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Stringency and Relaxation among the Halobacteria

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Accumulation of stable RNA and production of guanosine polyphosphates (ppGpp and pppGpp) were studied during amino acid starvation in four species of halobacteria. In two of the four species, stable RNA was under stringent control, whereas one of the remaining two species was relaxed and the other gave an intermediate phenotype. The stringent reaction was reversed by anisomycin, an effect analogous to the chloroamphenicol-induced reversal of stringency in the eubacteria. During the stringent response, neither ppGpp nor pppGpp accumulation took place during starvation. In both growing and starved cells a very low basal level of the two polyphosphates appeared to be present. In the stringent species the intracellular concentration of GTP did not diminish but actually increased during the course of the stringent response. These data demonstrate that (i) wild-type halobacteria can have either the stringent or the relaxed phenotype (all wild-type eubacteria tested have been shown to be stringent); (ii) stringency in the halobacteria is dependent on the deaminoacylation of tRNA, as in the eubacteria; and (iii) in the halobacteria, ppGpp is not an effector of stringent control over stable-RNA synthesis.

It was shown in 1952 that in wild-type *Escherichia coli* amino acid starvation causes the arrest of the synthesis not only of protein but also of stable RNA (sRNA) (16). This phenomenon was shown not to be an obligatory property of the cells, but rather to be a regulatory event, when a mutant of *E. coli* was found in which RNA synthesis was not arrested as a result of amino acid starvation (5). The phenomenon was defined as stringent control and mapped to a locus called *relA*; mutants lacking stringent control were defined as relaxed (2, 20). It is known today that relaxation is caused by mutations in at least three different loci (7). Numerous wild-type eubacterial species have been examined for the presence of a stringent reaction, and in all cases they were shown to be stringent. It has been shown over the years that many other aspects of cell physiology are under positive or negative stringent control (7). It was also shown that the stringent response is elicited when either the aminoacylation of any one species of tRNA (13) or the formylation of formylmethionyl-tRNA (18) is prevented. Apart from sRNA control, an aspect of stringency that has been studied extensively is the production of guanosine tetra- and pentaphosphates (ppGpp and pppGpp) that are produced in stringent but not in relaxed strains (6). It was long ago suggested (6), and recently demonstrated (17), that ppGpp acts as a negative effector in sRNA control and presumably in other aspects of stringency (7). Consistent with this notion is the fact that the *relA* gene product is a (p)ppGpp synthetase with activity requirements that closely match the physiology of the stringent response (11, 15). In some instances, however, stringent control of sRNA has been shown to take place in stringent bacteria in which the accumulation of ppGpp does not occur (1, 14, 19). It appears, therefore, that in the eubacteria stringent control can be acheived by means of two distinct mechanisms, one mediated by ppGpp and the other independent of ppGpp.

Information on stringent control has been obtained almost exclusively from work carried out with the eubacteria. Only two species of archaea, both methanogens, have been examined with respect to amino acid control of sRNA synthesis and ppGpp production, and both were found to have the relaxed phenotype (3). This finding raised the possibility that wild-type archaea may be consistently relaxed and that as a consequence stringent control could constitute an additional feature that distinguishes the eubacteria from the archaea. Confirmation of this possibility would have significant basic biological, taxonomic, and evolutionary implications, and it is therefore important to expand our knowledge of stringent control in the archaea. The purpose of the present study is to establish whether stringent control is present in the halobacteria, a subdivision of the archaea distant from the methanogens. To this end, four species of halobacteria have been examined for sRNA accumulation and production of guanosine polyphosphates.

MATERIALS AND METHODS

Bacterial strains. Three halobacterial strains were obtained from Deutsche Sammlung von Mikroorganismen (Göttingen, Germany): *Halobacterium halobium* (670), *Haloferax volcanii* (3757), and *Halococcus morrhuae* (1307). *Halobacterium salinarium* was kindly supplied by S. Zillig (Max-Planck-Institut für Biochemie, Martinsried, Germany). This strain was furnished under the name *H. halobium*; from a reexamination of its characteristics, however, it can be classified more appropriately as a member of the species *H. salinarium* (21). *Salmonella typhimurium* TA997 (*aroC5 purF145 hisD2655*) was obtained from R. Cortese (IRBM, Pomezia, Italy).

Sources of reagents. Nutrients and agar were furnished by Difco (Detroit, Mich.), pseudomonic acid (PA) was furnished by SmithKline Beecham Pharmaceuticals (Worthing, United Kingdom), and the other antibiotics were obtained from Sigma (St. Louis, Mo.). All other chemicals were obtained from Merck (Darmstadt, Germany). [^{3}H]threonine was from New England Nuclear (Du Pont de Nemours, Firenze, Italy); [^{14}C]uridine and $^{32}P_i$ were from Amersham (Amersham, United Kingdom).

Media and growth conditions. *H. halobium* and *H. salinarium* were grown in a salt medium (SM1) consisting of 25% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.5% KCl, 0.02% $CaCl_2$, 0.25% yeast extract, and 0.25% tryptone, pH 7. *H. volcanii* and *H. morrhuae* were grown in a different salt medium (SM2) consisting of 19.5% NaCl, 6.5% $MgCl_2$, 4.1% $MgSO_4 \cdot 7H_2O$,

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0.1% $CaCl_2 \cdot 2H_2O$, 0.05% KCl, 0.02% $NaHCO_3$, 0.06% NaBr, and 0.5% yeast extract, pH 7. All four species were grown with shaking and illumination at 37°C. *S. typhimurium* was grown in low-salt medium (0.25% yeast extract, 1% NaCl).

Analysis of RNA and protein syntheses. When the cultures growing exponentially in either SM1 or SM2 (see above) reached an optical density at 600 nm of about 0.2 (corresponding to about 7×10^7 cells per ml), [^{3}H]threonine (0.4 μCi/ml) and [^{14}C]uridine (0.025 μCi/ml) were added. After at least one doubling, starvation for isoleucine was accomplished by adding PA (4) to the cultures. Samples were then placed in 5% trichloroacetic acid at different time intervals. The bacterial precipitates were collected on Millipore filters, and the radioactivity was counted as previously described (8). The labeling periods used were sufficiently long to ensure that the RNA labeled was essentially sRNA.

Assay for guanosine polyphosphates. The method used to assay guanosine polyphosphates was that described by Gallant et al. (9). Bacteria were grown in modified versions of SM1 and SM2 in which the concentrations of yeast extract and tryptone were halved. The cells were labeled with $^{32}P_i$ (100 μCi/ml) at least one doubling before the addition of PA. PA was added when the optical density at 600 nm reached a value of about 0.4. At the appropriate times, 400-μl samples were removed from the culture, spun down in an Eppendorf centrifuge at 14×10^3 rpm for 2 min, and resuspended in the same volume of low-salt medium containing 1 N formic acid; resuspension in low-salt medium was necessary because the high salt concentrations present in SM1 and SM2 interfered with polyethyleneimine-cellulose (Macherey-Nagel; Brinkman Instruments, Inc.) chromatography. The tubes were vortexed immediately, and after an extraction period on ice of at least 30 min, samples were either used for chromatography or frozen at −20°C. Samples were centrifuged at 4°C for 10 min, and the clarified supernatants were applied to polyethyleneimine-cellulose plates. After spotting of the samples, the chromatograms were soaked in methanol for 5 min and dried. Plates were developed in a single dimension of ascending chromatography with 1.2 M KH_2PO_4 (pH 3.5) as the eluting solvent. The nucleotides ppGpp and pppGpp were revealed by autoradiography at −70°C with an intensifying screen, using Amersham Hyperfilm-MP film.

RESULTS

Amino acid control over sRNA synthesis was studied by starving cells for isoleucine by adding PA to the cultures and then monitoring the incorporation of radioactive precursors into RNA and protein. In preparation for this study, the MICs of this drug for the four different species used in this study had been determined and had been found to vary over a 20-fold range (see Table 1). A typical experiment carried out with *H. morrhuae* is reported in Fig. 1 and shows that this strain is stringent. An identical experiment carried out with *H. salinarium* is reported in Fig. 2 and shows that this strain has the relaxed phenotype. Table 1 summarizes the results of experiments with all four halobacterial species and shows that at the appropriate PA concentrations protein synthesis is shut down efficiently in all species. In two of the species, *H. morrhuae* and *H. volcanii*, sRNA synthesis is under stringent control. *H. halobium* is partially relaxed, whereas *H. salinarium* is fully relaxed.

A characteristic feature of stringent control over sRNA synthesis in the eubacteria is that the control does not occur when inhibition of protein synthesis is brought about by agents, such as chloroamphenicol, that do not interfere with amino-

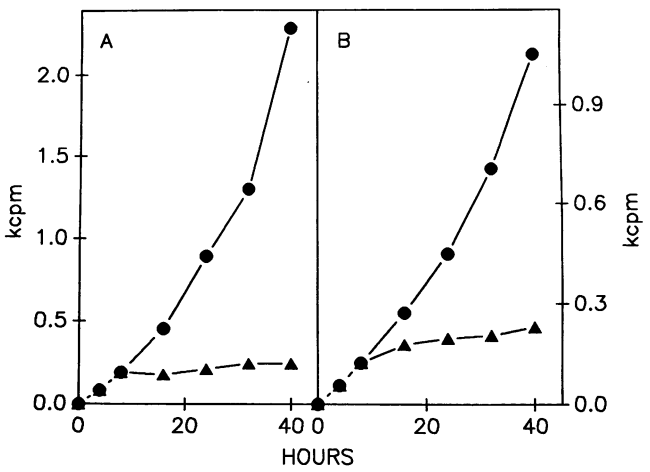

FIG. 1. Protein and RNA syntheses in *H. morrhuae*. The syntheses of protein (A) and RNA (B) were measured by the incorporation of [^{3}H]threonine and [^{14}C]uridine, respectively, into the acid-insoluble material of cells as described in Materials and Methods. Experiments were performed during either exponential growth (●) or starvation for isoleucine (▲); PA was added at 8 h.

acylation of tRNA (22). As shown in Table 1, the halobacteria are only slightly sensitive to chloroamphenicol. Furthermore, this drug appears to have a direct effect on sRNA synthesis, since sRNA accumulation is inhibited more than protein accumulation. The antibiotic anisomycin, however, is a potent inhibitor of protein synthesis in the halobacteria, and its mechanism of action is analogous to that of chloroamphenicol in the eubacteria (12). Table 1 shows that the effect of anisomycin on *H. volcanii* is similar to that of chloroamphenicol on the eubacteria, since the synthesis of sRNA is inhibited only moderately.

The two stringent species, *H. volcanii* and *H. morrhuae*, were then examined for the production of guanosine polyphosphates. In a preliminary experiment it was shown that the

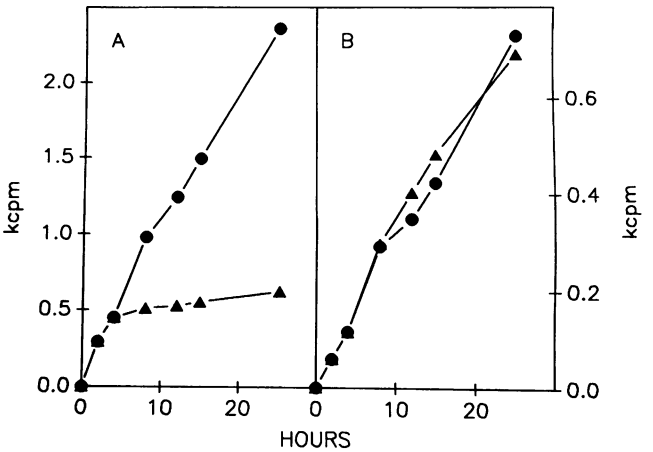

FIG. 2. Protein and RNA syntheses in *H. salinarium*. The syntheses of protein (A) and RNA (B) were measured by the incorporation of [^{3}H]threonine and [^{14}C]uridine, respectively, into the acid-insoluble material of cells as described in Materials and Methods. Experiments were performed during either exponential growth (●) or starvation for isoleucine (▲); PA was added at 4 h.

TABLE 1. Effect of different inhibitors on RNA and protein syntheses

Inhibitor	Concn[a] (μg/ml)	Species	Rate of synthesis[b] of:	
			RNA	Protein
PA	6	*H. morrhuae*	6.5	3.3
	0.4	*H. volcanii*	13.6	4.2
	8	*H. halobium*	65.7	2.1
	4	*H. salinarium*	94.3	8.9
Chloro-amphenicol	200	*H. volcanii*	15.5	21.4
	300	*H. halobium*	9.5	14.6
Anisomycin	6	*H. volcanii*	60.0	0.03

[a] The concentrations used were twice the MIC.
[b] The values reported are for the cultures treated with PA and are expressed as percentages of the rates of synthesis in the untreated cultures. Rates of synthesis were obtained by carrying out regression analyses on the exponential portions of the incorporation curves.

relA$^+$ strain S. typhimurium TA997, growing in a growth medium with the same organic composition as SM2 and amino acid starved by means of PA, produced large amounts of (p)ppGpp, as expected. As shown in Fig. 3, neither of the two halobacterial species accumulated either ppGpp or pppGpp as a result of PA addition, a finding that conflicts with those normally obtained with wild-type stringent eubacterial species. The search for (p)ppGpp was carried out over extended time periods: 30 h for *H. volcanii* and 40 h for *H. morrhuae*. As expected for a relaxed strain, *H. salinarium* did not accumulate (p)ppGpp during amino acid starvation (data not shown). However, faint spots with the mobility of ppGpp were seen in either the presence or absence of PA at all times and in all three species (in Fig. 3 such spots are particularly visible in lanes 2, 3, and 4). This suggests that a trace basal level of ppGpp is present in the archaeal halophiles and is produced by an alternate pathway, conceivably analogous to the *relA*-independent pathway described for *E. coli* (7).

In the course of the stringent response in the eubacteria, the production of ppGpp and pppGpp is accompanied by a concomitant significant drop in the level of GTP, whereas amino acid starvation of relaxed bacteria has no such effect. This is consistent with the notion that the two polyphosphates are produced at the expense of this nucleoside triphosphate (10). The data reported in Fig. 4 confirm the presence of this phenomenon in the control stringent eubacterium and show that it is absent in *H. volcanii*. In effect, in the halophile the level of GTP begins to rise immediately upon inception of amino acid starvation and rapidly increases by a factor of almost four. This finding is not totally unexpected, in view of the fact that in these cells neither sRNA nor the guanosine polyphosphates are being produced. Results very similar to these were obtained with *H. morrhuae*.

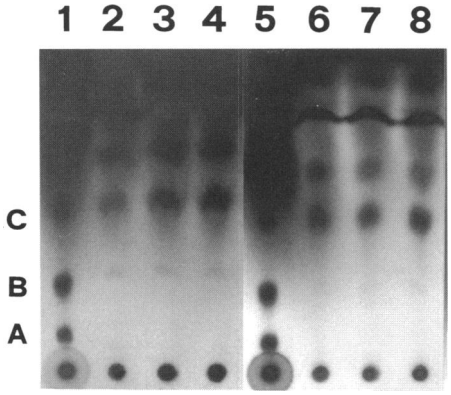

FIG. 3. Assay for guanosine polyphosphates in *H. morruhae* and *H. volcanii*. Bacteria were labeled with $^{32}P_i$, and a formic acid extract was prepared and analyzed by ascending chromatography as described in Materials and Methods. The locations of the nucleotides pppGpp (A), ppGpp (B), and GTP (C) were detected by autoradiography. Lanes 1 and 5, control (*S. typhimurium* TA997) at 5 min after addition of PA; lanes 2 to 4, *H. morrhuae* at 0, 20, and 40 min, respectively, after addition of PA; lanes 6 to 8, *H. volcanii* at 0, 10, and 20 min, respectively, after addition of PA.

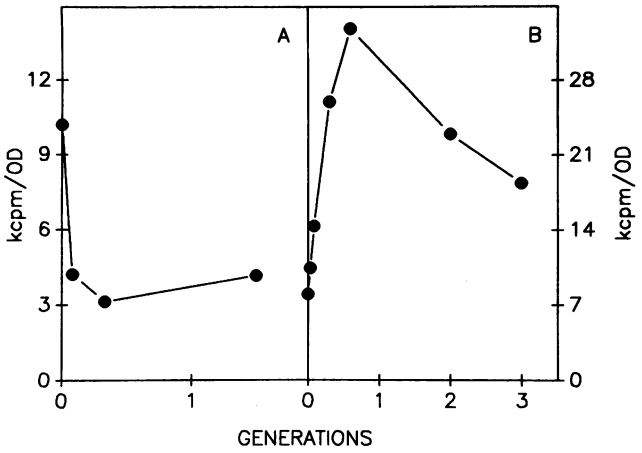

FIG. 4. GTP pools in *S. typhimurium* (A) and *H. volcanii* (B). The spots corresponding to GTP on the polyethyleneimine-cellulose chromatograms from the experiment shown in Fig. 3 were cut out and counted. The counts per minute were normalized to the corresponding optical densities at 600 nm (OD).

DISCUSSION

After several decades of intense study, a completely satisfactory explanation for the existence of the stringent response has not yet materialized. It seems reasonable to hypothesize that stringency is an event primarily designed to prevent an imbalance of cellular constituents when bacteria growing in the wild are transferred from a nutritionally rich to a nutritionally poor environment (13). To date, the stringent reaction has been shown to be present in all wild-type eubacterial species examined. In these organisms, therefore, stringency must confer a substantial selective advantage to the cells.

This report shows that in the halobacteria, sRNA accumulation during amino acid starvation can lead to a variety of behaviors, including fully stringent, intermediate, and fully relaxed phenotypes. When the phenotype is stringent, the reaction is caused by deaminoacylation of tRNA, since it is provoked by PA and not by anisomycin, in analogy to the eubacterial situation. In a previous study (3) two wild-type methanogens were shown to be relaxed. Thus, stringency can occur among the archaea, but its presence is not indispensable for the survival of the archaeal cell in the wild. The presence of an intermediate stringent phenotype, which is absent in the eubacteria, indicates that in the archaea RNA control may be evolving either from stringency towards relaxation or, as is more likely, from relaxation towards stringency.

In the halobacteria the level of the guanosine polyphos-

phates does not rise as a result of amino acid starvation. Thus, in these organisms, when the stringent reaction is present, it is not mediated by ppGpp. This is reminiscent of the case of the *hisT* mutants of *S. typhimurium*, in which stringent control over sRNA persists in the absence of (p)ppGpp accumulation (19). Stringent halobacteria possess only a ppGpp-independent stringent control mechanism, which could be analogous to the one that is present in the eubacteria in addition to the ppGpp-dependent mechanism.

When the stringent reaction is provoked in *H. volcanii* and *H. morruhae*, the level of GTP does not drop, as it does in the stringent eubacteria (10). This provides additional confirmation that neither (p)ppGpp nor other guanosine polyphosphates such as phantom spot (14) are produced during the stringent response in stringent halobacteria.

Work is presently under way to characterize the stringent reaction in the halobacteria at the molecular level and to determine whether other aspects of cell physiology are under stringent control in these organisms. It is likely that a better understanding of stringency in the archaea will also shed light on this still-mysterious phenomenon in the eubacteria.

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REFERENCES

1. **Acosta, R., and D. R. Lueking.** 1987. Stringency in the absence of ppGpp accumulation in *Rhodobacter sphaeroides*. J. Bacteriol. **169:**908–912.
2. **Alföldi, L., G. S. Stent, and R. C. Clowes.** 1962. The chromosomal site for the RNA control (R.C.) locus in *Escherichia coli*. J. Mol. Biol. **5:**348–355.
3. **Beauclerk, A. A. D., H. Hummel, D. J. Holmes, A. Böck, and E. Cundliffe.** 1985. Studies of the GTPase domain of archaebacterial ribosomes. Eur. J. Biochem. **151:**245–255.
4. **Böck, A., and O. Kandler.** 1985. Antibiotic sensitivity of archaebacteria, p. 525–544. *In* J. R. Sokatch and L. N. Ornston (ed.), The bacteria. Academic Press, Orlando, Fla.
5. **Borek, E., J. Rockenbach, and A. Ryan.** 1956. Studies on a mutant of *Escherichia coli* with unbalanced ribonucleic acid synthesis. J. Bacteriol. **71:**318–323.
6. **Cashel, M., and J. Gallant.** 1969. Two compounds implicated in the function of the RC gene of *Escherichia coli*. Nature (London) **221:**838–841.
7. **Cashel, M., and K. E. Rudd.** 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
8. **Donini, P.** 1970. Amino acid control over deoxyribonucleic acid synthesis in *Escherichia coli* infected with T-even bacteriophage. J. Bacteriol. **102:**616–627.
9. **Gallant, J., H. Erlich, B. Hall, and T. Laffler.** 1970. Analysis of the RC function. Cold Spring Harbor Symp. Quant. Biol. **35:**397–405.
10. **Gallant, J., and B. Harada.** 1969. The control of RNA synthesis in *Escherichia coli*. III. The functional relationship between purine ribonucleoside triphosphate pool sizes and rate of ribonucleic acid accumulation. J. Biol. Chem. **244:**3125–3132.
11. **Haseltine, W. A., and R. Block.** 1973. Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. Proc. Natl. Acad. Sci. USA **70:**1564–1568.
12. **Jimenez, A., and D. Vasquez.** 1979. Anisomycin and related antibiotics, p 1–19. *In* F. E. Hahn (ed.), Antibiotics. Springer-Verlag, Berlin.
13. **Neidhardt, F. C.** 1963. Properties of a bacterial mutant lacking amino acid control of RNA synthesis. Biochim. Biophys. Acta **68:**365–379.
14. **Pao, C. C., and B. T. Dyess.** 1981. Stringent control of RNA synthesis in the absence of guanosine 5′-diphosphate-3′-diphosphate. J. Biol. Chem. **256:**2252–2257.
15. **Pedersen, F. S., E. Lund, and N. O. Kjelgaard.** 1973. Codon specific, tRNA-dependent *in vitro* synthesis of ppGpp and pp pGpp. Nature (London) New Biol. **243:**13–15.
16. **Sands, M. K., and R. B. Roberts.** 1952. The effects of a tryptophane-histidine deficiency in a mutant of *Escherichia coli*. J. Bacteriol. **63:**505–511.
17. **Schreiber, G., S. Metzger, E. Aizenman, S. Roza, M. Cashel, and G. Glaser.** 1991. Overexpression of the *rel*A gene in *Escherichia coli*. J. Biol. Chem. **266:**3760–3767.
18. **Shih, A.-Y., J. Eisenstadt, and P. Lengyel.** 1966. On the relation between ribonucleic acid synthesis and peptide chain initiation in *E. coli*. Proc. Natl. Acad. Sci. USA **56:**1599–1605.
19. **Spadaro, A., A. Spena, V. Santonastaso, and P. Donini.** 1981. Stringency without ppGpp accumulation. Nature (London) **291:** 256–258.
20. **Stent, G. S., and S. Brenner.** 1961. A genetic locus for the regulation of ribonucleic acid synthesis. Proc. Natl. Acad. Sci. USA **47:**2005–2014.
21. **Tindall, B. J. (Deutsche Sammlung von Mikroorganismen).** 1993. Personal communication.
22. **Wisseman, C. L., Jr., J. E. Samdel, F. E. Hahn, and H. E. Hopps.** 1954. Mode of action of chloroamphenicol on assimilation of ammonia and on synthesis of proteins and nucleic acids in *Escherichia coli*. J. Bacteriol. **67:**662–673.