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From: Feitelson
Subject: ms. for spore paper (revised)
To: lederberg
cc: case

The following is a copy of the latest version of the spore paper. I rearranged some pages in the .doc extension, so it probably won't run on diablo. But I expect you will make fairly major changes to it.

Spores of Bacillus subtilis Can Not Be Transformed

James T. Case, Jerald S. Feitelson, and Joshua Lederberg

Abstract

Germinating spores of Bacillus subtilis can not be transformed to tryptophan or thymidine independence. Spores do not become competent until after germination and vegetative growth. One cell division is required for competence to be achieved.

1 INTRODUCTION

The original paper demonstrating transformation in Bacillus subtilis 1 employed germinating spores of auxotrophic strains as recipients of DNA. In the course of setting up a model system for transformation in soil, we have reexamined these findings.

The competent population of cells in the initial experiments are shown to be vegetative cells which have developed competence by incubation in a minimal medium during the transformation procedure. Our conclusion is that germinating spores are unable to undergo transformation.

2 MATERIALS AND METHODS

The B. subtilis strains which were used are shown in Table 1.

TABLE 1
Strains used in these experiments.

STRAIN	GENOTYPE
SB25	prototroph
SB168	trpR-S
SB591	thyR-S

Spores of B. subtilis strain SB168 and SB591 were prepared in Schaeffer medium by a method previously described 4 . Germination of the spores was done at 37RoSC following heat shock at 70RoSC for 5 minutes in Spizizen minimal medium 1 .

Ten ul of DNA (187ug/ml) isolated from strain SB25 were added

to the transformation mixtures and incubated with the cells for 1 hour at 37RoSC with slow shaking. The transformation mixtures were diluted and plated onto minimal medium plus all amino acids (SB591) or minimal medium plus all amino acids minus tryptophan (SB168). Viability counts were done on nutrient agar.

Transformation efficiencies were calculated as the percentage of transformants relative to the viable counts on nutrient agar. Competent cells produced by the procedure of Wilson and Bott 5 were used as controls for the relative transformation efficiencies.

3 RESULTS

In the first series of experiments, attempts were made to transform purified spores during the germination process. The spores were heat shocked as above and the DNA added immediately following dilution into the transformation mixture. The cells were incubated for one hour and then plated directly onto the appropriate media.

Table 2 shows the frequencies of transformation for both purified spores and competent cells of the two strains.

TABLE 2

STRAIN	VIABILITY	TRANSFORMANTS / ML.	TRANSFORMATION EFFICIENCY
SB168 (competent cells)	1.06 x 10R8S/ml	1.25 x 10R6S	1.18%
SB168 (spores)	8.0 x 10R6S/ml	0	0
SB591 (competent cells)	5.0 x 10R7S/ml	5.07 x 10R4S	0.1%
SB591 (spores)	2.0 x 10R6S/ml	0	0

The competent cell preparations showed reasonable transformation frequencies. No transformants appeared with the germinating spores. The experiment was repeated seven times, with consistently negative results for spore transformation.

Spizizen's original protocol 1 stated that the purpose of the heat shock was to inactivate any vegetative cells which may have been present in the spore suspension (1). In the present study, this was tested by heating a stationary phase culture of SB591 at 65RoSC for 5 min. and then subsequently plating on nutrient agar for determination of the viable counts. An unheated control was run also to determine relative viabilities. 75% of the vegetative cells were killed by this treatment. If a subpopulation of competent cells existed in the spore preparation, then this may have contributed to the frequencies of transformation that were observed in the original experiments.

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(1) Isolation of spores was done from stocks maintained on potato agar slants. No lysis of any remaining vegetative cells was done, as is now commonly practised.

McCarthy and Nestor 6 have shown that heating competent cell preparations at 50RoSC for 5 min. causes loss of over 90% of the original competence of the culture, which is not regained for several hours. This was confirmed by heating competent cells of SB168 as above and

then adding DNA. Unheated competent cells were run as controls. Only 4% of the cells remained competent after this procedure. Assuming that 10% of the cells in a normal competent cell preparation are competent [6], the frequency of competent cells which may be isolated from potato agar slants following heat shock is approximately 0.05%. Such a low level of competent cells strongly suggests that vegetative cells remaining in Spizizen's spore suspension could not contribute significantly to the frequencies of transformation achieved in the original protocol.

Spizizen [1] noted that following heat shock, the spores were suspended in glucose minimal medium supplemented with 0.1% yeast extract for four hours prior to transformation. We repeated this procedure and, in addition to following their growth turbidimetrically, determined the morphological characteristics of the cells by microscopic examination. As a negative control for transformation, spores were germinated in nutrient broth and then diluted into the transformation mixture prior to the addition of the DNA. Figure 1 shows the growth curves for SB168 in nutrient broth and glucose minimal medium. Microscopic examination of the spores incubated in minimal medium showed over 99% rounded forms after 90 min. at 37RoSC. Only 80% of the spores in the nutrient broth showed this phenotype.

Following the four hour incubation, the cells were diluted 1:10 in the transformation fluid containing 0.01% yeast extract and subjected to the transformation procedure. 0.1 ml of the suspensions were plated on the appropriate media and incubated overnight. The control spores gave less than 0.001% transformants while the spores incubated in the minimal medium gave 0.013% transformants.

Finally, SB168 spores were heat shocked and incubated at 37RoSC in the GM1 medium of Wilson and Bott [5]. Flask 1 was incubated for four hours and then immediately transferred to GM2. Flask 2 was allowed to grow for 90 min. past the end of log phase growth and then was transferred to GM2 (see Fig.2). Following 45 min. incubation in GM2, the cultures were observed microscopically and found to contain over 95% vegetative cells in both populations. These cells were then subjected to the transformation procedure. The results are shown in Table 3. A significant increase in the competence level was attained by the additional incubation in GM1.

TABLE 3

FLASK	VIABILITY	TRANSFORMANTS / ML.	TRANFORMATION EFFICIENCY
1	1.36 x 10R8S/ml	1.46 x 10R5S	0.11%
2	2.19 x 10R8S/ml	8.62 x 10R5S	0.39%

The growth curves show that following the maximal loss of turbidity, the Klett readings increased 4-fold, suggesting two doublings in the four hours of incubation. This is supported by the viability counts before and after the incubation periods.

4 DISCUSSION

Our inability to transform germinating spores lead to an investigation of the physical state of the cells when competence had been acquired. We have shown that spores do not become competent until after the spores have germinated and undergone at least one cell division. At this stage of growth they are vegetative cells, having gone far beyond the stages of germination. Other researchers reporting transformation of germinating spores give times for acquisition of competence of 10 hours [2] and 16-18 hours [3]. It is clear that by

this time the cells have become vegetative and more likely are approaching the stages of sporulation rather than germination.

These experiments lead to two conclusions.

1) The cells which were transformed in Spizizen's original protocol were cells which had undergone germination, vegetative division and were in the early processes of sporulation. Germinating spores per se cannot be transformed.

2) It is possible to achieve a moderate amount of competence in a bacterial culture in a considerably shorter time than has been reported in the literature, by the use of spores as the inoculum rather than an overnight culture of vegetative cells.

References

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