Bacterial Resistance to the Synergistic Antibiotics of the PA 114, Streptogramin, and Vernamycin Complexes

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A mutant of *Bacillus subtilis* was isolated that was resistant to the growth inhibitory activity of the synergistic antibiotics of the PA 114, streptogramin, and vernamycin complexes. *Escherichia coli* is naturally resistant to the action of these antibiotics. In both cases, it was shown that resistance was due to an inability of the bacteria to transport the antibiotics into the cell.

The antibiotics of the PA 114, streptogramin, and vernamycin complexes are unusually interesting because of the synergism in action observed between the separate components of the antibiotic mixtures. Each antibiotic complex exists as a mixture of at least two active compounds, denoted as A and B (E. L. Smith, J. Gen. Microbiol. 33:iii, 1963). The group A antibiotics isolated from the different complexes are similar in structure and contain an oxazole nucleus (6); the antibiotics of group B are likewise similar and are cyclic peptides (1, 7, 13, 19). Each compound is itself capable of inhibiting the growth of gram-positive bacteria, but the compounds are more active in combination (2, 8; P. Actor, H. Basch, and W. P. Jambor, Bacteriol. Proc., p. 94, 1963; D. Vazquez, J. Gen. Microbiol. 33:ix, 1963). Gram-negative bacteria are generally resistant to the action of these antibiotics (3, 8, 23).

Protein synthesis is the primary site of inhibition of growth by the antibiotics (9-11, 15, 16, 20-22). The antibiotics inhibit protein synthesis by interfering with the binding of aminoacyl-transfer ribonucleic acid (tRNA) to ribosomes (11). Protein synthesis in extracts derived from the resistant bacterium *Escherichia coli* is equally sensitive to the antibiotics as is protein synthesis in extracts derived from the susceptible *Bacillus subtilis* (10, 15). It thus appears that resistance to the antibiotics in *E. coli* is due to the inability of the drugs to penetrate the intact cell (10).

The present investigation is concerned with the mechanism of bacterial resistance to these antibiotics.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* ATCC 6051, a mutant of *B. subtilis* 6051 resistant to 25 μ g of streptogramin per ml, and *E. coli* B strain 163, which is auxotrophic for histidine, leucine, and methionine, were used.

Media and growth of bacteria. *B. subtilis* was grown in Brain Heart Infusion (Difco) supplemented with 0.125% glucose (w/v). *E. coli* was grown in a mineral salts medium (5) supplemented with 0.25% glucose (w/v), 25 μ g/ml each of histidine, leucine, and methionine, and 10 μ g of uracil per ml. Suspensions of the cells were grown at 37 C with vigorous shaking and were used during exponential growth.

Antibiotics. The antibiotics used were PA 114 (the crude mixture of A and B), the components PA 114 A and PA 114 B, vernamycin B α , and streptogramin. No attempt was made to isolate the components of streptogramin. The antibiotics are very insoluble in water, and were therefore used as nonsterile homogenized suspensions. When incorporated into solid medium, the antibiotics were dissolved in methyl alcohol and then added to the cooled agar just before pouring the plates.

Measurement of incorporation of radioactivity into RNA and protein. Uracil-2- 14 C (30 mc/mmol), and L-leucine-1- 14 C (23.3 mc/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Incorporation of 14 C-leucine and 14 C-uracil into trichloroacetic acid-insoluble material was used as an index of protein and RNA synthesis, respectively. The details of the method were previously described (9).

For *B. subtilis*, 0.2 μ C of 14 C-leucine per ml of culture was used; for *E. coli*, 0.05 μ C of 14 C-leucine and 0.1 μ C of 14 C-uracil per ml of culture were used.

Preparation of protoplasts of *B. subtilis*. Exponentially growing cells were harvested and were concentrated fivefold by suspension in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% magnesium acetate (w/v) and 25% sucrose (w/v). Muramidase

(lysozyme, Worthington Biochemical Corp., Freehold, N.J.), 50 $\mu\text{g}/\text{ml}$, was added, and the suspension was incubated without shaking for 10 min at 37 C.

A 2-ml amount of the protoplast suspension was added to 8 ml of Brain Heart Infusion containing 0.125% glucose, 0.1% magnesium acetate, and 25% sucrose. Antibiotic (or water to make up the volume) was added as indicated in the experiment. After 2 min, ^{14}C -leucine (0.2 $\mu\text{C}/\text{ml}$) was added, and samples were taken at intervals. The incorporation of the precursor into trichloroacetic acid-insoluble material was determined.

Ethylenediaminetetraacetic acid (EDTA) treatment of *E. coli* cells. Exponentially growing cells were treated with EDTA as previously described (4).

Preparation of extracts of *B. subtilis*. Cell extracts (S-30) were prepared and preincubated as described elsewhere (10). The extracts derived from *B. subtilis* resistant to 25 μg of streptogramin per ml were made from cells growing in the absence of the antibiotic.

In vitro synthesis of polyphenylalanine. The reaction mixture was that described by Kobayashi and Halvorson (14). A polyuridylic acid reaction mixture containing 50 μg of acid per ml was used, and in this system 200 μmoles of ^{14}C -phenylalanine were polymerized per ml of reaction. All other procedures used for the determination of the amount of peptide synthesized were those previously described (10).

Minimal inhibitory concentration required to inhibit growth of *B. subtilis*. An overnight culture of the appropriate strain of *B. subtilis* was diluted 1:100 by inoculation into a mineral salts medium (5) supplemented with 0.2% Casamino Acids (Difco) and 0.5% glucose. Portions (5 ml) of the inoculated medium were dispensed into 50-ml tubes, and selected amounts of antibiotic (dissolved in 25% ethyl alcohol) were added. The tubes were incubated overnight with vigorous shaking, and growth, as compared with control tubes not containing antibiotic, was determined. The concentrations of antibiotics given in Table 1 are those which completely inhibited growth, and are averages of two to four separate determinations.

RESULTS

Cross-resistance of a mutant of *B. subtilis* resistant to streptogramin. The mutant was isolated by directly plating 10^9 cells of strain 6051 onto each of several plates of Brain Heart Infusion Agar containing 25 μg of streptogramin per ml. About 1 of 10^9 cells was resistant to this high level of antibiotic. Several independent isolates were made and purified by a few cycles of growth in the presence and absence of the antibiotic. Only one of these isolates has been studied. This mutant is stable and retains its high degree of resistance after many successive subcultures in medium lacking antibiotic.

The cross-resistance of this mutant with other related and unrelated antibiotics was studied (Fig. 1 and Table 1). This streptogramin-resistant mutant was also resistant to PA 114, PA 114 B,

vernarmycin B α , erythromycin, and oleandomycin (Fig. 1). Table 1 gives the minimal inhibitory concentration of antibiotic required to inhibit growth of the susceptible and resistant strains.

FIG. 1. Cross-resistance of *Bacillus subtilis* resistant to 25 $\mu\text{g}/\text{ml}$ of streptogramin with other antibiotics. Seed Agar (BBL) was inoculated with the appropriate bacterial strain and poured over solidified Base Agar in 150-mm plastic petri dishes. After the agar had solidified, filter pads (diameter, 7 mm) containing the indicated amounts of antibiotics were placed on the agar. The plates were incubated at 37 C overnight. (A) *B. subtilis* 6051 (sensitive). (B) *B. subtilis* resistant to 25 $\mu\text{g}/\text{ml}$ of streptogramin. Amounts of antibiotic on each pad were: (1) 10 μg of PA 114; (2) 10 μg of streptogramin; (3) 10 μg of PA 114 B; (4) 10 μg of vernarmycin B α ; (5) 2 μg of erythromycin; (6) 2 μg of oleandomycin.

TABLE 1. *Minimal inhibitory concentration of antibiotic required to completely inhibit growth of Bacillus subtilis strains*

Bacterial strain	Antibiotic ($\mu\text{g/ml}$ of culture)			
	PA 114	PA 114 A	PA 114 B	Streptogramin
<i>B. subtilis</i> 6051 (sensitive)...	2.5	40.0	2.5	2.0
<i>B. subtilis</i> resistant to 25 $\mu\text{g/ml}$ of streptogramin.....	55.0	>200.0	>40.0	55.0

As has been shown previously (8), the growth of *B. subtilis* was inhibited better by PA 114 B than by PA 114 A. Crude PA 114 (the mixture of A and B) and streptogramin were also good inhibitors of growth. The mutant was resistant to PA 114 and streptogramin and at the same time was resistant to the individual antibiotics of the complex PA 114 A and PA 114 B. The actual amount of A or B required to inhibit growth could not be determined owing to a lack of a sufficient quantity of antibiotic.

The results are given for only one of the antibiotics studied. However, all of the experiments reported in this paper, except those with *E. coli*, were done with all of the antibiotic mixtures or separate components. The results obtained with all the other antibiotics were the same as those observed with the antibiotic described.

Inhibition of protein synthesis in intact cells and protoplasts of B. subtilis susceptible and resistant to streptogramin. The effect of streptogramin on protein synthesis in intact cells and protoplasts of the susceptible and resistant strains was determined by following the incorporation of ^{14}C -leucine into acid-insoluble components. For studies with intact cells, exponentially growing cells at 3×10^8 cells per milliliter were dispensed into flasks, and the indicated amount of antibiotic was added. The cultures were shaken at 37 C for 5 min to allow penetration of the drug. ^{14}C -leucine was added, and incorporation was determined at selected intervals.

For studies with protoplasts, the same procedure was followed, except that protoplasts were made as outlined in Materials and Methods and preincubation with antibiotic was for 2 min.

Figure 2 shows that protein synthesis in intact cells of the parent *B. subtilis* 6051 was highly sensitive to streptogramin, whereas the resistant strain was resistant to the action of the antibiotic at this concentration.

Figure 3 indicates, on the other hand, that protoplasts of both the susceptible and streptogramin-resistant strains were susceptible to the same concentration of streptogramin.

Inhibition of in vitro protein synthesis by antibiotics in extracts derived from susceptible and resistant cells. Because the antibiotics inhibit growth by interfering with protein synthesis, the effect of the mutation to resistance on the in vitro protein synthesizing complex of the cell was determined. The results summarized in Fig. 4 show that the protein-synthesizing systems derived from both susceptible and resistant strains of *B. subtilis* were equally susceptible to inhibition by PA 114 A. As has been shown previously with extracts of *E. coli* (9, 15), PA 114 B (and vernamycin B α) were ineffective in inhibiting polyphenylalanine synthesis.

Mechanism of resistance of E. coli to antibiotics. Intact *E. coli* cells are naturally resistant to the antibiotics of the PA 114, streptogramin, and the vernamycin group (8, 23). EDTA, which is known to make *E. coli* permeable to other compounds ordinarily unable to penetrate the cell (17), was used to study the mechanism of the resistance of this cell to the antibiotics.

Figure 5 shows the effect of PA 114 A on protein synthesis in intact cells (left), and the effect on protein synthesis (center) and RNA synthesis (right) in EDTA-treated cells. As has been demonstrated before, the antibiotic had no effect on protein synthesis in intact cells. However, protein synthesis in EDTA-treated cells was markedly inhibited by PA 114 A. The antibiotic had no effect on RNA synthesis in the EDTA-treated cells, which was the same as that observed during inhibition of protein synthesis by the antibiotics in susceptible *B. subtilis* cells (9).

DISCUSSION

The results of this investigation indicate that resistance to the antibiotics of the PA 114, streptogramin, and vernamycin complexes is due to an altered permeability of the mutant cell to the drugs. Intact *B. subtilis* mutant cells are resistant to the action of the antibiotics, but protoplasts derived from these same cells are not. This points to the important role played by the cell wall. However, it is also possible that the integrity of the cell wall is only indirectly related to resistance to antibiotics. For example, an intact cell wall may be important for normal functioning of the cell membrane.

Another line of evidence which indicates that resistance is due to the inability of the antibiotics to penetrate the cell surface of the mutant is obtained from the results observed with cross-resistance. The resistant *B. subtilis* strain is, by probably

FIG. 2. Effect of streptogramin on incorporation of ^{14}C -leucine into protein of intact cells of growing *Bacillus subtilis* strains sensitive and resistant to $25\text{ }\mu\text{g/ml}$ of streptogramin. See text for details of experiment. Symbols: $\circ$, control, no antibiotic; $\bullet$, 2 or $5\text{ }\mu\text{g/ml}$ of streptogramin.

FIG. 3. Effect of streptogramin on incorporation of ^{14}C -leucine into protein of protoplasts of *Bacillus subtilis* strains sensitive and resistant to $25\text{ }\mu\text{g/ml}$ of streptogramin. See text for details of experiment. Symbols: $\circ$, control, no antibiotic; $\bullet$, $5\text{ }\mu\text{g/ml}$ of streptogramin.

FIG. 4. Inhibition of polyuridylic acid-stimulated incorporation of phenylalanine into hot trichloroacetic acid-insoluble peptide by PA 114 A (○) and by PA 114 B (●). Extracts were prepared from *Bacillus subtilis* strains sensitive and resistant to 25 μg/ml of streptomycin. Per cent inhibition was obtained by comparing the amount of acid-insoluble radioactivity incorporated in the presence of the indicated quantity of antibiotic to the radioactivity incorporated in the absence of antibiotic.

FIG. 5. Effect of EDTA treatment of *Escherichia coli* cells on sensitivity to PA 114 A. See Materials and Methods and text for methods and experimental details. Symbols: ○, control, no antibiotic; Δ, 1 μg/ml of PA 114 A; ●, 5 μg/ml of PA 114 A; □, 10 μg/ml of PA 114 A.

a single mutational event, simultaneously resistant both to the chemically dissimilar A and B groups of antibiotics and to the unrelated macrolide antibiotics, erythromycin and oleandomycin.

It is unlikely that a single mutation would affect the activity of all these unrelated antibiotics, unless their mode of action or their mechanism of intracellular accumulation were the same. Al-

though all the antibiotics are able to inhibit protein synthesis, the precise point at which they do this is not the same (10, 18). Therefore, all of the antibiotics are probably concentrated by a single permeation mechanism located in the cell wall. Simultaneous resistance to all the antibiotics is thus due to a single change in this system, enabling the cells to exclude the drugs. Garrod and Waterworth (12) demonstrated cross-resistance between ostreogrycin, which is similar to PA 114, and erythromycin.

Two other possible mechanisms involving the protein synthesizing system or enzymes which inactivate the antibiotics, which could account for the resistance observed, have been excluded. First, the antibiotics are known to inhibit growth by interfering with protein synthesis. However, the *in vitro* protein-synthesizing system derived from the resistant mutant is as susceptible to inhibition by the antibiotics as is the system derived from the susceptible parent.

Second, I have been uniformly unable to demonstrate the presence in the resistant strain of an inducible or constitutive enzyme which could render the antibiotics inactive.

Related to resistance to these antibiotics is the observation that the growth of *E. coli* is not affected by the antibiotics (8, 23). However, protein synthesis by cell-free extracts derived from *E. coli* is susceptible to inhibition by the antibiotics (10, 15, 21). Treatment of growing *E. coli* cells with EDTA has been shown to render the cells permeable to substances which are ordinarily unable to penetrate the cell surface (17). The finding that protein synthesis in *E. coli* cells pretreated with EDTA is susceptible to inhibition by PA 114 A, whereas the untreated cells are not, gives further support to the previously advanced hypothesis (10) that *E. coli* is resistant because it can exclude the antibiotics.

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