Two classes of ethidium-bromide-resistant mutants of *Streptomyces lividans* 66

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Five spontaneous mutants of *Streptomyces lividans* TK64 resistant to 5 or 15 μ M ethidium bromide (EB) were isolated, and the corresponding mutations were mapped to two different chromosomal locations. Both types of mutations conferred unselected resistance to several basic dyes and norfloxacin. The strain with the low-level resistance exhibited wild-type levels of EB uptake and energy-dependent efflux, and the resistance mechanism is unclear. The highly resistant mutants, which additionally were resistant to phosphonium ions, had a reduced accumulation and an increased efflux of EB, reminiscent of a mammalian multidrug resistance efflux pump.

Keywords: *Streptomyces*, ethidium bromide, multidrug resistance

INTRODUCTION

DNA-intercalating agents, such as ethidium bromide (EB), acriflavine and acridine orange, are basic compounds containing complex hydrophobic rings that can insert between the base pairs in the double helix. These DNA intercalators appear to enter the cells freely and interfere with a large number of processes, such as replication, transcription, translation, recombination (Seto & Tomasz, 1977), RNA splicing (Tanner & Cech, 1985), maintenance of plasmids, and formation of Z-DNA (Walker *et al.*, 1985; Hardin *et al.*, 1988). They also generate frameshift mutations (Brenner *et al.*, 1961; Crick *et al.*, 1961). In Gram-positive filamentous bacteria of the genus *Streptomyces* they also induce extensive DNA deletions and amplifications (Schrempf, 1983; Hintermann *et al.*, 1984) at the terminal (unstable) regions of the linear chromosomes (Redenbach *et al.*, 1993). Little is known about how these agents cause plasmid curing or rearrangement of chromosomal DNA. These compounds cause cell death, but it is not known whether the lethality is due to mutagenicity or to some other toxic effect.

Many bacteria, including *Escherichia coli*, are resistant to DNA-intercalating compounds. Sensitive mutants have been isolated with a defective energy-dependent efflux system. Genes that appear to be involved in the efflux systems have been cloned from the chromosome or plasmids of *E. coli* and other bacteria (reviewed by Lewis, 1994; Ma *et al.*, 1994; Nikaido, 1994). The protein products encoded by these genes are cell membrane

transporters, and may be grouped into four families according to their supramolecular assembly, mechanism of action, and sequence homology (Nikaido, 1994). Each system confers resistance to remarkably broad (and often overlapping) spectra of agents, only some of which are structurally related. The most common targets for these pumps are basic dyes, hydrophobic antibiotics, quaternary ammonium compounds, fluoroquinolones and detergents.

In contrast, Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, are more sensitive to EB (Nikaido, 1994), and resistant mutants may be selected. Neyfakh *et al.* (1991) isolated *Bacillus subtilis* mutants (*bmr*) resistant to rhodamine-6G, which were cross-resistant to EB. The increased resistance appeared to be mediated by an elevated efflux. These authors cloned the resistance gene, and found it to encode a 389 amino acid protein with 12 putative transmembrane domains. The Bmr protein closely resembled the tetracycline-efflux (TetR) proteins encoded by Tn10, Tn721 and pBR322, in agreement with its putative role in drug efflux.

In *Staphylococcus aureus*, determinants of resistance to quaternary ammonium compounds and basic dyes have been found on various plasmids. The resistance determinant, *qacA*, on plasmid pSK1 also mediates an efflux system (Tennent *et al.*, 1985, 1989). *qacA* (Sasatsu *et al.*, 1989; Rouch *et al.*, 1990) encodes a 514 amino acid protein, which also resembles TetR and Bmr. This type of drug resistance, mediated by efflux, is similar to the so called 'multidrug resistance' in cancer cells conferred by an elevated level of membrane-bound P-glycoprotein that pumps out cytotoxic drugs of various kinds, including DNA-intercalating compounds.

Abbreviations: EB, ethidium bromide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

Here we report that wild-type *Streptomyces lividans* tolerates only low concentrations of EB, and resistance is not induced by pre-exposure to a sublethal level of EB. Spontaneous mutants of *S. lividans* resistant to elevated EB concentrations were isolated and characterized. These mutants were grouped into two classes with respect to their efficiencies of EB efflux and substrate spectra, and the chromosomal locations of the mutations. One class of mutants exhibited increased efflux of EB, and the other did not. These results revealed at least two separate genetic systems involved in multidrug resistance.

METHODS

Bacterial strains and plasmids. The *S. lividans* strains are listed in Table 1. *Streptomyces* cultures were grown in YM (0.3%, w/v, yeast extract, 0.3%, w/v, malt extract, 2%, w/v, agar), CM (YM plus 0.5%, w/v, peptone, 1%, w/v, glucose and 5 mM MgCl₂; Dyson & Schrempf, 1987), MM or R2 (Hopwood *et al.*, 1985) solid medium, or TSB liquid medium (Hopwood *et al.*, 1985). Required amino acid and antibiotic supplements were added to MM as described by Hopwood *et al.* (1985).

Chemicals. Lysozyme, agarose, streptomycin, spectinomycin, acridine orange, proflavine, acriflavine, methyl green, rhodamine-6G, benzalkonium chloride, toluidine blue O, norfloxacin and carbonyl cyanide *m*-chlorophenylhydrazide (CCCP) were purchased from Sigma; cetyltrimethylammonium bromide and tetraphenylphosphonium bromide from Aldrich; methyl triphenylphosphonium bromide from Fluka; EB and methylene blue from E. Merck; pyronine Y from Allied Chemical; crystal violet from J. T. Baker; and restriction enzyme *AseI* from New England Biolabs.

Drug sensitivity test. For survival counts, *S. lividans* spore suspensions were plated on YM plates containing EB and incubated in the dark at 30 °C; surviving colonies were counted after 2–3 d. Semi-quantitative tests were performed by placing 8 mm filter disks (Toyo Adventec, Toyo Roshi) containing 25 µl of the drugs in various concentrations onto solid YM agar seeded with 10⁶–10⁷ spores. The plates were incubated at 30 °C for 2–3 d and the diameters of the inhibition zones were measured.

Genetic mapping. Genetic mapping of the *ebr* mutations by conjugation was based primarily on the procedures of Hopwood *et al.* (1985). Spores of an *ebr* mutant were mixed with spores of *S. lividans* 3078 (about 10⁷ of each), and grown on R2 medium until sporulation occurred (7–10 d). The spores were plated on MM containing leucine, proline and spectinomycin to select for *his⁺ spc* recombinants. The genotypes of the recombinants were

further analysed by replica plating on diagnostic media containing various combinations of supplements.

Uptake and efflux of EB. The fluorescence technique used by Jones & Midgley (1985) and Midgley (1986) was adapted to measure the uptake and efflux of EB. Mycelium was collected from an early exponential culture in TSB medium, washed three times and resuspended in ice-cold MM (without glucose; filtered through a 0.2 µm polyestersulfone membrane) to an OD₆₀₀ of 0.3, and kept on ice.

For the measurement of EB accumulation, 2 ml of the suspended mycelium was transferred to a cuvette in a Hitachi F-4010 fluorescence spectrophotometer and stirred with a magnetic stir bar. Glucose (1 M) was added to a final concentration of 10 mM. To test the energy dependence of the accumulation and efflux, the oxidative phosphorylation uncoupler CCCP (20 mM) was added in parallel with glucose to a final concentration of 40 µM. Uptake was initiated when EB (2.5 mM) was added to a final concentration of 5 µM, and fluorescence was measured at 596 nm using a 530 nm excitation light. Mycelial aggregates formed during the measurement were intermittently broken up by repeated pipetting through a 1 ml pipette.

For efflux measurements, the washed mycelium (OD₆₀₀ 0.3) was pre-incubated in MM containing 5 µM EB, 40 µM CCCP, and 10 mM glucose at 30 °C for 1 h, washed three times in ice-cold MM (without glucose), and resuspended in 0.1 vol. MM (without glucose) immediately before assay. The cuvette was first filled with 1.8 ml MM (containing glucose and/or CCCP as necessary), and the measurement of fluorescence was started when 0.2 ml of EB-preloaded mycelium was added. When protoplasts were used for the assays, they were prepared from fresh cultures, and all the media contained 0.3 M sucrose.

RESULTS

Low resistance of *S. lividans* TK64 to EB

S. lividans TK64 exhibited various degrees of resistance to low concentrations (below 8 µM) of EB as a function of the medium composition (Fig. 1). The survival curves displayed a shoulder up to an EB concentration of 2 µM in YM medium, and 6 µM in the richer CM medium. Further supplementation of glucose (1%) to YM slightly increased the survival fraction of TK64, while removal of glucose from CM reduced the survival fraction. These results indicated that richer nutrients increased the resistance of TK64 to EB.

To test whether EB resistance was inducible, TK64 was grown for a 0.5–8 h on YM containing a sublethal

Table 1. *S. lividans* strains used in this study

Designation	Genotype/characteristics	Source/reference
1326	Wild-type	Hopwood <i>et al.</i> (1983)
3078	<i>his-2 leu-2 spc-1</i> pIJ303 SLP2 ⁺ SLP3 ⁺	Hopwood <i>et al.</i> (1983)
TK64	<i>pro-2 str-6</i> SLP2 ⁺ SLP3 ⁺	Hopwood <i>et al.</i> (1983)
YJ11	<i>pro-2 str-6 ebr-11</i> SLP2 ⁺ SLP3 ⁺	This study
YJ18	<i>pro-2 str-6 ebr-18</i> SLP2 ⁺ SLP3 ⁺	This study
YJ51	<i>pro-2 str-6 ebr-51</i> SLP2 ⁺ SLP3 ⁺	This study
YJ52	<i>pro-2 str-6 ebr-52</i> SLP2 ⁺ SLP3 ⁺	This study
YJ57	<i>pro-2 str-6 ebr-57</i> SLP2 ⁺ SLP3 ⁺	This study

Fig. 1. Sensitivity of *S. lividans* TK64 to low concentrations of EB. ○, YM agar; ●, YM agar plus 1% glucose; ▲, CM agar; △, CM minus glucose.

Fig. 2. Sensitivity of *S. lividans* TK64 and its *ebr* derivatives to EB. The survival fractions (on YM agar) were calculated as the ratios of the numbers of colony-forming units in the presence and absence of EB. □, TK64; ◆, YJ11; ○, YJ18; ■, YJ51; ●, YJ52; ▲, YJ57.

concentration (1 μ M) of EB before challenge with a lethal concentration of EB. No increase in the survival fraction was observed for EB concentrations up to 10 μ M.

Isolation of EB-resistant (*ebr*) mutants

The colony-forming ability of *S. lividans* TK64 spores on YM containing 10 and 15 μ M EB was reduced by about 6 orders of magnitude (Fig. 2). Five spontaneous resistant mutants were picked from amongst the surviving colonies, and designated YJ11, YJ18, YJ51, YJ52 and YJ57 (carrying mutations *ebr-11*, *ebr-18*, *ebr-51*, *ebr-52* and *ebr-57*, respectively). Four of the mutants exhibited essentially complete resistance to EB up to 15 μ M; the fifth mutant, YJ18, was partially sensitive to EB at concentrations above 5 μ M (Fig. 2). The four totally resistant mutants

Fig. 3. Genetic mapping of the *ebr* mutations in *S. lividans*. The circles represent the genetic maps of the two mating strains (inside, 3078; outside, the *ebr* mutant). The triangles indicate the selected alleles (*his*⁺ and *spc*-1).

(YJ11, YJ51, YJ52 and YJ57) were grouped as class I, and the partially resistant mutant, YJ18, as class II.

The morphology, pigmentation and growth characteristics of these mutants were indistinguishable from those of TK64. Total DNA from the *ebr* mutants, analysed by conventional restriction digestion and electrophoresis, showed no detectable amplifications. Digestion of the chromosomal DNA with the rare-cutting enzyme *Ase*I and separation by pulsed-field gel electrophoresis gave rise to the same 16 chromosomal fragments (Leblond *et al.*, 1993; Lin *et al.*, 1993) as in TK64. These results indicated that no gross genomic rearrangements had occurred in these *ebr* mutants, despite their prior exposure to the rearrangement-inducing EB.

Genetic mapping of the *ebr* mutations

The *ebr* mutations were mapped by conjugation. Each of the *ebr* mutants (*pro*-2 *str*-6) was mated with *S. lividans* 3078 (*his*-2 *leu*-2 *spc*-1) containing the conjugative plasmid pIJ303 (Hopwood *et al.*, 1983). His⁺ and spectinomycin-resistant (*spc*) recombinants were isolated and analysed for

Fig. 4. Sensitivities of the *ebr* mutants to other drugs. The filter disk assay was used to measure the mean diameter of the inhibition zones exerted by drugs applied to the filter disks. The diameter of the filters was 8 mm. □, TK64; ◆, YJ11; ○, YJ18; ■, YJ51; ●, YJ52; ▲, YJ57. The results are summarized in Table 2.

the segregation of the unselected markers. The frequency of *his⁺ spc* recombinants in each mating was $2-9 \times 10^{-3}$. From the allele ratios of the markers there were two possible locations for each of the *ebr* mutations: between *pro-2* and *spc-1* or between *his-2* and *str-6* for the class I mutations; and on the left or right of *spc-1* for the class II mutation (Fig. 3). The choice was made between each pair of locations by analysing the linkage between each *ebr* mutation and its neighbouring markers by minimizing the numbers of crossovers required for the recombinant genotypes (Hopwood *et al.*, 1985). Class I mutations were placed in the 9 o'clock region, and the class II mutation between *str-6* and *spc-1* near 6 o'clock.

Cross-resistance of the *ebr* mutants to other DNA-intercalating agents

EB resistance mutations generally affect susceptibility of the mutants to other drugs. The sensitivities of these mutants to three other DNA intercalators, acriflavine, proflavine and acridine orange, were similar to their sensitivity to EB: the class I mutants were completely resistant, and the class II mutant was partially resistant (Fig. 4; Table 2). The class I mutants were also more resistant to pyronine Y than the class II mutant. The class I mutants showed a slightly lower sensitivity to crystal violet and methyl green than TK64, while YJ18 was only slightly less sensitive to CV than TK64. The results indicated a biochemical difference in the resistance mechanisms in the two classes of *ebr* mutants, further justifying the classification.

The *ebr* mutants were as sensitive as TK64 to rhodamine-6G, SDS, CCCP and two quaternary ammonium compounds, cetyltrimethylammonium bromide and benzalkonium chloride. They were also equally resistant as TK64 to two basic dyes, toluidine blue O and methylene blue. YJ18 was as sensitive as TK64 to the phosphonium ions methyl-triphenylphosphonium bromide and tetraphenylphosphonium bromide, while YJ51 showed re-

sistance (Table 2). All *ebr* mutants exhibited elevated resistance to norfloxacin, a fluoroquinolone; the class I mutants were more resistant than the class II mutant (Fig. 4).

These results showed that the *ebr* mutations had relatively broad ranges of resistance spectra, like the multidrug resistance systems in other bacteria. TK64 is a derivative of the wild-type strain 1326 obtained through several steps of mutations (Hopwood *et al.*, 1983). The pattern of drug susceptibility of 1326 was identical to that of TK64, except for the streptomycin resistance conferred by a mutation in TK64 (Hopwood *et al.*, 1983).

Efflux studies

The uptake and efflux of EB by mycelial fragments of YJ18 and YJ51 were compared to those of TK64 using a fluorescence assay (Jones & Midgley, 1985; Midgley, 1986), in which the presence of intracellular EB was measured by the fluorescence produced by the EB-nucleic acid complexes (Fig. 5, left panels). Both TK64 and YJ18 accumulated EB in minimal medium (containing glucose), approaching a plateau in about 40 min. Addition of CCCP, an uncoupler of oxidative phosphorylation, resulted in an approximately 70% increase in accumulation, indicating the existence of an energy-dependent efflux system in these two strains. YJ51, on the other hand, accumulated much less EB and approached a plateau in less than 5 min in the absence of CCCP. It accumulated about three times more EB in the presence of CCCP. This indicated that the putative efflux system in YJ51 was significantly more efficient than those in TK64 and YJ18.

The presence of the efflux system was supported by efflux studies (Fig. 5, right panels), in which the efflux of EB was measured after removal of EB from the medium. TK64 and YJ18 pumped out EB at about the same rate, reaching a basal level in 40 min. In contrast, EB efflux in YJ51 was much faster and completed in less than 10 min. The efflux

Table 2. Summary of drug resistance patterns of the *ebr* mutants

See Fig. 4 for representative quantitative results.

Drug	Resistance/sensitivity*		
	TK64	Class I	Class II
Basic dyes			
EB	S	R	r
Acriflavine	S	R	r
Proflavine	S	R	r
Pyronine Y	S	R	r
Crystal violet	S	r	r
Methyl green	S	r	S
Toluidine blue O	R	R	R
Methylene blue	R	R	R
Rhodamine-6G	S	S	S
Phosphonium ions			
Methyltriphenylphosphonium	S	R†	S
Tetraphenylphosphonium	S	R†	S
Quaternary ammonium compounds			
Cetyltrimethylammonium bromide	S	S	S
Benzalkonium chloride	S	S	S
Detergent			
SDS	S	S	S
Uncoupler			
CCCP	S	S	S
Fluoroquinolone			
Norfloxacin	S	R	r

* S, sensitive; R, completely/highly resistant; r, partially/slightly resistant.

† Only YJ51 was tested.

in all three cultures was blocked by CCCP. These results confirmed a more efficient efflux system in YJ51, which was presumed to be the result of the *ebr-51* mutation and responsible for the elevated resistance of this strain to EB. Another class I mutant, YJ11, was tested; it exhibited a similar increase in EB efflux and a similar decrease in EB accumulation to those seen in YJ51. Similar results were seen when protoplasts were used for the assays instead of mycelial fragments (data not shown).

DISCUSSION

Wild-type *S. lividans* (represented by TK64) appears to possess at least one functional EB efflux system, which may be responsible for the limited resistance observed in rich medium (CM). On the other hand, the increased efflux shown in the class I *ebr* mutants appeared to be sufficient for complete resistance to 15 μ M EB. This type of resistance, mediated by increased efflux, has been well documented in *E. coli* and other bacteria (Midgley, 1987). The efflux system in wild-type *S. lividans* is not inducible by EB, so its biological role is not clear. Removal of

Fig. 5. Accumulation and efflux of EB in the *ebr* mutants. Left panels: accumulation of intracellular EB by the mycelium of YJ18, YJ51 or TK64 was measured in the presence (open symbols) or absence (filled symbols) of CCCP. Right panels: efflux of EB from the mycelium of YJ18, YJ51 or TK64 was measured in MM containing glucose in the presence (open symbols) or absence (filled symbols) of CCCP.

potentially toxic compounds is one possibility (Ferguson & Baguley, 1993). The preference for basic dyes as the substrate of the *ebr* system suggests that it may act to protect the nucleic acids and/or protein targets in the cell.

TK64 growing on rich media exhibited an increased EB resistance (Fig. 1). The difference between CM, which allowed the highest resistance (to 6 μ M), and YM, which allowed the least (to ≤ 2 μ M) may reflect the additional peptone, glucose and $MgCl_2$ in CM. Although the addition of glucose to YM increased the resistance slightly, CM depleted of glucose still allowed better resistance than YM. Thus, it appeared that the higher resistance in YM was mainly due to the peptone. The putative role of peptone in promoting resistance may be (i) through titration of EB by the peptides in it, (ii) by supporting faster growth and higher cellular DNA content, or (iii) through the induction of one or more efflux systems.

The two classes of *ebr* mutations differed in their respective genetic locations, and in the spectra and extent of drug resistance conferred. The class I mutants appeared to acquire increased resistance through elevated energy-dependent efflux. Although the increased efflux was demonstrated only in YJ51 and YJ11, it is likely to be present also in YJ52 and YJ57.

The class II mutant YJ18 did not exhibit a significant increase in EB efflux. The reproducible sigmoid shape of the EB survival curve of this mutant, with a shoulder extending to 5 μ M EB, is interesting. Resistance to EB at concentrations lower than 5 μ M could not be mediated by an uptake barrier, because YJ18 accumulated EB at this concentration at the same rate as TK64. The resistance may be mediated by a mutation in a cellular target of EB, resulting in loss of affinity. Proteins, in addition to nucleic acids, are targets of EB binding, a notable example being the RecA protein of *E. coli* (Thresher & Griffith, 1990). The sensitivity of YJ18 to EB above 5 μ M might be mediated by a secondary target of lower sensitivity.

Antibiotic-producing cultures of *Streptomyces* spp. frequently contain efflux systems specific for the antibiotics produced, such as ActII for actinorhodin (Caballero *et al.*, 1991), and Mmr for methylenomycin in *Streptomyces coelicolor* A3(2) (Neal & Chater, 1987), OtrB for oxytetracycline in *Streptomyces rimosus* (Ohnuki *et al.*, 1985), DrrA and DrrB for daunorubicin and doxorubicin, respectively, in *Streptomyces peucetius* (Guilfoile & Hutchinson, 1991), TlrC and TlrB for tylosin in *Streptomyces fradiae* (Rosteck *et al.*, 1991), and TcmA for tetracenomycin in *Streptomyces glaucescens* (Guilfoile & Hutchinson, 1992). Genes encoding these proteins are closely linked to the production genes. The efflux system(s) identified in this study represent(s) the first broad-spectrum system found in *Streptomyces*. The *ebr* mutations of both classes conferred resistance to most of the basic dyes tested, but not to quaternary ammonium ions, SDS or CCCP (Table 2). YJ51 exhibited a slight resistance to phosphonium ions. The broad-spectrum efflux systems characterized in other Gram-positive bacteria exhibited similar substrate spectra. The Bmr transporter of *B. subtilis* acts on basic dyes, fluoroquinolones, chloramphenicol and puromycin (Neyfakh *et al.*, 1991). In *Staphylococcus aureus* the QacA and QacC (Smr) systems transport basic dyes and quaternary ammonium compounds (Tennent *et al.*, 1989; Littlejohn *et al.*, 1991); the NorA system transports basic dyes, fluoroquinolones, phosphonium ions, puromycin and chloramphenicol (Yoshida *et al.*, 1990).

The *ebr* mutants were isolated on medium containing EB at concentrations that may have been high enough to induce chromosomal rearrangements (Gregory & Huang, 1964; Cramer *et al.*, 1986), so it was possible that the mutations resulted from the mutagenic action of EB. However, several observations argue against this possibility. Firstly, other than the acquired resistance observed in these mutants, there were no phenotypic changes, particularly the loss of chloramphenicol resistance or arginine prototrophy frequently associated with EB-

induced deletions. Secondly, no gross DNA arrangements (another EB-sensitive event) were seen in any of the *ebr* mutants. Thirdly, no new traits were segregated in the recombinant products of the crosses between the *ebr* mutants and 3078. Therefore, the *ebr* mutations appeared to exist before the EB selection.

Chou (1987) found that the *rec-46* mutant, JT46, of *S. lividans* that was defective in intraplasmid recombination exhibited slightly higher resistance to EB. However, the *ebr* mutants were as proficient in plasmid recombination as TK64 (Huang, 1989), indicating the absence of correlation between EB resistance and intraplasmid recombination.

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