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Bacillus subtilis 168 Genetic Transformation Mediated by Outgrowing Spores: Necessity for Cell Contact

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Transforming activity released in sequential genetic order during the first synchronous cycle of DNA replication during outgrowth of spores of *Bacillus subtilis* 168 was investigated. A transformation assay was used consisting of outgrowing spores as DNA donors and multiply marked competent cells as recipients. DNA synthesis inhibitors known to stop DNA release were used during and subsequent to DNA transfer to recipient cells. The released DNA sedimented with the outgrowing cells after low-speed centrifugation, and it was discovered that markers released both early and late were resistant to up to 500 μg of deoxyribonuclease per ml under conditions in which the transforming capacity of purified DNA was eliminated by 5 μg of the nuclease per ml. Inaccessibility to deoxyribonuclease was increased and maintained during the transformation event while detergents and proteolytic attack did not expose the released chromosome to nuclease action. The results indicate that tight physical contact between outgrowing spores and competent cells is required for transformation in this system.

Borenstein and Ephrati-Elizur (2) discovered that outgrowing cells originating from a culture of dormant *Bacillus subtilis* 168 spores release DNA in a sequential genetic order and in synchrony with the first replication cycle of the genome. Release of genetic material was followed by simply mixing samples from an outgrowing culture with appropriately marked competent cells. Markers closer to the origin of replication were transformed first, whereas markers away from the origin were transformed later, as a function of DNA replication time. These observations were used for genetic mapping (2). The generality of these original observations is nevertheless in question, given that nonsequential release of certain markers from particular *B. subtilis* strains has been observed (W. D. Crabb and U. Streips, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, H8, p. 97), suggesting different replication patterns between strains or a lag between replication and release which varies between chromosomal regions. A correlation between DNA synthesis and DNA release was indicated by the fact that inhibition of chromosome replication in the outgrowing culture impaired inhibition of transformation of competent cells (2). These observations, coupled with the sequential nature of the release, excluded the possibility that the transformation observed was due to lysis of the outgrowing cells, either in the liquid and/or during their residence on the agar plate after mixing with the comptent cells.

The physical nature of the DNA release during spore outgrowth has not been explored. This communication presents evidence that the released copy of the bacterial chromosome remains attached to the cell in a form inaccessible to deoxyribonuclease (DNase) yet available for transformation of competent cells. The results obtained indicate that tight physical contact between outgrowing spores and competent cells is required for transformation in this system.

(Parts of this report have already been presented [C. Orrego, M. Arnaud, and H. O. Halvorson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, H76, p. 147].)

MATERIALS AND METHODS

Bacterial strains. Genotypes of *B. subtilis* 168 strains used are given in Table 1.

Preparation of spores. Spores of *B. subtilis* 168 *trpC2* were obtained after growth and sporulation in double-strength nutrient broth (Difco) (15). Spores were extensively washed with distilled water. This was followed by successive treatments with lysozyme, 2% sodium dodecyl sulfate, 1 M NaCl, 0.1 M NaCl, distilled water, 30 μg of DNase I per ml in 0.1 M tris(hydroxymethyl)aminomethane (pH 8.1), 0.01 M $MgCl_2$, and several washes in distilled water. Refractile spores were further fractionated on a discontinuous Renografin gradient by successive layering of 2.5 ml of 100% Renografin, 10 ml of 75% Renografin, and the spore sample in 1.5 ml of water (5×10^9 spores). Refractile spores banded between the two bottom layers upon centrifugation at 5,000 rpm for 10 min,

TABLE 1. *Strains used*

B. subtilis strain	Genotype	Source and comments
168	*trpC2*	Our stock
ANS-1	Spontaneous revertant of 168	Our stock
BC109	*trpC2 thyA thyB*	J. Copeland (F. Rothman, initial source)
BC50	*purA16 leuA8 metB5 nic*	J. Copeland
BC50 333-1	*purA16 leuA8 metB5 nic nov*	BC50 resistant to 3 μg of novobiocin per ml in minimal plates; our stock

followed by 30 min at 15,000 rpm, in an SW25.1 Beckman rotor at 4°C. The spores collected from the gradient were washed five times with water. The final fraction contained at least 96% of the spores in their phase-bright state. The cellulose nitrate tube used for centrifugation was siliconized and then sterilized with UV light. Spore samples were sonically irradiated for 4 to 5 s previous and subsequent to fractionation on Renografin to disaggregate clumps of spores.

Media. Strains were maintained as colonies on Difco tryptose blood agar base or Difco sporulation medium plates (19). Vegetative growth was usually obtained in Difco Penassay broth supplemented with 20 μg of tryptophan and thymidine per ml. Transformants were revealed on plates containing the mineral salts medium of Anagnostopoulos and Spizizen (1) supplemented with 0.5% glucose, 1.5 g of glutamic acid per liter, a 20-μg/ml amount of the auxotrophic requirements not under selection, and 2 g of Difco assay medium per liter for the particular amino acid requirement for which selection was being performed.

Competent cells. Competent cell cultures of strain BC50 333-1 (Table 1) were prepared according to the method of Bott and Wilson (3). Cells were stored in 1-ml aliquots in 18% glycerol at −80°C until used in transformation experiments.

Germination and outgrowth. Germination and outgrowth of approximately 2×10^8 spores per ml were carried out in Difco Penassay broth (supplemented with 20 μg of tryptophan per ml) or in similarly supplemented brain heart infusion broth (Difco), containing 5 g of yeast extract (Difco) per liter (13), and incubated at 37°C on a rotatory shaker (250 rpm). When indicated, the spore suspension was heat activated for 7 min at 80°C before incubation at 37°C (zero time).

Transformation by outgrowing cells. Transformation was followed by withdrawing 0.3-ml aliquots from the spore culture into 0.03 ml of 10,400 μM 6-(*p*-hydroxyphenylazo)uracil (HPURA) followed by incubation for 3 min at 37°C to instantaneously inhibit DNA synthesis. This was immediately followed by transfer of a 0.1-ml sample into 1 ml of competent cells previously equilibrated to 37°C (104 μM final concentration of HPURA). Transformation was carried out for 15 min. The mixture was then diluted with 5 ml of the mineral salts medium, filtered through a membrane filter (type HA, 0.45-μm pore diameter; Millipore Corp.), and washed with 5 ml of the same medium, and the filter was transferred into a vial containing 2 ml of minimal medium with 3 μg of novobiocin per ml. The suspension was blended in a Vortex mixer for 1 min, and 0.1 ml was plated on plates containing 3 μg of novobiocin per ml. Plates were incubated for 24 to 48 h at 37°C. Experimental points were done in duplicates. The following controls were performed: reversion frequency of the competent cells; plating of the spore culture on the counterselecting plate; and replica plating of the transformants to confirm the phenotype. The final number of transformants was obtained after subtracting the transformants obtained with nongerminated spores. Transformation values are expressed per initial number of spores in the original culture per milliliter.

Presence of HPURA for the first 15 min of DNA-mediated transformation has been reported not to interfere with transformation (14). We observed a 46 to 73% inhibition depending on the genetic marker. This loss was acceptable given the rapid effect of HPURA on cessation of DNA synthesis in the donor cells, a prerequisite in our experiments. The use of a recipient cell resistant to the drug was abandoned due to the mutator effect of the HPURA resistance mutation (11).

Septa. Septa were counted by phase-contrast microscopy after mixing 1 drop of the cell suspension with 1 drop of saturated CsCl (13).

DNA synthesis. Spores were suspended in Penassay broth to which 5 μCi of tritiated thymidine per ml was added. One-milliliter aliquots were removed at different times and added to 5 ml of cold 10% trichloroacetic acid containing 100 μg of thymidine per ml. The samples were filtered through Whatman GF/C filters and washed, and the dried filters were counted in scintillation fluid.

Chemicals and enzymes. HPURA was obtained from B. W. Langley, ICI, England, and from G. Wright, University of Massachusetts Medical School, Worcester, Mass. Novobiocin was purchased from Sigma Chemical Co., Tween 80 was obtained from Nutritional Biochemicals Corp., and Triton X-100 was obtained from Research Products International. Renografin was a product from E. R. Squibb & Sons. DNase I was purchased from Worthington Biochemicals Corp., and protease K was from E. Merck AG, Darmstadt, Germany. Tritiated thymidine was obtained from New England Nuclear. Tritiated *B. subtilis* DNA was a gift from D. Dean and I. Mahler. DNA was stored in 0.15 M sodium chloride plus 0.015 M trisodium citrate, pH 7.0 (SSC) (16).

RESULTS

HPURA inhibition of DNA synthesis during outgrowth. A specific inhibitor of DNA synthesis was required to obtain rapid cessation of DNA release at the time of the transformation test. HPURA, a specific inhibitor of DNA polymerase III in *B. subtilis* (7), was used since it does not interfere with the initial stages of DNA-mediated transformation (14). HPURA only begins to impair the process of germination and outgrowth at the stage of cell swelling, as indicated by the turbidity curves (and confirmed by

microscopic observation) of two parallel cultures (Fig. 1A). DNA synthesis is completely inhibited by HPURA (Fig. 1B), whereas it only affects cell viability after 90 min into the cell outgrowth process (results not shown). In subsequent experiments, HPURA was used at a concentration of 104 μM during transformation of competent recipient cells by donor outgrowing cells.

Sequential release of DNA during cell outgrowth. Optimum conditions for synchrony of germination and outgrowth were found by using the very rich medium consisting of brain heart infusion with yeast extract previously used by Keynan et al. (13). These conditions led to rapid germination, as revealed by phase darkening of 93% of the spores during the first 15 min and appearance of septa beginning at 87 min, with 90% of the cells showing one or more septa 23 min later (Fig. 2B). In contrast, septa formation in Penassay broth took 63 min to be completed even though cell division also began to occur at 87 min (Fig. 2A). In either medium,

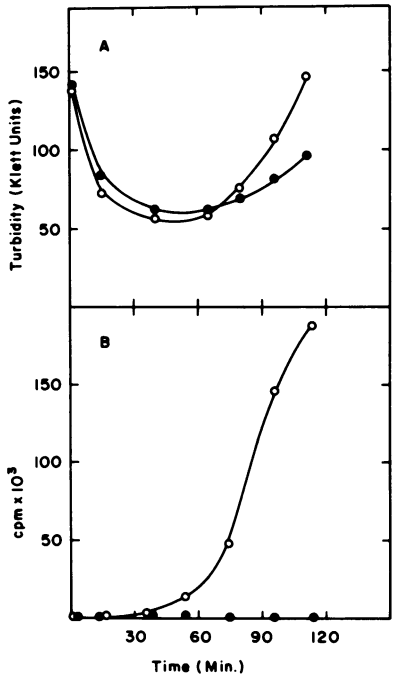

FIG. 1. *Effect of HPURA on germination, outgrowth, and DNA synthesis. B. subtilis 168 spores were incubated in supplemented Penassay broth after heat shock, and the cell suspension was divided into four portions. (A) Turbidity was followed in the presence (●) and absence (○) of 200 μM HPURA; (B) incorporation of tritiated thymidine into acid-insoluble material was determined in the presence (●) and absence (○) of the same concentration of the drug.*

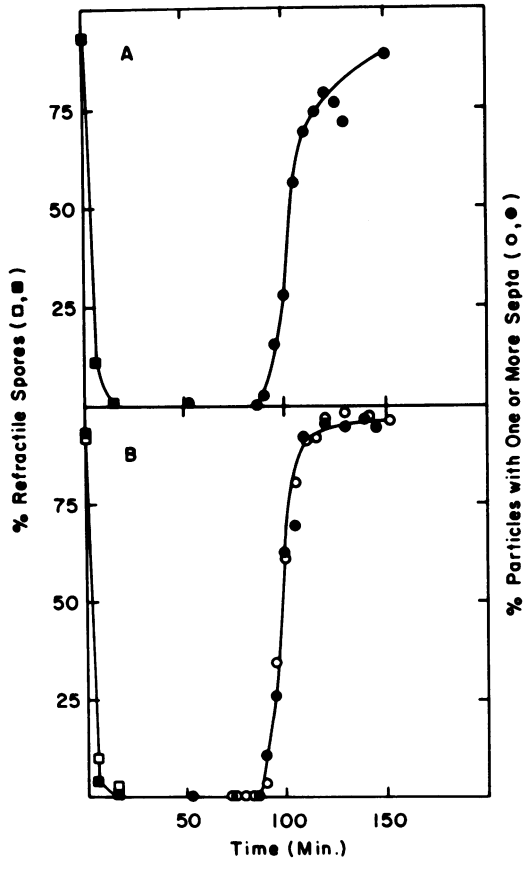

FIG. 2. *Synchrony of germination and septation during outgrowth of Renografin-fractionated spores. A total of 2.1 × 10⁸ B. subtilis 168 spores per ml were incubated in (A) supplemented Penassay broth and (B) brain heart infusion plus yeast extract with (○) and without (●) 0.5% glucose. Samples were taken for measurement of number of phase-bright bodies and septa per cell.*

colony-forming units did not increase up to 100 min of culture time (results not shown).

The process of spontaneous release of transforming DNA was followed by mixing samples taken at different times during outgrowth with a multiply marked competent cell resistant to novobiocin. This compound, known to inhibit semiconservative DNA synthesis (12), was present in all steps after use of HPURA, including the selecting plates. Lack of novobiocin in the agar plate led to germination of spores on plates, resulting in high numbers of transformants after mixing nongerminated spores and competent cells. Macromolecular synthesis supported by intracellular protein turnover up to the outgrowth stage of tryptophan-requiring *B. subtilis* spores in the absence of the amino acid require-

ment has already been documented (10). It is conceivable that in our experiments transformation in the absence of novobiocin is the result of the first and presumably only cycle of DNA replication, which most likely aborts soon afterwards due to the absence of the auxotrophic requirement.

Sequential release of genetic material by outgrowing spores was apparent from the emergence of the *purA16* gene, a marker close to the origin of replication (31), followed 15 min later by the *metB* gene, which is contiguous to the replication terminus (Fig. 3). Transformation for the early marker coincided with the beginning of the first cycle of chromosome replication, as detected by radioactive thymidine incorporation into acid-insoluble material (Fig. 1B). Transformation frequencies have not been normalized to values obtained with DNA from cells with completed chromosomes. Nevertheless, lower efficiencies of transformation obtained with late markers has already been reported to occur with cell-mediated transformation (2).

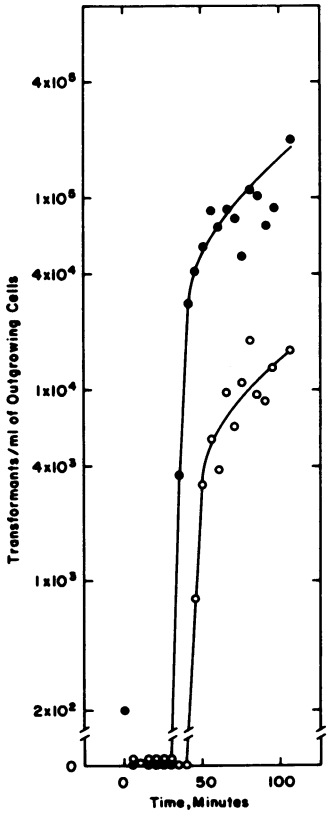

FIG. 3. *Sequential appearance of genetic markers in an outgrowing culture of B. subtilis 168. Competent recipient strain BC50 333-1. Symbols: (●) transformants for purA16; (○) transformants for metB.*

Transformation versus donor cell concentration. We wished to determine a range of donor cell concentrations which would elicit a linear response in transformation values. Donor cells from a 90-min culture were tested at different concentrations with a fixed level of recipient cells. An input of 2×10^7 donor cells per ml was found to be well within the above requirement (Fig. 4). This concentration was used in all subsequent experiments.

Inhibition of marker release by HPURA. The presence of HPURA from the beginning of germination prevents the appearance of the early *purA16* marker (Table 2), a confirmation that specific inhibition of DNA synthesis impairs the process of release as measured by transformation, suggesting that the chromosome becomes available for this process only as a result of concomitant replication.

DNase I resistance of released DNA. Attempts to stop the transformation reaction by addition of pancreatic DNase I to a mixture of competent and outgrowing cells revealed that the enzyme had no termination effect on trans-

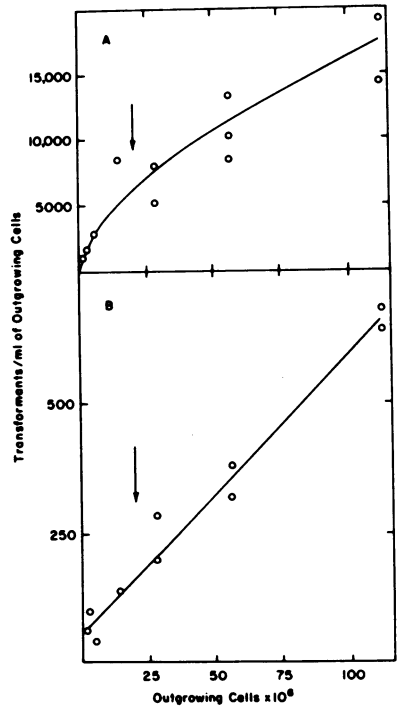

FIG. 4. *Transformation dependence of BC50 333-1 on outgrowing cells of strain 168. Spores were heat shocked, suspended in supplemented Penassay broth, and 90 min later tested for their capacity to transform for (A) purA16 and (B) metB. Arrows indicate the number of input outgrowing cells used in all other transformation experiments.*

TABLE 2. *Effect of HPURA on the release of DNA from outgrowing spores of BC109*[a]

Culture time (min)	Adenine transformants/outgrowing cells per ml		Viable cells/ml	
	No HPURA	Plus HPURA	No HPURA	Plus HPURA
0	0	0	6.9×10^7	3.8×10^7
60	5.0×10^3	0.1×10^3	3.4×10^7	5.6×10^7

[a] Heat-shocked BC109 spores were suspended in supplemented Penassay broth. The suspension was then separated into two parallel cultures with and without 208 μM HPURA and incubated at 37°C.

formation levels of any of the markers tested. DNase added to outgrowing cells (in the presence of HPURA) prior to mixing with competent recipients or during the initial 15 min of transformation did not disturb the number of transformants up to at least 500 μg of DNase per ml (Fig. 5A and B). Ninety-nine percent inhibition of DNA (phenol-extracted)-mediated transformation was obtained with 100 times less nuclease concentrations (Fig. 6A and B). Exposure to 2 mg of DNase per ml before transformation did affect subsequent transformation frequencies depending on the marker followed (Fig. 5A). Addition of competent cells to the mixture of outgrowing cells plus DNase (2 mg/ml) had a dramatic protective effect (Fig. 5B). It is unlikely that the protection was due to nuclease inactivation by the competent cell suspension: limiting concentrations of DNase were equally effective on hydrolysis of phenol-extracted DNA in the presence as well as in the absence of competent cells (Fig. 6A and B).

Lack of sensitivity to DNase was not due to inactivation of the enzyme under the conditions used or to masking from the nuclease by a cell density effect. Phenol-extracted, tritium-labeled *B. subtilis* DNA added to a 150-min culture of outgrowing spores in the presence of HPURA and simultaneously treated with DNase I under the same conditions as in the previous experiments showed extensive degradation of the labeled DNA to acid-soluble material (Table 3). DNase resistance was also shown to be independent of the concentration of outgrowing cells exposed to the enzyme (Table 4).

Attachment of the released DNA to the outgrowing cell. Outgrowing cells collected on nitrocellulose filters under conditions of DNA synthesis arrest retained their capacity to transform competent cells (Table 5). The corresponding cell-free filtrate showed about 1% of the original transformation of the cell suspension. Attachment of released DNA to the outgrowing cell was also evident from the cosedimentation of transforming activity with cells subjected to a centrifugal force and time of centrifugation just enough to pellet about 90% of the cells. A similar loss of transforming capacity by the resulting supernatant was observed. It was also clear that no transforming activity was disengaged from the cells into the medium as a result of DNase treatment (Table 6).

Attempts to unmask the released DNA. Genetic and chemical evidence indicates that the *B. subtilis* genome is found associated not only with the cell membrane but with cell wall polymers as well (4, 23). Cell wall preparations

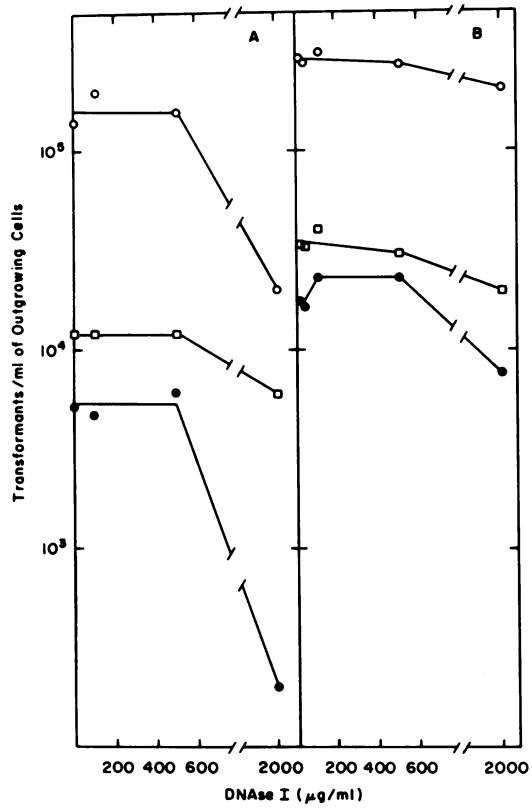

FIG. 5. *Treatment of outgrowing cells prior to (A) and during (B) transformation of BC50 333-1, with different concentrations of DNase. A total of 2.1×10^6 168 spores per ml were heat shocked and suspended in supplemented Penassay broth. (A) At 90 min, 0.25 ml of the cell suspension was incubated for 10 min with different concentrations of DNase I in 6 mM $MgSO_4$ and 1,040 μM HPURA at 37°C. The cells were filtered through Millipore filters and resuspended in twice the volume of minimal medium plus HPURA, and 0.2 ml was tested for transformation. (B) At 135 min, 0.1 ml of the outgrowing culture was tested for transformation in the presence of different concentrations of DNase I (in 6 mM $MgSO_4$ and 104 μM HPURA) for 15 min. Transformation of: (○) purA16; (□) leuA8; (●) metB. The outgrowing culture showed 1.7×10^8 colony-forming units/ml at 135 min.*

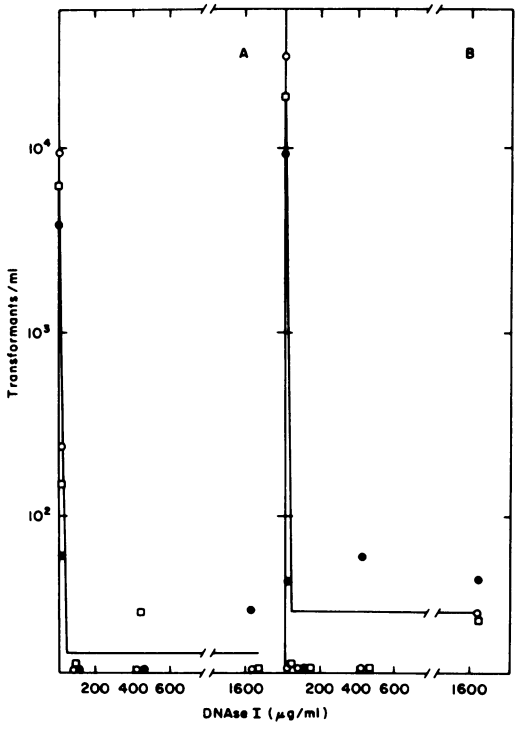

FIG. 6. *Treatment of phenol-extracted B. subtilis ANS-1 DNA with DNase I (A) prior to and (B) during transformation. (A) A 0.05-ml amount of DNA (450 μg/ml in 1× SSC) was incubated with varying concentrations of DNase I, 1,040 μM HPURA, and 6 mM $MgSO_4$ for 10 min at 37°C. One milliliter of competent cells of strain BC50 333-1 was then added, and transformation was carried out for 15 min at the same temperature before plating. The HPURA concentration was adjusted to 104 μM during the first 15 min of transformation. (B) Incubation with DNase (in 104 μM HPURA and 6 mM $MgSO_4$) was done during the initial 15 min, following mixing of DNA and competent cells. Transformation of:* (○) *purA16;* (□) *leuA8;* (●) *metB.*

have been found to be active in transformation assays and to preferentially contain genetic markers close to the origin and terminus of the chromosome (23). Experiments designed to remove nucleic acid material from cell wall fractions showed that the DNA is partially protected from DNase and from reaction with the mutagen chloroacetaldehyde (4, 23). Unmasking of the chromosome material was obtained by treatment with subtilisin followed by DNase (4). These observations encouraged us to treat outgrowing cells with nonionic detergents in the presence of protease K, an enzyme known to be refractive to detergents and completely free of DNase activity. Exposure of donor outgrowing cells to either Brij 58 or Tween 80 in the presence of protease K followed by DNase showed no detrimental effect but, on the contrary, a consistent slight enhancement on subsequent transformation of competent cells (Table 7). Independent controls (not shown) demonstrated that

TABLE 3. *Activity of DNase I on DNA added to an outgrowing culture of BC109 spores*[a]

B. subtilis ANS-1 [^{3}H]DNA (31 μg/ml) added to:	10-min DNase treatment (100 μg/ml)	Acid-precipitable radioactivity (cpm)	Degradation (%)
PAB	−	30,578	
PAB	+	1,044	96.6
PAB + HPURA (155 μM)	+	1,427	95.3
0-min culture	−	23,196	
0-min culture	+	4,641	80.0
150-min culture	−	30,104	
150-min culture	+	6,319	79.0

[a] Spores of BC109 were heat shocked, suspended in supplemented Penassay broth (PAB), and incubated at 37°C. Samples (0.2 ml) were taken at the indicated times, and ANS-1 [^{3}H]DNA, DNase I, HPURA (160 μM), and $MgSO_4$ (6 mM) were added, followed by incubation at 37°C for 10 min. The reaction was terminated by acid precipitation followed by collection of the insoluble material on filters.

TABLE 4. *Exposure of different concentrations of outgrowing cells to DNase I: effect on transformation*[a]

Cells/ml	DNase	Colonies/plate[b]	Adenine transformants[c]
2×10^8	−	557	1.06×10^5
2×10^8	+	550	1.04×10^4
4×10^7	+	225	3.9×10^4
2×10^7	+	536	1.02×10^5
1×10^7	+	535	1.02×10^5
2.8×10^6	+	626	1.19×10^5

[a] A total of 2.7×10^8 spores of strain 168 per ml were suspended in supplemented Penassay broth and incubated at 37°C. At 90 min a sample was taken and diluted to the indicated concentration in 87 μM HPURA and 5 mM $MgSO_4$, followed by incubation for 3 min at 37°C to stop DNA synthesis. DNase I was then added to a final concentration of 100 μg/ml, and the mixture was kept for 10 min at 37°C with mild rotatory agitation. The reaction was stopped by filtration through a Millipore filter. The collected cells were washed with minimal medium (plus 100 μM HPURA) and resuspended in 0.5 ml of the same. Cells were then tested for transformation.

[b] Average of two determinations. Transformation with ungerminated spores yielded 30 colonies per plate.

[c] Transformants are expressed per milliliter of original outgrowing culture and have not been corrected for transformation values obtained with ungerminated spores.

TABLE 5. *Effect of DNase I and of Millipore filtration on the transforming activity of outgrowing BC109 spores*[a]

Culture time (min)	Additional manipulation of the culture	Viable cells/ml	Adenine transformants/outgrowing cells per ml
0		1.6×10^9	0
30			0
120		1.2×10^9	4.4×10^3
120	DNase	1.6×10^9	7.8×10^3
160	Filtered cells	0.5×10^9	1.5×10^4
160	Cell filtrate	720	2.0×10^2
160	Filtered cells plus DNase	0.72×10^9	1.9×10^4
160	Cell filtrate plus DNase	220	0

[a] Heat-shocked BC109 spores were suspended in supplemented Penassay broth and incubated at 37°C. DNase I (100 μg/ml) treatment was performed in 175 μM HPURA and 4.5 mM $MgSO_4$. Cells were collected by Millipore filtration and resuspended in minimal medium prior to the transformation test.

TABLE 6. *Cosedimentation of outgrowing cells and transforming activity*[a]

Culture time (min)	Manipulation of the culture	Viable cells	Transformants[b]	
			Adenine	Methionine
0		4.1×10^8	29	4
100		0.8×10^8	247	12
	+DNase	0.8×10^8	384	25
	Supernatant from DNase-treated cell suspension	0.006×10^8	2	0
	Supernatant	0.05×10^8	12	1
	Supernatant + DNase	0.05×10^8	14	2

[a] *B. subtilis* 168 spores were suspended in supplemented Penassay broth and incubated at 37°C. At the indicated time the culture was centrifuged at 10,000 rpm in the Sorvall SS34 head (12,062 × *g*) for 5 min. DNase treatment (150 μg/ml) was performed in 3 mM $MgSO_4$ for 10 min at 37°C.

[b] Values correspond to actual number of colonies observed. No correction for zero-time transformation values has been introduced.

the protease enzyme is active in the presence of HPURA and both detergents, and that the latter did not impair cell viability at the indicated concentrations. No inhibition of DNase activity toward radiolabeled DNA was observed when enzymatic hydrolysis was performed in the presence of an outgrowing cell suspension which had been previously filtered to remove protease K (data not shown).

DISCUSSION

Release of DNA in cell populations of transformable as well as nontransformable bacteria has been well documented by now. In pneumococcal cultures release of transforming activity

TABLE 7. *Pretreatment of outgrowing cells with detergents and protease K: effect on DNase resistance of transformation*[a]

Culture time (min)	Manipulation of the culture		Colonies/plate[b]	Adenine transformants[c]
	Step 1	Step 2		
0			3	0
105			216	1.1×10^5
	0.1% Tween 80		145	7.2×10^4
	0.1% Tween 80 + protease K		178	8.9×10^4
	0.1% Tween 80 + protease K	+DNase	290	1.4×10^5
	0.1% Brij 58		321	1.6×10^5
	0.1% Brij 58 + protease K		180	9.0×10^4
	0.1% Brij 58 + protease K	+DNase	181	9.0×10^4

[a] A total of 2.0×10^8 spores of strain 168 per ml were suspended in supplemented Penassay broth and incubated at 37°C. At 105 min, 0.2-ml samples of the culture were incubated with the indicated concentration of detergents with or without protease K (200 μg/ml) and in the presence of 104 μM HPURA, for 20 min at 32°C, with mild rotatory agitation. The mixture was then diluted with Penassay broth + HPURA and filtered, and cells were washed and resuspended in 0.5 ml of the same medium. Exposure to DNase I (182 μg/ml) was performed in 5 mM $MgSO_4$ for 10 min at 37°C.

[b] Average of three determinations.

[c] Transformation frequencies have been corrected for the indicated zero-time value and are expressed per milliliter of the original cell suspension.

appears preferentially during the middle of exponential growth (17), in contrast to cultures of halophilic bacteria (21), *Micrococcus* (5), *Alcaligenes* (5), *Pseudomonas* (5), *Flavobacterium* (5), and *Neisseria* (6), where excretion of DNA occurs late during exponential growth or under unfavorable conditions. The transformable strain *B. subtilis* 168 is known to release transforming activity during early logarithmic growth and stationary phase (9, 25), at the time of development of competence (8, 20, 24, 25), and during outgrowth of germinated spores (2).

We find that transforming activity release during cell emergence of *B. subtilis* from the dormant state is resistant to relatively high concentrations of DNase, in contrast to all other processes of DNA excretion during other stages of the cell cycle, where the DNA is accessible to nuclease action at very low levels of the enzyme (8, 9, 20, 24, 25). The fact that transforming activity remains attached to the cell is not surprising. In almost all cases, and independent of the microorganism in question, transforming activity of cell-free filtrates or supernatants is invariably lower than that exhibited by an equivalent sample of the cell suspension (9, 17, 20, 25). The unusual feature of the DNA released from outgrowing spores is its resistance to high concentrations of DNase (at least 500 μg/ml) during the first cycle of replication. A combination of

detergent and proteolytic treatment known to expose DNA specifically attached to cell wall preparations of *B. subtilis* (4) did not unmask the transforming activity of outgrowing spores. The masking of the released chromosome does not appear to be due to structural elements remaining from the spore itself. Electron micrographs reveal that at 60 min of germination the cell has already emerged from the disintegrating spore coat and that cell walls look like their vegetative counterparts (18).

Development of competence occurs at a time when changes of autolytic enzyme activity and discrete reversible cell wall modifications are taking place (27–29). Autolysins external to the plasma membrane of *B. subtilis*, in particular those present during competence, have been postulated to alter the cell wall components of the recipient cells, thus facilitating irreversible DNA uptake (26, 30). We envision the process of DNA transfer presented in this communication as involving a tight contact between donor and recipient cell, with the latter providing some of the elements (probably the autolysins) necessary to trigger the unmasking of the donor's genetic material. Confirmation of the "cell-to-cell contact" hypothesis is suggested by the protection against very high concentrations of DNase (2 mg/ml) provided by the simultaneous presence of competent cells during nuclease treatment (Fig. 5A and B). Contact between donor and recipient further masks the genetic material from extracellular agents.

This paper deals with outgrowing spores as donors of genetic material. Spizizen in his first paper describing transformation of *B. subtilis* showed that at some stage during outgrowth germinated spores become competent for reception of genetic material (22). The facts that during a certain stage in their life cycle *B. subtilis* cells can act as donors as well as recipients of genetic material and that this transfer occurs only during close cellular contact raises the question of the possible occurrence in nature of such a genetic exchange and its mechanism. The latter, in its phenomenology, is somewhat reminiscent of the process of bacterial conjugation.

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