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Recombination-Deficient Mutants of *Bacillus subtilis*[1]

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Two mutant strains of *Bacillus subtilis* Marburg, NIG43 and NIG45, were isolated. They showed high sensitivities to gamma rays, ultraviolet light (UV), and chemicals. Deficiencies in genetic recombination of these two mutants were shown by the experiments on their capacity in transformation, SPO2 transfection, and PBS1 phage transduction, as well as on their radiation and drug sensitivities and their Hcr$^+$ capacity for UV-exposed phage M2. Some of these characteristics were compared with those of the known strains possessing the *recA1* or *recB2* alleles. Mapping studies revealed that the mutation *rec-43* of strain NIG43 lies in the region of chromosome replication origin. The order was *purA dna-8132 rec-43*. Another mutation, *rec-45*, of strain NIG45 was found to be tightly linked to *recA1*. The mutation *rec-43* reduced mainly the frequency of PBS1 transduction. On the other hand, the mutation *rec-45* reduced the frequency of recombination involved both in transformation and PBS1 transduction. The mutation *rec-43* of strain NIG43 is conditional, but *rec-45* of strain NIG45 is not. The UV impairment in cellular survival of strain NIG43 was gradually reverted at higher salt or sucrose concentrations, suggesting cellular possession of a mutated gene product whose function is conditional. In contrast to several other recombination-deficient strains, SPO2 lysogens of strains NIG43 and NIG45 were not inducible, indicating involvement of *rec-43*$^+$ or *rec-45*$^+$ gene product in the development of SPO2 prophage to a vegetative form. The UV-induced deoxyribonucleic acid degradation in vegetative cells was higher in *rec-43* and *rec-45* strains.

Recombination-deficient strains of *Bacillus subtilis* have been isolated and characterized in several laboratories (6, 8, 9, 11, 12, 14, 15, 25, 27, 29, 36). Studies on recombination in *B. subtilis* have a unique feature: the fate of transforming deoxyribonucleic acid (DNA) can be pursued in the course of integration into host genomes with different genetic capacities in recombination (5, 8, 40). To develop these studies, however, we still lack systematic characterization of *B. subtilis rec* strains with respect to ultraviolet light (UV) or gamma ray sensitivities, radiation-induced DNA degradations, drug sensitivities, prophage inducibilities, activities of DNA enzymes, including adenosine 5'-triphosphate-dependent deoxyribonuclease (ATP-dependent DNase), and other properties that have been well analyzed in typical *rec* strains of *Escherichia coli* (3, 16, 38). We describe here some results of such studies carried out on two recombination-deficient strains of *B. subtilis* isolated in our laboratory in comparison with some other known *rec* strains. The results of a genetic analysis of our mutants are also presented.

MATERIALS AND METHODS

Strains. A Marburg strain 15 (*argA15 trp-3*) was first obtained from the laboratory of Y. Ikeda and H. Saito (24). After single-colony purification from this strain, a strain designated NIG17 (*argA15 trp-3*) was obtained which has a stable recipient property for transformation. By treating cells of strain NIG17 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG, 500 μg/ml at pH 6.0) and testing randomly 433 surviving colonies for gamma ray sensitivity, several radiation-sensitive mutants were isolated. One, strain NIG45 (*argA15 trp-3 rec-45*), has a very high sensitivity to gamma rays and UV. Another radiation-supersensitive strain, NIG43 (*argA15 trp-3 rec-43*), was isolated by examining 94 colonies of NTG-mutagenized surviving cells of strain Sb36 (31), which had been derived from strain NIG17 through NTG mutagenesis but remained radiation resistant. Other strains used are presented in Table 1. Strain 168T was used for preparing radioactive or transforming DNA. All of the strains were maintained on potato extract slants at 4 C or in the culture medium containing 12.5% glycerol at −80 C. Strains were examined periodically for their specific properties.

Media. In all experiments, bacteria were grown at

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TABLE 1. *Bacterial strains and phages used*

Strain	Genotype	Origin
168T	*thy*	Y. Ikeda
15	*argA15 trp-3*	H. Saito
UVR	*thy met ind sul*	N. Munakata and H. Saito
UVS19	*thy ind uvs-19*	N. Munakata and H. Saito
NIG17	*argA15 trp-3*	Derived from strain 15 (H17)[a]
NIG43	*argA15 trp-3 rec-43*	NTG-induced from NIG17 (L43)[a]
NIG45	*argA15 trp-3 rec-45*	NTG-induced from NIG17 (M45)[a]
GSY1035	*metC3 hisA1 ura-1*	C. Anagnostopoulos
GSY1037	*metC3 ura-1 recA1*	C. Anagnostopoulos
GSY1061	*nic-38 trpC2 recB2*	C. Anagnostopoulos
BD170	*thr-5 trpC2*	D. Dubnau
BD191	*thr-5 trpC2 recB2*	D. Dubnau
BD193	*thr-5 trpC2 recD3*	D. Dubnau
BD194	*trpC2 recA1*	D. Dubnau
BD224	*thr-5 trpC2 recE4*	D. Dubnau
DB239	*thr-5 trpC2 recC7*	D. Dubnau
BD241	*thr-5 trpC2 recF16*	D. Dubnau
BD246	*thr-5 trpC2 recG13*	D. Dubnau
P19	*trpC2 osp-19*[b]	J. G. Coote
Ts[c]	*purA dna-8132 sacA321*	H. Yoshikawa
SPO1	Wild type	S. Okubo
SPO2	Wild type	T. Shibata
SPO2	*c1*	S. Okubo
M2	Wild type	H. Saito
PBS1	Wild type	C. Anagnostopoulos

[a] Formerly named.
[b] Tentative genotype of an oligosporogenous strain.
[c] Tentative name of a *dna* strain.

37 C, unless otherwise stated, in TF medium (21) or in Penassay broth (Difco). TF medium was prepared by enriching a modified Vogel-Bonner salt mixture with 0.1% Casamino Acids, 0.2% yeast extracts, 10 μg of each required amino acid or other substance per ml, and 0.4% glucose. The above Vogel-Bonner minimal salt mixture was prepared by dissolving 1 g of $(NH_4)_2SO_4$, 10 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of trisodium citrate $\cdot 2H_2O$ in distilled water, neutralizing with KOH (about 1.94 g required), and finally making the volume 1,000 ml with distilled water. Phosphate buffer solution (67 mM, pH 7.0) or TF medium without glucose was used as the diluent where indicated. Nutrient broth (0.8%) (Difco) was solidified with Wako agar (1.5%) and used for determination of the number of viable cells. For extraction of cellular DNA, strain 168T was cultured in the above salt mixture supplemented with 0.5% polypeptone, 0.05% yeast extract, 0.4% glucose, and 20 μg of thymine per ml.

Irradiation. Cells collected in the exponential phase of growth were washed, resuspended in phosphate buffer, and kept at 0 C. UV irradiation was carried out with a 15-W germicidal lamp (National Co. Ltd.) at a dose rate of about 15 ergs/mm^2 per s. Irradiation with gamma rays was carried out using a 6,000-Ci ^{137}Cs source at a dose rate of about 40 kR/h.

Host-controlled reactivation. The host-controlled reactivation experiments were conducted with a virulent phage M2 (17) provided by H. Saito. UV-exposed (900 ergs/mm^2) phage particles in phosphate buffer were titered on different strains using the usual soft-agar method.

Transformation. An overnight culture of each recipient strain in TF medium was diluted 30 times with fresh TF medium and incubated at 37 C with reciprocal shaking. At indicated times, portions of the culture were sampled. After adding defined amounts of transforming DNA, they were incubated for 60 min under gentle shaking. The cellular uptake of DNA was terminated by adding 12 μg of DNase I/ml and 6 mM $MgSO_4 \cdot 7H_2O$ into the sample and by incubating the culture for 20 min. After appropriate dilutions with TF medium without glucose, a portion of the sample was plated on selective agar consisting of minimal salt mixture (containing 0.4% glucose) supplemented with 20 μg of amino acid/ml, 0.0016% broth, and 1.5% agar (Difco). Transformant colonies were scored after incubating the plates for 48 h at 37 C. In experiments using strain NIG43, plates were incubated for 4 days because the development of transformant colonies was slow.

The above procedure, which gave a fairly good number of very reproducible transformants without artificial modification of cellular growth physiology, was adopted for all of the experiments. Maximal competency was observed at the end of the logarithmic phase of growth for each strain. The frequency of transformation from arginine dependence to arginine

independence was usually 10^{-4}/cell in wild strain NIG17 with a saturating amount of DNA. The properties of the strains remained stable over 5 years.

Transforming DNA was isolated by the phenol method (34). The DNA concentration was determined by the diphenylamine method (2).

Transfection. Phages SPO1 (wild) and SPO2 (*c1*, clear-plaque mutant) were kindly provided by S. Okubo and used as described by Okubo et al. (26, 28), but the purification step by CsCl centrifugation was omitted. Their DNA was combined with the competent culture of each strain in TF medium at 37 C for 60 min. After terminating the DNA uptake with DNase, the number of infectious centers was scored using strain 168T.

Transduction. Phage PBS1-mediated general transduction was carried out as described by I. Takahashi (37). PBS1 phages were obtained from C. Anagnostopoulos. Recipient cells grown in Penassay broth were combined with phages at a multiplicity less than one and incubated for 30 min. The cells were then collected by centrifugation, resuspended in TF medium without glucose, and plated on selective agar. Recipient strains were checked as to their active flagella by their motility on semisolid agar (Penassay broth containing 0.4% agar [Difco]). For preparation of the phage lysate, cells of strain 168T grown exponentially in Penassay broth were infected with PBS1 phages and incubated for an additional 60 min and kept at 37 C overnight. Cell debris was discarded after low-speed centrifugation, and the lysate was sterilized by chloroform and treated with DNase. Phages were diluted in absorption buffer (39) and titered on strain 168T after preabsorption of the phage in tryptose blood base agar.

Bacterial sensitivities to drugs. Log-phase cultures of strains were inoculated on small areas of broth agar containing each drug at different concentrations. These plates were incubated overnight at 37 C and scored for bacterial growth.

Mitomycin C (MC) was purchased from Kyowa Hakko Co. Ltd. Methyl methane sulfonate (MMS) was purchased from Tokyo Kasei Co. Ltd. and dissolved at 0.086% in 0.07 phosphate buffer to obtain a 10 mM solution.

Selection of recombinants. Transductant or transformant colonies were picked and grown in Penassay broth or on broth agar and inoculated or replicated on selective agar. For selection of Rec^+ recombinants, broth agar containing 0.05 μg of MC/ml was used. Colonies appearing on the plate were designated as Rec^+. Temperature-resistant recombinants were selected as clones that developed on broth agar at 48 C. For selection of oligosporogenic recombinants, lactate-glutamate agar (4) was employed. Colonies pigmented brown on this plate were designated as Osp^+.

Prophage SPO2 induction. Wild SPO2 phages were obtained from T. Shibata. SPO2 lysogens were prepared from the center of the turbid plaques formed on each strain. Establishment of lysogenization was confirmed by observing resistance to the SPO2 clear-plaque mutant and spontaneous inducibility of the lysogens.

Exponential cultures at about 25 Klett units were filtered with a membrane filter (Millipore, 0.45-μm, type HA) and resuspended in prewarmed fresh medium of the same volume. The bacteria were then exposed to UV in a thin layer of about 0.35 mm from a 5-W germicidal lamp (Sankyo Co. Ltd.) and incubated with vigorous shaking at 37 C. After 3 h of incubation, a few drops of chloroform were added and the cultures were shaken gently for 15 min. Cell debris was removed by low centrifugation, and the phages in the resulting supernatant fraction were titered on strain 168T as indicator. The liquid medium was NY (26) broth, and phage titration was done by the method of Okubo and Romig (26).

DNA degradation in intact cells. To study degradation of cellular DNA, bacteria were labeled with [^{3}H]thymidine. An overnight bacterial culture in TF medium was diluted five times with the same medium supplemented with 2 μCi of [^{3}H]thymidine/ml and 300 μg of deoxyadenosine/ml and incubated with shaking. When the cellular concentration reached about 10^8/ml, cells were washed twice with phosphate buffer and resuspended in fresh TF medium of the same volume containing 50 μg of unlabeled thymidine/ml and incubated for 40 min to deplete their metabolic pools of labeled thymine. The cells were then washed twice with phosphate buffer and resuspended in TF medium. For UV irradiation, 5 ml of cold bacterial suspension was added in a petri dish (9-cm diameter) and exposed to the UV lamp. The irradiated bacterial suspension was then supplemented with 50 μg of unlabeled thymidine/ml and incubated with shaking at 37 C, and 1.0-ml samples were withdrawn at 40-min intervals, chilled, and then combined with 1.0-ml portions of 10% trichloroacetic acid solutions. After keeping the mixture in ice water for more than 1 h, it was centrifuged at 1,500 $\times$ *g* for 15 min, and the radioactivity in 0.5 ml of the supernatant solution was determined. For measurement of the total radioactivity in the sample, the precipitate was heated to 90 C for 30 min together with the remaining 1.5 ml of supernatant solution (5% trichloroacetic acid). After the extract was cooled to 0 C and centrifuged, the radioactivity in the 0.2-ml supernatant solution was measured.

RESULTS

Radiation and drug sensitivities. The lack of genetic recombination capacity in a mutant strain is usually accompanied by a variety of phenotypic differences from the parent strain. Recombination-deficient strains of *E. coli* are usually sensitive to UV, gamma rays, and certain chemicals. The recombination repair processes are postulated to be efficient mechanisms assuring cellular resistance to a number of DNA-damaging agents. To isolate recombination-deficient mutant strains of *B. subtilis*, cells of the wild-type strain NIG17 were treated with NTG, and colonies of mutagenized cells were examined for their sensitivities to gamma rays. Two strains, NIG43 and NIG45, showed the most remarkable radiation sensitivities.

Survival of these strains is shown in Fig. 1a for gamma rays and in Fig. 1b for UV. The typical recombination-deficient strains of *B. subtilis* described earlier by Hoch and Anagnostopoulos (14) were obtained and their radiation sensitivities were compared. We showed that the strain GSY1061 (*recB2*) was considerably more sensitive than wild strains for both gamma rays and UV irradiation. Strains GSY1037 (*recA1*), NIG43, and NIG45 were more sensitive than the others for two types of radiation. The sensitivities of these six strains to MC and MMS were also studied (Table 2). The drug sensitivities were almost parallel to the radiation sensitivities for all strains studied.

We have utilized recombination-deficient strains of *B. subtilis* for the detection of new chemical mutagens in our environment by a

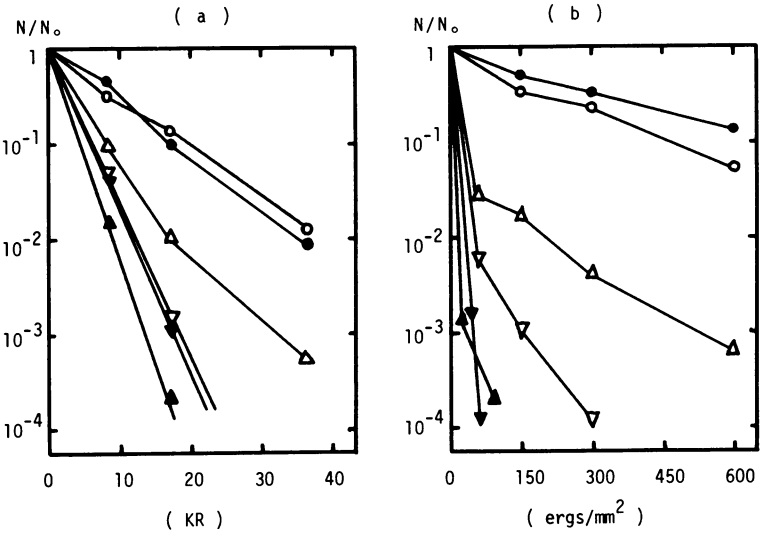

FIG. 1. *Radiation inactivation of Rec⁺ and Rec⁻ strains of B. subtilis. Exponential cells grown in TF medium were washed, resuspended in phosphate buffer, and exposed to radiation. (a) Gamma rays; (b) UV. Symbols:* ●, *NIG17;* ▼, *NIG43;* ▲, *NIG45;* ○, *GSY1035;* ▽, *GSY1037;* △, *GSY1061.*

TABLE 2. *Drug sensitivities of B. subtilis strains*[a]

Drug (μg/ml)	Sensitivities of bacterial strains:					
	NIG17	NIG43	NIG45	GSY1035	GSY1037	GSY1061
MC						
0.1000	–	–	–	–	–	–
0.0500	+	–	–	+	–	–
0.0250	+	–	–	+	–	–
0.0125	+	–	–	+	–	–
0.0063	+	–	–	+	+	+
0.0032	+	–	–	+	+	+
0.0016	+	+	+	+	+	+
0.0008	+	+	+	+	+	+
MMS						
880	–	–	–	–	–	–
660	+	–	–	+	–	–
440	+	–	–	+	–	–
220	+	–	–	+	–	–
110	+	–	–	+	–	–
55	+	–	–	+	+	+
27.5	+	+	+	+	+	+

[a] Bacterial cells exponentially grown in Penassay broth were applied to small areas on broth agar containing each drug. Plates were incubated overnight at 37 C and scored for their growth.

sensitivity test called "rec-assay," in which chemicals giving increased lethal effects on Rec$^-$ over Rec$^+$ cells were selected and further examined for their possible mutagenic capacity (19). Utilization of the strain NIG45 (= M45) led to the detection of at least 12 new chemical mutagens with different mutation specificities (19, 20, 21; Y. Shirasu, M. Moriya, K. Kato, A. Furuhashi, and T. Kada, Mutat. Res., in press). Strain NIG45 has the widest spectrum, including frameshift mutagens such as ICR-191D, sodium-*p*-dimethylaminobenzenediazosulfonate, 2,4-dinitrophenyl thiocyanate, or 5-nitro-1-naphthonitrile, to which other strains do not show enhanced sensitivities.

Host-controlled reactivation in strains NIG43 and NIG45. Though recombination-deficient strains are usually sensitive to both X or gamma rays and UV, they can reactivate UV-irradiated infective phages. This implies that their UV sensitivity is not due to lack of ability to excise pyrimidine dimers. To examine this aspect, phage M2 was exposed to UV and infected on wild and radiation-sensitive strains. The results obtained (Table 3) indicate that strains NIG43 and NIG45 are both Hcr$^+$, as is strain NIG17. For comparison, the capacities of an *hcr* strain UVS19 (23) and its parent wild strain were also described.

TABLE 3. *Capacity for host cell reactivation*[a]

Indicator strains	PFU/ml of phage M2 exposed to UV (ergs/mm^2)		Surviving fraction (%)
	0	900	
NIG17	4,830	167	3.46
NIG43	5,545	37	2.40
NIG45	3,750	193	5.14
UVR	8,470	625	7.40
UVS19	3,400	<10	<0.294

[a] UV-irradiated (900 ergs/mm^2) M2 phages in phosphate buffer were titered on wild and mutant bacteria with the usual soft-agar method.

Transformation in strains NIG43 and NIG45. In *B. subtilis*, both DNA-mediated transformation and phage-mediated transduction involve recombination events. We carried out transformation and PBS1 transduction experiments with our mutant strains. It is known that, if genetically homologous donor and recipient strains are used, the frequency of transformation is not always correlated with that of general transduction mediated by phage PBS1 (8, 9, 15). Concerning two genes controlling recombination ability that were genetically mapped (14), *recA1* mutant strains showed low frequencies of transformation but normal frequencies of PBS1 transduction. On the other hand, *recB2* strains showed low frequencies both in transformation and in PBS1 transduction. Therefore, the *recA1* strains were assumed to be deficient in a step relevant to recombinational pathways specific to transformation, and the *recB2* strains were assumed to be deficient in a step relevant to both transformation and PBS1 transduction. The possible existence of specific pathways in transformation and PBS1 transduction has been suggested (9). Table 4 describes the results of transformation of three strains, NIG17, NIG43 and NIG45, examined at the approximate time of their maximal competent phase. In these strains, the maximal competency was always observed at the late-logarithmic phase of growth. It is noted here that both strains NIG43 and NIG45 grew remarkably more slowly in TF medium than did other strains. The colony-forming units (CFU) per milliliter of each competent culture of these two strains was about five to ten times lower than that of wild strains. The frequency of transformation from arginine auxotrophy to arginine independence, expressed as the number of transformants per recipient cells, was strongly reduced in strain NIG45, but it was normal in strain NIG43 [Table 4, (a) values].

Transfection in strains NIG43 and NIG45. The frequency of transformants can be lowered without impairment of recombination by losing either cellular competency or competent cell fractions in the population. To determine whether our mutants retain the capacity to incorporate exogenous DNA, transfection experiments were done, and the results are shown in Table 4. The number of infectious centers resulting from SPO2 DNA, which does not require recombination to produce mature phages, could be considered as a measure of the capacity to incorporate (27). In our experiments, the frequencies of transformation of the strains NIG43 and NIG45 normalized by the number of SPO2 infectious centers at their most competent phase were clearly reduced [value (a)/(c) in Table 4]. In case of the wild strain, NIG17, the highest apparent competency for SPO2 transfection developed about 30 min earlier than that for transformation and disappeared rapidly, although the competency for the incorporation of exogenous phage DNA may coincide with that for transforming DNA. We compared the frequency of transformation at the maximal competent phase for SPO2 transfection. In the case of SPO1 DNA, which requires recombination events for transfection, the establishment of the transfectants was constantly less than that of SPO2 DNA in strain NIG45 [value (b)/(c) in

Table 4]. This observation implies that SPO1 DNA requires function of the *rec-45*$^+$ gene product for transfection. These results indicate that, based on the degree of the uptake on infectious SPO2 DNA, establishment of transformants or transfectants with SPO1 DNA was clearly reduced in strain NIG45. In strain NIG43, the absolute number of transformants was low, and the number of CFU was reduced to about the same extent. Therefore, the frequency of transformation remained almost constant and was comparable with that of the wild strain. On the other hand, the frequency of SPO1 and SPO2 transfectants per CFU was higher than that of the wild strains. Because the culture of strain NIG43 may contain cells that are deficient in colony formation, the results may be explained by supposing that development of transfectant phage particles may be possible, even in organisms that do not form colonies. The frequency of transformation normalized by the SPO2 transfection frequency was, therefore, reduced in strain NIG43 compared with that of the wild strains.

Transduction in strains NIG43 and NIG45. Results obtained in transduction experiments are described in Table 5. Frequencies of transduction obtained with strains NIG43 and NIG45 as recipients were much lower than those obtained with the wild strain, NIG17. The low transduction frequency may possibly be explained either by recombination deficiency or by loss of active flagella from the cell, since phage PBS1 is flagellotropic (18). To test the latter possibility with our strains, recipient cells with or without phage infection were streaked on semisolid agar (Penassay broth containing 0.4% agar). The motility was positive with control samples but negative with infected ones. In other experiments with our strains, the phage absorption tests were all positive. Thus, the low frequency of transduction cannot be explained

TABLE 4. *Transformation and transfection of Rec$^+$ and Rec$^-$ strains during their growth in TF medium*

Recipient strain	Time of culture (h)	Cells/ml ($\times 10^7$)	Arg$^+$ transformants[a]/cell ($\times 10^{-7}$) (a)	SPO1 transfectants[b]/cell ($\times 10^{-7}$) (b)	SPO2 transfectants[b]/cell ($\times 10^{-7}$) (c)	a/c (%)	b/c (%)
NIG17	4.5	98	459	10.65	18.95	24.2 (100)[c]	0.56 (100)
	5.0	92	582	6.41	0.924	629.0	6.93
	5.5	84	99.4	1.667	<0.595	>1,670.0	>28.0
NIG43	5.0	1.7	490	45.45	84.80	5.78	0.536
	5.5	2.1	639	75.6	95.12	6.73 (27.8)	0.795 (142)
	6.0	6.3	417	45.20	35.70	11.7	1.265
NIG45	5.0	1.4	18.5	3.707	59.26	0.312	0.0625
	5.5	2.3	36.9	2.174	65.20	0.567 (2.34)	0.0334 (5.97)
	6.0	7.6	48.3	3.947	12.5	0.386	0.316

[a] Concentration of transforming DNA was 2.5 μg/ml.
[b] Transfection was done with SPO1 DNA (8.4 μg/ml) and SPO2 DNA (5.5 μg/ml).
[c] Numbers in parentheses indicate percentages.

TABLE 5. *PBS1-mediated transduction of Rec$^+$ and Rec$^-$ strains*[a]

Expt	Strain	Selective marker	Transductants/ml	Recipients/ml ($\times 10^9$)	Transduction frequency/cell	
					$\times 10^{-9}$	%
I	NIG17	Arg$^+$	1,953	3.47	564	100
	NIG43	Arg$^+$	<3.3	1.45	<2.3	<0.4
	NIG45	Arg$^+$	20	1.49	13.5	2.4
II	NIG17	Arg$^+$	4,303	11.55	372	100
	NIG43	Arg$^+$	3.3	4.83	0.68	0.2
	NIG45	Arg$^+$	75	4.95	15.2	4.1

[a] Recipient cells grown in Penassay broth were combined with phage lysates (propagated on strain 168T) at a multiplicity less than one and incubated at 37 C for 30 min. Cells were then collected and resuspended in TF medium without glucose. A portion of the sample was plated on selective agar to detect arginine-independent transductants.

by the loss of flagella but may have resulted from a recombination deficiency in the cells.

We concluded from all of the above results on host-controlled reactivation, transformation, transfection, and transduction that our gamma ray-sensitive strains NIG43 and NIG45 have genetic defects in their recombination capacity. Strain NIG45 is defective in both transformation and PBS1 transduction, and strain NIG43 is defective especially in PBS1 transduction. These results support the possible existence of separate pathways in transformation and PBS1 transduction, as suggested earlier by Dubnau et al. (9).

Chromosomal location of the rec mutations. To elucidate the linkage relationships of the *rec* mutations of strains NIG43 and NIG45 with known markers on the *B. subtilis* chromosome, a series of transformation and PBS1 transduction experiments were performed. Mitomycin sensitivity was used as an index of recombination deficiency.

Linkage was found by PBS1 transduction between *rec-43* of the strain NIG43 and *purA* of a temperature-sensitive mutant (10). Results on three-factor transductional crosses between *purA dna-8132* and *rec-43* suggested an order of *purA dna-8132 rec-43* and a close linkage between *dna-8132* and *rec-43* (Table 6 and Fig. 2). An additional experiment indicated that the co-transfer index between *rec-43* and *dna-8132* was 74.9% by transformation.

The mutation in strain NIG45 was found to be weakly linked to *pyrA*. The *recA1* mutation has previously been mapped in this region (14). The relationship between *rec-45* and *recA1* was then examined. DNA extracted from *rec*$^+$ or *recA1* cells was used to transform strain NIG45 to MC resistance and also to arginine independence. MC-resistant transformants were first selected at low concentration of drug and then confirmed as to their resistance to 0.05 μg of MC/ml, allowing the growth of wild-type cells. The ratio of the number of MC-resistant transformant colonies to that of arginine-independent transformant colonies was found to be very much smaller in experiments using *recA1* DNA than in those using *rec*$^+$ DNA [Table 7(A)]. These results clearly indicate that the MC-sensitive mutation of strain NIG45 is closely linked to the *recA1* mutation. According to the recombination index method (1) based on the frequency of Arg$^+$ transformants (*argA15* is not linked to *recA1* by transformation), the results of our transformation experiments suggested a distance of 1.69% between the *rec-45* mutation of strain NIG45 and the *recA1* mutation. In a similar cross with DNA bearing a spore marker *osp-19* (4), light-brown colonies were observed among MC-resistant, fully pigmented transformant colonies, showing a linkage value of 73.3% between *rec-45* and *asp-19* [Table 7(B) and Fig. 3]. Results of three-point transductional crosses showed an order of *ura-1 osp-19 recA1* (Table 8). These results suggest that the mutation *rec-45* of strain NIG45 is located at the distal end of *osp-19* with a linkage value of 73.3% by transformation (Fig. 3). In the present study, a more precise order of the location of *rec-45* with respect to that of *recA1* was not determined.

Mapping studies utilized mitomycin sensitivity as an index of recombination deficiency of both mutants. To see whether this property really represents the phenotypes with recombination deficiency, a number of mitomycin-resistant revertants and transformants of strains NIG43 and NIG45 were studied. Typical mitomycin-resistant transformants of strain NIG43 or NIG45 obtained with DNA of strain 168T regained simultaneously the wild-type radiation resistance and the recombination capacities (Table 9). Further, by incubating single-cell clones of strain NIG43 or NIG45 in Penassay broth, MC-resistant revertants of each culture were scored on broth agar containing

TABLE 6. *Three-factor cross by PBS1-mediated transduction involving purA, dna-8132, and rec-43*[a]

Donor genotype	Recipient genotype	Transductants			Order implied by the results
		Primary selection	Class	No.	
rec-43	*purA dna-8132*	Pur$^+$	Pur$^+$ Ts$^-$ Rec$^+$	89	*purA dna-8132 rec-43*
			Pur$^+$ Ts$^+$ Rec$^+$	15	
			Pur$^+$ Ts$^+$ Rec$^-$	17	
			Pur$^+$ Ts$^-$ Rec$^-$	0	

[a] All of the experiments were carried out at 30 C, except for selecting Ts$^+$ recombinants. Pur$^+$ transductant colonies were formed on selective minimal agar containing 2% sucrose (Lepesant et al. [22]). Plates were incubated at 30 C. The donor strain carried *argA15* and *trp-3* markers in addition to *rec-43*. The recipient strain carried *sacA321* in addition to *purA* and *dna-8132*.

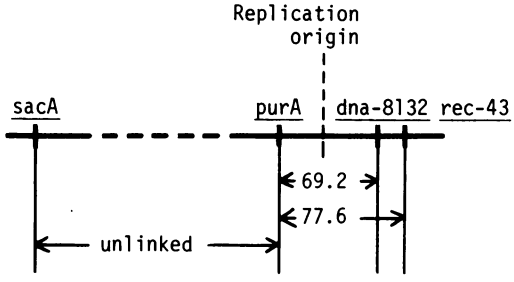

FIG. 2. *Map of the purA-dna-8132 region of B. subtilis genome constructed by PBS1 transduction experiments, showing the position of marker rec-43. Distances (averaged from several independent experiments) are expressed as percentage of recombination.*

TABLE 7. *Transformation of NIG45 to MC resistance with DNA carrying the rec⁺, recA1, or osp-19 marker*[a]

(A)

Donor genotype	Transformants/ml		
	Arg⁺	MC^r	MC^r/Arg⁺
rec⁺	433	273	0.63 (a)
recA1	253	6	0.024 (b)

(b/a) × 100 = 3.8

(B)

Donor genotype	Transformants		
	Primary selection	Class	No.
osp-19	MC^r	MC^r Osp⁺	89 (a)
		MC^r Osp⁻	40 (b)

[a/(a + b)] × 100 = 69.0

[a] A competent culture of NIG45 was transformed with a saturating amount of DNA for 1 h. Uptake of the DNA was terminated with DNase. The transformed culture was then incubated for an additional 2 h, plated on broth agar containing 0.0063 μg of MC/ml, and incubated for 2 days. Colonies developed were picked up and checked as to their growth on broth agar containing 0.05 μg of MC/ml. MC^r denotes the growth on this selective agar. Portions of transformed and incubated cultures were plated on selective agar to observe Arg⁺ transformants. The MC^r Osp⁺ transformant colonies were pigmented brown on 0.05 μg of MC/ml of broth agar. The number of MC^r or Arg⁺ transformants was scored on duplicate plates.

0.05 μg of MC/ml, allowing growth of wild-type cells. The strain NIG43 produced these revertants at a frequency of 2.34×10^{-8}/cell averaged from seven independent cultures. In 10 independent cultures of the NIG45 clones, resistant colonies developed at a frequency ranging from 3.50×10^{-9} to below 1.30×10^{-9}/cell, showing a reversion frequency of at most 1.93×10^{-9}/cell. Four independent revertants from each mutant were examined for their radiation sensitivities and recombination capacities. Three revertants of each mutant completely regained wild-type properties (Table 9). The variation in transformation frequencies may be due to different competent phases for each experiment. All of these results indicate that the mitomycin-sensitive mutations (*rec-43* and *rec-45*) are also responsible for enhanced radiation sensitivities and recombination deficiencies and that both are single mutations.

Conditional properties of the rec-43 mutation. Strain NIG43 was found to regain the wild-type UV resistance in the presence of NaCl (Fig. 4) or sucrose (10%). This recovery was observed not only with the original *rec-43* strain NIG43 but also in a *rec-43* transductant with a different genetic background. Thus, the effect is not dependent on the genetic background in which the *rec-43* mutation resides. The degree of recovery with salt or sucrose was higher in the range of lower UV dose. The phenomenon was much clearer in the repair for UV than for gamma rays or certain chemicals. We could not demonstrate the recovery in PBS1 transduction because the frequency of recombination in transduction was strongly reduced in the presence of NaCl.

A repairing effect at high osmotic pressure was reported in auxotrophic, UV-sensitive, or temperature-sensitive mutations of different organisms (13, 30, 35) and was considered to result from restoration of the enzymatic activity of a mutated protein (30). Thus, the mutated *rec-43* gene product may be conditional and

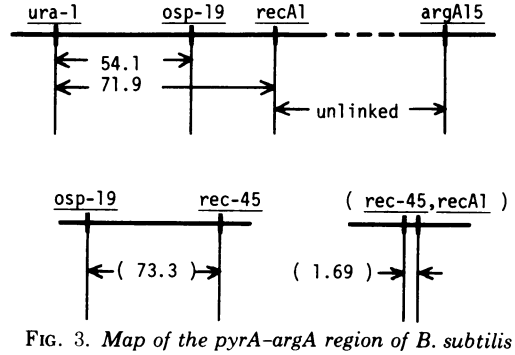

FIG. 3. *Map of the pyrA-argA region of B. subtilis genome constructed by transformation and PBS1 transduction experiments, showing the position of the marker rec-45. Distances (averaged from several independent experiments) are expressed as percentage of recombination. Distances in parentheses are from transformational crosses. The order of rec-45 mutation with respect to recA1 and osp-19 is unclear.*

TABLE 8. *Three-factor crosses by PBS1-mediated transduction involving ura-1, osp-19, and recA1*

Donor genotype	Recipient genotype	Transductants			Order implied by results
		Primary selection	Class	No.	
osp19	*ura-1 recA1*	Ura$^+$	Ura$^+$ Osp$^+$ Rec$^-$	54	*ura-1 osp-19 recA1*
			Ura$^+$ Osp$^-$ Rec$^-$	12	
			Ura$^+$ Osp$^-$ Rec$^+$	20	
			Ura$^+$ Osp$^+$ Rec$^+$	1	

TABLE 9. *Transformation and PBS1 transduction of MC-resistant derivatives of NIG43 and NIG45*[a]

Strain	Origin	Frequency (Arg$^+$ transformants or transductants/cell)	
		Transformation ($\times 10^{-6}$)	Transduction ($\times 10^{-8}$)
NIG17	Wild type	79.0	27.4
NIG44	MC^r transformant of NIG43	119.5	196.0
NIG48	MC^r transformant of NIG45	39.2	42.8
NIG17	Wild type	5.59	77.28
NIG50	Spontaneous MC^r revertant of NIG43	68.0	143.0
NIG51	Spontaneous MC^r revertant of NIG43	80.0	416.0
NIG53	Spontaneous MC^r revertant of NIG43	180.0	503.0
NIG54	Spontaneous MC^r revertant of NIG45	4.72	95.6
NIG56	Spontaneous MC^r revertant of NIG45	27.5	574.0
NIG57	Spontaneous MC^r revertant of NIG45	4.81	216.0

[a] Transformation and transduction were carried out as described in Tables 4 and 5. All derivatives carry double auxotrophic markers *argA15* and *trp-3*.

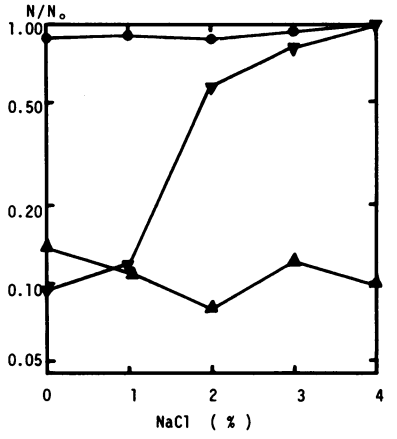

FIG. 4. *Effect of NaCl on the cellular survivals of Rec$^+$ and Rec$^-$ strains of B. subtilis after UV irradiation. Bacterial cell suspensions were irradiated with UV (50 ergs/mm^2), and the number of surviving cells was scored on broth agar containing the indicated amount of NaCl. Symbols: ●, NIG17; ▼, NIG43; ▲, NIG45.*

present in the cell. Broth culture of *rec-43* strain may contain a considerable fraction of nonviable cells because, at the same optical density, a mutant culture contains one-fifth the number of CFU per milliliter that a wild-type culture contains. In the presence of NaCl (3%) both in the culture and in broth agar, this number was elevated considerably (to about one-half).

Prophage induction. One of the remarkable properties of some *rec* mutants is the inability to destroy the repressor system of inducible prophages (3). Our mutants were lysogenized with SPO2 and induced with UV irradiation. Results indicated that the prophage was inducible in *rec$^+$* strains but not in either NIG43 or NIG45 (Fig. 5). However, the plating efficiencies of phage SPO2 on these mutants were similar to those found on the wild-type strain, and SPO2 transfection was not reduced in both mutants (Table 4). All of the *rec$^+$* transformants of strains NIG43 and NIG45 regained the inducibility. Either *rec-43* or *rec-45* was introduced by transduction into a series of other strains, and the absence of inducibility of these *rec$^-$* transductants was shown. Thus, both *rec-43$^+$* and *rec-45$^+$* gene products seem to be necessary for destroying the repressor system of SPO2 prophage. On the other hand, lack of phage inducibility may be due to death of the cells after UV and not the postulated role of the wild-type *rec-43* and *rec-45* gene products in SPO2 induction. These products may not be needed for phage development since transfection of the mutants with SPO2 DNA is normal. It is also possible to suppose that the mutants might make a product inhibiting SPO2 after UV exposure. The mutation *rec-45* reduced considerably the frequency of spontaneous induction.

Additional experiments showed that SPO2 lysogens of *rec-43* and *rec-45* strains were also not inducible with mitomycin and that, among six lysogenized mutants carrying *recA1*, *recB2*,

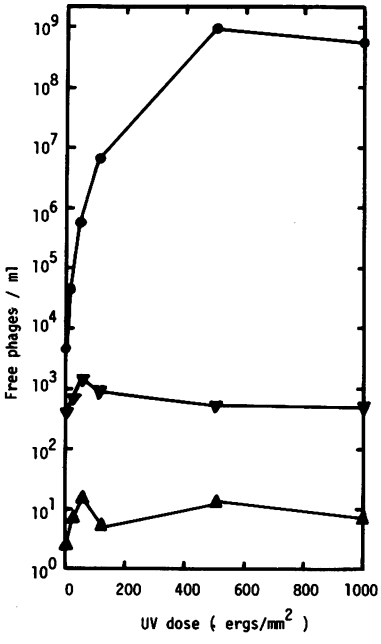

FIG. 5. *Prophage induction in Rec⁺ and Rec⁻ lysogenic strains of B. subtilis. SPO2 lysogens were irradiated with UV and incubated for 3 h. Free phages induced were titered on strain 168T as indicator. The number of CFU per milliliter before UV irradiation was 5.9 × 10⁷, 1.9 × 10⁷, and 2.6 × 10⁷ for rec⁺, rec-43, and rec-45 strains, respectively. Symbols: ●, NIG17, ▼, NIG43; ▲, NIG45.*

recD3, *recE4*, *recF16*, or *recG13* (7), three (those with *recE4*, *recF16*, or *recG13*) were not inducible. The *recC7* strain BD239 was confirmed to carry an inducible prophage that was immune to SPO2 infection, as pointed out by Garro et al. (Microb. Genet. Bull., no. 38, p. 9–10, May 1975). The lysogenic strains of the mutant carrying *dna-8132* isolated by Hara and Yoshikawa (10) produced phages upon the temperature shiftup. It was noted here that the frequency of spontaneous induction in a *recE4* lysogenic strain was extremely low.

UV effect on cellular DNA degradation. *E. coli* strains with a *recA* mutation were not inducible and exhibited very extensive or "reckless" DNA degradation after UV irradiation (3). So that this property could be examined in our *rec* strains of *B. subtilis*, cells of different strains were labeled with [³H]thymidine, and the release of acid-soluble radioactive materials was measured after UV irradiation. Figure 6 shows that cellular degradation of DNA was initiated by UV irradiation and that the extent of released radioactivities was higher in the strains NIG43 and NIG45 than in wild-type strain NIG17.

DISCUSSION

We have isolated two recombination-deficient strains of *B. subtilis* on the basis of sensitivity to ionizing radiation and have determined the chromosomal location of the mutations. The existence of deficiencies in genetic recombination of these mutants was conclusively shown by different types of experiments involving transformation, transfection, and transduction, as well as by studies on their capacities to reactivate UV-exposed phages and on their sensitivities to different mutagenic agents. Aside from their remarkable sensitivities to radiations, the characteristic aspects of these mutations may reside in their high sensitivities to various mutagenic chemicals, including frameshift-type mutagens. This property has been utilized efficiently for selection of DNA-damaging and mutagenic chemicals, and a number of new mutagens was detected (19, 20, 21; Shirasu et al., in press). The high sensitivities to radiation and to chemicals of strains NIG43 and NIG45 were not correlated with lack of either an ATP-dependent DNase acting on

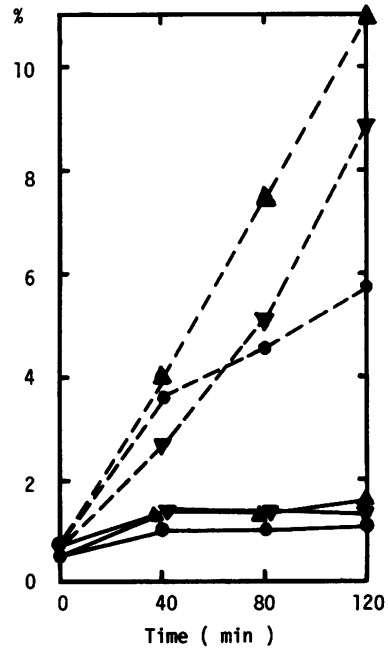

FIG. 6. *Effect of UV on DNA degradation in Rec⁺ and Rec⁻ cells of B. subtilis. Cells labeled with [³H]thymidine were exposed to UV (900 ergs/mm²) and incubated at 37 C with shaking. Fraction of degradated labels solubilized in cold trichloroacetic acid was indicated as the percentage of the total counts. Symbols: ●, NIG17; ▼, NIG43; ▲, NIG45; ——, minus UV; -----, plus UV.*

native DNA or of an ATP-independent DNase specific for denatured DNA, as formerly shown (32), but with the inability of SPO2 phage induction. In the case of *E. coli* carry λ, phage induction seems to be controlled by *recA* (3), whereas in *B. subtilis* carrying SPO2 our data suggested that there exist at least three *rec* regions, namely *rec-43* or *recG13*, *recF16*, and *rec-45* or *recE4*, controlling the phage induction.

Among them, the rate of spontaneous induction was lowest in the *rec-45* and *recE4* strains. Though *E. coli recA* strains usually show "reckless" degradation of DNA after UV exposure, that of NIG43 or NIG45 was rather modest, probably owing to low DNase activities in *B. subtilis*.

Dubnau et al. (7, 8) reported recently on several recombination-deficient strains with biochemical and genetic characterizations. The mutation *recG13*, which was mapped near the replication origin, reduces the frequency of PBS1 transduction but not that of transformation. These properties and mapping results are analogous to those of our strain NIG43. Our recent experiments revealed that the linkage value between *rec-43* and *recG13* was about 8.1% by the recombination index method in transformation crosses. On the other hand, the *rec-45* may be a mutation that is comparable to *recE4* of Dubnau et al. (7, 8) because we tentatively mapped this in the region of *recA1*. The linkage value between *rec45* and *recE4* was about 2.5% by the recombination index method in transformation crosses. The mutation *recE4* reduced both the transformation and PBS1 transduction frequencies, although the transduction frequency was normal in the *recA1* mutant; thus, the *recE4* phenotype is similar to that of our NIG45 strain. At present, the exact relative map positions of *rec-45* and *recE4* have not yet been established. It seems that the *recA* region may have multiple genes suppressing or modifying the recombination functions. Our preliminary results indicate that certain moderate radiosensitive revertants from strain NIG45 (one formerly designated R10 by Sadaie and Kada [33]) and some of the revertants derived from strain NIG43 were produced by mutations in the *recA* region.

In the case of *rec-45*, mapping studies were carried out first by means of transduction. Using the *rec-45* strain as a donor, strains GSY1035 (*rec*$^+$ *ura-1*) and GSY1037 (*recA1 ura-1*) were transduced to uracil independence. Among these Ura$^+$ transductants from strain GSY1037, no MC-resistant colonies were observed. On the other hand, a small number of MC-sensitive recombinants developed in transductant colonies obtained with the Rec$^+$ recipient. The linkage between *rec-45* and *ura-1* calculated by this cross was greater than 91%. This phenomenon was not observed with the *recA1* strain and may be similar to the case of the *recB2* mutation. In this case, integration of the DNA segment bearing the *recB2* seemed to be delayed, and heterozygotic clones were found (14).

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