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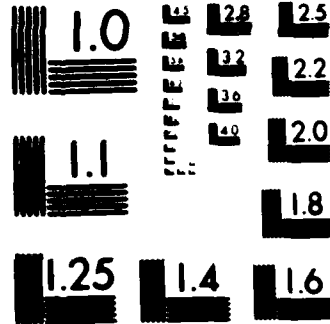
MECHANISMS OF RESISTANCE IN MICROBIAL SPORES(U)
MICHIGAN STATE UNIV EAST LANSING DEPT OF MICROBIOLOGY
AND PUBLIC HEALTH P GERHARDT 14 NOV 86 ARO-19787 6-LS
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Final Technical Report

U.S. Army Research Office
Contract DAAG29-83-K-0057
ARO Proposal 19787-LS

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MECHANISMS OF RESISTANCE IN MICROBIAL SPORES

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14 November 1986

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER ARO Proposal 19787.6-LS		2. GOVT ACCESSION NO.		3. REPORT'S CATALOG NUMBER	
4. TITLE (and Subtitle) MECHANISMS OF RESISTANCE IN MICROBIAL SPORES			5. TYPE OF REPORT & PERIOD COVERED Final Technical Report 14 Mar 1983 - 13 Sep 1986		
7. AUTHOR(s) Philipp Gerhardt			8. CONTRACT OR GRANT NUMBER(s) DAAG29-83-K-0057		
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology and Public Health Michigan State University East Lansing, Mi 48824			6. PROGRAM ELEMENT PROJECT TASK AREA & WORK UNIT NUMBERS		
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Research Office Post Office Box 12211 Research Triangle Park, NC 27709			2. REPORT DATE 14 Nov 1986		
			13. NUMBER OF PAGES		
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)			15. SECURITY CLASS (of this report) Unclassified		
			15a. DECLASSIFICATION/DOWNGRADING SCHEDULE		
16. DISTRIBUTION STATEMENT of this Report Approved for public release; distribution unlimited					
17. DISTRIBUTION STATEMENT of the contract entered in Block 20 (if different from Report) NA					
18. SUPPLEMENTARY NOTES The view, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.					
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Spores, Microbes, Resistance					
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) See next page					

20. ABSTRACT

Bacterial spores possess extraordinary resistance against destruction by heat and other deleterious agents, so extreme safeguards must be taken to prevent spore-caused infections, food spoilage and food poisoning. A determination of the physicochemical bases and physiological mechanisms accounting for spore resistance is the goal of this project. The rationale of approach is to employ biophysical probes that are invasive but not destructive to the cellular and molecular configurations conferring resistance in intact spores. Genetic, biochemical and microbiological techniques are also employed. The project involves collaboration by teams of investigators at two universities. Substantial progress has been accomplished during the contract period, with a number of articles published or in press.

At the University of Rochester, dielectric characterization of forespores indicated that the dehydration of a forespore during stage III of sporogenesis may be the result of ion movements out of the forespore into the sporangium. Mureins in the spore cortex were shown to be highly elastic, flexible, and well designed for their roles in compressing the spore protoplast. The heat resistance (HR) of fully demineralized spores was measured in order to assess the underlying resistance due to dehydration and other factors. Remineralization by Ca, Mn, Mg and K (but not Na) was found to increase HR in order of effectiveness and dependent on killing temperature. Thus, mineralization is clearly an important factor in sporal heat resistance, but the relationship with other factors is complex.

At Michigan State University, four morphotypes of *B. megaterium* spores, obtained by progressive divestment of the integument layers, were found to differ little in HR or germinability but greatly in permeability and lysozyme sensitivity (Lsz^S). Use of such Lsz^S spores enabled quantification by differential permeability measurement of the unequal distribution of water within the spores, the protoplast containing 27-29% (wet weight basis). A new and better method was devised to determine protoplast water content (PWC) by means of buoyant density sedimentation in a permeating medium. Thereby, the PWC was determined with 28 types among 7 *Bacillus* species spanning a 3,000-fold range in heat resistance, which was altered by acid demineralization and specific remineralization and also by thermal adaptation. These factors caused changes in PWC and thereby changes in HR between limits of 57 and 28% in PWC. Outside these limits, however, these factors correlated independently with HR. Thus, protoplast dehydration, mineralization and thermal adaptation all contribute to sporal heat resistance in a complex relationship, but dehydration predominates.

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STATEMENT OF THE PROBLEM STUDIED

Bacterial spores possess extraordinary resistance against destruction by heat and other deleterious agents, so extreme safeguards must be taken in medicine and industry to cope with this property. For example, surgical instruments must be sterilized by autoclaving to prevent the introduction of tetanus or gangrene spores into wounds, and foods must be pressure-heat canned to prevent clostridial spoilage and the survival of toxigenic botulism spores. If it were not for bacterial spores, or if their resistance could be circumvented, such precautionary measures could be greatly lessened.

Bacterial spores furthermore exemplify the general biological phenomenon of dormancy or cryptobiosis, the deathlike state of suspended activity first demonstrated in 1702 by Leeuwenhoek with rotifers and nematodes. Cryptobiosis occurs also in the primitive tardigrade animals, the cysts and larvae of certain crustaceans and insects, the seeds of plants, the spores of fungi, and certain viruses. But in bacteria the phenomenon occurs in extreme degree: for example, spores of thermoactinomycetes have been revived after survival in archaeological deposits for almost 2,000 years. Bacterial spores thus provide an excellent unicellular, prokaryotic model for studying the extended maintenance of the organized structure that characterizes living organisms.

Despite these practical and theoretical implications, and a century or research, how spores achieve resistance remains undefined. In seeking to explain the resistance of spores, it is necessary to discern both the physiochemical bases for stabilization of essential organelles and macromolecules and the physiological mechanisms for attainment of the stabilization. For example, a low water content in the sporal protoplast might account in part for the stabilization, and an inward pressure exerted by the cortex expressing water from the protoplast might account for the attainment of this state. Furthermore multiple bases and mechanisms probably prevail. To attain thermostability, a labile molecule or organelle must be immobilized in some manner, such as by removal or structuring of the surrounding water or by restriction of motion in molecular structure imposed by mechanism such as contraction and cross-bonding. Not only is it necessary to account for the resistance of the vital organelles and molecules within the protoplast, but also of the enzymes outside the protoplast which are

required for initiation of the germination process. Moreover, possible differences between the nature of resistance to one type of agent (e.g., moist heat) and another (e.g., dry heat or radiation) must be considered.

A key to solution of the problem appears to lie with unique in situ properties which are lost when the spore is analyzed by the usual extraction and fractionation techniques. The crucial properties appear essentially biophysical in character. Consequently, it appears necessary to examine cellular and molecular configurations conferring resistance. We have developed methods of nondestructively probing the spore interior, particularly dielectric, light refractometric and molecular permeation techniques. We also have explored the use of other biophysical techniques, some quite fruitful such as ultrasonic analysis.

This biophysical approach is combined in the present project with the use of genetic, biochemical and microbiological techniques. The isolation of coatless mutants and the chemical stripping of integument layers are seen as particularly valuable for obtaining structurally simplified spore models. The chemical exchange of specific single cations in place of the naturally occurring mixture is equally valuable.

These complementary approaches are brought together by long-standing collaboration between the teams of investigators in laboratories at Michigan State University and the University of Rochester. Each laboratory shares its special skills and facilities with the other, the same spore models and preparations are used as much as possible to enable correlations, and ideas are exchanged regularly.

Furthermore, this collaborative project is part of an international inter-disciplinary program for research on spore resistance. The program has included seminar-workshop meetings, publication of progress reports in special issues of Spore Newsletter, exchange of individual scientists for short and long-term working visits, and sharing of spore preparations.

The present project succeeds a prior USARO contract and precedes a current one. Substantial progress has been accomplished toward accomplishment of the general goal, which is to explain the physiocochemical bases and the physiological mechanisms accounting for the heat resistance of microbial spores.

PARTICIPATING SCIENTIFIC PERSONNEL

At Michigan State University: Philipp Gerhardt, Teofila C. Beaman, James A. Lindsay, Satoshi Nakashio, Tomihiko Koshikawa, L. P. Lin, H. Stuart Pankratz, Thomas R. Corner.

At University of Rochester: Robert E. Marquis, Gary R. Bender, Edwin L. Carstensen, Sally Z. Child.

SUMMARIES OF RESULTS AND LIST OF PUBLICATIONS

Reproduced below are the reprint or preprint abstracts of published papers from USARO-supported research during the contract period, together with the journal reference citations.

At Michigan State University:

Koshikawa, T., T.C. Beaman, H.S. Pankratz, S. Nakashio, T.R. Corner, and P. Gerhardt. 1984. Resistance, germination, and permeability correlates of *Bacillus megaterium* spores successively divested of integument layers. *J. Bacteriol.* 159: 624-632.

A variant strain that produced spores lacking exosporium was isolated from a culture of *Bacillus megaterium* QM-B1551. Two additional spore morphotypes were obtained from the parent and variant strains by chemical removal of the complex of coat and outer membrane. Among the four morphotype spores, heat resistance did not correlate with total water content, wet density, refractive index, or dipicolinate or cation content, but did correlate with the volume ratio of protoplast to protoplast plus cortex. The divestment of integument layers exterior to the cortex had little influence on heat resistance. Moreover, the divestment did not change the response of either the parent or the variant spores to various germination-initiating agents, except for making the spores susceptible to germination by lysozyme. The primary permeability barrier to glucose for the intact parent and variant spores was found to be the outer membrane, whereas the barrier for the divested spores was the inner membrane.

Beaman, T.C., T. Koshikawa, H.S. Pankratz, and P. Gerhardt. 1984. Dehydration partitioned within core protoplast accounts for heat resistance of bacterial spores. *FEMS Microbiol. Letters* 24:47-51.

Water distributed unequally within the dormant bacterial spore was quantified for the first time by use of three different lysozyme-sensitive morphotype spores of *Bacillus megaterium*. The extent of protoplast dehydration (27-29 g water/100 g wet protoplast) was sufficient to account for the heat resistance of such spores.

Nakashio, S. and P. Gerhardt. 1985. Protoplast dehydration correlated with heat resistance of bacterial spores. *J. Bacteriol.* 182:571-578.

Water content of the protoplast in situ within the fully hydrated dormant bacterial spore was quantified by use of a spore in which the complex of coat and outer (pericortex) membrane was genetically defective or chemically removed, as evidenced by susceptibility of the cortex to lysozyme and by permeability of the periprotoplast integument to glucose. Water content was determined by equilibrium permeability measurement with ³H-labeled water (confirmed by gravimetric measurement) for the entire spore, with ¹⁴C-labeled glucose for the integument outside the inner (pericytoplasm) membrane, and by the difference for the protoplast. The method was applied to lysozyme-sensitive spores of *Bacillus stearothermophilus*, *B. subtilis*, *B. cereus*, *B. sturtevantii*, and *B. megaterium* (four types). Comparable lysozyme-resistant spores, in which the outer membrane functioned as the primary permeability barrier to glucose, were employed as controls. Heat resistances were expressed as D_{120} values. Protoplast water content of the lysozyme-sensitive spore types correlated with heat resistance exponentially in two distinct clusters, with the four *B. megaterium* types in one alignment, and with the four other species types in another. Protoplast water contents of the *B. megaterium* spore types were sufficiently low (26 to 29%, based on wet protoplast weight) to account almost entirely for their lower heat resistance. Corresponding values of the other species types were similar or higher (30 to 35%), indicating that these spores depended on factors additional to protoplast dehydration for their much greater heat resistance.

Lindsay, J.A., T.C. Beaman, and P. Gerhardt. 1985. Protoplast water content of bacterial spores determined by buoyant sedimentation. *J. Bacteriol.* 163:735-737.

Protoplast wet densities (1.315 to 1.400 g/ml), determined by buoyant density sedimentation in Metrizamide gradients, were correlated inversely with the protoplast water contents (26.4 to 55.0 g of water/100 g of wet protoplast) of nine diverse types of pure lysozyme-sensitive dormant bacterial spores. The correlation equation provided a precise method for obtaining the protoplast water contents of other spore types with small impure samples and indicated that the average protoplast dry density was 1.460 g/ml.

Beaman, T.C., and P. Gerhardt. 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. *Appl. Environ. Microbiol.* 52: (December, in press).

Twenty-eight types of lysozyme-sensitive spores among seven Bacillus species representative of thermophiles, mesophiles and psychrophiles were obtained spanning a 3000-fold range in moist-heat resistance expressed as D_{100} values. The resistance within species was altered by demineralization of the native spores to protonated spores and remineralization of the protonated spores to calcified spores, and by thermal adaptation at maximum, optimum and minimum sporulation temperatures. Protoplast wet densities and thereby protoplast water contents were obtained by buoyant density sedimentation in Nycodenz gradients. Increases in mineralization and thermal adaptation caused reductions in protoplast water contents between limits of about 57 and 28% (wet-weight basis), and thereby correlated with increases in spore heat resistance. Above and below these limits, however, increases in mineralization and thermal adaptation correlated with increases in spore resistance independently of unchanged protoplast water contents. All three factors evidently contributed and were necessary for heat resistance of the spores, but dehydration predominated.

At University of Rochester:

Marquis, R.E., G.R. Bender, E.L. Carstensen, and S.Z. Child. 1983. Dielectric characterization of forespores isolated from Bacillus megaterium ATCC 19213. J. Bacteriol. 153:436-442.

Isolated stage III forespores of *Bacillus megaterium* ATCC 19213 in aqueous suspensions were nearly as dehydrated as mature spores, as indicated by low dextran-impermeable volumes of ca. 3.0 ml per g (dry weight) of cells compared with values of ca. 2.6 for mature spores and 7.3 for vegetative cells. The forespores lacked dipicolinate, had only minimal levels of calcium, magnesium, manganese, potassium, and sodium, and were more heat sensitive than vegetative cells. The effective homogeneous conductivities and dielectric constants measured over a frequency range of 1 to 200 MHz indicated that the inherent conductivities of the forespores were unusually low, in keeping with their low mineral contents, but that the forespores could be invaded by environmental ions which could penetrate dielectrically effective membranes. Overall, our findings support the view that the dehydration of a forespore during stage III of sporogenesis may be the result of ion movements out of the forespore into the sporangium.

Marquis, R.E., G.R. Bender, E.L. Carstensen, and S.Z. Child. 1983. Electrochemical and mechanical interactions in bacillus spore and vegetative mureins, p. 43-83. In R. Hakenbeck, J.V. Holtje, and J. Labischinski (ed.) The target of penicillin. Walter de Gruyter, Berlin.

1. Insoluble mureins of B. megaterium spores appear to be highly elastic, flexible and well designed for their roles in containing the high hydrostatic pressures of the spore core.
2. The electrochemical properties of spore mureins can be predicted at least roughly from a knowledge of their chemical structure and the known behavior of vegetative mureins.

Marquis, R.E. and G.R. Bender. 1985. Mineralization and heat resistance of bacterial spores. J. Bacteriol. 161:789-791.

The heat resistances of the fully demineralized H-form spores of *Bacillus megaterium* ATCC 19213, *B. subtilis* var. *niger*, and *B. stearothermophilus* ATCC 7993 were compared with those of vegetative cells and native spores to assess the components of resistance due to the mineral-free spore state, presumably mainly from dehydration of the spore core, and to mineralization. Mineralization greatly increased heat resistance at lower killing temperatures but appeared to have much less effect at higher ones.

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Marquis, R.E., E.L. Carstenson, G.R. Bender, and S.Z. Child. 1985. Physiological biophysics of spores, p. 227-240. In G.J. Dring, D.J. Ellar, and G.W. Gould (ed.), Fundamental and applied aspects of bacterial spores. Academic Press, London.

Information obtained by use of simple, nondestructive, physical techniques is surveyed to obtain a clearer picture of the nature of dehydration during sporogenesis, the physical states of spore electrolytes and the relationship of heat resistance to mineralization. Major dehydration during stage III of sporogenesis seems to depend on osmotic-metabolic mechanisms involving the inverted, outer, forespore membrane. Subsequent dehydration and the maintenance of the dehydrated state appear to depend on cortical peptidoglycan elasticity. Minerals in the spore core are immobilized, but those in enveloping structures of many spores are mobile. Heat resistance appears to be acquired incrementally in association with dehydration and specific mineralization.

Bender, G.R., and R.E. Marquis. 1985. Spore heat resistance and specific mineralization. Appl. Environ. Microbiol. 50:1414-1421.

Spores of *Bacillus megaterium* ATCC 19213, *Bacillus subtilis niger* and *Bacillus stearothermophilus* ATCC 7953 were converted to fully demineralized, but viable, H forms by controlled acid titration. H forms were more heat sensitive than were native forms, but z values were greater for killing of H spores than those for native spores. Therefore, the differences in heat sensitivity between native and H forms decreased with increasing killing temperature. The increase in heat sensitivity associated with demineralization did not appear to be due to damage to cortex lytic enzymes of the germination system because it could not be moderated by decontaminating heated H spores and plating them on medium with added lysozyme. H spores could be remineralized by means of back titration with appropriate base solutions. The remineralized spores, except for the Na form, were then more heat resistant than were H spores. Ca and Mn were more effective in restoring resistance than were Mg and K. Generally, the remineralized forms (except for the Na form) had z values greater than those of the native forms but still less than those of the H forms. At lower killing temperatures, the reinstatement of resistance could be related to the extent of remineralization. However, at higher killing temperatures, only a fraction of the mineral was effective in restoring resistance, and higher levels of remineralization did not result in greater resistance. Mineralization is clearly an important factor in spore heat resistance, but the relationship between resistance and mineralization is complex and dependent on killing temperature.

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