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

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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.

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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 μM), while a class II mutant (one member, YJ18) is resistant to only 5 μM EB. The two classes of mutants were mapped at different chromosomal loci and exhibited

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Abbreviations: EB, ethidium bromide; MDR, multidrug resistance; MFS, major facilitator superfamily.

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 frameshift 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* promoter sequences.

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 YJ18 (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 YJ18 were inoculated in 200 ml TSB broth to give an OD₆₀₀ 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 (CTTCTCCAGCAGGCAGCG, in *ebrS*) and PS7 (AGCATCAGCACGGCGAGC, in *ebrC*) were used to prepare by 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 [γ -³²P]ATP using polynucleotide kinase. The same primer was used for sequence determination in parallel.

Gene disruption. The suicide plasmid pLUS944 was digested with *AseI* and *BglII*. The linearized DNA was used to transform protoplasts of TK64 and YJ18 according to Oh & Chater (1997). After overnight incubation at 30 °C, thiostrepton-resistant transformants were selected by overlaying the agar plates with 200 µg thiostrepton ml⁻¹.

Induction of *ebrC* and *ebrS* promoters. The basic design of Salah-Bey (1995) was adopted. About 1.5 × 10⁷ 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

Strain/plasmid	Genotype/description	Source/reference
Bacterial strains		
<i>S. lividans</i> 1326	Wild-type, SLP2 ⁺ SLP3 ⁺	Hopwood <i>et al.</i> (1983)
<i>S. lividans</i> TK64	<i>pro-2 str-6</i> SLP2 [−] SLP3 [−]	Hopwood <i>et al.</i> (1983)
<i>S. lividans</i> YJ18	TK64 containing <i>ebrS-18</i> mutation	Lee <i>et al.</i> (1996)
<i>S. lividans</i> YJ18-8	YJ18 containing $\Delta(ebrS-ebrC)::tsr$ mutation	This study
<i>S. lividans</i> LF13	TK64 containing $\Delta(ebrS-ebrC)::tsr$ mutation	This study
<i>S. lividans</i> LF16	TK64 containing $\Delta ebrS::tsr$ mutation	This study
<i>S. lividans</i> LF18	TK64 containing $\Delta ebrS::tsr$ mutation	This study
<i>S. lividans</i> LF24	TK64 containing $\Delta(ebrS-ebrC)::tsr$ mutation	This study
<i>S. coelicolor</i> M145	SCP1 [−] SCP2 [−]	Hopwood <i>et al.</i> (1983)
Plasmids		
pIJ487	<i>Streptomyces</i> promoter-probe vector containing <i>tsr</i> and promoterless <i>neo</i>	Ward <i>et al.</i> (1986)
pIJ702	<i>Streptomyces</i> plasmid pIJ101 containing <i>melC</i> and <i>tsr</i>	Katz <i>et al.</i> (1983)
pMRC20	pIJ487 containing the <i>ebrC</i> promoter inserted upstream of <i>neo</i>	This study; Fig. 4A
pMCR30	pIJ487 containing the <i>ebrS</i> promoter inserted upstream of <i>neo</i>	This study; Fig. 4A
pLUS914D	<i>E. coli</i> vector pMTL23 containing the <i>ebrS-ebrC</i> operon with a deletion of complete <i>ebrC</i> and most of <i>ebrS</i>	Lee <i>et al.</i> (2003)
pLUS944	<i>E. coli</i> vector pMTL23 containing the <i>ebrS-ebrC</i> operon with a deletion of most <i>ebrS</i>	This study; Fig. 3A
pLUS947	pIJ702 harbouring the 4.0 kb <i>SphI</i> fragment containing wild-type <i>ebrS</i>	This study

for 5–10 days. For each drug, 25 µg was loaded onto the paper disc. For salts, the following quantities were loaded to each disc: 5 µmol CaCl₂, MgCl₂ and MgSO₄; 0.3 µmol ZnCl₂; and 0.75 mmol CuCl₂.

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>). 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 Omnislid (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 YJ18

Lee *et al.* (2003) showed that *ebrC* is involved in the elevated MDR displayed by the *ebr* mutant YJ18, presumably because of a frameshift mutation in the adjacent repressor gene, *ebrS*. To confirm that *ebrC* was indeed derepressed in YJ18, dot-blot hybridization using an *ebrC* probe was performed to analyse the *ebrC* transcripts present in YJ18 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 YJ18 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 YJ18 and TK64, but nevertheless it was considerably higher in YJ18. These results supported the notion that *ebrC* is overexpressed in YJ18 because of the defective *ebrS*. The higher expression of the mutant allele *ebrS18* in YJ18 also suggested that *ebrS* represses its own transcription.

The same conclusions were reached from S1 protection mapping (Fig. 2), which showed increased transcription of

Fig. 1. Expression of *ebcC* and *ebcS* in TK64 and YJ18. Total RNA was isolated at four different growth stages of TK64 and YJ18 growing in TSB liquid medium: early exponential (EE; OD₆₀₀ 0.6–0.7), mid-exponential (ME; OD₆₀₀ 1.7–1.8), late-exponential (LE; OD₆₀₀ 3.9–4.2) and stationary (S; OD₆₀₀ 1.2–7.8) phases. The RNA was blotted onto a nylon membrane and hybridized with a ³²P-labelled DNA probe of the *ebcC*, *ebcS* and 16S rRNA genes of *S. lividans*. *Neurospora crassa* (Nc) and *E. coli* (Ec) RNAs were included as controls. The weak hybridization signals of the *ebcS* probe were electronically enhanced to reveal the low expression in YJ18.

ebcC in YJ18 in all growth phases from 17 nt upstream of the predicted *ebcC* coding sequence. A putative –10 region of typical prokaryotic promoters (GACAAT) is found further upstream. The transcription start site for *ebcS* could not be detected by S1 mapping because of the low expression level.

Disruption of *ebcS* causes elevated MDR

To confirm that the elevated *ebcC* expression in YJ18 was the result of the defect in *ebcS*, an independent inactivation mutation in *ebcS* was created in TK64. A suicide vector pLUS944 was constructed that contained a 6 kb fragment spanning the *ebcS*–*ebcC* operon, in which most of the *ebcS* coding sequence was replaced by the thiostrepton resistance gene (*tsr*) (Fig. 3A). The vector was used to transform TK64. Thiostrepton-resistant transformants were isolated, in which the wild-type *ebcS* allele had been replaced by the Δ *ebcS* deletion allele on the suicide vector as shown by Southern hybridization analysis (Fig. 3B). Two independent Δ *ebcS* transformants (LF16, LF18) of TK64 were isolated on solid medium containing 20 μ M EB.

Our model also predicted that introduction of a wild-type copy of *ebcS* on a high-copy-number plasmid would convert YJ18 to a wild-type (EB-sensitive) phenotype. To test this, the 4.0 kb *Sph*I fragment containing the wild-type *ebcS* gene was inserted into the *Streptomyces* plasmid vector pIJ702 (Katz *et al.*, 1983). The resultant plasmid, pLUS947, was

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 μ M EB.

The *ebcS* 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 pristinaamycins I and II, among other unrelated drugs (Salah-Bey *et al.*, 1995). To test the drug inducibility of *ebcC*, a reporter system was constructed. The 0.3 kb *Bgl*II–*Bam*HI fragment containing the *ebcS*–*ebcC* intergenic region was inserted into the *Bgl*II site of pIJ487 (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 *ebcC* promoter was oriented toward the promoterless reporter gene, *neo*; in pMRC30, the *ebcS* 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 μ g 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 *ebcC* and *ebcS* expression. The last six drugs are targets of the *EbcC* efflux system (Lee *et al.*, 1996, 2003). Some salts (CaCl₂, MgCl₂, MgSO₄, ZnCl₂ and CuCl₂) were also tested.

None of the compounds induced the *ebcC* promoter in pMRC20 (data not shown). Induction of the *ebcS* 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 *ebcS* promoter. In the control experiments, TK64 (Fig. 4B) and TK64/pIJ487 (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 *ebcC* promoter, and both CaCl₂ and rifampicin induced the *ebcS* promoter. In addition, MgCl₂, which is not an inducer in TK64, induced the *ebcS* 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

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 $\Delta(e'brS-ebnC)$ mutants LF13 and LF24 (not shown) were minute compared to those of TK64 and the $\Delta ebrS$ 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 *ebnC*, but not *ebnS*, 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

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Fig. 3. Insertional inactivation of *ebrS*. (A) The expected cross-overs are shown between the suicide vector pLUS944 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.

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

Fig. 4. Induction of the *ebrCS* promoters. (A) Reporter system for the *ebrS* and *ebrC* promoters. The promoter-probe plasmid pLJ487 (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 pLJ487 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.

monovalent ions (K^{+} and Na^{+}), all as chlorides, were tested for their ability to suppress the $\Delta(ebrS-ebrC)$ 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. 5. Colony growth deficiency of the $\Delta(ebrS-ebrC)$ mutants. Diluted spore suspensions were plated on NE medium containing various concentrations of CaCl_2 (A), MgCl_2 (B) and KCl or NaCl (C) and incubated for 3 days at 30 °C. The results for LF24 and LF18 were similar to those for LF13 and LF16, respectively, and are not shown. Variations in the colony numbers in each series were insignificant (data not shown).

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 & Schrepf, 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 & Schrepf, 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. tanshiensis* 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. bikiniensis* 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 (Guilfoile & 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 YJ18 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 *tet* 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 ColE1 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 (YJ18) is no more resistant to rifampicin and $\Delta(\textit{ebrS-ebrC})$ mutants (LF13, YJ18-8) 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 $\Delta(\textit{ebrS-ebrC})$ mutants (but not $\Delta\textit{ebrS}$ 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 $\Delta(ebrS-ebrC)$ 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 $\Delta(ebrS-ebrC)$ colonies. Both Ca^{2+} and Mg^{2+} are extracellular signalling cations. As in other bacteria, Ca^{2+} 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.

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