TRANSFORMATION IN BACILLUS CEREUS: A CRITIQUE[1]

Ivan D. Goldberg and Darrel D. Gwinn
Biological Sciences Laboratory, Fort Detrick
Frederick, Maryland 21701

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In recent years several laboratories including our own have become interested in obtaining a genetic exchange system for Bacillus cereus. Such a system would enable one to study the genetics of sporogenesis in the bacterial species in which this process has been most extensively investigated. We were therefore most interested in the report by Felkner and Wyss (1964) that described a procedure for making B. cereus 569 competent and for the isolation of the "competence factor" involved. Our attempts to transform our own auxotrophs of B. cereus 569 by the above reported procedure were unsuccessful.

An examination of the strains used by Felkner and Wyss in their study revealed that they were not B. cereus by the following criteria: They were auxotrophic for tryptophan and could be transduced to prototrophy by phage SP-10 that had been propagated on Bacillus subtilis W-23-S^r; they became competent when grown according to the procedure devised by Anagnostopoulos and Spizizen (1961) for B. subtilis and could be transformed to prototrophy with deoxyribonucleic acid (DNA) isolated from B. subtilis W-23-S^r; and when DNA from one of the strains was melted, the melting curve was almost identical to that obtained with the DNA of B. subtilis 168 ind^-. Our studies clearly indicate that the strains used by Felkner and Wyss were not B. cereus but were, in fact, B. subtilis.

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[1] In conducting the research reported herein, the investigators adhered to "Guide for Laboratory Animal Facilities and Care" established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources NAS-NRC.

Materials and Methods: B. subtilis strains 168 ind$^-$ and W-23-S^r and B. cereus NRRL B-569 were from the Fort Detrick culture collection. Strains designated B. cereus 569 (Pollock), 569-S^r, and 569-smooth were obtained from I. C. Felkner. These latter strains will be referred to throughout this paper as "B. cereus". Auxotrophs of B. cereus strains ATCC 6464, NRRL B-569 and ATCC 9139 were isolated by the procedure of Goldberg et al. (1965). DNA was extracted from B. subtilis strains 168 ind$^-$, W-23-S^r and the strains obtained from Felkner according to the procedure of Marmur (1961). Because of their resistance to lysozyme and detergents, DNA was extracted from authentic B. cereus strains by the procedure of Goldberg and W. Braun (unpublished results). Spores (1×10^3/ml final concentration) were added to 2-liter Erlenmeyer flasks containing 200 ml of penassay broth (Difco Antibiotic Medium #3). The flasks (usually 6) were shaken on a reciprocating shaker at a speed of 90 strokes/min for 9 hr at 31 C. The cells were harvested and spheroplasts were obtained by a modification of the autolysis procedure of Church and Epstine (1962). The cells were resuspended in a solution containing 5% Carbowax 20,000 (Union Carbide Chemical Co., Cleveland, Ohio), 0.05 M Tris, 0.1 M versene, and 0.15 M NaCl at pH 7.6 (total volume = approx 10 ml). After 1.5 to 2.0 hr incubation at 37 C, 20% of the cells had spheroplasted (microscopic observation), presumably as the result of the activity of an intracellular enzyme. The suspension was centrifuged at 12,100 x g in the Sorvall RC-2 centrifuge, and the pellet was resuspended at the same concentration in a mixture of 0.1 M Tris, 0.2 M versene, and 0.3 M NaCl, pH 7.6. To each 10 ml of this suspension, 0.4 ml of sodium lauryl sulfate (saturated solution in 45% ethanol) was added, and the mixture was heated at 60 C for 15 min. The crude nucleic acid was deproteinized by treatment with chloroform-isoamyl alcohol by the procedure of Marmur (1961).

DNA was determined by the method of Burton (1956). The melting

points (Tm) of the DNA samples were determined by the method of Marmur and Doty (1962).

Cells were grown for plate transformation (Gwinn and Thorne, 1964) by inoculating spores into nutrient broth + 0.3% yeast extract (NBY) (Thorne, 1962) and shaking for 16 hr at 37 C. Cells (0.1 ml) and 0.1 ml DNA that had been extracted from B. subtilis W-23-S^r were spread directly onto minimal 10 plates (Thorne and Stull, 1966). Transformants were scored after 16 to 24 hr incubation at 37 C. Where maximum competence for transformation was desired, the procedure devised by Anagnostopoulos and Spizizen (1961) for B. subtilis 168 was used. Transformants were scored on minimal 10 agar after 16 to 24 hr incubation at 37 C.

Phage SP-10 was assayed in phage assay agar (PA) seeded with spores of B. subtilis W-23-S^r (Thorne, 1962). The phage was propagated for transduction on B. subtilis W-23-S^r as described by Taylor and Thorne (1966). Transductions were performed as described by Thorne (1962), except that the transduction mixtures were plated on minimal 10 agar. Transductants were scored after 24-36 hr incubation at 37 C. Antiserum against SP-10 was prepared by subcutaneous injection of rabbits.

Results: After the report by Felkner and Wyss (1964) describing the transformation of "B. cereus" 569 for a streptomycin-resistance marker, we attempted to transform auxotrophs of this strain that were available in our laboratory with DNA isolated from wild type B. cereus NRRL B-569. These attempts were unsuccessful even though we carefully followed the procedure outlined in the paper. Routine examination of the strains used in the paper, which were kindly provided to us by I. C. Felkner, revealed that they all required tryptophan for growth on minimal 10 agar. This tryptophan requirement together with certain morphological considerations led us to question whether the strains used by Felkner and Wyss might be B. subtilis instead of B. cereus and, more specifically, B. subtilis 168 ind$^-$. To test this possibility, we

grew "B. cereus" 569-S^r and 569 (Pollock) in NBY broth and tested them by plate transformation with DNA isolated from B. cereus NRRL B-569 and from B. subtilis W-23-S^r. As Table 1 shows, these trp^- strains

TABLE 1. Transformation of "B. cereus" strains by DNA's extracted from B. cereus NRRL B-569 and B. subtilis W-23-S^r

Recipient (trp^-)	DNA source	trp^+ transformants/0.1 ml
"B. cereus" 569-S^r	No DNA	2
	B. cereus NRRL B-569	1
	B. subtilis W-23-S^r	144
"B. cereus" 569 (Pollock)	No DNA	0
	B. cereus NRRL B-569	2
	B. subtilis W-23-S^r	862

Recipient cells were grown in NBY broth for 16 hr at 37 C. Transformation was done by spreading 0.1 ml cells and 0.1 ml DNA directly onto minimal 10 agar plates. The concentration of B. cereus DNA was approx 21 μg/plate; the concentration of B. subtilis DNA was approx 30 μg/plate. Plates were counted after 48 hr incubation at 37 C.

were transformed to prototrophy by the B. subtilis DNA but not by the B. cereus DNA. In order to get a measure of the degree of competence that could be achieved with the "B. cereus" strains compared to a highly transformable strain of B. subtilis, two of the "B. cereus" strains and

TABLE 2. Comparison of the transformation of B. subtilis and "B. cereus" strains by B. subtilis W-23-S^r DNA.

Recipient cells		W-23-S^r DNA	trp^+
Mutant	No. per ml	(370 μg/ml)	transformants/ml
B. subtilis 168 ind^-	1.6×10^8	0	0
	1.6×10^8	0.1	3.0×10^6
"B. cereus" 569-Pollock (trp^-)	1.2×10^8	0	0
	1.2×10^8	0.1	8.7×10^5
"B. cereus" 569-smooth (trp^-)	1.4×10^8	0	0
	1.4×10^8	0.1	2.7×10^5

Recipient cells and DNA were combined in tubes (final volume = 1.0 ml) and incubated on the shaker at 37 C for 60 min. After treatment with deoxyribonuclease (50 μg/ml), the mixtures were assayed for transformation to trp^+ on minimal 10 agar.

B. subtilis 168 ind⁻ were grown by the procedure of Anagnostopoulos and Spizizen (1961) and were transformed to prototrophy with DNA isolated from B. subtilis W-23-S^r. Table 2 reveals that the strains in question became quite competent in this medium. It should be pointed out that there is a variation in the degree of competence achieved with various stocks of B. subtilis 168 ind⁻. The data shown in the table are for our most highly transformable stock. In another test for genetic relatedness, the same strains were transduced to prototrophy by phage SP-10 that had been propagated on B. subtilis W-23-S^r (Table 3). The

TABLE 3. Comparison of the transduction of B. subtilis and "B. cereus" strains by phage propagated on B. subtilis W-23-S^r.

Recipient	No. of cells/ml	Phage SP-10	Phage antiserum	trp$^+$ transductants/ml
B. subtilis 168 ind⁻	1.2×10^9	0	0	0
	1.2×10^9	0.5	0.1	0
	1.2×10^9	0.5	0	1.4×10^3
"B. cereus" 569 smooth (trp⁻)	5.2×10^8	0	0	0
	5.2×10^8	0.5	0.1	0
	5.2×10^8	0.5	0	1.2×10^3
"B. cereus" 569 Pollock (trp⁻)	1.4×10^9	0	0	0
	1.4×10^9	0.5	0.1	0
	1.4×10^9	0.5	0	7.4×10^2

Recipient cells were grown in NBY broth for 6 hr at 37 C. Cells (0.5 ml) and phage SP-10 (7.5×10^8 plaque-forming units (PFU) contained in 0.5 ml) were combined in test tubes and incubated on the shaker for 45 min at 37 C. Transductants to tryptophan independence were scored on minimal 10 agar. Where antiserum treatment is indicated, phage and antiserum were preincubated together for 15 min prior to contact with cells. SP-10 was propagated on B. subtilis W-23-S^r. Lysates were treated with 50 μg/ml deoxyribonuclease prior to use in transduction experiments.

transduction frequencies (number of transductants/plaque-forming unit) obtained with "B. cereus" 569 smooth and 569 (Pollock) were in the same range as the frequency obtained with B.subtilis 168 ind⁻. It should be noted that SP-10, which does not produce plaques on B. subtilis 168 ind⁻ (Thorne, 1962), did not plaque on the strains used by Felkner and Wyss

(not shown). In a final experiment to demonstrate that the strains indeed were B. subtilis and not B. cereus, the Tm's of DNA isolated from authentic B. cereus NRRL B-569, B. subtilis 168 ind⁻ and "B. cereus" 569-S^r were compared (fig. 1). The melting curves for "B. cereus" 569-S^r and B. subtilis 168 ind⁻ were almost superimposable (Tm = 85.5 and 85.9 respectively); whereas authentic B. cereus DNA melted at a much lower temperature (Tm = 82.4).

Discussion: Although interspecies transformation has been demonstrated for antibiotic markers in Bacillus (Dubnau et al., 1965; Goldberg et al., 1966), it could not be shown for auxotrophic markers. It seems extremely unlikely that the high frequency genetic exchange that occurred between B. subtilis and the strains used by Felkner and Wyss could have occurred if they had, in fact, been B. cereus. Moreover, the

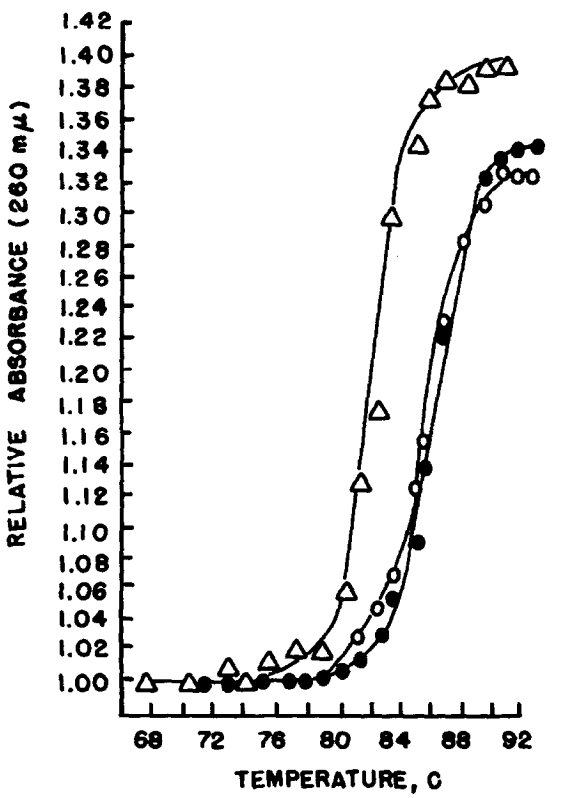

FIGURE 1. DNA melting point determinations. DNA samples were dissolved in standard saline-citrate, pH 7.0

O—O, B. subtilis 168 ind⁻
●—●, "B. cereus" 569-S^r
Δ—Δ, B. cereus NRRL B-569

degree of genetic exchange that we observed between B. subtilis and these strains was such that it provides almost conclusive evidence that they are indeed B. subtilis. This conclusion is strengthened by the fact that the DNA of "B. cereus" 569-S^r has a Tm typical of B. subtilis whereas authentic B. cereus DNA melts at a lower temperature. Although it would be difficult to prove, we feel that the strains in question are probably derivatives of B. subtilis 168 ind^-. The colonial morphologies of the auxotrophs on NBY agar and minimal 10 agar supplemented with tryptophan, and of the transformants and transductants on minimal 10 agar, were strikingly similar to the colonial morphologies of our isolates of B. subtilis 168 ind^-. Wyss (personal communication) has noted that the cells of his strains do not have typical B. cereus morphology. The fact that these strains are typtophan-requiring auxotrophs is also highly suggestive of B. subtilis 168 ind^-. The work of Felkner and Wyss (1964) has been widely cited as the first demonstration of a competence factor in Bacillus cereus (Charpak and Dedonder, 1965; Tomasz and Mosser, 1966; Spizizen et al., 1966; Dobrzanski and Osowiecki, 1967; Akrigg et al., 1967). Actually, this work may be the first report of the demonstration of a competence factor in B. subtilis.

We have been unsuccessful in attempts to render auxotrophs of several strains of B. cereus including NRLL B-569, ATCC 6464, and ATCC 9139 competent for transformation via a variety of procedures. Numerous mutants auxotrophic for various markers were grown in several different media and screened for transformants via plate transformation. In addition, the procedure devised for B. subtilis 168 (Anagnostopoulos and Spizizen, 1961) as well as the procedures used to obtain competence in B. licheniformis (Gwinn and Thorne, 1964; Leonard and Mattheis, 1965) were tried for various auxotrophs of several strains of B. cereus without success.

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