Thiostrepton-Resistant Mutants of *Bacillus subtilis*: Localization of Resistance to the 50S Subunit

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Summary. A number of thiostrepton-resistant mutants of *Bacillus subtilis* were obtained. The *thi* mutations map proximally to *strA*. Effects of thiostrepton on polyphenylalanine synthesis with ribosomes and S-100 fractions from parent and mutant strains indicated that resistance was localized to the ribosomes. Furthermore, effects of thiostrepton on binding of [3H]GTP to ribosomes and 50S subunits from thiostrepton-sensitive and -resistant strains localized the site of resistance to the 50S subunit. In addition, revertants from thiostrepton-resistance to thiostrepton-sensitivity were obtained. Ribosomes and 50S subunits from these thiostrepton-sensitive revertants were sensitive to thiostrepton similar to parental sensitive *B. subtilis*.

Introduction

The antibiotic thiostrepton inhibits reactions central to ribosome function and protein biosynthesis (Pestka, 1971; Pestka and Bodley, 1975). Thiostrepton has been shown to be a strong inhibitor of 50S ribosomal function (Weisblum and Demohn, 1970a). It inhibits initiation (Mazumder, 1973; and Lockwood and Maitra, 1974), EF-Tu dependent reactions (Kinoshita, Liou, and Tanaka, 1971; Cundliffe, 1971; Modolell *et al.*, 1971a; Cannon and Burns, 1971; Watanabe and Tanaka, 1971; Watanabe, 1972; and Weissbach *et al.*, 1972), translocation reactions dependent on EF-G (Pestka, 1970; Pestka and Brot, 1971; Watanabe and Tanaka, 1971; Tanaka, 1972; and Weissbach *et al.*, 1972), and termination catalyzed by the release factors (Brot *et al.*, 1974). In addition, Goldthwaite and Smith (1972) showed that the ribosomes from one thiostrepton-resistant mutant of *Bacillus subtilis* (*bry-2*) were resistant to inhibition by thiostrepton.

The present study was undertaken to define genetically and biochemically the alterations in thiostrepton-resistant mutants of *B. subtilis*.

Materials and Methods

**Bacterial Strains.** The parent strain *cysA14* was a derivative of *B. subtilis* strain 168 and was described previously (Goldthwaite, Dubnau and Smith, 1970). *B. subtilis* strain BD170 *strA*, used as a recipient for transformation, was created by transforming BD170 cells with DNA isolated from a prototrophic 168 *strA1* strain (Goldthwaite, Dubnau and Smith, 1970). The solid (TBAB) and liquid (BY) media used in the growth of cells and transduction and transformation assays have been described (Smith and Smith, 1973). The parent strain *B. subtilis cysA14* was used to obtain thiostrepton-resistant mutations.

**Transduction and Transformation.** PBS 1 transducing bacteriophage lysates were obtained and transductions were carried out as described previously (Goldthwaite, Dubnau and Smith, 1970). The preparation of competent cells and transforming DNA and transformation procedures were as described previously (Dubnau, Goldthwaite, Smith and Marmur, 1967), except that RNAse and phenol extraction steps were omitted from the preparation of DNA. Antibiotic selections, including overlays and expression times, were as described (Goldthwaite, Dubnau and Smith, 1970; Smith and Smith, 1973).

**Large Scale Growth of cysA14 Strains of B. subtilis.** Cells of each strain were inoculated from nutrient agar slants into Difco Bacto antibiotic medium 3 at 37° with vigorous aeration. Initial overnight cultures were grown in 100 ml of medium, then transferred to four overnight cultures of 1,500 ml each. Thiostrepton (0.05 μg/ml) was present in these overnight cultures of the thiostrepton- and bromamycin-resistant mutants. The 6 liters of overnight culture were used to inoculate 100 liters of medium. Growth of cells was monitored on a Klett-Summerson Photoelectric Colorimeter to late log phase. Upon reaching late log phase (100 Klett units), the cells were quickly cooled to 5° with ice, sedimented in a Sharples centrifuge, washed three times in 0.05 M KCl, 0.01 M Tris-acetate, pH 7.5, 0.01 M magnesium acetate, then frozen and stored in the vapor phase of a liquid nitrogen refrigerator. At various stages of this procedure, bacterial samples were withdrawn and assayed to confirm the presence of the *cysA* and *thi* markers.
Preparation of S-30 Fraction from B. subtilis Cells. Each 10 grams of frozen log-phase cells were resuspended in 15 ml of Buffer A (0.05 M KCl, 0.01 M Tris-acetate, pH 7.5, 0.01 M magnesium acetate, and 0.003 M 2-mercaptoethanol). Cells were disrupted in a cold French pressure cell at approximately 15,000 lbs/in². One-tenth volume of a 10 mg/ml benzotriazole solution in Buffer A was added to the extract. The extract was centrifuged in the SS-34 rotor of the Sorvall RC-2B centrifuge for 10 min at 18,000 rpm at 5°C. The pellet was discarded. The supernatant is considered the S-30 fraction.

Preparation of Ribosomal Subunits from the S-30 Fraction. The S-30 fraction was centrifuged in a Spincos Type 65 rotor at 50,000 rpm for 90 min at 5°C. The supernatant is considered the S-100 fraction. The ribosome pellet was washed once in a small amount of Buffer B (2.0 M NH₄Cl, 0.01 M Tris-Cl, pH 7.5, 0.01 M magnesium acetate, and 0.006 M 2-mercaptoethanol) and centrifuged in the Spincos Type 65 rotor at 50,000 rpm for 90 min at 5°C. The supernatant was discarded. The pellet was resuspended in a small volume of Buffer A and then centrifuged in the SS-34 rotor of the Sorvall RC-2B centrifuge for 10 min at 5,000 rpm at 5°C. The pellet was discarded. The supernatant contained the ribosomes used throughout these studies.

Preparation of Ribosomal Subunits from the S-30 Fraction. The S-30 fraction was dialyzed against 0.1 mM magnesium acetate, 40 mM potassium acetate, 10 mM Tris-acetate, pH 7.2, for 6 hours with several changes of buffer at 5°C. Sucrose gradients of 56 ml (5 to 30%, w/v) in the same buffer were prepared in tubes for the Spincos SW25.2 rotor and equilibrated for 6 hours at 5°C. The dialyzed S-30 was layered onto the gradients. Centrifugation was performed in the Spincos SW25.2 rotor at 21,200 rpm for 17 hrs at 5°C. Gradients were analyzed spectrophotometrically and the ribosomal 30S and 50S subunit peaks were pooled as previously described (Pestka and Himittka, 1971; Pestka and Nirenberg, 1966).

Protein Synthesis and GTP Binding to Ribosomes. Poly U dependent polyphenylalanine synthesis was determined with ribosomes and S-100 fraction as previously reported (Pestka, 1968). The binding of [3H]GTP to ribosomes and 50S subunits was performed as described previously (Pestka and Brot, 1971). The conditions for each assay are given in the legend to each of the tables.

Materials. Streptomycin was obtained from Eli Lilly: thiostrepton was a gift from Miss Barbara Stearns of Squibb. DNAase (RNAse-free) was from Worthington Biochemical Corp. [3H]GTP was from Amersham-Searle and had a specific activity of 14.5 Ci/mmole.

Results

Isolation of Thiostrepton-Resistant Mutants. Overnight cultures of B. subtilis 168 cysA14 were plated onto TBAB agar containing 1 µg/ml thiostrepton. Five resistant mutants were obtained. Each was obtained from a different overnight culture. One of these mutations, thi-3, had a thiostrepton LD50 (a concentration of antibiotic sufficient to prevent colony formation by 50%) of 2 µg/ml, compared to 168 cysA14 bry-2 (Smith and Smith, 1973) which had an LD50 of 4.2 µg/ml. In these experiments, the parent, cysA14, had an LD50 of 0.01 µg/ml.

Mapping of thi Mutations. The five cysA thi strains were used as recipients in transduction crosses, with PBSI transducing bacteriophage grown on 168 strA as donor (Table 1A). In all the crosses, high linkage between thi, cysA and strA was observed, confirming previous mapping of bry-2 (Smith and Smith, 1973). No recombination was found between the thi and cysA, and very little between thi and strA as shown previously for bry-2 and these markers. The thi mutations, on the basis of the few recombinants, map proximally to strA. To map further these mutations, DNA was prepared from the cysA14 strA strains and was used to transform BD170 (irp-2, thr-5) strA competent cells (Table 1B). DNA from 168 cysA14 bry-2 was also used to transform BD170 strA. The results indicate, on the basis of the high numbers in the 101 and 110 recombination classes, that the thi markers, as well as bry-2, are between cysA and strA.

Reversion of Thiostrepton Resistance. The mutation to bryamycin resistance in B. subtilis confers a marked sensitivity to erythromycin (Goldthwaite and Smith, 1972). This phenomenon was used to select thiostrepton-sensitive revertants from cysA14 thi-2, -3, and -4. Overnight cultures of the cysA14 thi strains were plated on TBAB agar containing 0.05 µg/ml of erythromycin, a concentration which allows the cysA14 parent to grow but completely inhibits the growth of the cysA14 thi strains. Colonies appearing on the plate were tested for their markers. Those that were cysteine-requiring were tested further. Nine "revertants", three from each of the thi strains, were selected and were shown to have parental (cysA14) sensitivity to thiostrepton. There is a possibility that the thiostrepton-sensitive strains are not true revertants, but result from second-site suppression. To check this, DNA was prepared from three of the revertants, one from each initial thi parent, and high levels of DNA were used to transform BD170, selecting for thiostrepton resistance. No thi transformants over background were obtained, although these DNA preparations had normal levels of thr⁺ transforming activity (Table 2). This suggests that these strains are true revertants, but the putative suppressor mutations could be closely linked to thi. The revertants, however, grow much faster than their thi parents, with division times similar to the parental cysA14.

Effect of Thiostrepton on Polyphenylalanine Synthesis with B. subtilis Ribosomes and S-100 from Parent and Mutant Strains. The incorporation of [14C]Phe from [14C]Phe-tRNA into polyphenylalanine was determined with ribosomes and supernatant from parental, thi, bry, and mic strains (Table 3). Polyphenylalanine synthesis with parental ribosomes was evaluated with S-100 from the parent as well as from each of the mutants. In addition, phenylalanine incor-