JOURNAL OF BACTERIOLOGY, Dec. 1990, p. 7306–7309
0021-9193/90/127306-04$02.00/0

Vol. 172, No. 12

Isolation and Characterization of *Bacillus stearothermophilus* 30S and 50S Ribosomal Protein Mutations

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Received 28 February 1990/Accepted 4 September 1990

***Bacillus stearothermophilus* mutations which confer resistance to or dependence on a variety of ribosome-targeted antibiotics have been isolated. Many of these mutations produce ribosomal proteins with altered mobilities in a two-dimensional gel electrophoresis system. This collection of altered thermophilic ribosomal proteins will be useful in examining ribosomal structure and function.**

Ribosomes and ribosomal subunits from *Bacillus stearothermophilus* form high-quality crystals suitable for analysis by X-ray and electron diffraction methods (1, 10, 20, 28, 30; Z. Berkovitch-Yellin, W. Bennet, H. Hansen, K. von Bohlen, N. Volkmann, H. G. Wittmann, and A. Yonath, J. Cryst. Growth, in press). Diffraction analysis of *B. stearothermophilus* crystallized ribosomes has revealed important details of ribosomal structure, including a previously unrecognized tunnel structure that may serve as a path for elaboration of nascent polypeptide chains through the body of the particle (1, 28, 31). Ribosomal particles that contain altered forms of or lack individual ribosomal proteins would be useful in extending the high-resolution crystallographic analysis of ribosome structure. We describe here the isolation of *B. stearothermophilus* mutants resistant to or dependent on the presence of antibiotics that affect protein synthesis.

A variety of antibiotic-resistant mutants were derived from *B. stearothermophilus* 799 (Table 1). The standard growth medium (SGM) consisted of 1% Bacto Tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, and 8.5 μM $MnCl_2$. For solid medium, 1% agar was added. Cultures were incubated at 60°C, and liquid cultures were shaken at 350 rpm. Spontaneous antibiotic-resistant mutants were isolated by growing 10-ml cultures of strain AL1 to stationary phase (10^9 cells per ml), concentrating the cells by centrifugation (10,000 × *g* for 10 min), resuspending the cells in 1 ml of SGM liquid medium, and spreading 0.1-ml portions of this cell suspension onto SGM plates containing an appropriate antibiotic. The following antibiotics were provided at the indicated final concentrations: thiostrepton, 1.0 to 2.5 μg/ml; erythromycin, 1 μg/ml; tylosin, 0.1 μg/ml; oleandomycin, 4 μg/ml; chalcomycin, 4 μg/ml; niddamycin, 0.4 μg/ml; spiramycin, 20 μg/ml; and streptomycin, 50 to 100 μg/ml. Following incubation for 36 to 48 h, resistant colonies were streak purified to yield single-cell clones.

Streptomycin-dependent mutants were also isolated from strain AL1 (19), either spontaneously or following nitrosoguanidine mutagenesis. After growth of the cells on SGM plates lacking antibiotic for intervals of 2, 4, and 6 h, the agar medium was lifted away from the bottom of the culture dish and 0.2 ml of streptomycin solution (10 mg/ml) was added to the dish. The plates were stored for 24 h at 4°C to allow for antibiotic diffusion prior to their incubation at 60°C for 36 to 48 h. Strains dependent on streptomycin for growth were identified by replicating colonies obtained from the streptomycin selection plates onto SGM plates with and without streptomycin. Spontaneous streptomycin-independent revertants were derived from streptomycin-dependent strains by incubating 10^9 cells of a dependent strain on SGM plates without streptomycin.

Ribosomes were isolated from the antibiotic-resistant mutants as previously described (2, 23), and the ribosomal proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) as described by Geyl et al. (9). *B. stearothermophilus* 30S ribosomal proteins are numbered with reference to their position in 2D PAGE (14). *B. stearothermophilus* 50S ribosomal proteins are numbered with reference to their immunological and amino acid sequence homology to *Escherichia coli* 50S proteins (7, 15). L and S designate large and small subunit proteins, respectively. Ribosomal proteins from *B. stearothermophilus* are designated B to differentiate them from *Bacillus subtilis* proteins, which are designated Bs, and from *E. coli* proteins, which are designated E.

Five independent spontaneous thiostrepton-resistant (Tsr^r) *B. stearothermophilus* mutants lacked protein BL11 when analyzed by 2D PAGE (70S ribosomes in Fig. 1A and 50S subunits in Fig. 2A). Thiostrepton resistance in *Bacillus megaterium* and *B. subtilis* also results in the loss of a homologous protein (3, 25). To exclude the possibility that the ribosomal binding site for BL11 might be altered, preventing the protein from being associated with 50S particles, reconstitution experiments were performed with purified wild-type BL11 (8, 11, 17) (Fig. 1C) and with 50S subunits from strain TST-1. Purified wild-type BL11 protein was incorporated into TST-1 50S subunits by using the second step of the reconstitution procedure described by Nierhaus and Dohme (21) (Fig. 2B). The purified BL11 protein was bound to mutant 50S particles in stoichiometric amounts as judged by densitometric analysis of the electropherograms (data not shown).

The activities of ribosomes from TST-1 and wild-type strain 799 and of BL11-reconstituted TST-1 ribosomes were compared in a poly(U)-programmed in vitro polyphenylalanine synthesis system, as described by Nierhaus and Dohme (21). Ribosomes from TST-1 showed a significantly reduced

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TABLE 1. *B. stearothermophilus* strains

Strain	Genotype[a]	Phenotype	
		Antibiotic selection	Altered ribosomal protein
AL1 (799)	Wild type		
TST-1	*tsr-1*	Thiostrepton resistant	L11 (missing)
TST-2	*tsr-2*	Thiostrepton resistant	L11 (missing)
AL100	*ery-1*	Erythromycin resistant	L4
AL110	*nid-8*	Niddamycin resistant	L4
AL120	*ole-1*	Oleandomycin resistant	L4
AL130	*spi-1*	Spiramycin resistant	L4
AL140	*cal-1*	Chalcomycin resistant	
AL150	*tyl-1*	Tylosin resistant	
AL201	str^d-2 str^i-2	Streptomycin independent	S5
AL211	str^d-3 str^i-3	Streptomycin independent	S4
SB12	str^d-6 str^i-6	Streptomycin independent	L14
SB44	str^d-7 str^i-7	Streptomycin independent	L19
SB85	str^d-8 str^i-8	Streptomycin independent	S4
SB111	str^d-9 str^i-9	Streptomycin independent	S4

[a] d and i, Dependent and independent, respectively.

protein synthesis capacity when compared with wild-type ribosomes (Table 2). However, the activity of the mutant ribosomes was restored to wild-type levels following reconstitution with wild-type protein BL11. These results indicate that added wild-type BL11 protein is incorporated at its normal site in the TST-1 50S ribosomal subunit and that reconstitution with BL11 restores normal synthetic function.

Twenty spontaneous erythromycin-resistant (Ery^r) mutants were isolated. Ribosomes from these strains contained an altered BL4 protein with an increased negative net charge (Fig. 3A). *B. subtilis* erythromycin-resistant mutations affect a nonhomologous ribosomal protein, BsL17 (22, 23). The BL4 and BsL17 proteins are homologous to the *E. coli* ribosomal proteins EL4 and EL22, respectively (7, 12, 13). Erythromycin resistance mutations in *E. coli* that affect both EL4 and EL22 have been isolated (26). Similar protein alterations in BL4 were found in ribosomes of mutants resistant to the related macrolide antibiotics oleandomycin and niddamycin (Fig. 3B and C). *B. stearothermophilus* strains resistant to spiramycin, another macrolide antibiotic, showed a small but reproducible alteration in the mobility of protein BL4, indicative of an increased positive net charge (Fig. 3D). However, mutants resistant to the macrolides tylosin and chalcomycin did not give rise to ribosomes with detectable protein alterations (Fig. 3E and F). *B. subtilis* strains resistant to these antibiotics contain altered forms of BsL17 (22).

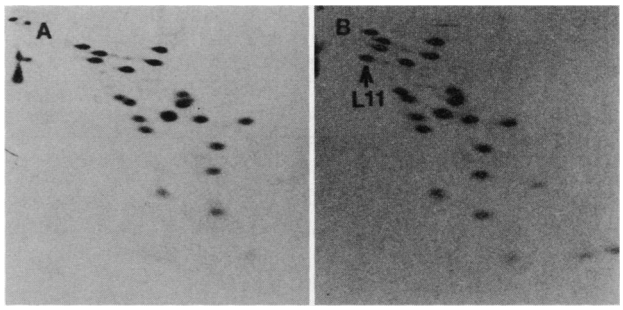

FIG. 2. 2D PAGE of the 50S ribosomal subunit proteins. (A) Thiostrepton-resistant strain TST-1; (B) TST-1 50S subunits reconstituted with wild-type BL11 protein.

Streptomycin-independent revertants isolated from streptomycin-dependent strains of *E. coli* and *B. subtilis* contain alterations in a variety of ribosomal proteins (for example, see references 4, 5, and 12). Although streptomycin specifically binds to the 30S subunit, streptomycin-independent revertants of *E. coli* may contain alterations in either 30S or 50S subunit proteins (4). To investigate the effects of streptomycin-independent mutations in *B. stearothermophilus*, spontaneous streptomycin-dependent (Str^d) strains were isolated, and from these strains, drug-independent revertants were derived. The independent revertants showed alterations only in 30S ribosomal proteins BS4 and BS5, corresponding to S4 and S5 of *E. coli* (Fig. 4A and B).

Streptomycin-dependent *B. stearothermophilus* mutants were also isolated from nitrosoguanidine-mutagenized strain AL1. Spontaneous streptomycin-independent revertants were selected from these streptomycin-dependent strains, and ribosomal proteins from the revertants were analyzed by 2D PAGE. Selected altered protein patterns are shown in Fig. 4C to F. Several streptomycin-independent revertants contained alterations in ribosomal proteins BS4 (Fig. 4C and D) or BL19 (Fig. 4F). Another streptomycin-independent revertant (strain SB12) was missing 50S ribosomal protein BL14 (Fig. 4F). However, BL14 must be present in a highly altered form, since antibodies raised against wild-type protein BL14 strongly reacted with 50S subunits from mutant SB12 (data not shown). Several independent revertants did not have any apparent protein alterations, as determined by 2D PAGE. These results demonstrate that the classes of streptomycin-dependent mutations obtained by spontaneous and chemical mutagenesis are not identical. Further, the classes of drug-independent revertants derived from these backgrounds are also distinct.

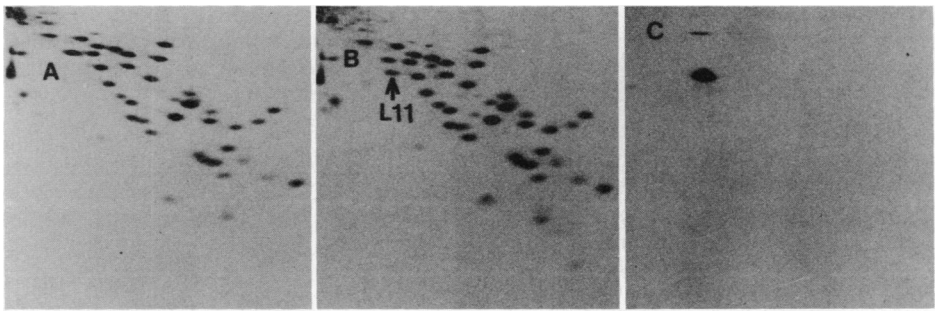

FIG. 1. 2D PAGE of *B. stearothermophilus* 70S ribosomal proteins. (A) Thiostrepton-resistant strain TST-1; (B) wild-type strain AL1; (C) BL11 protein purified from the wild-type strain. The position of protein BL11, absent in TST-1, is indicated in panel B.

TABLE 2. Poly(U)-directed polyphenylalanine in vitro protein synthesis with BL11-deficient and BL11-reconstituted 50S ribosomal subunits

Expt no. and 50S component(s) varied during reconstitution[a]	Incorporation (cpm)
1	
Wild type	32,810
TST-1	16,100
TST-1 + BL11 (wild type)	32,400
2	
Wild type	35,000
TST-2	19,300
TST-2 + BL11 (wild type)	33,550

[a] Procedures for the isolation of ribosomal subunits, protein BL11, and in vitro reconstitution are described in the text. In vitro protein synthesis reactions employing isolated wild-type or Tsr^r 50S subunits were carried out as described by Nierhaus and Dohme (21). All reaction mixtures contained *E. coli* 30S subunits (A_{260}, 0.7). A_{260} for all components was 0.5.

We have established that *B. stearothermophilus* is sensitive to a variety of antibiotics which interfere with protein-synthesizing reactions involving either 30S or 50S ribosomal subunits. *B. stearothermophilus* is thus the first thermophilic eubacterium demonstrated to have an antibiotic sensitivity and a ribosomal protein target pattern similar to those of mesophilic gram-positive and gram-negative bacteria (3–6, 16, 22, 23). Taken together, the results of these genetic studies suggest that the organization of *B. stearothermophilus* ribosomes is generally similar to that of ribosomes of mesophilic eubacteria, with subtle differences that may be related to the extreme thermal stress that is routinely exerted on these structures.

The mutants described here have already been of value in ribosomal-structure investigations. The thiostrepton mutants lacking protein BL11 have been used for in vitro reconstitution and crystallization experiments (20, 29). L11-deficient ribosomes have been used as a core for reconstitution with heavy-atom-derivatized (gold cluster or tetrairidium derivatives) L11 protein molecules. These particles have diffraction intensities that differ significantly from those of native crystals (24).

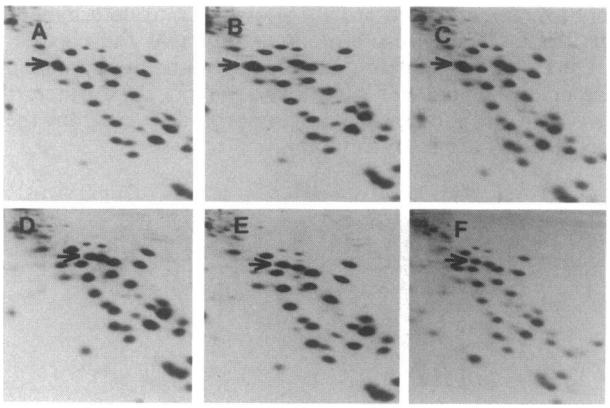

FIG. 3. 2D PAGE of 70S ribosomal proteins from *B. stearothermophilus* mutants resistant to various macrolide antibiotics. (A) Erythromycin-resistant strain AL100; (B) oleandomycin-resistant strain AL120; (C) niddamycin-resistant strain AL110; (D) spiramycin-resistant strain AL130; (E) chalcomycin-resistant strain AL140; (F) tylosin-resistant strain AL150. Protein BL4 is indicated by an arrow on each gel.

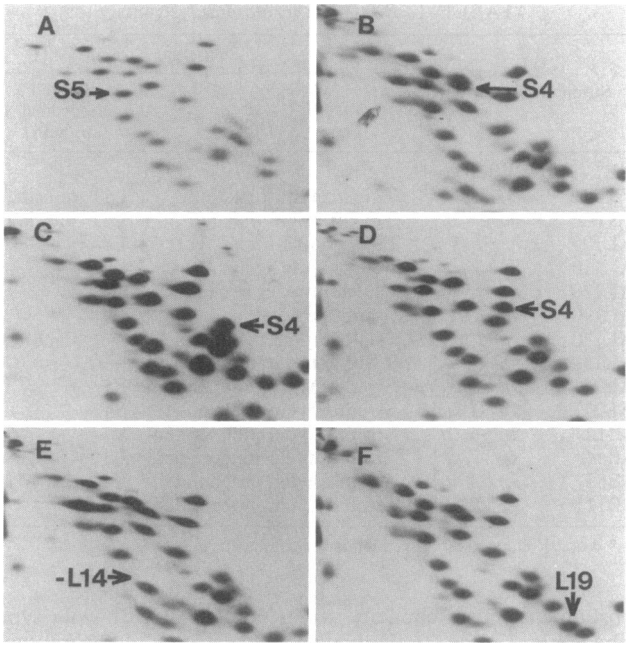

FIG. 4. 2D PAGE of 70S ribosomal proteins from streptomycin-independent *B. stearothermophilus* mutants. (A) AL201, a streptomycin-independent revertant derived from a spontaneous streptomycin-dependent strain and containing an altered BS5 protein; (B) AL211, a strain similar to AL201 except that it contains an altered BS4 protein; (C and D) SB85 and SB111, respectively, two examples of nitrosoguanidine-induced streptomycin-dependent strains from which spontaneous antibiotic independence was selected and which contain altered ribosomal protein BS4; (E) SB12, selected identically to SB85 and SB111 but lacking ribosomal protein BL14; (F) SB44, selected identically to SB85 and SB111 but containing an altered BL19 protein. The positions of the altered proteins are indicated. In the case of SB12, the position of the wild-type BL14 protein is indicated.

Other procaryotes from which high-quality three-dimensional ribosomal subunit crystals have been obtained are *Halobacterium marismortui*, a halophilic archaebacterium (18), and *Thermus thermophilus*, a gram-negative heterotrophic thermophile (27; Berkovitch-Yellin et al., in press). It is possible that the high-salt conditions or the elevated temperatures to which these organisms are exposed have selected for a highly ordered and stable ribosomal structure that readily forms single three-dimensional crystalline arrays in vitro (20). However, among these organisms, *B. stearothermophilus* appears to be particularly suitable for genetic manipulation of ribosomal structure and function. The further study of crystallized *B. stearothermophilus* ribosomes lacking specific proteins will be of substantial value in finding the solution to and the biological interpretation of ribosomal higher-order structure.

We thank H. G. Wittmann and A. Yonath for support and helpful discussions. We also thank M. Stoffler-Meilicke for the immunological analysis of strain SB12 50S subunits with anti-BL14 antibodies.

This work was supported by grants from the Max-Planck-Gesellschaft and the National Science Foundation.

LITERATURE CITED

1. **Arad, T., J. Piefke, S. Weinstein, H. S. Gewitz, A. Yonath, and H. G. Wittmann.** 1987. Three-dimensional image reconstruction from ordered arrays of 70S ribosomes. Biochimie **69**:1001–1006.

2. **Cronenberger, J. H., and V. A. Erdmann.** 1975. Stimulation of peptide polymerization by blocking of sulphydryl groups in *Escherichia coli* ribosomal proteins. J. Mol. Biol. **95**:125–137.
3. **Cundliffe, E., P. Dixon, M. Stark, G. Stoffler, R. Ehrlich, M. Stoffler-Meilicke, and M. Cannon.** 1979. Ribosomes in the thiostrepton-resistant mutants of *Bacillus megaterium* lacking a single 50 S subunit protein. J. Mol. Biol. **132**:235–252.
4. **Dabbs, E. R.** 1978. Mutational alterations in 50 proteins of the *Escherichia coli* ribosome. Mol. Gen. Genet. **165**:73–78.
5. **Dabbs, E. R.** 1982. Selection in *Bacillus subtilis* giving rise to strains with mutational alterations in a variety of ribosomal proteins. Mol. Gen. Genet. **187**:297–301.
6. **Dabbs, E. R.** 1986. Mutant studies of the prokaryotic ribosome, p. 733–748. *In* B. Hardesty and G. Kramer (ed.), Structure, function and genetics of ribosomes. Springer-Verlag, New York.
7. **Fahnestock, S. R., W. A. Strycharz, and D. M. Marquis.** 1981. Immunochemical evidence of homologies among 50S ribosomal proteins of *Bacillus stearothermophilus* and *Escherichia coli*. J. Biol. Chem. **256**:1011–1016.
8. **Gewitz, H.-S., C. Glotz, P. Goischke, B. Romberg, J. Mussig, A. Yonath, and H. G. Wittmann.** 1987. Reconstruction and crystallization experiments with isolated split proteins from *Bacillus stearothermophilus* ribosomes. Biochem. Int. **15**:887–892.
9. **Geyl, D., A. Bock, and K. Isono.** 1981. An improved method for two-dimensional gel-electrophoresis: analysis of mutationally altered ribosomal proteins of *Escherichia coli*. Mol. Gen. Genet. **181**:309–312.
10. **Glotz, C., J. Mussig, H.-S. Gewitz, I. Makowski, T. Arad, A. Yonath, and H. G. Wittmann.** 1987. Three-dimensional crystals of ribosomes and their subunits from eu- and archaebacteria. Biochem. Int. **15**:953–960.
11. **Hamel, E., M. Koka, and T. Nakamoto.** 1972. Requirement of an *Escherichia coli* 50S ribosomal protein component for effective interaction of the ribosome with T and G factor and with guanosine triphosphate. J. Biol. Chem. **247**:805–814.
12. **Hasenbank, R., C. Guthrie, G. Stoffler, H. G. Wittmann, L. Rosen, and D. Apirion.** 1973. Electrophoretic and immunological studies on ribosomal proteins of 100 *Escherichia coli* revertants from streptomycin dependence. Mol. Gen. Genet. **127**:1–18.
13. **Higo, K.-I., T. Itoh, T. Kumazaki, and S. Osawa.** 1980. Ribosomal protein from mesophilic and thermophilic bacteria, p. 655–666. *In* S. Osawa, H. Ozeki, H. Uchida, and T. Yura (ed.), Genetics and evolution of RNA polymerase, tRNA, and ribosomes. Elsevier/North-Holland Publishing Co., New York.
14. **Isono, S., and K. Isono.** 1975. Purification and characterization of 30-S ribosomal proteins from *Bacillus stearothermophilus*. Eur. J. Biochem. **50**:483–488.
15. **Kimura, M., J. Kimura, and K. Ashmann.** 1985. The complete primary structure of ribosomal proteins L1, L14, L15, L23, L24 and L29 from *Bacillus stearothermophilus*. Eur. J. Biochem. **150**:491–497.
16. **Leighton, T.** 1974. Sporulation specific translational discrimination in *Bacillus subtilis*. J. Mol. Biol. **86**:855–863.
17. **Liljas, A., and M. E. Newcomer.** 1981. Purification and crystallization of a protein complex from *Bacillus stearothermophilus* ribosomes. J. Mol. Biol. **153**:393–398.
18. **Makowski, I., F. Frolow, M. A. Saper, M. Shoham, H. G. Wittmann, and A. Yonath.** 1987. Single crystals of large ribosomal particles from *Halobacterium marismortui* diffract to 6 Å. J. Mol. Biol. **193**:819–822.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mussig, J., I. Makowski, K. von Bohlen, H. Hansen, K. S. Bartels, H. G. Wittmann, and A. Yonath.** 1989. Crystals of wild-type, mutated, derivatized and complexed 50S ribosomal subunits from *Bacillus stearothermophilus* suitable for X-ray analysis. J. Mol. Biol. **205**:619–621.
21. **Nierhaus, K. H., and F. Dohme.** 1979. Total reconstruction of 50S subunits from *Escherichia coli* ribosomes. Methods Enzymol. **59**:443–449.
22. **Sharrock, R. A., T. Leighton, and H. G. Wittmann.** 1981. Macrolide and aminoglycoside antibiotic resistance mutations in the *Bacillus subtilis* ribosome resulting in temperature-sensitive sporulation. Mol. Gen. Genet. **183**:538–543.
23. **Tipper, D. J., C. W. Johnson, C. L. Ginther, T. J. Leighton, and H. G. Wittmann.** 1977. Erythromycin resistant mutations in *Bacillus subtilis* cause temperature sensitive sporulation. Mol. Gen. Genet. **150**:147–159.
24. **Weinstein, S., W. Jahn, H. Hansen, H. G. Wittmann, and A. Yonath.** 1989. Novel procedures for derivatization of ribosomes for crystallographic studies. J. Biol. Chem. **264**:19138–19142.
25. **Wienen, B., R. Erhlich, M. Stoffler-Meilicke, G. Stoffler, I. Smith, D. Weiss, R. Vince, and S. Pestka.** 1979. Ribosomal protein alterations in thiostrepton- and micrococcin-resistant mutations of *Bacillus subtilis*. J. Biol. Chem. **254**:8031–8041.
26. **Wittmann, H. G., G. Stoffler, D. Apirion, L. Rosen, K. Tanaka, M. Tamaki, R. Takata, S. Dekio, E. Otaka, and S. Osawa.** 1973. Biochemical and genetic studies of two different types of erythromycin resistant mutants of *Escherichia coli* with altered ribosomal proteins. Mol. Gen. Genet. **127**:175–189.
27. **Yonath, A., C. Glotz, H. S. Gewitz, K. S. Bartels, K. von Bohlen, I. Makowski, and H. G. Wittmann.** 1988. Characterization of crystals of small ribosomal subunits. J. Mol. Biol. **203**:831–834.
28. **Yonath, A., K. R. Leonard, and H. G. Wittmann.** 1987. A tunnel in the large ribosomal subunit revealed by three-dimensional image reconstruction. Science **236**:813–816.
29. **Yonath, A., M. A. Saper, F. Frolow, I. Makowski, and H. G. Wittmann.** 1986. Characterization of single crystals of the large ribosomal particles from a mutant of *Bacillus stearothermophilus*. J. Mol. Biol. **192**:161–162.
30. **Yonath, A., M. A. Saper, I. Makowski, J. Mussig, J. Piefke, H. D. Bartunik, K. S. Bartels, and H. G. Wittmann.** 1986. Characterization of single crystals of the large ribosomal particles from *Bacillus stearothermophilus*. J. Mol. Biol. **187**:633–636.
31. **Yonath, A., and H. G. Wittmann.** 1989. Challenging the three-dimensional structure of ribosomes. Trends Biochem. Sci. **14**: 329–335.