor M145</td>
<td>SC1- SC2-</td>
<td>Hopwood et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIJ487</td>
<td>Streptomyces promoter-probe vector containing tsr and promoterless neo</td>
<td>Ward et al. (1986)</td>
</tr>
<tr>
<td>pIJ702</td>
<td>Streptomyces plasmid pIJ101 containing melC and tsr</td>
<td>Katz et al. (1983)</td>
</tr>
<tr>
<td>pMCR20</td>
<td>pIJ487 containing the ebrC promoter inserted upstream of neo</td>
<td>This study; Fig. 4A</td>
</tr>
<tr>
<td>pMCR30</td>
<td>pIJ487 containing the ebrS promoter inserted upstream of neo</td>
<td>This study; Fig. 4A</td>
</tr>
<tr>
<td>pLUS914D</td>
<td>E. coli vector pMLT23 containing the ebrS-ebrC operon with a deletion of complete ebrC and most of ebrS</td>
<td>Lee et al. (2003)</td>
</tr>
<tr>
<td>pLUS944</td>
<td>E. coli vector pMLT23 containing the ebrS-ebrC operon with a deletion of most ebrS</td>
<td>This study; Fig. 3A</td>
</tr>
<tr>
<td>pLUS947</td>
<td>pIJ702 harbouring the 4.0 kb Sphi fragment containing wild-type ebrS</td>
<td>This study</td>
</tr>
</tbody>
</table>

Microarray analysis. Genomic DNA from a stationary culture was purified by the salting-out procedure (Pospiech & Neumann, 1995) and sonicated to <2 kb. Four to six micrograms of sonicated genomic DNA was mixed with 12 μg random hexamers (of 72 mol% G+C content) in a total volume of 25 μl and heated at 100 °C for 10 min. The mixture was quickly cooled on ice before adding the remaining reaction components: 1.5 μl Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech), 4 μl Klenow fragment (NEB), 5 μl Klenow buffer, 0.5 μl dNTP (4 mM dATP, 4 mM dTTP, 10 mM dGTP and 0.2 mM dCTP) and 14 μl H2O. The random-primed labelling reaction was carried out for 2–3 h at 37 °C. The reaction mixture was added to 0.5 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and filtered through a Microcon-30 concentrator (Millipore). The dilution and filtration cycle was repeated twice. The two DNA pools to be compared were mixed in a hybridization mixture containing 3.68 × SSC, 0.18 % (w/v) SDS and 1 μg yeast tRNA (total 16.3 μl), heated at 100 °C for 5 min and applied to a UniS S. coelicolor PCR array [http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays](http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays). The two DNA pools to be compared were mixed in a hybridization mixture containing 3.68 × SSC, 0.18 % (w/v) SDS and 1 μg yeast tRNA (total 16.3 μl), heated at 100 °C for 5 min and applied to a UniS S. coelicolor PCR array (http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays). Hybridization took place under a glass coverslip in a humidified Omnislide (Thermo Hybaid) at 60 °C for 12–14 h. The slides were washed, dried, and scanned in a GenePix 4000B (Molecular Devices). Mean signal intensity and local background measurements were obtained for each spot on each array by using GenePix Pro 6.0 software, and analysed using Stanford Microarray Database (Gollub et al., 2003). The dataset for each array was normalized using the signals for the S. coelicolor ribosomal protein genes on the array and a mean signal for each gene was calculated. Microarray data were visualized using the program TREEVIEW (Eisen et al., 1998) at a setting of 1.5. In this visualization, red shows hybridization above the dataset norm, green shows hybridization below the dataset norm and black shows mean hybridization close to the norm.

RESULTS

ebrC is overexpressed in ebr mutant Y18

Lee et al. (2003) showed that ebrC is involved in the elevated MDR displayed by the ebr mutant Y18, presumably because of a frameshift mutation in the adjacent repressor gene, ebrS. To confirm that ebrC was indeed derepressed in Y18, dot-blot hybridization using an ebrC probe was performed to analyse the ebrC transcripts present in Y18 and its ebr+ parent TK64 (Fig. 1). Liquid cultures were collected at different stages of growth, and RNA was extracted for analysis. The results showed that ebrC expression in TK64 in all growth phases was at a very low level that was nevertheless significantly higher than the background level in the control samples (Neurospora crassa and E. coli RNA). In contrast, ebrC expression in Y18 was highly induced in all growth phases and strongest at stationary phase. Similar analysis using an ebrS probe revealed a very low level of expression of ebrS in both Y18 and TK64, but nevertheless it was considerably higher in Y18. These results supported the notion that ebrC is overexpressed in Y18 because of the defective ebrS. The higher expression of the mutant allele ebrS18 in Y18 also suggested that ebrS represses its own transcription.

The same conclusions were reached from S1 protection mapping (Fig. 2), which showed increased transcription of...
ebrC in YJ18 in all growth phases from 17 nt upstream of the predicted ebrC coding sequence. A putative ~10 region of typical prokaryotic promoters (GACAAT) is found further upstream. The transcription start site for ebrS could not be detected by S1 mapping because of the low expression level.

**Disruption of ebrS causes elevated MDR**

To confirm that the elevated ebrS expression in YJ18 was the result of the defect in ebrS, an independent inactivation mutation in ebrS was created in TK64. A suicide vector pLUS944 was constructed that contained a 6 kb fragment spanning the ebrS-ebrC operon, in which most of the ebrS coding sequence was replaced by the thiostrepton resistance spanning the pLUS944 was constructed that contained a 6 kb fragment.

RNA was isolated at four different growth stages of TK64 and YJ18 growing in TSB liquid medium: early exponential (EE; OD 600 0.6–0.7), mid-exponential (ME; OD 600 1.7–1.8), late-exponential (LE; OD 600 3.9–4.2) and stationary (S; OD 600 1.2–7.8) phases. The RNA was blotted onto a nylon membrane and hybridized with a 32P-labelled DNA probe of the ebrC, ebrS and 16S rRNA genes of *E. coli* (Nc) and *Neurospora crassa*. Neurorspora crassa. *Neurospora crassa*. The weak hybridization signals of the ebrS probe were electronically enhanced to reveal the low expression in YJ18.

**The ebrS promoter is induced by rifampicin and calcium**

Some bacterial MDR genes are induced by target drugs. For example, in *Streptomyces pristinaespiralis*, *ptr* transcription is induced by the substrates pristinamycins I and II, among other unrelated drugs (Salah-Bey *et al.*, 1995). To test the drug inducibility of ebrC, a reporter system was constructed. The 0.3 kb *Bgl*II–*Bam*HI fragment containing the ebrS-ebrC intergenic region was inserted into the *Bgl*II site of pJ487 (Ward *et al.*, 1986), a promoter-probe vector. Two recombinant plasmids were produced with the promoter sequence inserted in opposite orientations (Fig. 4A). In pMRC20, the ebrC promoter was oriented toward the promoterless reporter gene, neo; in pMRC30, the ebrS promoter was oriented toward neo. These two plasmids were introduced into TK64 by transformation. Spores of the transformants were seeded onto a solid medium containing 30 µg kanamycin ml⁻¹. Paper discs containing the test drugs were applied to the plates. Induced expression of the neo gene was indicated by the appearance of bacterial growth around the discs (Salah-Bey & Thompson, 1995).

Eight drugs (rifampicin, chloramphenicol, tetracycline, nalidixic acid, methyltriphenylphosphonium, proflavin, norfloxacin, EB) were tested for induction of ebrC and ebrS expression. The last six drugs are targets of the EbrC efflux system (Lee *et al.*, 1996, 2003). Some salts (CaCl₂, MgCl₂, MgSO₄, ZnCl₂ and CuCl₂) were also tested.

None of the compounds induced the ebrC promoter in pMRC20 (data not shown). Induction of the ebrS promoter in pMRC30 was exerted by CaCl₂ and rifampicin at non-lethal concentrations (Fig. 4B). Interestingly, the induction effects of these two compounds appeared to be synergistic (Fig. 4B, middle panel). Other than these, no other compounds showed any significant induction of the ebrS promoter. In the control experiments, TK64 (Fig. 4B) and TK64/pJ487 (not shown) failed to grow on the kanamycin plates in the presence or absence of any of the test compounds, ruling out the possibility that the elevated resistance was caused by a physiological effect exerted by the test compounds.

pMRC20 and pMRC30 were also introduced into S. *lividans* 1326, the ancestor of TK64, and similar results were observed. None of the chemicals tested induced the ebrC promoter, and both CaCl₂ and rifampicin induced the ebrS promoter. In addition, MgCl₂, which is not an inducer in TK64, induced the ebrS promoter in 1326 (Fig. 4B, bottom panel). The known genetic difference between the two strains is in two plasmids (SLP2 and SLP3 in 1326), proline requirement (*pro-2* in TK64) and streptomycin resistance introduced into YJ18 by transformation. Thiostrepton-resistant transformants, YJ18/pLUS947, were isolated and tested for EB sensitivity. The results showed that these transformants had, as expected, become EB sensitive (3.7% survival) compared to YJ18 (100% survival) on 20 µM EB.
The reason for this interesting discrepancy is not clear.

The ebrS-ebrC pair is implicated in metal ion metabolism

The fact that rifampicin and calcium induced the ebrS promoter suggested that they might be involved in the EbrC efflux system. To test this possibility, the Δ('ebrS-ebrC) mutation was introduced into TK64 using the ebrS-ebrC suicide vector pLUS914D (Lee et al., 2003) in which the ebrS-ebrC sequence was replaced by the tsr gene. Two independent Δ('ebrS-ebrC) mutants of TK64 (designated LF13 and LF24) were used for further investigation. Similarly, a Δ(ebrS-ebrC) mutant of YJ18, designated YJ18-8, was created.

All the Δ('ebrS-ebrC) mutants exhibited normal colony growth on the isolation medium R5. The presence of rifampicin in the medium reduced both the numbers and sizes of the colonies formed by these mutants (data not shown). YJ18 did not exhibit an increased resistance to rifampicin. Neither did the ebrC deletion mutants of TK64 (LF13) and YJ18 (YJ18-8) exhibit any increased sensitivity. These results indicated that rifampicin was not a target of the EbrC efflux system.

CaCl₂, on the other hand, had a more complicated effect (Fig. 5A). In the absence of supplemental CaCl₂, the colonies of the Δ('ebrS-ebrC) mutants LF13 and LF24 (not shown) were minute compared to those of TK64 and the ΔebrS mutants LF16 and LF18 (not shown) on NE medium. Addition of CaCl₂ at 20–300 mM stimulated growth of LF13 and LF24, resulting in a striking increase in colony size with no significant change in colony number. Such growth stimulation was barely detectable in the other four strains. This result indicated that ebrC, but not ebrS, causes a growth defect on NE medium that can be suppressed by supplemental CaCl₂.

MgCl₂ exhibited a similar effect as CaCl₂ (Fig. 5B). The poor growth of the LF13 and LF24 colonies was also suppressed by addition of extra MgCl₂ up to 150 mM. The suppression by MgCl₂ appeared to be less effective than that by CaCl₂, requiring a concentration of 50 mM for full suppression. MgSO₄ exhibited essentially the same suppression effect (data not shown) as MgCl₂, indicating that the suppression
was exerted by the cations (Mg$^{2+}$ and Ca$^{2+}$) and not the anions (Cl$^{-}$ or SO$_4^{2-}$).

At high concentrations (500 mM), both CaCl$_2$ and MgCl$_2$ exerted a growth inhibition on all strains, resulting in a reduction in colony size, but insignificant (<30%) reduction in colony numbers for all the strains tested. This indicates that there is no apparent involvement of the *ebrS*-ebrC system in resistance to the toxicity of these metal ions.

**Specificity of the suppression**

To investigate the specificity of the metal cations, three other divalent cations (Zn$^{2+}$, Cu$^{2+}$ and Mn$^{2+}$) and two monovalent ions (K$^+$ and Na$^+$), all as chlorides, were tested for their ability to suppress the Δ(*ebrS*-ebrC) mutation effect in LF13 and LF24. Interestingly, none of the divalent ions could suppress the mutation (data not shown), but surprisingly KCl at 37.5 mM and NaCl at 100 mM promoted relatively good growth of LF13 and LF24 colonies (Fig. 5C). Thus, the suppression effect is not specific to divalent cations.

---

**Fig. 3.** Insertional inactivation of *ebrS*. (A) The expected crossovers are shown between the suicide vector pPLUS444 and the *S. lividans* chromosomal sequence. The suicide vector contains a deleted *ebrS* (replaced by the *tsr* gene, hatched box) and an intact *ebrC* gene. The open boxes represent the cloned DNA containing *ebrS* and *ebrC* (left and right arrows). The hybridization probes (dashed lines) were the 1.7 kb *ebrS*- and *ebrC*-containing insert plus the *E. coli* vector plasmid (pUC19 series). The *Bam*HI (○) and *Sac*I (●) restriction sites, the expected hybridizing restriction fragments and their sizes (in kb) are indicated. (B) Confirmation of the replacement in the transformants. Genomic DNA of the transformants was digested with *Bam*HI and *Sac*I, and subjected to Southern hybridization. d.c., A representative transformant arising by double crossing over; s.c., a representative transformant arising by a single crossover.

**Fig. 4.** Induction of the *ebrCS* promoters. (A) Reporter system for the *ebrS* and *ebrC* promoters. The promoter-probe plasmid pJ487 (Ward et al., 1986) harbours an intact *tsr* gene and a promoterless *neo* gene (kanamycin resistance). A transcriptional terminator (ter) is located upstream of the *neo* gene. The 0.3 kb *Bgl*II–*Bam*HI fragment containing the *ebrS*-*ebrC* promoter region (boxed arrowheads) was inserted at the *Bgl*II cloning site (Bg) of pJ487 in two orientations: in pMRC20, the *ebrC* promoter (filled arrowhead) was oriented toward the promoterless reporter gene, *neo*; in pMRC30, the *ebrS* promoter (open arrowhead) was oriented toward *neo*. pMRC20 and pMRC30 were transferred into *S. lividans* 1326 (or TK64). (B) Induction of the *ebrS* and *ebrC* promoters. Spores of the transformants were spread onto NE medium containing 30 μg kanamycin ml$^{-1}$. Solutions of each test compound were applied to a paper disc and placed on top of the spread spores. The plates were incubated at 30°C for 5 days. Induction of the *neo* gene resulted in growth of the transformants around the disc. Only results from selected inducers are shown. Rif, rifampicin; NF, norfloxacin; PF, proflavin.
The growth retardation of LF13 and LF24 under Ca\(^{2+}\) - and Mg\(^{2+}\)-limited conditions was also observed on another solid medium, PYM, but not on three other complex media (LB, R5 and HAU). Colonies of the two mutants growing on PYM were retarded to a lesser extent than on NE medium, and supplementation of additional Mg\(^{2+}\) increased the colony size (data not shown).

Genetic instability is not related to the ebr systems

*Streptomyces* chromosomes undergo spontaneous deletions of terminal sequences at high frequencies. Some terminally located markers, such as a chloramphenicol-resistance gene (Dyson & Schrempf, 1987) are lost at frequencies of about $10^{-3}$ to $10^{-2}$ per spore. The reason for this high genetic instability is not known.

This genetic instability is elevated by exposure to DNA intercalating compounds, such as EB (e.g. Crameri *et al.*, 1986; Dyson & Schrempf, 1987). Therefore, it is possible that the observed 'spontaneous' instability may also be induced by external agents taken up by the cell, some of which may also be substrates for the MDR efflux systems. If so, the ebr mutants with elevated drug efflux might be more genetically stable. Two ebr mutants, YJ18 and YJ51, were compared with the ebr\(^{+}\) parent TK64 for spontaneous appearance of chloramphenicol-sensitive variants. Spores of YJ18, YJ51 and TK64 were plated on chloramphenicol-free plates and tested for the appearance of chloramphenicol-sensitive variants by replica plating. The observed frequencies for the ebr mutants (0.0–0.1 \% for YJ18 and 0.4–0.5 \% for YJ51) did not differ significantly from that for the ebr\(^{+}\) TK64 (0.1–0.4 \%). Therefore, the observed spontaneous genetic instability is not mediated by the target drugs of ebrC.
in YJ18 or an overexpressed unidentified MDR system in YJ51.

**Conservation of ebrC and other MDR pump genes among Streptomyces**

Orthologues of ebrS and ebrC (SCO5517 with 100 % amino acid identity and SCO5516 with 99 % amino acid identity) have been previously identified on the chromosome of *S. coelicolor* (Lee et al., 2003). This pair is also present on the *S. avermitilis* chromosome (SAV2727 with 61 % amino acid identity and SAV2728 with 81 % amino acid identity) at a similar chromosomal location in the same synteny. A putative orthologue of ebrC (36 % amino acid identity), not accompanied by an ebrS homologue, is also found in the unpublished *Streptomyces scabies* chromosome sequence (http://www.sanger.ac.uk/Projects/S_scabies/; not yet annotated).

To explore the extent of conservation of MDR pump genes, hybridization of genomic DNA from 15 *Streptomyces* species to 129 of the 133 genes of *S. coelicolor* classified as multidrug pumps (TransportDB; http://www.membranetransport.org/) on microarrays was analysed (Fig. 6A). The dataset, when calibrated to the mean hybridization signals for the ribosomal proteins, showed that a mean of 89.2 % of the putative MDR genes in *S. coelicolor* are present in the other *Streptomyces* species (hybridization signal \( \geq \) mean \(-1\) SD; dark green to red signals), with the lowest being in *Streptomyces clavuligerus* (78 %) and highest in *Streptomyces argenteolus* (92 %). The result indicated that most of the MDR pump genes are conserved among *Streptomyces* species.

Of the 133 designated MDR genes in *S. coelicolor*, 79 (about 60 %) found a putative orthologue in *S. avermitilis* using a maximum expectation value of \(10^{-20}\) as criterion. The locations of these orthologue pairs were mapped on the *S. coelicolor* and *S. avermitilis* chromosomes and compared (Fig. 6B). The results showed that about 90 % of the putative MDR genes are located in the highly conserved central region on the two chromosomes (SCO1196-6804 in *S. coelicolor* and SAV1625-7142 in *S. avermitilis* (Ikeda et al., 2003), which contain essentially all the housekeeping genes. These MDR genes display significant conservation in chromosomal locations (parallel connection lines) and orientation (86 % of the pairs being in the same orientation; red lines in the inverted regions plus blue lines elsewhere). These results indicate that the majority of the MDR genes in *Streptomyces* are evolutionarily conserved.

**Fig. 6.** Conservation of the MDR genes among *Streptomyces* species. (A) Genomic DNA of 15 *Streptomyces* species was labelled and hybridized to the UniS *S. coelicolor* PCR array. Hybridization signals to 129 of the 133 putative MDR pump genes (from TransportDB database) present on the microarray were shown. *S. coelicolor* M145 genomic DNA was used as the reference sample. The microarray data are represented on a colour scale, with green, black and red representing gene copy numbers less than, equal to or greater than the mean signal level of the ribosomal protein genes. The MDR genes of *S. coelicolor* are arranged in the order from left to right. *Streptomyces* species: 1, *S. clavuligerus* ATCC 27064; 2, *S. griseus* ATCC 10137; 3, *S. scabies* ISP 5078; 4, *S. lipmanii* ATCC 27357; 5, *S. argenteolus* ATCC 23967; 6, *S. tannashiensis* ATCC 23967; 7, *S. hydrogenans* ATCC 19631; 8, *S. avermitilis* ATCC 31267; 9, *S. cattleya* ATCC 35852; 10, *S. venezuelae* ATCC 10595; 11, *S. rochei* 7434-AN4; 12, *S. antibioticus* IMRU 3720; 13, *S. bikinniensis* ATCC 11062; 14, *S. rimosus* ATCC 10970; 15, *S. aureofaciens* ATCC 10762. The blue horizontal bars mark the highly conserved regions between *S. coelicolor* and *S. avermitilis* (Ikeda et al., 2003). Details of the microarray results are available as Supplementary Fig. S1 with the online version of this paper. (B) Syntenic conservation between MDR genes on the *S. coelicolor* and *S. avermitilis* chromosomes. The 133 putative MDR pump genes in *S. coelicolor* were used to search for homologues in the *S. avermitilis* chromosome using TBLASTN (National Center for Biotechnology Information) with a maximum expectation value of \(10^{-20}\). The best hit was taken from each of the 93 positive results as an orthologue. Each orthologue pair was mapped to the *S. coelicolor* (upper black bar) and *S. avermitilis* (bottom black bar; flipped to match) chromosomes using the ACT program (Release 2, Sanger Centre), and are connected by blue (same orientation) and red (opposite orientation) lines. An inversion relative to oriC is indicated by the convergent arrows. Seven syntenic orthologous gene pairs are in these regions. The blue horizontal bars mark the highly conserved regions between *S. coelicolor* and *S. avermitilis* (Ikeda et al., 2003).
**DISCUSSION**

**Co-regulation of ebrS and ebrC expression by EbrS**

Divergent transcription of the ebrS-ebrC gene pair from a 65 bp promoter region was found in this study to be co-regulated by a common repressor – EbrS. Regulation by a divergently transcribed local repressor gene is common among bacterial MDR systems. The repressors in many cases, like EbrS, belong to the TetR family (reviewed by Grkovic et al., 2002). Mutation in the repressor gene is also a common basis for acquired drug resistance. In Streptomyces, divergently oriented regulator-pump pairs have also been found in antibiotic biosynthesis gene clusters, such as tcmR/tcmA in the tetracenomycin cluster (Guillole & Hutchinson, 1992), actII-orf1/actII-orf2 in the actinorhodin cluster (Fernandez-Moreno et al., 1991), rifP/rifQ in the rifamycin cluster (August et al., 1998) and lanJ/lanK in the landomycin cluster (Westrich et al., 1999). In the pqrAB operon of S. coelicolor, mutations in a TetR-type regulatory gene, pqrA, result in derepression of the efflux gene, pqrB, and elevated resistance to paraquat (Cho et al., 2003). PqrA is also its own repressor. Deletion of the pqrAB operon in wild-type S. coelicolor caused supersensitivity to paraquat, indicating that pqrB is expressed at a low level that confers a low level of resistance to paraquat.

**Biological significance of calcium- and magnesium-induced ebrS expression**

In this study we showed that the ebrS promoter is induced by high concentrations of Ca$^{2+}$ in TK64 and by high concentrations of Ca$^{2+}$ and Mg$^{2+}$ in 1326. This raises two questions: (i) why do the two strains exhibit different responses to Mg$^{2+}$; and (ii) why do these ions induce the EbrS repressor and shut down the EbrC pump?

The answer to the first question may only be speculated on, but discrepancy in the induction pattern has also been demonstrated previously in the pristinamycin MDR gene (ptr) in three Streptomyces species (Salah-Bey et al., 1995). It is notable that the ptr gene is induced by 15 antibiotics (including pristinamycins I and II and rifampicin) and four non-antibiotic chemicals including CaCl$_2$ (Salah-Bey et al., 1995). With respect to the second question, one may rule out Ca$^{2+}$ itself as the target of the EbrC efflux pump, because the ebrS mutant Y118 did not exhibit elevated resistance to excessive Ca$^{2+}$.

**Mechanism of calcium and magnesium induction**

In strains TK64 and 1326, Ca$^{2+}$ may act by binding directly to EbrS and prevent its self-repression. The same goes for Mg$^{2+}$ in 1326. Mg$^{2+}$ has been previously shown to complex with tetracycline and the complex binds to the repressor TetR. The binding causes a conformational change in the TetR protein so that it can no longer bind to the tetA operator, freeing the tetA promoter for transcription (Hinrichs et al., 1994). Alternatively, Ca$^{2+}$ and Mg$^{2+}$ (and probably rifampicin) may act through a common global stress-related adaptive regulon as suggested by Salah-Bey & Thompson (1995) and Salah-Bey et al. (1995) for ptr gene regulation.

The biological significance of the induction of the ebrS promoter by rifampicin and its synergistic effect with Ca$^{2+}$ is unclear. Rifampicin is known to increase the copy number of some plasmids, such as CoI1 in E. coli, the replication initiation of which is controlled by RNAs. It is not known whether rifampicin would also affect the copy number of pIJ487 in Streptomyces, which is already high (Kieser et al., 1982). It is noteworthy that rifampicin is not a target of the EbrC efflux system, because the ebrS mutant (Y118) is no more resistant to rifampicin and Δ(ebrS-ebrC) mutants (LF13, Y118-8) are not supersensitive. In contrast, rifampicin is both a target and an inducer for the pristinamycin MDR gene (ptr) of S. pristinaespiralis (Salah-Bey et al., 1995).

**Implication of the EbrC pump in waste management**

Despite the presence of multiple MDR genes in their genomes, Streptomyces species, like many other Gram-positive bacteria, are sensitive to a multitude of drugs, suggesting that many of the MDR genes are repressed under the tested conditions. It is interesting that none of the five target drugs of EbrC tested in this study showed any induction effect on the expression of EbrC, and the inducing drug, rifampicin, is not a target of the EbrC system. These results are consistent with our previous notion that defence against drugs is probably not the natural biological role of most of the MDR systems in Streptomyces (Lee et al., 2003). It has been postulated that a likely function of these MDR systems is the efflux of undesirable intracellular metabolites (Köhler et al., 1999; Neyfakh, 1997).

Discovery of the growth defect in the Δ(ebrS-ebrC) mutants (but not ΔebrS mutants) supports such a postulate. The growth defect of the mutants is attributed to lack of the transporter gene ebrC and not of the regulator gene ebrS. It is unlikely that the mutants are deficient in any or all of the four suppressing cations, because any of the ions alone is sufficient to restore normal growth. We propose that, under the experimental conditions, the constitutively expressed EbrC pump (Fig. 1) is involved in the efflux of undesirable metabolite products, and that the accumulation of such products retards growth of the mutant colonies on solid medium.

We suggest that many or most of the MDR systems in Streptomyces species are involved in similar intracellular waste treatment tasks. In particular, Streptomyces species and closely related actinomycetes possess large repertoires of gene clusters encoding a wide variety of secondary metabolites in their large genomes. It is likely that more undesirable byproducts are produced in these complex genomes, and it has been beneficial to develop various
efficient efflux pumps specific for these wastes during evolution. Specificity is important, as it is disadvantageous to remove useful materials from the cells.

MDR genes involved in waste management would be expected to lie in the ‘core region’ of the *Streptomyces* chromosomes, which consist mostly of housekeeping genes, whereas the ‘arm regions’ are dominated by ‘conditionally adaptive’ genes (Bentley *et al.*, 2002; Ikeda *et al.*, 2003). Of the 79 putative orthologue pairs of MDR pump genes shared by *S. coelicolor* and *S. avermitilis*, 90% are in the conserved *Streptomyces*-specific core region. These gene pairs are also conserved in chromosomal location and orientation (77%). Moreover, none of the MDR genes are located in the putative horizontally transferred islands (Bentley *et al.*, 2002). These results support a housekeeping role for the MDR systems.

The waste management model predicts that the many MDR genes are conserved evolutionarily. In this regard, it has been previously shown that homologues of *ebrA*, *ebrB* and *ebrC* appear to be widespread among *Streptomyces* based on hybridization analysis (Lee *et al.*, 2003). Our microarray and synteny analyses in this study further showed that most of the MDR genes found in *S. coelicolor* are conserved among other *Streptomyces* species.

**MDR is probably a fortuitous side effect of the waste management systems**

Why then do the MDR pumps display a very relaxed specificity toward extrinsic drugs? We propose that because most of the extrinsic drugs are not the *bona fide* substrates, they may be exported at very low efficiencies and with low specificity, and overexpressed MDR pumps (through derepression or increased gene copy numbers) are required to achieve sufficient resistance.

In the waste management model, the relevant MDR pumps are expected to be expressed constitutively at a level sufficient for effective removal of undesirable metabolic intermediates (see constitutive expression of *ebrC* in Fig. 1), and yet insufficient for providing protection against commonly tested drugs (Lee *et al.*, 1996, 2003). In this regard, it is worthwhile to recall that *Streptomyces* species are generally drug-sensitive, despite the presence of numerous MDR homologues in their genomes (more than 100 in the *S. coelicolor* chromosome). Other bacteria that are relatively susceptible to antibiotics, such as *Staphylococcus aureus* and *Bacillus subtilis*, also contain a multitude of MDR genes (Jack *et al.*, 2000; Neyfakh *et al.*, 1991; Ohki & Murata, 1997). If the role of most MDR systems were the exclusion of extrinsic toxic compounds, the large number of these systems in *Streptomyces* species would appear to be redundant and unnecessary in view of the wide and relaxed substrate specificity they display.

The mechanism of suppression of the growth defect of the Δ(*ebrS-ebrC*) colonies is not clear. It is possible that the added cations exert a physiological effect that reduces the production of the toxic metabolites, and thereby suppresses growth retardation of the Δ(*ebrS-ebrC*) colonies. Both Ca²⁺ and Mg²⁺ are extracellular signalling cations. As in other bacteria, Ca²⁺ is expected to be involved in many cellular processes in *Streptomyces*, and its low cytoplasmic concentration is stringently controlled by dedicated influx and efflux systems (Dominguez, 2004), such as the putative cation transport system encoded by a three-gene cluster (SCO3716–8) in the *S. coelicolor* genome (Yonekawa *et al.*, 2001). Alternatively, the suppressing cations may act as counter-ions for a transport system that functionally replaces the EbrC pump.

**ACKNOWLEDGEMENTS**

We thank David Hopwood for critical comments and suggestion for improvement of the manuscript. This research was supported by research grants from National Science Council, ROC (NSC94-2320-B-273-005 to Y.-J. C.; NSC98-2811-B-010-0017 and NSC90-2811-B-010-001 to R. K.; NSC93-2312-B-010-004 and NSC94-2321-B010-005 to C. W. C.), and a National Lectureship Award (2001-2004) from Ministry of Education, ROC, to C. W. C.

**REFERENCES**


Edited by: T. Ninhiba