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RPPR Final Report
as of 28-May-2019

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Major Goals: The objectives of the proposed work are to determine parameters of metabolic activity in dormant spores of Bacillus species, and whether dormant spore metabolic activity alters spore properties such as resistance to heat and chemical agents, as well as ease of germination. Spores of Bacillus species as well as closely related Clostridium spores are major causes of food spoilage and food borne illness, as well as a number of serious diseases, largely because of these spores' dormancy and resistance. It has long been accepted dogma that spores have no metabolic activity. However, this dogma was called into question by a 2012 paper (Segev et al. 2012. Cell 148:139-149) that provided evidence that there was massive rRNA and mRNA degradation as well as significant transcription in dormant Bacillus subtilis spores held at 37 or 50°C for 3-8 d.
Our laboratory followed-up the Cell paper and found evidence of slow metabolic activity in spores stored at 37-50° C, but only in spent sporulation medium, not during storage in water, and no ATP was detected. The current proposal seeks to extend this initial study much further to establish the magnitude of this metabolism and its effects on dormant spore properties, using primarily spores of B. subtilis and B. megaterium. The specific goals of the new research are as follows. 1) Identify and quantitate low mol wt catabolites in spores, including the levels of malate recently reported to be significant in spores. This analysis will utilize 13C- and 31P-NMR, as well as sensitive enzymatic assays for ATP. 2) For any new catabolites identified, determine the fate of such catabolites: a) during extended storage of spores at physiological temperatures; and b) during normal spore germination. 3) If malate is found in spores at high levels, the levels of enzymes involved in malate metabolism will be determined in dormant spores. 4) 3- Phosphoglyceric acid (3PGA) is a major catabolite in spores of Bacillus and Clostridium species, and its catabolism to acetate in the first min of germination generates ATP and NADH. 3PGA is also catabolized slowly in dormant spores in some conditions, and we will determine if it is catabolized to acetate, or is the phosphate removed giving glyceric acid. 5) Determine the rates of degradation of rRNA during dormant spore incubation at physiological temperatures under conditions where spore germination is prevented. 6) Measure levels of mRNAs or mRNA remnants in spores during extended incubation at either 4 or 37°C to determine if specific mRNAs are degraded or synthesized. We will also examine levels of mRNAs in spores prepared under different conditions to learn if levels of these RNA species in dormant spores vary depending on sporulation conditions. 7) Since the spore core's low water content is likely a major factor in low enzymatic activity in the spore core, we will increase core water content by alterations in spore cortex structure and/or sporulation at different temperatures, and examine the effects of these changes on dormant spore metabolism. 8) Examine the effects of dormant spore metabolism on spore properties by examining the germination and resistance of spores in which there has been either no dormant spore metabolism (held at 4°C), or extensive dormant spore metabolism (held for extended periods at physiological temperatures along with other changes found to promote dormant spore metabolism). These studies should provide important new information on how bacterial spores prepare for dormancy, act during dormancy and whether metabolic changes during dormancy alter spore properties.

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Accomplishments: See uploaded pdf document

Training Opportunities: Have had two undergraduates and a recent college graduate who participated in the research under this contract, all of whom are URM students, and all are coauthors on at least one publication.
1- Stephen Abini-Agbomson, worked as a UConn Undergraduate, now a graduate student in Biophysics at NYU (1 publication).
2-Janelle Robinson, worked as a rising senior at Northeastern University (1 publication).
3 - Joshua Green, worked as a recent graduate of Southern CT State University - going to Temple School of Podiatric Medicine this coming fall (2 publications).

Results Dissemination: 6 Refereed publications from the work supported under this contract as follows.

1. Korza, G., B. Setlow, L. Rao, Q. Liu and P. Setlow. 2016. Changes in Bacillus spore small molecules, rRNA, germination and outgrowth after extended sub-lethal exposure to various temperatures: evidence that protein synthesis is not essential for spore germination. J. Bacteriol. 198:3254-3264 (Special commentary paper, and a press release).
2. Korza, G., S. Abini-Agbomson, B. Setlow, A. Shen and P. Setlow. 2017. Levels of L-malate and other low molecular weight metabolites in spores of Bacillus species and Clostridium difficile. PLoS One. 12:e0182656.
3. Setlow, P., S. Wang and Y.-Q. Li. 2017. Germination of spores of the orders Bacillales and Clostridiales. Annu. Rev. Microbiol. 71:459-477.
4.Setlow, P. 2019. Observations on research with spores of Bacillales and Clostridiales species. J. Appl. Microbiol. 126:348-358.
5. Korza, G., E. Camilleri, J. Green, J. Robinson, K. Nagler, R. Moeller, M. Caimano and P. Setlow. 2019. Analysis of messenger RNAs in spores of Bacillus subtilis. J. Bacteriol. 201:e-00007-19. (Spotlight paper in this issue of J. Bacteriol.)
6. Camilleri, E., G. Korza, J. Green, J. Hui, Y.Q. Li, M.J. Caimano and P. Setlow. 2019. Properties of aged spores of Bacillus subtilis. J Bacteriol in press.

Have given 11 seminars on results from work under this contract as follows:

1-3) April 2016 - 3 seminars in the UK, 1) Food Research Institute in Norwich; 2) International Spore meeting in London; and 3) British Biodefense Research Establishment at Porton Down, Salisbury.
4) June 2016 - Keynote presentation at Wind River Conference in CO.
5) Feb 2017 - Seminar at the 3M Corporation in St. Paul, MN.
6) Aug 2017 - Presentation at the ARO Research Conference at Moffett Field in CA.
7-8) Nov 2017 - Two seminars at the University of Helsinki in Finland.
9) Mar 2018 - Keynote presentation at the Dutch Royal Microbiology Society Annual Meeting in the Netherlands.
10) Nov 2018 - Seminar at the Army Research Lab in MD.
11) Mar 2019 - Seminar at Amherst College.

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Gave seminar at the Army Research Laboratory in November of 2018, and while there talked to a number of Army Scientists about their research.

PARTICIPANTS:

Participant Type: PD/PI
Participant: Peter Setlow
Person Months Worked: 15.00
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

Funding Support:

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Participant Type: Other Professional
Participant: George Korza
Person Months Worked: 15.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

Participant Type: Other Professional
Participant: Emily Camilleri
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

Participant Type: Faculty
Participant: Barbara Setlow
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

In the first two years of this project we made a number of important discoveries as follows:

1.We found that a recent report in a high-profile journal (1) that when spores germinate (or are even dormant) they can obtain energy from catabolism of L-malate is wrong, as analyzing spore small molecules by several techniques including ^{13}C-NMR, we showed that spores of several species have levels of malate $\leq$ 0.5 μmol/spore, at least 60-fold lower than erroneously reported in the "high profile" paper. The absence of L-malate from spores was in agreement with much older work from the Setlow lab and others.

2. We found that *B subtilis* spores incubated at 50-75°C for 20 hr to 5 d had lost all detectable intact rRNA. However, the rRNA was not degraded to free ribonucleotides but to large "chunks". There was no detectable ATP in the *B. subtilis* spores incubated under these conditions, and there was no decrease in spore viability. Importantly: 1) the spores did not germinate during these incubations; 2) germination of the rRNA-less spores was identical to that of rRNA replete spores; 3) the return to vegetative growth of these rRNA-less spores in a rich media was indistinguishable from that of spores containing normal rRNA levels; and 4) all spore rRNA was resynthesized in the period between the end of germination and the beginning of outgrowth. These findings essentially debunk the idea promoted in several high-profile papers that: 1) there is active metabolism in dormant spores (2); and 2) that protein synthesis is essential for spore germination (1) (and see results of experiments on spore mRNA carried out in the final year of support).

In the last period of support on this project our focus has been on analysis, identification and quantitation of mRNAs in dormant *Bacillus subtilis* spores and some determination of what these mRNAs do and don't do. Previous literature has indicated that spores of *Bacillus* and *Clostridium* species have many 100s and even thousands of mRNA species (2,3-10). The papers describing these results have then made a variety of speculations on what these mRNAs might do to allow spores to get a "leg up" on the process of spore germination and return to life. For a variety of reasons, I had felt this was all completely overblown, in particular as mRNA-like RNA was identified in spores > 50 years ago (11,12) and there were data indicating that this RNA was degraded rapidly when spores germinated (13).

In any event, we set out to do RNA-seq analysis of both highly purified *B. subtilis* spores as well as spores that were not so well purified, in hopes of learning if many supposedly spore mRNAs were actually contaminants. We started doing RNA-seq in triplicate on RNA from which rRNA was removed, all from highly purified spores. This work found ~ 500 mRNAs, but with relative abundances that varied > 1000-fold, when the relative mRNA abundances are expressed as RPKM values – reads per kilobase of transcript per million reads (Table 1,2). We also knew when we got these data that the amount of mRNA nt/*B. subtilis* spore was only ~ 10^6 (14), assuming mRNA is 3% of total RNA, which is close to values determined directly for mRNA like RNA in growing *B. subtilis* cells and in spores (12,15,16). Using this value for mRNA nt and RPKM values and mRNA lengths allowed us to calculate that only ~ 46 mRNAs in *B. subtilis* spores are present at > 1 molecule/spore with all the rest at lower abundance – most at < 0.05 molecules/spores. This finding immediately indicated that the low abundance mRNAs were likely unimportant in a spore population. Equally importantly, almost all of the abundant spore mRNAs are from genes that: i) are transcribed late in the developing forespore late in spore development by RNA polymerase with the forespore-specific σ factor, σ^G; and ii) encode gene

products almost all of which have been identified by proteomic analysis in dormant spores (17,18). Thus, it seems almost certain that these spore mRNAs are not important in directing protein synthesis early in spore germination outgrowth – indeed several proteins encoded by abundant spore mRNAs are lethal in outgrowing cells if not degraded. Our current hypothesis for the role of these spore mRNAs is that they are present to deal with the fact that the dormant spore lacks a number of enzymes of nucleotide biosynthesis, which are lost in sporulation and not synthesized until later in spore outgrowth (14). Consequently, spore mRNAs appear to be degraded soon after initiation of germination and the resultant ribonucleotides are then used for new RNA synthesis. We also showed in this work that less well purified spores and purified spores extracted at pH 13 at 70^{o}C, which should chemically degrade any RNA not in the spore core, all had similar levels of the same mRNAs. This indicated that spore mRNAs are not contaminants in spore preparations. In addition, the spore mRNAs were stable for at least a week in dormant spores held at 4^{o}C.

The work described above has just been published in the Journal of Bacteriology as a paper of special interest. We have now just followed this paper with a second one which asks a very simple question, which is, what happens to spore mRNA and spores, if a sporulating culture is allowed to incubate much longer than the usual 2-3 d? We approached this problem by incubating sporulating cultures on plates for 2-98 d and measuring spore properties of viability, Ca-dipicolinic acid (Ca-DPA) content, germination, outgrowth, resistance and mRNA and ribonucleotide levels. The results of the work were as follows – again for wild-type *B. subtilis*. 1) Spores from plates incubated for 2-98 d exhibited no evidence of continued sporulation, although there was some germination on plates incubated > 1.5 months. 2) both young and old spores had identical Ca-DPA content. 3) Highly purified spores from plates incubated 2-98 d had identical viability on rich medium and poor medium plates. 3) Spores of all ages exhibited identical return to vegetative growth via germination and outgrowth in rich and minimal media (Fig. 1A,B). 4). The resistance of spores of all ages to dry heat and UV radiation was almost identical, although with NaOCl resistance slightly higher in the older spores (Fig. 2B-D). 5) Resistance of spores to wet heat rose dramatically as spores aged, with the 98 d spores much more wet heat resistant that the younger spores (Fig. 2A). 6) The levels of both the abundant and less abundant mRNAs in spores fell precipitously as spores aged, such that the 98 d spores had ≤ 1% of the mRNA level of the 2 d spores. This analysis used RNA that had not undergone rRNA removal, because rRNA was also degraded (Fig. 3; Table 3). The mRNAs were not, however, degraded to mononucleotides but to fragments. This was also the case for spore rRNAs which were also degraded > 99% but to large fragments as determined by analysis of the reads of rRNAs in RNA-seq as well as gel electrophoresis (Table 3; Fig. 3).

Overall, these results indicate that spore mRNAs are not essential for spore viability, or for spore germination and outgrowth. This is then a further indication that the function of spore mRNA is to be degraded when spores germinate to generate ribonucleotides for new RNA synthesis as noted above. A second conclusion from this work, especially given the loss of all rRNA as well as mRNA from spores but with no notable effect on spore germination and outgrowth, is that there is no requirement for protein synthesis in order to complete spore germination as was suggested a few years ago in a paper in a high-profile journal (2). A paper on the aged spore results is also now in press in J. Bacteriology (18).

An obvious question from the work above on mRNAs in *B. subtilis* spores is whether the findings on this topic are the same for spores of other spore formers. We are continuing this

work with spores of: 1) *Bacillus atrophaeus*, a close relative of *B. subtilis* spores which are used in many sterilization assurance tests; 2) *Bacillus licheniformis*, a close relative of *Bacillus anthracis*; 3) *Bacillus megaterium*; 4) *Geobacillus stearothermophilus*, also used as an indicator for sterilization assurance and a thermophile; 5) *Paenibacillus polymyxa*, evolutionarily far removed from *B. subtilis*; and 6) *Clostridiodes difficile*, a serious pathogen and far removed from *B. subtilis* evolutionarily. This work is now being supported by other lab funds, but was begun with ARO support.

References

1.Sinai et al. 2013. The molecular timeline of a reviving bacterial spore. Mol. Cell 57:695-707.
2.Segev et al. 2012. RNA dynamics in aging bacterial spores. Cell 148:139-149.
3. Bassi et al. 2013. Array-based transcriptional analysis of *Clostridium sporogenes* UC9000 during germination, cell outgrowth and vegetative life. Food Microbiol 33:11-23.
4. Bassi et al. 2016. Transcriptome analysis of *Bacillus thuringiensis* spore life, germination and cell outgrowth in a vegetable-based food model. Food Microbiol 55:73-85.
5. Bettegowda et al. 2006. The genome and transcriptomes of the anti-tumor agent *Clostridium novyi*-NT. Nat Biotechnol 12:1573-1580.
6. Dembek et al. 2013. Transcriptional analysis of temporal gene expression in germinating *Clostridium difficile* 630 endospores. PLoS One 8:e64011.
7. Nicholson WL et al. 2012. Transcriptomic responses of germinating *Bacillus subtilis* spores exposed to 1.5 years of space and simulated martian conditions on the EXPOSE-E experiment PROTECT. Astrobiol 12:469-487.
8. Nagler K et al. 2016. Identification of differently expressed genes during *Bacillus subtilis* spore outgrowth in high-salinity environments using RNA sequencing. Front Microbiol 7:1564.
9. Van Melis CC et al. 2011. Characterization of germination and outgrowth of sorbic acid-stressed *Bacillus cereus* ATCC 14579 spores: phenotype and transcriptome analysis. Food Microbiol 2:275-283.
10. Arrieta-Ortiz ML et al. 2015. An experimentally supported model of the *Bacillus subtilis* global transcriptional regulatory network. Mol Syst Biol 11:839.
11. Deutscher MP et al. 1968. Biochemical studies of bacterial sporulation and germination. XXI. Protein-synthesizing systems from vegetative cells and spores of *Bacillus megaterium*. J Biol Chem 243:5117-5125.
12. Jeng Y and Doi R. 1974. Messenger ribonucleic acid of dormant spores of *Bacillus subtilis*. *J* Bacteriol 119:514-521.
13. Keijser BJF et al. 2007. Analysis of temporal gene expression during *Bacilllus subtilis* spore germination and outgrowth. J Bacteriol 189:3624-3634
14. Setlow P, Kornberg A. 1970. Biochemical studies of bacterial sporulation and germination. XXIII. Nucleotide metabolism during spore germination. J Biol Chem 245:3645-3652.
15. Brown S, Coleman G. 1975. Messenger ribonucleic acid content of *Bacillus amyloliquefaciens* throughout its growth cycle compared with *Bacillus subtilis* 168. J Mol Biol 96:345-352.
16. Midgley JE. 1969. The messenger ribonucleic acid content of *Bacillus subtilis*. Biochem *J* 115:171-181.
17. Swarge BN, et al. 2018. "One-Pot" sample processing method for proteome-wide analysis of microbial cells and spores. Proteomics Clin Appl 12:e1700169.
18. Korza G, et al. 2019. Analysis of messenger RNAs in spores of *Bacillus subtilis*. J Bacteriol 201:e00007-19.

Table 1*

Average molecules/spore for different abundance mRNAs in highly purified *B. subtilis* spores

Avg RPKM Group	A Genes #[1]	B Length nt[2]	C RPKM	D Reads gene[3]	E RelmRNAs/ gene[4]	F mRNA nt/ spo[5]	G mRNA nt/ spo(□=10^6)[6]	H mRNA/spo[7]
>7000	29(29)	339	$1.7x10^5$	57,630	4433	$4.4x10^7$	$9.84x10^5$	100
2000-7000	7(6)	404	2789	1127	87	$2.5x10^5$	$5.6x10^3$	2
567-2000	10(6)	500	979	490	38	$1.9x10^5$	$4.3x10^3$	0.9
111-566	24(8)	565	221	125	10	$1.4x10^5$	$3.1x10^3$	0.2
1-110	432(28)	472	28	13	1	$1.1x10^5$	$2.5x10^3$	0.02
	Total = 302							

*RPKM values are averages of RNA-seq analyses carried out on 3 independent RNA preparations from highly purified dormant *B. subtilis* spores (spo) prepared on plates for 2 d at 37°C as described in Methods. Values in Columns B-E and H are averages for all mRNAs in each group. Values in Columns A, F and G are totals for all genes or mRNAs in this group.

[1]Values in parentheses are numbers of σ^G–dependent genes. There may be more σ^G-dependent genes in the two lowest groups

[2]The numbers of nucleotides in mRNAs in original RNA-seq data are the coding sequence plus the stop codon. Consequently, the values in this column have been increased by 50 nt to take into account the 3'- and 5'-untranslated regions in mRNAs.

[3]Calculated as (C x B/1000) to correct for the fact that RPKM values are based on reads per kb of transcript.

[4]ReLmRNA/gene are relative levels of spore mRNA that were calculated as D/13, the average Reads/gene for the mRNAs of the group with the lowest average RPKM value.

[5]Values were calculated as (E x A x B) for each group.

Table 2*

Characteristics of the 46 most abundant dormant *B. subtilis* spore mRNAs.

Gene name	Length-nt[1]	# Reads	RPKM[2]	Sigma Factor
		RPKM > 7000		
sspA	210	2479	10848	Sigma F&G
sspE	255	159687	577971	Sigma F&G
sspF	186	26976	131260	Sigma G
sspJ	141	20095	133439	Sigma G
sspK	153	3947	23696	Sigma G
sspM	105	2878	25626	Sigma G
sspN	147	32488	201551	Sigma F&G
tlp	252	56102	202343	Sigma F&G
sspO	147	16078	102766	Sigma F&G
sspP	147	13486	86361	Sigma F&G
yfhD	192	36871	177547	Sigma F&G
yhcN	570	4689	7464	Sigma F&G
yhcQ	654	9699	13540	Sigma G
yhcV	423	74504	161470	Sigma F&G
yhdB	243	22294	82876	Sigma G
yizC	198	16362	75142	Sigma G
ykzE	177	51891	271743	Sigma G
ykzP	156	81390	480746	Sigma G
ymfJ	258	24076	85309	Sigma F&G
yozQ	294	3085	9528	Sigma G
ypzF	147	133054	827125	Sigma G
ypzG	153	110878	652271	Sigma G
yqfX	390	16853	39843	Sigma G
yrzQ	132	11482	81387	Sigma F&G
yrzR	192	3663	17332	Sigma F&G
ytzC	273	80936	269462	Sigma F&G
ytzL	159	26849	157337	Sigma F&G
yuzA	237	2512	9616	Sigma G
yxeD	354	2697	7175	Sigma G
		RPKM = 2000-7000		
sspB	204	809	2972	Sigma F&G
sspH	180	593	2415	Sigma G
yhcM	456	1457	4044	Sigma F&G
yraE	198	54	3249	Sigma G
yrrD	525	1853	2674	Sigma G
yusG	237	628	2087	UK
yusN	333	942	2082	Sigma F&G
		RPKM = 567-2000		
pdhA	1116	823	683	Sigma A
sscA	87	124	1767	Sigma K
sspI	216	148	843	Sigma F&G
yhcO	969	1502	1429	Sigma E&K
ykzD	138	108	714	Sigma G
yoyE	126	137	1462	Sigma G
ypzI	132	119	850	Sigma F&G

yraD	300	284	843	Sigma G
yrzB	282	208	567	UK
yutC	633	516	636	Sigma G

*Data are averages from RNA-seq analyses on RNA from 3 independent highly purified spore preparations prepared on plates, all as described in Methods. Genes with names emboldened are those for which the protein product has been identified in dormant spores (42,43). RNA polymerase σ factors that direct the transcription of individual genes were identified using information in subtiwiki and the literature (26), including information on gene expression in sporulation and correlation of a gene's expression with that from a gene definitively established as under the control of a specific sigma factor. For the *yusG* and *yrzB* genes, their σ factor dependence is unknown (UK). Two genes highlighted with the same color are almost certainly co-transcribed as seen by their appropriate genomic arrangement and the complete coverage of both genes by RNA-seq reads, with no gaps in intergenic spaces (Fig. S2F; and data not shown).

[1]Note that the mRNAs for coding genes are invariably longer than the coding sequences shown; this is ~ 50 nt for these 46 most abundant mRNAs (data not shown).

[2]The average standard deviations for the average KPRM values for the most abundant 29 mRNAs were 8%, 21% for the next most abundant 7 mRNAs and 17% for the next most abundant 10 mRNAs.

Table 3*

mRNA Reads, rRNA reads and /total Reads for 23S+16S rRNAs in young and aged spores

2 day spores	47 d spores	98d spores
Total mRNA Reads/23S+ 16S rRNA Reads x 10^6		
19,041 (100)[1]	566 (3)	163 (0.9)
23S+16S rRNA Reads		
94,820,503	77,821,429	83,264,436

*Spores of various ages were prepared, purified RNA extracted, RNA-seq carried out and the data analyzed as described in Methods.

[1]Values in parentheses are the percentage of total mRNA Reads/23+16S rRNA Reads relative to the value in 2 d spores.

Fig. 1A,B. Germination and outgrowth of *B. subtilis* 2, 47 and 98 d spores in rich or minimal media. Spores harvested from sporulation plates after 2, 47 or 98 d were purified as described in Methods. Spores were then incubated at 37°C in either A) L-broth plus L-valine or B) Spizizen's minimal medium plus L-alanine as described in Methods, and spore germination, outgrowth and vegetative growth were followed by measuring the OD_{600} of the cultures. The symbols used are: ○, 2 d spores; ●, 47 d spores; and △, 98 d spores. This experiment was carried out twice with essentially identical results.

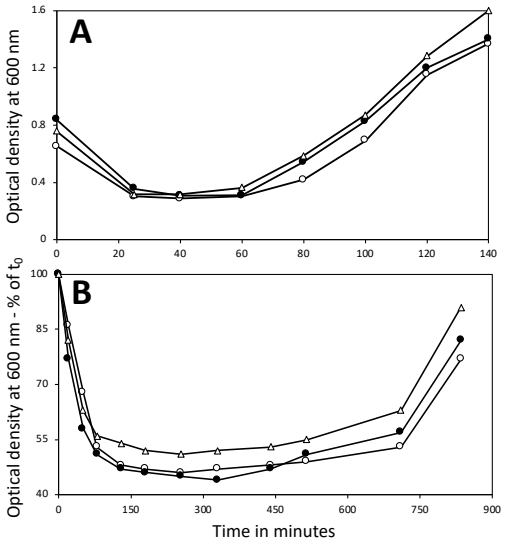

Fig. 2A-D. Resistance properties of *B. subtilis* spores incubated for 2, 47 or 98 d. Spores harvested from sporulation plates incubated for 2, 6, 15, 47 or 98 d were purified as described in Methods. The purified spores' survival when exposed to A) wet heat, B) dry heat, C) hypochlorite, or D) UV radiation were then measured as described in Methods, and essentially identical results were obtained in duplicate experiments. The symbols for the ages of the spores used are: ○, 2 d; ●, 6 d; △, 15 d; 47 d, ▲; and, 98 d, □.

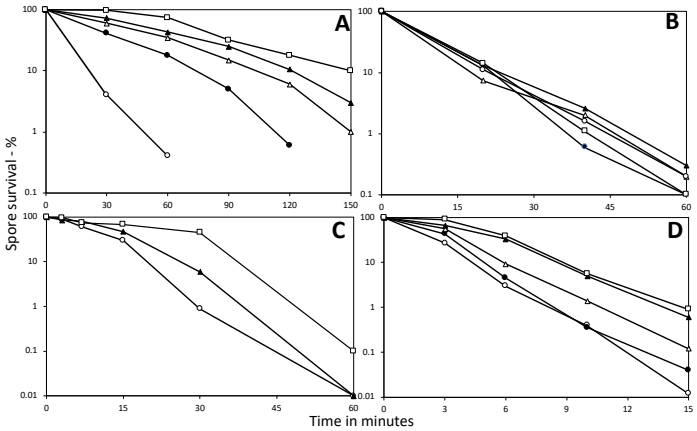

Fig. 3A,B. Electrophoretic analysis of RNA in *B. subtilis* spores incubated for 2 - 98 d. Spores were harvested from plates incubated for 2 - 98 d and were purified. RNA was extracted from the purified spores, and analyzed either by agarose gel electrophoresis with co-electrophoresed nt size markers (panel A) or on an Agilent TapeStation 4200 Automated Gel Electrophoresis System (High Sensitivity RNA Assay) using in silico size markers (panel B). Bands labeled "a" and "b" in panels A and B denote the migration positions of intact 23S and 16S rRNA, respectively; note the software sizing of detected rRNA peaks in panel B is not accurate compared to the expected size of the bands detected in panel A. Samples run in panels A and B were from separate experiments and had RNA from two separate spore preparations harvested after 2 d. In panel B, the 47 and 98 d RNA lanes were from a different TapeStation run than the 2d, 6d and 15d RNA lanes.