Genetic and Physiological Studies on the Site of Action of Distamycin A

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Two new genetic loci of *Bacillus subtilis* are identified by mutations that confer resistance to distamycin A and to other antibacterial agents. The chromosomal region where they map probably contains a cluster of genes whose products are related to membrane structure and function. Some of the biological effects of distamycin A are still in evidence in the resistant mutants indicating that the drug possibly acts at multiple sites. Most biological effects of the drug (including the phenotypic correction of a morphopoietic mutation) are likely to be due to the interaction of distamycin A with membrane (or surface) structures.

Distamycin A (3) is a basic oligopeptide antibiotic with antiviral (6) and antiphagic (8) activities at low concentration (10^{-5} to 10^{-6} M), and antibacterial activity at doses 10 times higher. The compound binds to deoxyribonucleic acid (DNA) (14) forming two types of complexes (15), but none of the reported *in vivo* activities could be demonstrated to be due to this affinity. Even the inhibition of DNA uptake, genetic transformation, and phage DNA transfection in *Bacillus subtilis* have been shown to be due to an interaction between distamycin and competent cells rather than with donor DNA (15). This interpretation fits with other experimental work (8, 19) that indicated the cell surface as the probable site of action of the antibiotic.

In the present work we describe the isolation and characterization of *B. subtilis* mutants resistant to high doses of distamycin A, and the effect of such a mutation upon distamycin binding, DNA transformation inhibition, and phage lysis inhibition.

We also present data on phenotypic repair by distamycin A of a conditional mutant of *B. subtilis* defective in spore germination.

MATERIALS AND METHODS

Bacterial and phage strains. Bacterial strains used in this study are listed in Table 1. The distamycin-resistant and acriflavin-resistant mutants were obtained by plating cultures, mutagenized with nitroguanidine as described by Adelberg et al. (2), on PY agar plates containing 200 μ g of distamycin A per ml or 10 μ g of acriflavin per ml. The distamycin resistance marker was transferred by DNA transformation to PB 202 and all work has been carried out with this derivative.

SPP1 bacteriophage (16) has been used in phage lysis experiments.

PBS-1 bacteriophage (20) has been used for transduction experiments.

Chemicals and media. Distamycin A (Farmitalia Corp., Milan, Italy) is stored under vacuum and dissolved before use at a concentration of 500 μ g/ml in water, or in glycerol to obtain higher concentrations, and then stored at -20°C . The radioactive compound [*formyl*- ^{14}C]distamycin (14.9 $\mu\text{Ci/mg}$) was a gift of Farmitalia Corp. Acriflavine, ethidium bromide, and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo.; mytomicin C from Schuchardt Corp., Munich; chloramphenicol from Serva, Heidelberg; and streptomycin from Squibb, Rome.

The following media were employed: NB (nutrient broth, Difco), PY (Penassay broth, Difco), TY (5), minimal medium of Davis and Mingioli (7), and transformation medium MT (17). When necessary, the media were solidified with 2% Difco agar.

Susceptibility to various chemicals was tested on nutrient agar plates containing increasing concentrations of the drug, added to the melted medium cooled to 45°C . The susceptibility was determined as the minimum inhibitory concentration after 20 h of incubation at 37°C .

Experimental techniques. Spores were prepared as described (9); competent cells were prepared and transformation experiments were performed as previously reported (15). [*Formyl*- ^{14}C]distamycin binding to bacterial cells was evaluated by incubating different concentrations of the cells in the presence of 5 μ g of distamycin A per ml in MT medium at 37°C for 15 min. The radioactivity that remained associated with the cells after one, two, three, and four washings (centrifuged at $8,000 \times g$ for 5 min) in MT medium was measured.

Turbidity measurements on liquid cultures have been carried out with a biophotometer (De Bonet Maury et Jouan, Brion-Leroux, Paris, France), or with a Klett-Summerson photoelectric colorimeter

TABLE 1. *B. subtilis* strains used

Strain	Genotype ^a	Origin
PB 168	<i>trpC2</i>	SB 168, J. Lederberg
PB 202	<i>tyrA1 hisB2 trpC2 aroB2</i>	SB 202, J. Lederberg
PB 1652	<i>lys-3 trpC2 metB10</i>	BR 151, F. Young
PB 1682	<i>aroD120 trpC2</i>	SB 120, E. Nester
PB 3372	<i>pheA12 argA3 ery</i>	BC 26, J. Copeland
PB 3361	<i>purB6 leu-8 metB5 trpC2 hisA1 lys-21 thr-5 ery-110</i>	HLL 39, R. Schaeffer
PB 1686	<i>trpC2 dst-3</i>	PB 168 + NTG
PB 1689	<i>tyrA1 hisB2 trpC2 aroB2 dst-3</i>	PB 202 + DNA PB 1686
PB 1717	<i>tyrA1 hisB2 trpC2 aroB2 acfB6</i>	PB 202 + NTG
PB 1718	<i>tyrA1 hisB2 trpC2 aroB2 acfB10</i>	PB 202 + NTG
PB 2388	<i>hisB2 trpC2 met ts-Gsp-IV-a4</i>	See (9)
PB 2398	<i>hisB2 trpC2 met ts-Gsp-1</i>	See (9)

^a Symbols: *trp*, *tyr*, *his*, *aroB*, *aroD*, *met*, *lys*, *phe*, *arg*, *pur*, *leu*, *thr*, requirement for tryptophan, tyrosine, histidine, shikimic acid, phenylalanine and tryptophan, methionine, lysine, phenylalanine, arginine, adenine, leucine, threonine, respectively; *ery*, *dst*, *acf*, resistance to erythromycin, distamycin A, acriflavine, respectively; *ts-Gsp*, thermosensitivity in spore germination.

(green filter). The data are given as transmittance (T percent) or as Klett units.

Transduction mapping. Linkage relationships between *dst* and *acf* mutations with biochemical markers of known location were determined by PBS-1 transduction following the procedure described by Hoch et al. (10). After selection on appropriate selective media, transductants were isolated on the same media before testing for unselected markers. Susceptibility to distamycin and acriflavine was tested on nutrient agar plates containing 100 and 6 µg/ml, respectively.

Mapping data are expressed as percentages of recombination according to the convention: percentage of recombination = (1-marker co-transfer) × 100.

RESULTS

Selection and characterization of distamycin-resistant mutants. Several distamycin A-resistant (*dst*) mutants of *B. subtilis* were obtained from mutagenized PB 168 cultures plated on nutrient agar plates containing 200 µg of distamycin A per ml. One of the mutants (PB 1686) bearing the mutation *dst-3* was used for the subsequent work. The marker *dst-3* was transferred by transformation into PB 202. Figure 1 (a and b) shows the growth curves of PB 202 and its *dst* derivative (PB 1689) in the presence of a range of doses of distamycin A. The growth of the parental strain is inhibited by 50 µg of the antibiotic per ml, whereas the mutant is affected only at doses higher than 100 µg/ml.

Strain PB 1689 was also tested for its susceptibility to other antibacterial agents, in a search for possible cross-resistances. The data are reported in Table 2 and show that the distamycin-resistant strain is resistant also to acriflavine.

Because of the cross-resistance to acriflavine of the *dst 3* strain, we selected acriflavine-resistant mutants and checked their cross-resistance to distamycin and other antibacterial compounds. The two mutants, PB 1717 (*acfB6*) and PB 1718 (*acfB10*), showed cross-resistance to distamycin and to ethidium bromide (Table 2).

A mutation that confers resistance to acriflavine, here referred to as *acfA* for reasons that will be apparent below, has been described by Ionesco et al. (12). This mutation is located between *pheA* and *lys* on the *B. subtilis* map. Bishop and Brown (4) have described a locus *ebr*, linked to *hisA*, that confers resistance to ethidium bromide and cross-resistance to acriflavine.

It was then of interest to determine if our *dst* and *acf* markers would map in *ebr*, in *acfA*, or somewhere else.

Chromosomal location of the *dst* and *acfB* mutations. Genetic mapping studies, performed with PBS-1 phage-mediated transduction, were carried out to determine the location of the *dst* and *acfB* resistance markers.

The results of the crosses reported in Table 3 and 4 allow us to place the *dst-3* and *acfB6* markers in the same chromosomal region of *acfA*, but at different positions. The mutation *dst-3* is closer to *pheA* and unlinked to *lys*, whereas *acfB6* is closer to *lys* and unlinked to *pheA*. Figure 2 represents the map of the *metB-argA* region of the *B. subtilis* chromosome with all the map distances calculated from our crosses, plus the location of the *acfA* locus described by Ionesco et al. (12).

Distamycin binding to *B. subtilis* cells. [¹⁴C]distamycin binding to competent cells of PB 202 and its derivative, PB 1689, was evalu-

FIG. 1. Growth curves of PB 202 (a) (parental) and PB 1689 (b) (dst) measured in PY medium at 37 C in presence of distamycin A at the concentrations of 0 (A), 5 (B), 10 (C), 50 (D), 100 (E), and 250 (F) µg/ml.

TABLE 2. Cross-resistance of strains PB 1689, PB 1717, and PB 1718 to different antimicrobial compounds^a

Drug	PB 202 parental	PB 1689 dst-3	PB 1717 acfB6	PB 1718 acfB10
Distamycin A	20	200	200	200
Mytomycin C	0.04	0.03	0.04	0.03
Streptomycin	10	5	5	5
Chloramphenicol	5	5	5	5
Tetracycline	5	4	5	5
Acridine	4	10	8	20
Ethidium bromide	2	2	7	5

^a The resistance to the compounds tested is expressed as minimum inhibitory concentration (µg/ml).

TABLE 3. Mapping analysis of the *dst3* mutation by two-factor transduction crosses, with strain PB 1689 as donor^a

Recipient strain	Recombination		
	Selection	Classes	No.
PB 3361	lys ⁺	lys ⁺ dst	0
		lys ⁺ dst ⁺	65
	leu ⁺	leu ⁺ dst	8
PB 3372	arg ⁺	leu ⁺ dst ⁺	72
		arg ⁺ dst	0
	phe ⁺	arg ⁺ dst ⁺	60
		phe ⁺ dst	176
PB 1682	aro ⁺	phe ⁺ dst ⁺	395
		aro ⁺ dst	67
		aro ⁺ dst ⁺	225

^a dst⁺ indicates wild-type phenotype.

ated as described above. Figure 3 shows the nanomoles of [¹⁴C]distamycin retained by various concentrations of competent cells of PB 202 and of PB 1689 after one, two, three, and four washings.

The *dst* mutant seems to bind more distamycin than the wild-type strain: in this experiment they bound 2.6×10^5 and 8.5×10^5 molecules per cell, respectively. The binding increases linearly with cell concentration, and the highest cell concentrations bind approximately 5 and 2% of the radioactive compound initially present in the assay. At each washing a decreasing fraction of the drug is released, indicating that the binding is a rather tight one and not a spurious contamination.

A binding coefficient (nanomoles of distamycin/ 10^8 cells) can be calculated from each

TABLE 4. Mapping analysis of *acfB6* mutation by two-factor transduction crosses, using strain PB 1717 as donor^a

Recipient strain	Recombinants		
	Selection	Classes	No.
PB 1652	lys ⁺	lys ⁺ met acf	109
		lys ⁺ met acf ⁺	51
	met ⁺	met ⁺ lys acf	6
		met ⁺ lys acf ⁺	132

^a *acf*⁺ indicates the wild-type phenotype.

linear slope in Fig. 3. Such values can be normalized by the binding coefficient calculated from the first slope (data after the first washing): plotting such a relative binding coefficient versus the number of washings, we obtain the curves depicted in Fig. 4 that represent a measure of the rate of distamycin release from bacterial cells.

The mutant strain PB 1689 releases the bound drug at a rate 1.7 times higher than the parental strain PB 202.

The normalized plot in Fig. 4 is independent of the absolute amount of antibiotic bound, and the difference between the two slopes must correspond to a different affinity of the bacterial cells for the antibiotic.

For this kind of measurements we used competent cell cultures since they are a homogeneous population of a short, nonseptated bacilli of uniform length, allowing for a precise evaluation of the cell titer. We attempted to perform analogous measurements with competent cell cultures of mutant *acfB6*, but such cells lyse at the first washing and are also abnormal in other respects, as we will describe in the following section.

DNA transformation inhibition. The effect of distamycin A on DNA transformation is probably due to an interaction of the drug with the cell envelope (15). Therefore it was of interest to test the effect of the drug on the distamycin-resistant strain.

We have tested the effect of a range of doses of

FIG. 3. Binding of distamycin to competent cells of PB 202 and PB 1689 using different concentrations of cells. Curves 1, 2, 3, and 4 represent sequential washings of the cells (see Materials and Methods).

distamycin A on transformation efficiency: both parental and *dst-3* strains show the same response to distamycin, indicating that the mutation does not affect the receptor(s) for DNA.

We were unable to obtain competence with the *acfB6* mutant. The competent cells of the mutant are very prone to lysis, even in media of high ionic strength, such as MT, in spite of their 100% viability when seeded on agar plates.

SPP1 bacteriophage lysis inhibition by distamycin A. The infection of germinating *B. subtilis* spores in TY medium by SPP1 bacteriophage leads to an abrupt lysis 180 min after the initiation of infection, even at low multiplicity of infection. Before lysis the presence of the phage does not cause any alteration of the outgrowth as judged by the growth curve (Fig. 5).

FIG. 2. Transduction map of the *metB-argA* region of the *B. subtilis* chromosome. The arrow heads indicate the unselected markers. Recombination distances of *acfA* were taken from (12).

FIG. 4. Rate of distamycin release from PB 202 and PB 1689 cells. The binding coefficients calculated from curves 2, 3, and 4 of Fig. 3 have been normalized by the coefficient calculated from curves 1 (see text).

In presence of sublethal doses of distamycin A (2 and 3 $\mu\text{g}/\text{ml}$), lysis is completely suppressed (Fig. 5). A fourfold dilution of the culture with drug-free medium (that reduces the concentration of distamycin A below the minimal protection dose) results in continuation of bacterial growth to plateau values without any sign of lysis. Nevertheless, infection had been successful since the viability of the culture, judged from the colony-forming ability, was reduced to 1% and all cells in the culture are infective centers. If the phage-infected, distamycin A-protected culture is washed in MT medium it lyses, freeing plaque-forming units in numbers comparable to those obtained in the non-protected control.

Thus, distamycin A protects *B. subtilis* cells from phage lysis, possibly by osmotic stabilization of the cells envelopes, but it is ineffective on both phage infection and multiplication.

Identical results were obtained with the distamycin resistant-mutant indicating that antibacterial and lysis inhibition effects of the drug are independent.

These results differ from the ones obtained by Di Marco et al. (8) on the system phage T1-

Escherichia coli K-12, where both phage adsorption and cell lysis were prevented by distamycin A.

Phenotypic repair of a morphopoietic germination mutant. Some membrane-active agents phenotypically repair morphopoietic mutants in a number of systems (see below).

Distamycin seemed to us a good candidate as a membrane-active agent because of its chemical structure and its biological properties. We therefore tested its effect on a series of temperature-sensitive mutants in the process of spore germination (*Gsp* mutants) that, when germinated at a nonpermissive temperature, give rise to morphologically aberrant forms (9). We studied the morphology of the spore outgrowth at 46 C in presence of distamycin A in the parental strain PB 1424 and in a number of *Gsp* mutants. PB 1424 outgrows normally in the presence of 3 μg of distamycin A per ml, and after 2 h only bacillary forms are visible (see Fig. 6A). Strain PB 2388 is a *Gsp* mutant that at nonpermissive temperature initiates an abortive septation. The cells formed at 46 C are spherical and are much bigger than the ones formed at 35 C (Fig. 6B). In the presence of 3 μg of distamycin A per ml at 46 C the spores of this mutant germinate regularly into septated bacillary forms identical to the ones shown in Fig. 6A. Conversely, another mutant tested, PB 2398, defective at an earlier stage of the germination process, stops its outgrowth at 46 C at

FIG. 5. Effect of distamycin A upon SPP1 bacteriophage lysis in germinating spores. Heat-activated spores of PB 202 were suspended in TY medium at 37 C with agitation and infected with SPP1 at a multiplicity of infection of 3. Without distamycin (A); in presence of 1, 2, and 5 μg of the drug per ml (B, C, and D, respectively), the curve (E) described the fourfold dilution of the (C) one in the same medium without distamycin. The (F) curve is the control of germinating spores without distamycin and phage.

FIG. 6. Morphology of germinating spores of PB 1424 (A), PB 2398 (B), and PB 2388 (C) after 120 min of growth in nutrient broth at 46 C. Bar indicates 10 μ m.

the "swollen spore" stage both in the absence and presence of a range of doses of distamycin A (Fig. 6C).

DISCUSSION

The basic oligopeptide distamycin A interacts (14, 15, 21) with DNA in vitro, but most of the biological effects it displays seem to be unrelated to this property and due to other interactions between the antibiotic and surface structures (15, 19).

We have isolated a *B. subtilis* mutant resistant to high doses of distamycin A that is also resistant to acriflavine; and independently isolated acriflavine-resistant mutants show the same cross-resistance to distamycin plus a resistance to ethidium bromide. Two loci, *ebr* and *acfA*, involved in acriflavine and ethidium bromide resistances have been mapped by other authors (4, 12). The mutations described in this paper, *acfB* and *dst*, seem to be unrelated to the ones already reported, and map between *lys* and

pheA (as *acfA*, but with different linkage values). This region of the *B. subtilis* chromosome also contains other resistance markers possibly due to altered drug permeability such as *asaA* (1) and *strC* (18). The hypothesis is that it might contain a cluster of genes whose products are membrane components or related to membrane biosynthesis.

We have studied distamycin binding to competent cells of parental and *dst* strains. The mutant cells bind as much distamycin as the parental one (or more), but they release the drug at a much higher rate, indicating a different affinity or a lack of permeability. Other biological effects of the drug, such as inhibition of DNA transformation and of phage-induced lysis, are unaffected by the *dst* mutation, suggesting that the antibacterial effect might operate through a different route as compared to the other biological effects. *acfB* mutants have a distinct phenotype, are very prone to lysis, and never develop a competence state.

We therefore propose that the biological effects of distamycin A upon *B. subtilis* cells can be subdivided into at least two groups: those abolished by a mutation (*dst*) that possibly alters the permeability to the drug, and those due to an interaction of the drug with surface structures.

In view of this, we searched for other biological effects of distamycin A as a possible membrane-active agent in analogy to the findings of Ingram and Fisher (11) who found that filamentous bacterial mutants, defective in cell septation, can be induced to septate by compounds that act on the cell membrane (Na-oleate, lysolecithin, long-chain alcohols), and those of King and Grula (13) who found that, in *Micrococcus lysodeikticus*, pantoyl lactone can prevent the septation inhibition produced by D-cycloserine. These authors postulated a physical effect of these compounds on the cytoplasmic membrane, suggesting that a membrane, altered in its chemico-physical properties by the presence of such compounds, might repair cell-wall lesions or mimic to some extent cell-wall functions.

We have found that distamycin A is effective in phenotypically correcting a morphopoietic mutant defective in spore outgrowth, unable to complete the first septation.

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