PHLEOMYCIN AND BLEOMYCIN RESISTANCE IN *ESCHERICHIA COLI*

GEOFFREY W. GRIGG and CHRISTINA M. COLLIS

Laboratory for Molecular Biology,
CSIRO Division of Biotechnology,
North Ryde, NSW 2113, Australia

(Received for publication August 24, 1988)

Phleomycins (PLMs) and bleomycins (BLMs) are closely-related families of glycopeptides produced as Cu(II) chelates by *Streptomyces verticillus*. The drugs induce extensive DNA strand breakage *in vivo* and *in vitro* and this is assumed to account for their antibiotic and antitumor activity[1].

In this paper we describe the properties of some *Escherichia coli* mutants selected for resistance to PLM. The PLM resistance of some other strains is also reported. Table 1 sets out the genotypes of the bacterial strains used.

PLM-resistant mutants were selected by plating stationary phase bacteria onto blood agar base (Oxoid) plates (10^8 bacteria/plate) containing PLM A9331-648† (PLM-648). PLM-648 is a mixture of Cu(II)-containing PLMs-G, -E and -D[2]. Plates were incubated overnight at 37°C. Colonies were then picked off and streaked onto blood agar base plates to check for homogeneity of growth rate between the cells of each colony. The phenotypes of these PLM-resistant strains were investigated.

From the K-12 parental strains used in this study, mutants were readily isolated on plates containing 8 μg/ml PLM with a frequency of about 10^{-7}. Because of the high molecular weight of PLM (about 1,500) and the charged nature of the molecule, it was anticipated that many mutants would exhibit a reduced permeability to the drug. Therefore randomly-selected mutants were examined for characteristics indicative of a change in cell envelope structure, such as altered sensitivities to a range of bacteriophages and to the detergent sodium deoxycholate[3]. Sensitivities to phages T1, T2, T3, T4 and the male specific phage MS2 were tested by cross-streaking bacteria and phage at right angles to each other and examining for lysis at the intersections of the streaks. Hfr character of presumptive Hfr strains was similarly checked by cross-streaking against the F^- strain JC2918 on minimal GT[4] medium lacking either

Table 1. Bacterial strains.

Strain	Sex	Genotype	Source
AB259	HfrH	*thi*	Laboratory stock
AB259 PLM^r-1	Not Hfr	As AB259; PLM-resistant	This study
AB259 PLM^r-10	Not Hfr	As AB259; PLM-resistant	This study
AB259 PLM^r-3	HfrH	As AB259; PLM-resistant	This study
AB259 PLM^r-3A	HfrH	As AB259; PLM-resistant	This study
AB2322	HfrC	*met*, (λ)	University of Melbourne
AB2322 PLM^r-1	Not Hfr	As AB2322; PLM-resistant	This study
AB2322 PLM^r-10	Not Hfr	As AB2322; PLM-resistant	This study
JC2918	F^-	*thr-1, leu-6, pro-12, his-4, arg-E8, lacY1, galK2, ara-14, xyl-5, mtl-1, tsx-33, rpsL31, supE44*	R. HULL University of Adelaide
JC2918 PLM^r-3	F^-	As JC2918; PLM-resistant	This study
AB712	F^-	*thr, leu, thi, pro, str, lacY, xyl, ara, gal, mtl*, (λ)	Laboratory stock
AB712 PLM^r-3A	Hfr	As AB712; PLM-resistant	This study
KL16	Hfr	*thi*	W. WOODS
KL16 PLM^r-7	Hfr	As KL16; PLM-resistant	This study
KL16 PLM^r-9	Hfr	As KL16; PLM-resistant	This study
KMBL54	F^-	*pyrF, thy, lac, thi*	W. BERENDS
129	F^-	As KMBL54; *gshA*	W. BERENDS

† A gift from Dr. W. BRADNER, Bristol Laboratories, Syracuse, U.S.A.

Table 2. Cell envelope-related properties and PLM sensitivity of wild type and PLM-resistant strains of *Escherichia coli* K-12.

Strain	PLM sensitivity	Phage sensitivity					Hfr status	Deoxycholate sensitivity
		T1	T2	T3	T4	MS2		
AB2322 (HfrC)	+	+	+	+	+	+	Hfr	+
AB2322 PLM^r-1	R	R	R	R	+	R	Not Hfr	R
AB2322 PLM^r-10	R	R	R	R	+	R	Not Hfr	R
AB259 (HfrH)	+	+	R	+	+	+	Hfr	+
AB259 PLM^r-1	R	R	R	R	+	R	Not Hfr	R
AB259 PLM^r-3	R	−	+	+	+	−	Not Hfr	−
AB259 PLM^r-3A	R	−	+	+	+	−	Hfr	+
AB259 PLM^r-10	R	R	R	R	+	R	Not Hfr	R
AB712 (F$^-$)	+	+	+	+	+	−	F$^-$	+
AB712 PLM^r-3A	R	R	+	+	+	−	F$^-$	+
AB712 PLM^r-4B	R	−	+	+	+	−	F$^-$	R
KL16 (Hfr)	+	+	+	+	+	+	Hfr	+
KL16 PLM^r-7	R	+	+	+	+	−	Hfr	+
KL16 PLM^r-9	R	+	+	+	+	−	Hfr	+

+: Normal (wild type) sensitivity, R: more resistant than wild type, −: not tested.

Table 3. Categories of PLM-resistant mutants described in this paper.

Class	Apparent cause of PLM resistance	Example	Phenotype of example
I	Cell envelope alteration	AB2322 PLM^r-1	Phage and deoxycholate resistance; loss of Hfr character
II	Ubiquinone deficiency	KL16 PLM^r-9	Resistance to heat inactivation, slight sensitivity to UV and other DNA damaging agents, Suc$^-$
III	Excision-repair deficiency (*uvrA*)	Bs-12[a]	UV sensitivity; inability to reactivate UV-irradiated phage T1
IV	Glutathione deficiency (*gshA*)	129	See ref 11

[a] See ref 4.

proline, threonine or leucine but containing streptomycin (250 μg/ml). To measure resistance to deoxycholate, stationary phase broth-grown cultures were diluted and plated for single colonies on nutrient agar containing concentrations of sodium deoxycholate ranging from 0~0.5%.

As expected, the majority of K-12 mutants examined displayed altered surface properties as judged by these criteria. Some examples are shown in Table 2. AB2322 PLM^r-1 and PLM^r-10 and AB259 PLM^r-1 and PLM^r-10 had acquired resistance to a number of phages including MS2, had lost their ability to transfer genetic material, and were more resistant than their parental strains to deoxycholate. Clearly these strains, designated Class I in Table 3, suffered from a major change to cell wall structure. Of the other K-12 mutants, AB259 PLM^r-3 had lost its Hfr character and AB712 PLM^r-4B had become resistant to deoxycholate, again probably indicating some change in the cell envelope.

The remaining K-12 mutants were apparently normal with respect to those surface characteristics tested. Of these, a small group (KL16 PLM^r-7 and PLM^r-9) displayed an intriguing phenotype including considerable resistance to heat inactivation at 52°C and a slight sensitivity to UV and other DNA-damaging agents. The level of resistance to PLM varied according to the nature of the basic group of the molecule, but was identical for PLMs and BLMs with corresponding basic groups. One of these mutants (PLM^r-9) was the subject of a more detailed study[5] and was shown to be defective in the

biosynthesis of ubiquinone, an inner membrane constituent which functions in aerobic electron transport. Reduced permeability of the inner membrane rather than the cell envelope may account for the PLM resistance of this group of mutants, designated Class II in Table 3. Ubiquinone-deficient and other respiratory mutants are often unable to grow on succinate as sole carbon source[6] (Suc$^-$ phenotype). That this is a common mechanism of resistance to PLM is indicated by the fact that, out of 134 PLM-resistant mutants of KL16, isolated as above, 99 (74%) were found to be Suc$^-$.

In contrast to the K-12 strains, it proved to be impossible to isolate single-step mutants of *E. coli* B on plates containing 5 μg/ml PLM. However, mutants were readily obtained on plates containing 1 μg/ml PLM at a frequency of $10^{-6} \sim 10^{-7}$. When these strains were cultured and spread on plates containing 5 μg/ml PLM, resistant clones did appear with a frequency of $10^{-6} \sim 10^{-7}$. It is apparent that mutation to resistance to higher concentrations of PLM requires two independent mutations in *E. coli* B.

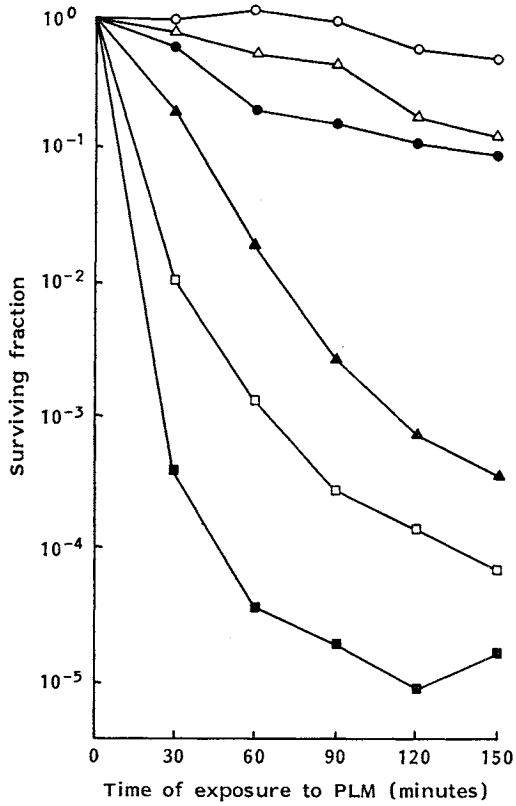
The principal novelty of the single-step *E. coli* B PLM^r mutants, Class III in Table 3, is that they appeared to have the phenotype of excision repair-defective strains. Of 8 resistant isolates examined, all were UV-sensitive and were unable to reactivate UV-irradiated phage T1[4]. Moreover their UV-sensitivity was not increased by known inhibitors of excision repair such as coumarin and caffeine. In agreement with this, the known *uvr* strains Bs-8 and Bs-12 were resistant to PLM[4].

These data indicate that in *E. coli* B the inability to carry out excision repair is associated with resistance to low levels of PLM, though the reason for this is not clear. The same is not true in K-12 strains of *E. coli*, in which *uvr* mutants are at least as sensitive to PLM as the wild type strains[7].

Finally, the absence of cofactors required for PLM-induced DNA breakage in cells may also result in PLM resistance. The *in vitro* reaction of PLM or BLM with DNA requires molecular oxygen and a reducing agent[8,9]. Glutathione is the principal intracellular reducing agent in *E. coli*[10]. Accordingly, the glutathione-deficient strain 129[11] was tested for sensitivity to PLM together with its parent strain KMBL54. Log

Fig. 1. Survival of the *gshA* strain 129 (open) after treatment with various doses of PLM, compared with the *gsh*$^+$ parental strain (closed).

PLM (μg/ml): ○, ●: 0.2, △, ▲: 0.5, □, ■: 5.



phase, minimal GT-glucose grown cultures were diluted 10-fold into minimal glucose medium containing PLM-648 (0.2, 0.5 or 5 μg/ml) and shaken at 37°C. Samples were removed at intervals and plated for viable counts. The results (Fig. 1) clearly indicated that glutathione deficiency leads to increased cell survival at the PLM levels used. This is consistent with the observation that DNA breakage induced by low levels of PLM is greatly reduced in the same glutathione-deficient strain[12]. The glutathione-deficient strain is described as Class IV in Table 3.

In conclusion, our results show that various factors, including envelope structure, ubiquinone levels, excision-repair capacity of the cell, and the intracellular reducing environment can all affect sensitivity of PLM in *E. coli*. These conclusions are summarised in Table 3.

Acknowledgment

We thank Dr. M. J. SLEIGH for supplying the data on PLM sensitivity of the glutathione-deficient strain.

References

1) WARING, M.: DNA modification and cancer. Annu. Rev. Biochem. 50: 159~192, 1981
2) GRIGG, G. W.; R. M. HALL, N. K. HART, D. R. KAVULAK, J. A. LAMBERTON & A. LANE: Amplification of the antibiotic effects of the bleomycins, phleomycins and tallysomycins: Its dependence on the nature of the variable basic groups. J. Antibiotics 38: 99~110, 1985
3) HOLLAND, I. B.; E. J. THRELFALL, É. M. HOLLAND, V. DARBY & A. C. R. SAMSON: Mutants of *Escherichia coli* with altered surface properties which are refractory to colicin E2, sensitive to ultraviolet light and which can also show recombination deficiency, abortive growth of bacteriophage λ and filament formation. J. Gen. Microbiol. 62: 371~382, 1970
4) GRIGG, G. W.: Induction of DNA breakdown and death in *Escherichia coli* by phleomycin: Its association with dark repair processes. Mol. Gen. Genet. 104: 1~11, 1969
5) COLLIS, C. M. & G. W. GRIGG: A mutant of *Escherichia coli* showing resistance to phleomycin and bleomycin and to heat inactivation is defective in ubiquinone synthesis. J. Bacteriol., to submitted
6) COX, G. B. & F. GIBSON: Studies on electron transport and energy-linked reactions using mutants of *Escherichia coli*. Biochim. Biophys. Acta 346: 1~25, 1974
7) NAKAYAMA, H.: Phleomycin-induced lethality and DNA degradation in *Escherichia coli* K12. Mutat. Res. 29: 21~33, 1975
8) EHRENFELD, G. M.; J. B. SHIPLEY, D. C. HEIMBROOK, H. SUGIYAMA, E. C. LONG, J. H. VAN BOOM, G. A. VAN DER MAREL, N. J. OPPENHEIMER & S. M. HECHT: Copper-dependent cleavage of DNA by bleomycin. Biochemistry 26: 931~942, 1987
9) FREEDMAN, J. H.; S. B. HORWITZ & J. PEISACH: Reduction of copper(II)-bleomycin: A model for *in vivo* drug activity. Biochemistry 21: 2203~2210, 1982
10) APONTOWEIL, P. & W. BERENDS: Glutathione biosynthesis in *Escherichia coli* K12: Properties of the enzymes and regulation. Biochim. Biophys. Acta 399: 1~9, 1975
11) APONTOWEIL, P. & W. BERENDS: Isolation and initial characterization of glutathione-deficient mutants of *Escherichia coli* K12. Biochim. Biophys. Acta 399: 10~22, 1975
12) SLEIGH, M. J. & G. W. GRIGG: Sulphydryl-mediated DNA breakage by phleomycin in *Escherichia coli*. Mutat. Res. 42: 181~190, 1977