

FINAL REPORT ON RESEARCH GRANT NASA - NsG-693

"Studies on trace elements in the sporulation of bacteria and the germination of bacterial spores"

Period of the grant: 6/64 - 12/67

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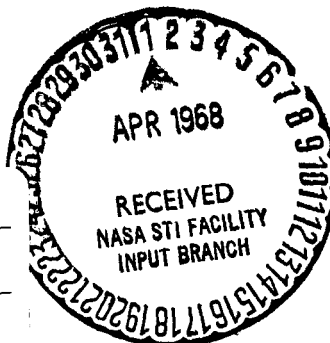
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I. General statement with regard to the relationship between studies on the morphogenesis of bacterial spores and space science.

The change from a bacterial vegetative cell possessing the usual attributes of living systems with regard to sensitivities to various physical and chemical agents to a bacterial spore having unusual resistant properties is a unique transformation in biological material. Such a change, subject to vigorous control and quantitation, represents a model system for studying morphogenesis or differentiation at a very basic level. These highly resistant spore structures representing the highest degree of resistance among terrestrial living things have often been speculated upon as possible likely candidates as earth representatives of extraterrestrial life. Present origin of life theories consider the possibility that living substances may have been carried to earth on extraterrestrial bodies. Whenever this possibility is considered, bacterial spores are cited as the living form which may be able to withstand the various extreme environmental conditions to which such a body would be exposed.

Also, in plans to explore life in outer space, much consideration is given to spore resistance in the necessary sterilization of interplanetary vehicles and in the possibility that one of the likely forms which might be discovered would be bacterial spore-like life.

Therefore, studies on the morphogenesis of bacterial spores have some bearing on exobiology or space biology.

II. List of Personnel Engaged on the Project.

	Period
Dr. Ralph A. Slepecky, Principal Investigator,	6/64 - 12/67
Miss Zita Celkis, Technician	6/64 - 6/67
Mr. Jeffrey Buckman, Research Assistant	6/64 - 7/65
Mr. Howard Gruft, Graduate Student	6/64 - 8/66
Mr. John Gillis*, Graduate Student	6/64 - 6/65
Mr. Jere Northrop, Research Fellow	6/65 - 12/67
Mr. Wayne Crosby, Research Assistant	2/66 - 12/67
Mr. Robert Longworth, Research Assistant	6/66 - 6/67

* Present address: Missile and Space Division, General Electric Co., P.O. Box 8555, Philadelphia, Pa.

III. Publications during the period of the report. (Some of the publications were supported also by an NSF Grant on a related topic)

- Rogolsky, M. and Slepecky, R.A.. Elimination of a genetic determinant for sporulation of Bacillus subtilis with acriflavin. Biochem. Biophys. Res. Commun., 16:204-208 (1964). (Research Article).
- Kolodziej, B. and Slepecky, R.A. Trace metal requirements for sporulation of Bacillus megaterium. J. Bacteriol., 88:821-830 (1964). (Research Article).
- Rogolsky, M. and Slepecky, R.A. Induction of asporogeny in Bacillus subtilis with acriflavine. Bact. Proc., 1965 p. 36 (An abstract).
- Gillis, J.R., Rosas del Valle, M., Vinter, V. and Slepecky, R.A. Sporulation studies in a synchronous culture of Bacillus megaterium. Bact. Proc., 1965:37 (An abstract).
- Gruft, H., Buckman, J. and Slepecky, R.A. Amino acid replacement of the manganese sporulation requirement of Bacillus megaterium in a synchronous system. Bact. Proc., 1965:37 (An abstract).
- Slepecky, R.A. Spores III. American Society for Microbiology News, 31:66-67. (1965) (A book review).
- Vinter, V. and Slepecky, R.A. Direct transition of outgrowing bacterial spores to new sporangia without intermediate cell division. J. Bacteriol., 90:803-807 (1965). (Research Article).
- Remsen, C.C., Lundgren, D.G. and Slepecky, R.A. Inhibition of spore septum and forespore membrane development in Bacillus cereus by beta-phenethylalcohol. J. Bacteriol., 91:324-331 (1966) (Research Article).
- Slepecky, R.A. The use of combined sonication-germicide treatment in surgical instrument cleaning. Hospital Topics, 44:133-134 (1966) (Research Article).
- Northrop, J. and Slepecky, R.A. Temperature induced sporulation mutants in Bacillus subtilis. Bact. Proc., 1966:16 (An abstract).
- Imanaka, H. and Slepecky, R.A. Enzyme synthesis during sporulation of Bacillus megaterium in a synchronous growth system. IX International Congress for Microbiology. Abstracts of Papers 1966:151.
- Northrop, J., and R.A. Slepecky. Sporulation mutations induced by heat in Bacillus subtilis. Science, 155:838-839 (1967) (Research Article).
- Imanaka, H., Gillis, J.R. and Slepecky, R.A. Synchronous growth and sporulation of Bacillus megaterium. J. Bact., 93:1624-1630 (1967) (Research Article).
- Ellar, D.J., Lundgren, D.G., and Slepecky, R.A. Fine structure of the mesosome and its involvement in cell wall synthesis and sporulation of synchronously dividing Bacillus megaterium. Bact. Proc., 1967:24 (An abstract).
- Imanaka, H. and Slepecky, R.A. Patterns of enzyme synthesis during sporulation of Bacillus megaterium. Bact. Proc., 1967:28 (An abstract).
- Imanaka, H. and Slepecky, R.A. The inhibition of the sporulation of Bacillus megaterium. VII Intnt'l. of Biochemistry, Abstracts 1967.
- Ellar, D.J., Lundgren, D.G. and Slepecky, R.A. Fine structure of Bacillus megaterium during synchronous growth. J. Bact., 94:1189-1205 (1967) (Research Article).

- Rogolsky, M. and Slepecky, R.A. The response of sporogenesis in Bacillus subtilis to acriflavine. Can. J. Microbiology, (in press) 1967 (Research Article).
- Slepecky, R.A. Synchrony and the formation and germination of bacterial spores. "The Cell Cycle: genetic and developmental aspects." (Eds. G.M. Padilla, G.L. Whitsan and I.L. Cameron) Academic Press 1968. (In press) (An invited review).
- Northrop, J. and Slepecky, R.A. Kinetics of mutation in a non-replicating system. (In preparation).
- Crosby, W. and Slepecky, R.A. Analysis of metals in spores of Bacillus megaterium (In preparation).

IV. Resumé of the research done on the project.

Earlier reports indicated the areas of study and the various approaches that have been used in an attempt to understand the relationships between trace elements and the formation of spores and their subsequent dormancy and germination. The areas studied may be regulated to the following categories: a) the manganese sporulation requirement, b) trace elements and germination in a highly purified system, c) comparison of the germination of asynchroneously grown with synchronously grown spores and d) temperature induced sporulation mutants.

The manganese sporulation requirement (Gruft, H., Buckman, J., and Slepecky, R.A., Bact. Proc. 1965:37 (an abstract)) has been covered in the 1 June 1965 and 30 January 1967 reports. It can be stated that a sporeformer needs manganese at a proper concentration before growth ceases in order to sporulate. There appears to be three levels of manganese required for the morphogenesis: one for growth; one for forespore formation; and one for spore formation. The third level can be replaced by amino acids suggesting that it is required for a protease. Our evidence to date for the activation of a protease has been largely circumstantial based on studies with crude extracts and our attempts to isolate a specific protease have been unsuccessful to date. The current hypothesis for the need for the manganese at other times also revolves around the activation of particular enzymes. These previous studies were concerned with various additions of manganese to manganese deficient cells at various times of the growth cycle. At present, we are trying to correlate these results with data obtained by measuring the manganese levels of the various cell stages using atomic absorption spectrophotometry (AAS).

Concurrently, other studies in our lab are concerned with the pattern of formation of various enzymes, many of which are suspected to be manganese activated, during the cycle. The previous studies on the Mn requirement, based on the additions of Mn to Mn deficient cells at various times of the growth cycle, are being extended by measuring the Mn levels of the various cell stages. We hope to compare Mn uptake patterns with known enzymatic patterns established with out synchronous system. We are currently measuring the Mn levels using the atomic absorption spectrophotometer (AAS) and we have extended this approach to other metals as well.

In anticipation of experiments on the germination of Mn-low spores and on the location and fate of the important metals of spores, we have developed a simple reproducible AAS method for spore metal analysis. Parallel assays by means of the AAS were done using spores of B. megaterium treated by: a) acid digestion, b) dry ashing at 550C, c) acid treatment, and d) no treatment. Comparison of the results indicate a high degree of correlation and reproducibility with Mg, Mn and Zn. Results with Ca (with and without lanthanum treatment) and Fe have been inconclusive and we are attempting to resolve the assays of these metals. Typical results are shown in the following Table:

Table 1. Assay of metals in B. megaterium spores after various treatments.

metal	Treatment			
	untreated ¹	acid ² treated	acid digested ³	dry ⁴ ashed
Mg	1.60	1.93	2.00	2.10
Mn	3.04	3.18	3.43	3.36
Zn	0.20	0.23	0.26	0.24
Ca ⁵				
+La	7.10	7.10	3.02	---
-La	4.65	5.76	5.37	---
Fe ⁶	1.30	2.19	2.84	2.46

¹ Whole spore suspension in distilled deionized water.

² Spores + HCl to pH 1.

³ Digested with HNO₃ & H₂SO₄.

⁴ 550C for 3 hr.

⁵ Lanthanum is added to suppress PO₄ interference.

⁶ Elution studies indicate that Fe is not all released by acidification to pH 1. Figures in table refer to PPM.

It appears that Ca and Fe can not be determined directly but that prior treatment is required. In subsequent studies we used acid treated spores.

The preliminary results with the use of the AAS to examine spore metal content show much promise. By using a suitable suspension of clean spores in the proper liquid phase, we have been able to analyze directly the content of such biologically important metals as Na, K, Ca, Mg, Mn, Zn and Fe in the 0.1 to 10 PPM range. Subsequently, with this type approach we may be able to assess the importance of these metals in maintaining the high dormancy found in bacterial spores. The following table gives the metal content of different batches of B. megaterium spores:

Table II. Metal content of B. megaterium spores.*

Na	K	Mg	Mn	Zn	Fe	Ca**
--	--	0.42	0.72	0.03	0.51	1.19
--	--	0.30	0.48	0.03	0.43	1.05
--	--	0.53	0.65	0.15	0.92	1.70
0.57	0.81	0.31	0.56	0.21	0.57	1.15
0.57	0.78	0.31	0.54	0.19	0.56	1.14
0.64	0.58	0.33	0.66	0.14	0.54	1.09
--	--	0.28	0.34	0.22	0.94	0.93

* Acid treated with HCl to pH 1. Figures are per cent of dry weight. Each line of figures in the table represents a different batch of spores.

** Analysis done in the presence of lanthanum.

As can be seen from Table II fairly good agreement of the metal content of various batches of spores has been obtained. Even though previous results indicated difficulties with Ca and Fe with regard to different amounts of metal being analyzed by the various treatments, approximately the same amount of each metal is being analyzed by one particular treatment from each batch of spores.

A series of preliminary experiments were carried on with the aim of determining the relative binding of the metals in the spore. Essentially these experiments consisted of examining spore supernatants

after each stage treatment during a sequence of treatments of the spores: suspension in deionized water for 10 days; 0.02M EDTA, pH 5.2 for 2 hrs; acidification to pH 2.5 for 2 hrs; acidification to pH 1.75 for 2 hrs; acidification to pH 1.30 for 2 hrs. Following these treatments the spores were examined for residual metal. From this type of experiment the following tentative conclusions can be made with regard to the relative binding of metals in spores:

Calcium--is either tightly bound or in an inaccessible region of the spore. Only 6% is eluted during storage in distilled water, and EDTA (known to be an effective chelator of Ca) removes only 12% of the total calcium. Most of the spore Ca (70%) is removed by acidification to pH 1.30, while 11% remains bound to the residual spore material.

Iron--appears to exist in at least two distinct regions (or forms) within the spore. EDTA removes 37% of the total iron, while acidification following the EDTA treatment replaces only small amounts of this metal. However, the residual spore material still contains 40% of the total Fe.

Potassium--69% of the K was removed from the spores by simple elution for 10 days in deionized water. Consistently smaller amounts of the metal were removed during each succeeding step. Potassium is probably bound largely to the surface of the spores; this is a natural result of the great excess of K ions in the growth medium (1500 ppm).

Magnesium--is rather easily removed from the spore structure. During the 10 day elution period in water, the spores lose 32% of their Mg, and an additional 37% is removed by EDTA. After acidification, only 1.9% of the Mg remains in the spore residual material. Although Mg is an essential cation for normal metabolism, it appears to be much more loosely bound than calcium in the spore. This may indicate a replacement (i.e. 'antagonism') of Mg by Ca ions. The Mg/Ca ratio of these spores is 0.272.

Manganese--is found unevenly distributed throughout all the elution fractions, indicating several roles or locations of Mn within the spore. Eleven percent is eluted in water, 26% chelated by EDTA, 54% removed by acidification, and 9% remains in the residual spore material.

Zinc--is either loosely bound or located near the surface of the spore. Almost 90% of the total zinc is removed by a combination of water elution and EDTA treatment. This loose association is not due to the same mass binding effect as was noted with K, for the growth medium contains only 4 ppm Zn. Some zinc (4%) is found in the residual material after acidification.

From the data presented, it appears that each of the metals analysed has a characteristic pattern of elution from the intact spore. Based upon their ease of removal from B. megaterium spores and their distribution in the eluted fractions, these eight metals can be grouped in the following classification:

- A. Loosely bound, eluted from spores during storage in distilled water (K, Na, Zn and perhaps Mg).
- B. Removed by a chelator of divalent cations, EDTA (Fe, Zn, and Mg).
- C. Replaced by moderate acidification (range of pH 2.5 to 1.3) (Ca and Mn).
- D. Tightly bound, found in the residual spore material after acidification (Fe and Ca).
- E. Distributed in several fractions, possibly indicating multiple locations or roles in the spore (Mg and Mn).

With regard to the phase concerned with trace elements and germination, it appears that molar ionic effects, in either a potassium phosphate buffered system or a sodium chloride unbuffered system, supplemented with the physiological germinants, L-alanine and inosine, were solely caused by potassium or sodium, and that any extraneous heavy metal or alkaline earth metal contamination displayed either inert or inhibitory activity in the germination process. The validity of the purification procedures was confirmed by atomic absorption spectrophotometry. We are checking these results further since such a finding strengthens the ionic germination ideas of Rode and Foster (Arch. Mikrobiol. 43:201 (1962) and Nature 194:1300 (1962)) and may simplify further studies of ion involvement. For example, we can ask the question, without being concerned with other ions, whether the sodium and potassium are acting at the surface or internally.

Our recent studies have shown that spores formed in a synchronous growth system exhibited a faster rate of germination than those grown in an asynchronous system. We have examined this phenomenon further by comparing the germination of both types of spores at various times after formation. Asynchronous spores did not exhibit large differences in germinative ability, and the variations were not consistent. From this, it was concluded that the effect of age on germinative ability could not be observed clearly using the asynchronous system. The most probable reason for the small and inconsistent variation

was the heterogeneity of ages in any sample from an asynchronous system. On the other hand, the results of the experiments using synchronous systems appeared to be intrinsically similar, if not parallel. Synchronously formed sporangia germinated most rapidly and most completely. As the sporangia became older and free spores began to be released, germinative ability decreased. These studies are preliminary but are being continued for they may lead to more knowledge on dormancy and the breaking of that unusual state.

Lastly, we have been concerned with temperature induced sporulation mutants in Bacillus subtilis. This work was related in the 1 January 1966 report and an abstract and a paper has been published (Northrop, J., and Slepecky, R.A., Bact. Proc. 1966 and Science 155:838 (1967)). As indicated in the previous report these studies may support the hypothesis of cytoplasmic genetic determinants (plasmids or episomes) for sporulation previously presented from this laboratory (Rogolsky, M., and Slepecky, R.A., BBRC 16:204-208 (1964)) and they may be germane to the problem of dormancy and the elimination of spores by high temperatures, a problem of much concern to NASA in its planetary quarantine program.

V. Comments of Principal Investigator.

Syracuse University, as other privately endowed institutions, can provide only limited support of an active research program. The funds from this grant have been extremely important in the execution of the project as outlined in the various reports and renewal requests as well as aiding in the overall laboratory aim of elucidating the morphogenesis of spores.

The funds of this grant have supported interested graduate and undergraduate students and have helped the principal investigator in directing students towards careers in experimental biology in various fields. As an example, one student became extremely interested in the biological aspect of NASA's Space Biology Program after reading many of the reports circulated to this grantee. The main result of his interest was his seeking a permanent position in Space biology after graduation. He is now employed with General Electric Co. (See Sect. II) and is actively engaged in their space program. Currently he is involved in the biology satellite program as well as coauthoring a handbook for engineers on NASA planetary sterilization procedures.

The principal investigator is grateful to NASA for the aid in carrying out his research program.