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Isolation and Partial Characterization of *Bacillus subtilis* Mutants Impaired in DNA Entry

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Transformation-deficient mutants of *Bacillus subtilis* have been identified either by screening for a nuclease-deficient phenotype on methyl green-DNA agar or for nontransformability on transforming DNA-containing agar. After purification of the mutations causing a reduction in the entry of DNA, a set of isogenic entry-deficient strains was obtained. In addition to being entry deficient to various extents, the strains usually were less capable of association with DNA than the entry-proficient parent. Likewise, the specific transforming activity in the purified mutant strains continued to be less than that in the wild type. With the possible exception of one strain, no evidence was obtained that the mutant strains were impaired in recombination. Since the breakdown of transforming DNA to acid-soluble products correlated fairly well with the residual capacity of the strains to take up DNA, nucleolytic activity is likely to be involved in the entry of DNA in *B. subtilis*.

On the basis of the observation that shortly after entry transforming DNA is reextractable from competent cells of *Streptococcus pneumoniae* only as single-stranded DNA and that the amount of acid-soluble products generated from donor DNA is equal to the amount of high-molecular-weight DNA rendered resistant to DNase I during entry, as early as 1962 Lacks (12) postulated that competent cells of *S. pneumoniae* possess a cell surface-located nuclease which effects the entry of transforming DNA by degrading one strand of the DNA duplex molecule while the complementary strand is pulled in. Subsequent experiments with the *S. pneumoniae* transformation system have provided excellent evidence for the correctness of this hypothesis. By means of selection on methyl green-DNA agar plates, Lacks and co-workers (14) isolated mutants which fail to produce a decoloration zone around the colonies on such plates. These mutants have been designated *noz* (for no zone) and appear to be almost completely DNA entry deficient. The mutants lack the major, membrane-located endonuclease (15, 16). Genetic analysis has further indicated that the *noz* mutations are in all probability located in the gene specifying the major endonuclease (15).

Because shortly after entry transforming DNA is also reextractable as single-stranded DNA from *Bacillus subtilis* recipients (5, 6, 20): and because the entry of DNA is also accompanied by the generation of acid-soluble breakdown products (7, 10), a mechanism of DNA entry similar to that of *S. pneumoniae* might also be operating in *B. subtilis*. Some support for this idea was obtained from the observation that EDTA completely prevents entry of donor DNA in *B. subtilis* (19) as well as the appearance of acid-soluble breakdown products of transforming DNA (4). In addition, spheroplasting of competent cells of *B. subtilis* (previously separated from the noncompetent fraction by Reno grafin gradient centrifugation) liberates DNase activity in the spheroplast supernatant fluid. This activity, probably exonucleolytic in nature, is probably located at the cell surface and strictly parallels the development of competence (10).

To establish whether Lacks' model for the entry of transforming DNA also applies to *B. subtilis*, we have engaged in a program of isolating transformation-deficient mutants having a nuclease-deficient phenotype on methyl green-DNA agar. In addition, transformation-deficient mutants have been screened on transforming DNA-containing agar. This report describes the isolation of such mutants and some of their properties.

MATERIALS AND METHODS

Strains. All strains used in this study were derived from *B. subtilis* 168; they are listed in Table 1.

Media. Minimal medium consisted of Spizizen (22) minimal salts plus 0.5% glucose, 0.02% casein hydrolysate (Difco Laboratories), and 14 μg of each of the growth factors per ml, except nicotinic acid and riboflavin which were added at 0.4 μg/ml. Rich minimal medium contained all constituents of the minimal medium plus 10 μg of each of the 20 amino acids and the four DNA bases per ml, if not already present in

TABLE 1. Genetic background and general properties of the *B. subtilis* strains used

Strain	Genotype[a]	Comments and references
OG-1	Prototrophic	
2G-8	*tyr-1 thy*	
3G-18	*trpC2 ade met*	Transformation proficient
8G-5	*trpC2 ade his met nic rib tyr-1 ura*	Transformation proficient (2)
7G-305	*trpC2 ade his met rib*	Transformation-proficient derivative of 8G-5, isolated on methyl green-DNA agar (this study)
7G-315	*trpC2 ade met rib*	Transformation-deficient derivative of 7G-305, isolated on methyl green-DNA agar (this study)
7G-319	*trpC2 ade*	
7G-320	*trpC2 ade his met nic rib*	
7G-321	*trpC2 ade his met nic ura*	Transformation-deficient derivatives of 8G-5, isolated on transforming DNA-containing agar (this study)
7G-322	*trpC2 ade his met nic ura*	
7G-325	*trpC2 ade his met nic ura*	
7G-326	*trpC2 ade his met nic rib ura*	
7G-73	*trpC2 met nic ura*	MMS-sensitive, recombination-deficient derivative of 8G-5 (3)
1G-34	*trpC2*	Strongly recombination-deficient strain carrying the *rec-4* mutation from GSY 908 (C. Anagnostopoulos, 17) in a background isogenic to 8G-5 (J. van Randen)

[a] Abbreviations have been explained previously (2, 3).

minimal medium. Starvation medium consisted of minimal salts plus 0.5% glucose. Minimal agar consisted of Spizizen minimal salts containing 1.5% agar (Difco) plus 1% glucose and the required growth factors. Nutrient agar contained 2.5% nutrient broth (Difco) and 1.25% agar. TY broth (pH 7.2) contained 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. TY agar, used for plating phage SPP1, consisted of TY broth plus 1.5% agar. Penassay broth (antibiotic medium no. 3, Difco) was used for growing bacterial strains in the transduction experiments. Unless stated otherwise all chemicals were obtained from BDH (Poole, England).

Mutagenesis. An overnight culture of the wild type in minimal medium was diluted threefold in rich minimal medium. The culture was grown for 2 h at 37°C and subsequently exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (50 μg/ml; Aldrich Chemicals Co., Inc., Milwaukee, Wis.) for 60 or 90 min at 37°C. The cells were washed twice in nutrient broth and diluted 10-fold in this medium. Segregation was for 4 to 5 h at 37°C.

Selection of transformation-deficient mutants having a nuclease-deficient phenotype. After segregation appropriate dilutions of the mutagenized culture were plated on nutrient agar containing methyl green and DNA (13). The plates were prepared in the following way. A solution of methyl green (4 g/liter; Merck & Co., Inc.) was extracted five times with chloroform (0.1 of the original volume). Calf thymus DNA (8 mg/ml; sodium salt, BDH) in 0.15 M NaCl plus 0.015 M trisodium citrate (SSC) was sterilized by the addition of chloroform (0.1 of the original volume). The chloroform was evaporated at 68°C.

To 160 ml of nutrient agar (pH 7.2) 40 ml of the DNA solution was added together with 8 ml of methyl green solution; 12.5 ml of the mixture was used per dish. At the pH chosen methyl green loses its color in nutrient agar not containing DNA. Therefore on this medium wild-type cells, by virtue of nucleases diffusing into the agar, produce a decoloration zone which is visible after 24 h of incubation at 37°C and becomes pronounced after 40 h. Clones showing a decreased decoloration capacity were isolated and tested for their transformability.

Selection of transformation-deficient mutants on agar plates containing transforming DNA. The method described by Joenje et al. (9) was used for selection of transformation-deficient mutants. Colonies of the mutagenized culture were replica plated from nonselective agar onto selective agar lacking tryptophan but spread with $trpC2^+$ DNA (approximately 30 μg per plate). The plates were incubated at 37°C. The colonies that failed to grow on the selective medium were picked up from the master plate and assayed for their transformability in liquid medium.

DNA preparations. Transforming DNA was isolated by a modification of Kirby's method (11) as described by Venema et al. (24). Unlabeled DNA was isolated from the prototrophic strain OG-1. Tritium-labeled DNA was obtained from strain 2G-8 grown in minimal medium containing [*methyl*-^{3}H]thymidine (1 mCi/100 ml; specific activity, 20.7 mCi/mg; Radiochemical Centre). The specific activity of the DNA preparations obtained was 1.5×10^5 to 3.0×10^5 cpm/μg of DNA.

Transformation. Cells were made competent by the method of Buitenwerf and Venema (3), with some modifications. Overnight cultures were grown and diluted in rich minimal medium. Transformation was for 30 min at 34°C. Uptake of DNA was terminated by the addition of DNase I (BDH; 40 μg/ml in 10 mM $MgSO_4$) and 5 min of additional incubation.

Total association, entry, and breakdown of transforming DNA. Total association, entry, and breakdown of transforming DNA by competent cultures were determined as described previously by Buitenwerf and Venema (3), with some modifications.

The total amount of DNA associated with the cells, both in DNase I-sensitive and in DNase I-resistant form, was determined by exposing the cultures to 1.5

μg of [*methyl*-^{3}H]thymidine-labeled *trpC2*$^+$ DNA per ml for 30 min at 34°C. Samples (1 ml) of the transformation mixture were added to 1 ml of ice-cold starvation medium containing 0.5 mg of highly polymerized calf thymus DNA (BDH) and 4 mM EDTA and gently mixed. One milliliter of the mixture was layered on top of a stepwise sucrose gradient (6 ml of 5% sucrose on top of 8 ml of 10% sucrose in minimal salts containing 20 mM EDTA) at 4°C and centrifuged for 30 min at 5,000 × *g* and 4°C. After decanting of the supernatant and careful removal of residual fluid, the pellet was suspended in 1 ml of minimal salts containing 100 μg of DNase I, 100 μg of lysozyme (Merck), and 10 mM $MgSO_4$. After incubation for 30 min at 37°C, a sample (0.6 ml) was mixed with 10 ml of scintillation fluid to determine the radioactivity as described previously (3).

The entry of DNA was measured in the same way, except that before centrifugation 1 ml of the transformation mixture was added to 1 ml of starvation medium of 37°C containing 80 μg of DNase I per ml and 15 mM $MgSO_4$, followed by vigorous agitation and subsequent incubation for 10 min at 37°C.

Breakdown of transforming DNA to acid-soluble products was determined by adding 1 ml of the transformation mixture and 1 ml of carrier DNA (highly polymerized calf thymus DNA, 1 mg/ml at 0°C) to 2 ml of ice-cold 6% perchloric acid. After 60 min at 0°C the mixture was centrifuged for 30 min at 6,000 × *g* and 4°C. Samples of the supernatant (0.6 ml) were mixed with scintillation fluid to determine the radioactivity.

Transformation frequencies were determined in the samples treated with DNase I after washing twice with starvation medium. The total number of viable cells was determined by plating the transformation mixture on nonselective agar.

Transduction. A transducing lysate of phage SPP1 was produced by collecting the confluent lysis obtained essentially by the soft agar plating method of Ferrari et al. (8). Strain OG-1 was used as donor. To increase the quantity of transducing SPP1 lysate, strain OG-1 was grown overnight in Penassay broth containing 20 mM $MgCl_2$ with moderate aeration. The overnight culture was diluted 50-fold in fresh medium, and after 2 h of incubation at 37°C with vigorous aeration, the culture was infected with 5×10^5 PFU of SPP1 per ml. Incubation at 37°C was continued until the decrease in optical density at 450 nm had stopped. The phages were collected by precipitation with polyethylene glycol 6000 followed by CsCl gradient centrifugation by the method of Bron et al. (1). The phage suspension (titer, 2×10^{12} PFU/ml) was stored at 4°C.

Transduction was carried out as follows. Overnight cultures of the recipient strains in Penassay broth were diluted in fresh medium to an optical density at 450 nm of 0.5 and grown for 3.5 h with moderate aeration at 37°C. One milliliter of this culture was subsequently mixed with 7.5 ml of Penassay broth (37°C), 0.5 ml of 1 M $MgCl_2$ containing 250 μg of DNase I, and 1 ml of the transducing lysate (diluted in Penassay broth to about 3×10^{10} PFU/ml). The mixture was incubated at 37°C for 30 min with shaking and centrifuged for 10 min at 6,000 × *g*, and the pellet was suspended in 1 ml of starvation medium. Transductants (*trpC2*$^+$) were scored on selective minimal agar.

Sensitivity to UV, MMS, and mitomycin C. Sensitivity of the bacterial strains to irradiation with UV and to treatment with methyl methane sulfonate (MMS) and mitomycin C was determined as described by Buitenwerf and Venema (3). The lethal dose of mitomycin C was determined as that concentration which inhibited growth on the mitomycin C-containing agar completely (<1/500 colonies). The minimal inhibiting concentration was determined as that concentration of mitomycin C producing a just noticeable effect on growth (diameter or number of colonies).

RESULTS

Isolation and partial characterization of transformation-deficient mutants showing a nuclease-deficient phenotype on methyl green-DNA agar. Since exonuclease activity can easily be masked by endonuclease activity produced by colonies on methyl green-DNA agar (13), we attempted to obtain a mutant considerably impaired in its capacity to decolorize the methyl green-DNA agar, to improve the conditions for the isolation of mutants impaired in exonucleolytic activity. Such a mutant was isolated, and the mutation was subsequently purified by transformation of the wild type 8G-5 with the mutant DNA with selection for *tyr-1*$^+$ transformants having the mutant phenotype on methyl green-DNA agar. The properties of the purified mutant strain (7G-305) are given in Table 2. Little, if any, change has occurred in the interaction of strain 8G-5 with transforming DNA as the result of the introduction of the mutation causing the nuclease-deficient phenotype.

Strain 7G-305 was mutagenized, and mutants were screened for a further decreased capacity to decolorize methyl green-DNA agar. Two different phenotypes were obtained: mutants which completely failed to produce a decoloration zone after 4 days of incubation at 37°C (Noz mutants) and mutants which had a phenotype intermediate between Noz and that of the parental type (7G-305). Both the Noz and the intermediary decolorizing mutants were assayed for their transformability and, if transformation was strongly reduced, also for their capacity to associate with DNA (Table 2). The results show that the Noz mutants were not (AJ-1) or only slightly impaired in transformation, and that this impairment in mutant AM-26 correlated with its decreased capacity to associate with DNA. In addition, mutant AM-26 appeared to be not impaired in DNA entry. Therefore the Noz mutants are not (AM-26) or slightly, if at all, impaired in DNA entry, suggesting that the nuclease that is believed to be involved in DNA entry probably does not contribute to the decoloration of methyl green-DNA agar. Only 10 out of 103 intermediary decolorizing mutants appeared to be strongly impaired in transformation. Six of these mutants, AJ-12, AK-16, AK-21, AK-28, AM-13, and AM-24, were strongly deficient in total DNA association (Table 2).

TABLE 2. Transformation, specific transforming activity, and total association, entry, efficiency of entry, and breakdown of transforming DNA in transformation-deficient mutants derived from strain 7G-305 and showing decreased decoloration on methyl green-DNA agar

Strain or mutant	Phenotype on methyl green-DNA agar[a]	Transformation frequency[b] (10^6 *trpC2*$^+$/CFU)	^{3}H radioactivity (10^6 cpm/CFU)			Efficiency of entry[c] (%)	Specific transforming activity[d] (*trpC2*$^+$/cpm)
			Total DNA association[e]	Entry[f]	Breakdown[g]		
8G-5	Wild type	15,100[h]	241	53.6	163	100	362
7G-305	Moderate	35,300	545	101	317	83.3	186
AJ-1	Noz	22,200	590				
AK-3	Noz	11,300					
AL-3	Noz	6,300					
AM-26	Noz	4,500	62.7	11.4	25.7	81.9	513
AJ-12	Intermediate	444	8.5				
AK-10	Intermediate	1.5	40.7	1.4	3.3	15.3	1.0
AK-12	Intermediate	426	147	16.7	112	51.3	39.5
AK-16	Intermediate	1.5	4.2				
AK-21	Intermediate	679	14.1				
AK-28	Intermediate	651	8.9				
AL-15	Intermediate	285	85.5	4.9	9.8	25.7	102
AM-13	Intermediate	593	7.5				
AM-23	Intermediate	703	53.5	8.1	19.4	68.5	111
AM-24	Intermediate	375	7.5				

[a] The wild type produced clear decoloration zones after 2 days at 37°C. Strain 7G-305 was a decoloration mutant producing clear zones after 3 days at 37°C. Mutants that failed to produce a decoloration zone after 4 days at 37°C were designated Noz (for no zone). Mutants having an intermediate decoloration phenotype still had a definite decoloration capacity, but less than that of strain 7G-305.

[b] Number of DNase I-resistant *trpC2*$^+$ transformants per colony-forming unit (CFU) after 30 min of incubation at 34°C with transforming *trpC2*$^+$ [^{3}H]DNA.

[c] Fraction of total cell-associated radioactivity rendered resistant to DNase I, expressed as percentage of the wild-type value.

[d] Number of DNase I-resistant *trpC2*$^+$ transformants per counts per minute of transforming *trpC2*$^+$ [^{3}H]DNA rendered resistant to DNase I.

[e] Total amount of radioactivity associated with cells both sensitive and resistant to DNase I.

[f] Amount of DNase I-resistant radioactivity associated with the cells.

[g] Acid-soluble radioactivity in the transformation mixture, derived from transforming DNA.

[h] The values given are the averages of two (mutants assayed only for total association) to four experiments.

This deficiency almost completely accounted for the reduced transformability of these mutants. Mutant AK-16 might be entry deficient, but, because of its very low total DNA association capacity, this mutant was not investigated further.

The mutants AK-10, AK-12, AL-15, and AM-23, also having an intermediary decoloration capacity, were still capable of associating a considerable amount of DNA, although their transformability was severely reduced (Table 2). These mutants have been examined in greater detail. In addition to the total DNA association capacity, the DNase I-resistant fraction of the cell-associated DNA (entry) and breakdown of the donor DNA to acid-soluble products were determined. Mutants AK-10 and AL-15 were to various degrees impaired in the entry of DNA, AK-10 most strongly so. In addition the specific transforming activity was also very low in the latter mutant. Contrary to the parental strain 7G-305 and the mutant AK-12, the mutants AK-10 and AL-15 were also strongly impaired in their capacity to generate acid-soluble products from transforming DNA.

Properties of transformation-deficient mutants screened on transforming DNA-containing agar. Colonies that failed to be transformed on transforming DNA-containing agar were picked, subjected to the competence regimen in liquid medium, and assayed for transformation. Thirty mutants were deficient in transformation. Those mutants for which the ratio of total DNA association to transformation frequency was decreased more than 10-fold relative to the wild type were also investigated with respect to DNA entry, their capacity to degrade the donor DNA to acid-soluble products, and their specific transforming activity. All mutants were impaired in total DNA association and in DNA entry, although to various extents (Table 3). The inhibition of DNA entry was most pronounced in mutant 8-23. All mutants also showed a reduced capacity to degrade transforming DNA to

TABLE 3. Transformation, specific transforming activity, and total association, entry, efficiency of entry, and breakdown of transforming DNA in transformation-deficient mutants, isolated on transforming DNA-containing agar

Mutant	Transformation frequency[a] (10^6 *trpC2*$^+$/CFU)	^{3}H radioactivity (10^6 cpm/CFU)			Efficiency of entry[b] (%)	Specific transforming activity[c] (*trpC2*$^+$/cpm)
		Total DNA association[d]	Entry[e]	Breakdown[f]		
3G-18	12,700[g]	171	68	196	100	293
1-29	0.3	16.6	1.2	7.0	18.1	0.2
1-37	0.8	56.2	1.5	15.1	6.8	0.6
2-37	190	32.9	7.3	25.9	55.8	32.9
4-9	5.8	13.9	0.9	9.3	16.3	6.8
4-18	10.3	9.3	0.6	2.3	16.1	27.5
4-25	36.0	13.5	0.4	2.9	7.5	130
5-3	23.3	21.5	0.5	4.7	5.8	70.3
5-27	80.2	21.8	1.6	5.2	18.4	94.6
6-56	<0.1	3.9	0.3	3.7	19.3	<0.1
8-23	10.3	141	1.5	6.3	2.8	11.8
8-33	6.5	10.2	0.4	3.6	9.8	36.4
8-46	16.5	8.9	0.8	2.6	22.6	33.2

[a,b,c,d,e,f] See footnotes *b*, *c*, *d*, *e*, *f*, and *g*, respectively, of Table 2.
[g] The values given are the averages of two to six experiments.

acid-soluble products. The level of DNA degradation paralleled the level of DNA entry fairly well in these mutants. Additionally all mutants showed various decreases of the specific transforming activity of the entered DNA. This decrease was very marked in the mutants 1-29, 1-37, and 6-56. Apparently, no quantitative relationship exists between the impairment in the specific transforming activity and the reduction in DNA entry.

Effect of purification of mutations causing entry deficiency on the interaction of the mutant strains with transforming DNA. To establish whether the impairment of the various properties in the mutants was the consequence of one mutation or several, some of the mutations causing a decrease in DNA entry were transferred into the wild-type background by transformation.

The mutation in AK-10 was transferred into the 7G-305 background by congression: after the isolation of an *his*$^+$ transformant of AK-10 having the relevant AK-10 properties, strain 7G-305 was transformed by DNA of this transformant, followed by selection for *his*$^+$ transformants of strain 7G-305 having the AK-10 phenotype on selective minimal agar containing methyl green and DNA. The resulting entry-deficient strain was denoted 7G-315. The mutations of the mutants isolated on transforming DNA-containing agar were transferred into strain 8G-5 also by congression: *tyr-1*$^+$ transformants of 8G-5 were selected, which were subsequently assayed for deficiency in DNA entry. After purification of the mutations derived from the mutants 8-23, 1-29, 4-25, 6-56, 1-37, and 5-3, the 8G-5 recipients have been designated 7G-319, 7G-320, 7G-321, 7G-322, 7G-325, and 7G-326, respectively.

The properties of the purified mutants with respect to total DNA association, entry, breakdown, and specific transforming activity are listed in Table 4. The DNA association capacity of the mutants was little affected by purification in the strains 7G-315, 7G-319, 7G-321, and 7G-322. Strain 7G-320 appeared to have lost its impairment in DNA association completely, resulting in a much stronger impairment in the efficiency of DNA entry. This strain retained its strong impairment to break down transforming DNA. Strain 7G-325 and to a lesser extent strain 7G-326 showed a considerably reduced capacity to associate with DNA, resulting in a reduction of the efficiency of DNA entry. Purification of the mutations causing an impairment in DNA entry in the strains 7G-315, 7G-319, and 7G-320 resulted in a considerable increase in the specific transforming activity of the DNA: strain 7G-319 attained almost the wild-type level; 7G-315 still showed a moderate impairment, whereas 7G-320 continued to be strongly impaired.

Binding of DNA in the presence of EDTA. The capacity to associate with DNA of the entry-deficient strains was determined in the presence of 30 mM EDTA, which is known to prevent the entry, but not the binding, of DNA (19). Comparison of the DNA binding in the presence of EDTA with the total association of DNA during transformation (in the presence of Mg^{2+} ions, Table 4) showed that the capacity to associate with DNA increased only slightly in strain 7G-315. In strain 7G-325 the total DNA association increased about ninefold in the presence of EDTA, indicating that DNA bound to this mutant may be detached from the cell surface when nucleolytic activity is not inhibited.

TABLE 4. Transformation, specific transforming activity, and total association, entry, efficiency of entry, breakdown, and binding in the presence of EDTA of transforming DNA by purified transformation-deficient strains

Strain	Transformation frequency[a] (10^6 *trpC2*$^+$/CFU)	^{3}H radioactivity (10^6 cpm/CFU)				Efficiency of entry[b] (%)	Specific transforming activity[c] (*trpC2*$^+$/cpm)
		Total DNA association[d]	Entry[e]	Breakdown[f]	DNA binding[g]		
8G-5	15,100[h]	241	53.6	163	471	100	362
7G-315	194	84.2	3.5	11.7	292	18.7	68.8
7G-319	87.5	152	0.6	3.1	331	1.8	180
7G-320	4.1	229	0.5	4.9	523	1.0	12.4
7G-321	46.4	35.9	0.3	3.5	50.1	3.8	155
7G-322	<0.1	10.2	0.2	1.3	26.7	8.8	<0.1
7G-325	0.3	5.7	0.2	5.1	106	15.8	2.0
7G-326	20.7	11.3	0.3	4.7	32.4	12.0	121

[a,b,c,d,e,f] See footnotes *b*, *c*, *d*, *e*, *f*, and *g*, respectively, of Table 2.

[g] Total amount of radioactivity associated with the cells after preincubation (15 min) with 30 mM EDTA and subsequent incubation for 30 min at 34°C with 1.5 μg of transforming [^{3}H]DNA per ml.

[h] The values given are the averages of three to five experiments.

Sensitivity to UV irradiation, MMS, and mitomycin C. Since mutants specifically impaired in the postentry processing of transforming DNA (recombination) are often sensitive to UV irradiation and radiomimetic agents, we have tested the sensitivity of the purified mutants to UV irradiation, MMS, and mitomycin C. With the exception of strain 7G-315, which was slightly more sensitive to UV irradiation than the wild type, the UV sensitivity of the remainder of the strains was similar to that of the wild type (Fig. 1).

The mutant strains were as sensitive to MMS as the wild type (Fig. 2). In addition, strain 7G-315 was the only strain that was slightly sensitive to mitomycin C (Table 5). From these data the conclusion can be drawn that, contrary to the highly sensitive reference strain 7G-73, the mutants are not significantly impaired in postreplication repair.

Phage SPP1-mediated transduction. Ferrari et al. (8) have demonstrated that in all recombination-deficient *B. subtilis* mutants tested both SPP1 transduction and transformation are impaired. Therefore, the purified mutant strains were tested with respect to their transducibility with SPP1 (Table 6). All mutant strains were closely similar to the wild-type strain with respect to their transducibility mediated by phage SPP1. The reference strain 1G-34, which is strongly recombination deficient (17), showed no detectable level of transduction.

DISCUSSION

We have not been able to isolate entry-deficient mutants of *B. subtilis* that have completely lost their capacity to decolorize methyl green-DNA agar (mutants showing the Noz phenotype). This is in striking contrast to *S. pneumoniae*, in which *noz* mutants are entry deficient (14). Two entry-deficient mutants, AK-10 and AL-15, have been obtained in the class of decoloration mutants still showing a reduced capacity to decolorize methyl green-DNA agar. The rea-

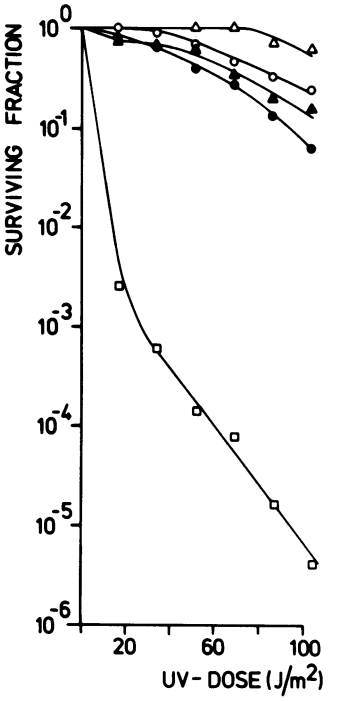

FIG. 1. Cell survival as a function of UV dose. Samples of exponentially growing cultures were irradiated for various intervals with UV and assayed for colony-forming ability. Symbols: ○, 8G-5; ●, 7G-315; △, 7G-322; ▲, 7G-326; □, 7G-73, a highly sensitive strain (3). The curves for the strains 7G-319, 7G-320, 7G-321, and 7G-325 were almost identical to that of 8G-5.

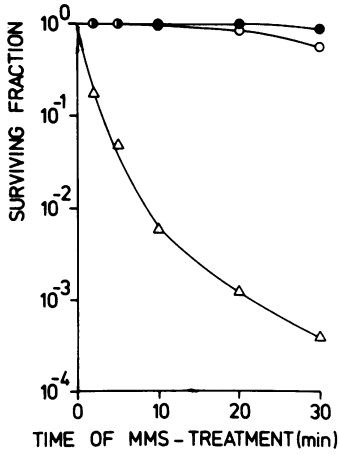

FIG. 2. Cell survival as a function of time of exposure to MMS. Samples of exponentially growing cultures were exposed for various times to 7.5 mM MMS and assayed for colony-forming ability. Symbols: ○, 8G-5; ●, 7G-326; △, 7G-73. The curves for strains 7G-319, 7G-320, and 7G-321 were identical to that of 8G-5, and the curves for strains 7G-315, 7G-322, and 7G-325 were identical to that of 7G-326.

son why we were unable to observe entry-deficient mutants among the Noz phenotypes, although two entry-deficient mutants showing a decreased nucleolytic activity were isolated, remains unclear. One possibility is that the Noz phenotype was due to a changed cell envelope preventing proper diffusion of the nucleases into the surrounding agar. An alternative explanation is that the nuclease activity that is thought to be involved in DNA entry is diffusing into the agar, but does not contribute to decoloration of methyl green-DNA agar. Some support for the latter possibility may be derived from the observation

TABLE 5. Sensitivity to mitomycin C[a]

Strain	Lethal concn[b] (ng/ml)	Minimal inhibiting concn[c] (ng/ml)
8G-5	75	50
7G-315	37	25
7G-319[d]	75	50
7G-73	5	1

[a] The colony-forming ability was assayed on nutrient agar containing various concentrations of mitomycin C.

[b] The concentration inhibiting growth completely.

[c] The concentration of mitomycin C resulting in a just observable inhibition of growth (number or diameter of colonies).

[d] The mutants 7G-320, 7G-321, 7G-322, 7G-325, and 7G-326 showed the same sensitivity to mitomycin C as did 7G-319.

TABLE 6. SPP1 transduction in the purified mutant strains and the wild type

Strain	Transduction frequency[a] (10^6 *trpC2*$^+$/CFU)
8G-5	2.0 ± 0.8 (9)[b]
7G-315	1.3 ± 1.1 (4)
7G-319	2.7 ± 1.5 (3)
7G-320	1.2 ± 0.7 (4)
7G-321	2.4 ± 0.4 (4)
7G-322	1.5 ± 0.6 (4)
7G-325	3.4 ± 1.4 (4)
7G-326	1.9 ± 0.8 (4)
1G-34	<0.05 ± 0.0 (5)

[a] Ratio of the number of DNase I-resistant *trpC2*$^+$ transductants and the original number of colony forming units (CFU) added to the transduction mixture.

[b] The values given are the averages of three to five experiments. The numbers of experiments from which the standard deviations were calculated are given within parentheses.

that the entry-deficient strains, which had been isolated on transforming DNA-containing agar (the strains listed in Table 4, except for 7G-315), showed the wild-type phenotype on methyl green-DNA agar. In addition, some of these mutants (7G-321, 7G-322, 7G-325, and 7G-326) appeared to be impaired in a competence-associated nucleolytic activity when assayed on DNA-containing sodium dodecyl sulfate-polyacrylamide gels, whereas 7G-315 showed the wild-type activity (submitted for publication). These results and the low frequency of entry-deficient mutants among the intermediary decolorizing phenotypes lead to the conclusion that the methyl green-DNA decoloration method does not appear to be very efficient for the isolation of entry-deficient mutants in *B. subtilis*. The high proportion of transformation-proficient mutants showing an intermediate phenotype on methyl green-DNA agar suggests that nuclease activity not involved in transformation also contributes to the decoloration.

By determining the efficiencies of DNA entry of mutants unable to be transformed on transforming DNA-containing agar plates, entry-deficient mutants were more successfully obtained. Both in these mutants and in the entry-deficient intermediary decolorizing mutants the breakdown of DNA was fairly well correlated with the extent of DNA entry even after purification of the mutations causing entry deficiency (Table 4), suggesting that nuclease activity is involved in DNA entry.

Purification of the mutations by transformation had a marked beneficial effect on the specific transforming activity of the strains 7G-315

(mutant AK-10), 7G-319 (mutant 8-23), and 7G-320 (mutant 1-29; Tables 2, 3, and 4); the specific transforming activity of 7G-319 was restored almost to the range of the wild-type value. This seems to indicate that the unpurified mutants possessed additional mutations interfering with the postentry processing of DNA. The incomplete recovery of the specific transforming activity upon mutant purification and the low value of this characteristic in the purified strains 7G-320, 7G-322, and 7G-325 may either suggest that the physical state of the DNA entering these strains renders it insuitable for recombination with the recipient chromosome or that residual mutations inhibiting recombination continue to the present in the purified mutants. However, the presence of mutations interfering with recombination renders *B. subtilis* very sensitive to UV irradiation or radiomimetic agents or both (for a review, see reference 18). Since the purified mutant strains, with the exception of strain 7G-315, were as resistant as the wild type to UV irradiation (Fig. 1), MMS (Fig. 2), and mitomycin C (Table 5), and since the frequency of phage SPP1-mediated transduction was very much similar to that of the wild type, whereas it is reduced in all tested recombination-deficient mutants of *B. subtilis* (8), the latter possibility is unlikely. In addition it may be questioned whether the radioactivity which remains associated with the cells after treatment with DNase I reflects in a quantitative way the amount of DNA which has entered the cells. Resistance to removal by DNase I of only a small amount of bound but not entered DNA would be insignificant in the wild type, but would result in an apparent low specific transforming activity in mutants in which only very small amounts of DNA enter the cells (for instance, strains 7G-322 and 7G-325).

That the purification procedure reduced the total DNA association capacity of strain 7G-325 (mutant 1-37) may be explained on the basis of the elimination of a secondary mutation partly compensating for the loss of the capacity of this mutant to associate with DNA. The partial restoration of the DNA-binding capacity of strain 7G-325 in the presence of EDTA may indicate that nucleolytic activity at the cell surface of this strain, which is inhibitable by EDTA, might be responsible for its reduced capacity to associate with DNA. The question may be raised whether the low total DNA association values of the strains 7G-322, 7G-325, and 7G-326 are still related to transformation or should be considered as basal levels not related to this process. We believe that these values are meaningful, because of the properties of a strain which showed at least five times less DNA association than strain 7G-325 and which still showed residual transformation (data not shown). The dramatic increase in total DNA association in strain 7G-320 (mutant 1-29) after purification of the mutation causing entry deficiency is probably due to the elimination of an additional mutation inhibiting the binding of DNA.

Except for strain 7G-320, the impairment of DNA entry to various extents in the mutant strains was accompanied by a decrease in their total DNA association capacity. This marked pleiotropic effect continued to exist after purification of the mutations in DNA entry, particularly in the strains 7G-321, 7G-322, 7G-325, and 7G-326. The binding of DNA never reached the wild-type level in conditions preventing the entry and breakdown of DNA (Table 4). This seems to suggest that the presumptive nuclease required for DNA entry might also be involved in the binding of DNA. However, the properties of strain 7G-320, which is binding proficient but entry deficient, show that, at the genetic level, the two functions are separable. The fact that the two functions are often jointly impaired may be explained on the assumption that the presumptive genes for binding and entry of DNA are coordinately transcribed.

The entry-deficient strains described in this study are different from the vast majority of transformation-deficient mutants of *B. subtilis* described before, because almost all of those mutants are recombination deficient and sensitive to UV irradiation or radiomimetic agents or both (18). Only a few transformation-deficient mutants that have been isolated on transforming DNA-containing agar appeared to be not or only slightly sensitive to UV irradiation, MMS, or mitomycin C. Strain 7G-97, described by Buitenwerf and Venema (3), is only slightly sensitive to UV irradiation and completely resistant to MMS and mitomycin C. Because it is capable of associating with an appreciable amount of DNA and is impaired in DNA entry, it might be similar to the strains 7G-319 and 7G-320 described in this study. Tichý and co-workers (23) have described four entry-deficient mutants that are fairly capable of associating with DNA, but are completely nontransformable. In at least two of these mutants a very low amount of donor radioactivity was rendered resistant to DNase I. However, because the purification of these mutants was not described, no definite comparison with our mutants can be made. Some of the mutants described by Polsinelli and co-workers (21) and by Mazza and co-workers (17) may be similar to the mutants described in this study, because they are completely resistant to UV irradiation and mitomycin C. However, because no data concerning the total DNA association and DNA binding are available for these mutants, a more detailed comparison with the mutant strains described here is not possible.

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