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# Characterization of *Bacillus subtilis* Recombinational Pathways

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**Recombination in *Bacillus subtilis* requires the products of numerous *rec* loci. To dissect the various mechanisms which may be involved in genetic recombination, we constructed a series of isogenic strains containing more than one mutant *rec* allele. On the basis of their impairment in genetic exchange, the various loci (represented by specific *rec* alleles) were classified into different epistatic groups. Group α consists of *rec* genes represented by *recB*, *recD*, *recF*, *recG*, *recL*, and *recR* mutations, while group β comprises the *addA* and *addB* mutations. Group γ consists of the *recH* and *recP* mutations. These results suggest that *B. subtilis* has multiple pathways for genetic recombination and that the products of the genes within the α, β, and γ epistatic groups are involved in these alternative recombination pathways. The RecA protein is required in all three pathways of intermolecular recombination.**

In *Bacillus subtilis*, recombination-deficient mutants with increased sensitivities to DNA-damaging agents have been isolated (see reference 25 for a review). Genetic studies of these mutants have defined 12 different genes (25). The biochemical activities of some of those genes, e.g., *recE* (24) and *addABC* (11, 12, 27, 29), have been defined. The *recF* and *recM* loci have only been preliminarily characterized (1–3, 17).

The *B. subtilis recE*, *recM*, and *recF* genes are the homologs of the *Escherichia coli recA*, *recR*, and *recF* genes, respectively (2, 26, 31). At the recent International Conference on the *Bacillus subtilis* Genome (2–5 September 1990, Paris, France), it was suggested that the *recA* and *recR* designations be adopted for the genes previously referred to as *recE* and *recM* in *B. subtilis*. We have followed this recommendation in this communication.

The *B. subtilis* RecA protein has two distinct activities: (i) it acts as a recombinase (14, 15, 24) and (ii) it stimulates derepression of the SOS response (9, 23). Recently, it was shown that the removal of DNA damage by the *addA*, *addB*, *recF*, *recH*, *recL*, and *recP* gene products is strictly dependent on *recA* gene product activity (6). Thus, *recA* could be a master gene whose product controls, or contributes to, all types of recombination and recombination repair.

Functions associated with the AddABC enzyme (also designated Exo V) include a processive ATP-dependent double-strand exonuclease, a single-strand exonuclease, a DNA-dependent ATPase, and an ATP-dependent double-strand-unwinding enzyme (11, 27, 29, 32). The AddBCD protein is the *B. subtilis* counterpart of the *E. coli* RecBCD enzyme (19, 32).

Blocks in both DNA repair and genetic exchange are observed when the *recF15* mutation is placed into the *addA5* genetic background (3). We had assumed that the *recF* and *addA* mutations define different epistatic groups, tentatively termed the α and β groups, respectively (3, 17). Furthermore, a clear defect in *B. subtilis* genetic exchange can be detected only when more than one alternative pathway is affected.

In this paper, we report the classification of *B. subtilis* Rec$^-$ mutant strains, other than *recA* mutant strains, into three epistatic groups (α, β, and γ). Our data suggest that the products of the genes within these epistatic groups may define alternative recombination pathways.

## MATERIAL AND METHODS

**Bacterial strains, plasmids, and phages.** All *B. subtilis* strains used are listed in Table 1 and are isogenic with strain YB886. Construction of the *recF* double mutant strains and strains BG189 and BG128 has been previously reported (4, 20). Transfer of the *recR*::*cat* insertion allele (termed *recR1*) from plasmid pBT53 to the *B. subtilis* chromosome was done as described previously (4). The *addA5* mutation was moved into the "prophage-free" single mutant strains by allele transfer (gene conversion) as described previously (4). The chromosomal allele of *addA*$^+$ cells transformed with plasmid pADD5 was efficiently converted to the plasmid genotype as reported by Iglesias and Trautner (18).

With the exception of the *recA*, *recF*, *addA*, and *recR* mutations, the presence of the *rec* mutation was confirmed by plasmid-mediated selection as previously described (4, 20). The presence of the *recA*, *recF*, *addA*, and *recR* mutations was ascertained by determining the complementing activity of the plasmid-borne *recA* (pBT61), *recF* (pBT7), *addA* (pBT200), and *recR* (pBT52) genes.

The plasmids used were pBC30 (4), pBG59 (8), p1962 (1), pBT7 (3), pBT61 (17), and pBT52 and pBT53 (2). Plasmid pADD5 is a pBT200 derivative bearing the *addA5* mutation (3).

The *B. subtilis* bacteriophage SPP1 was used (8).

**Transduction and transformation of bacteria.** Phage-mediated plasmid transduction was performed as described by Deichelbohrer et al. (8). *B. subtilis* competent cells were prepared as described by Rottländer and Trautner (28). Plasmid transformants were selected on agar medium containing neomycin or chloramphenicol at 5 μg/ml. In the *recR1* mutant strains, a neomycin-resistant plasmid (pBG59) was used because, by construction, they were resistant to chloramphenicol. Met$^+$ recombinants were selected by plating on minimal agar containing all nutritional requirements except for methionine (28).

**Recombination frequencies.** Relative transformation and transduction frequencies were used as a measure of recombination. *B. subtilis* competent cells (about $5.0 \times 10^7$ cells

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TABLE 1. *B. subtilis* strains[a]

| Single mutant genotype (strain) | Strain with the following double mutant genotype: | | |
|---|---|---|---|
| | *recF15* | *addA5* | *recR1* |
| *rec*$^+$ (YB886) | NA | NA | NA |
| *recB3* (BG117) | BG135 | BG169 | BG256 |
| *recD41* (BG121) | BG139 | BG171 | ND |
| *recA4*[b] (YB1015) | BG145 | BG163 | ND |
| *recF15* (BG129) | NA | BG143 | BG257 |
| *recG40* (BG123) | BG141 | BG175 | BG258 |
| *recH342* (BG119) | BG137 | BG177 | BG259 |
| *recL16* (BG107) | BG159 | BG179 | BG161 |
| *recR13* (BG127) | BG161 | BG181 | NA |
| *recR1* (BG128) | BG257 | BG265 | NA |
| *recP149* (BG101) | BG155 | BG185 | BG263 |
| *addA5* (BG125) | BG143 | NA | BG265 |
| *addB72* (BG126) | ND | BG189 | ND |

[a] The isogenic background of all strains was *trpC2 metB5 amyE sigB xin-1 att*SPβ. Strain BG189, by construction, was resistant to phleomycin (3). For the YB series of strains, see Friedman and Yasbin (16); for the BG series of strains, see Alonso et al. (3, 4), Kupsch et al. (20), and this work. NA, not applicable; ND, not done.

[b] This allele was known previously as *recE4*.

per ml) were transformed as described by Rottländer and Trautner (28). The yield of Met$^+$ or plasmid transformants was corrected for DNA uptake and cell viability as described by Alonso et al. (3). DNA uptake, which is taken as a measurement of competence, was measured as described previously (28). Cell viability was defined as the number of CFU at an $A_{560}$ of 1.0 (wild type, $1.0 \times 10^8$ CFU/ml). Cell viability ranged from $1.6 \times 10^7$ to $8.3 \times 10^7$ CFU/ml for the double mutant strains.

## RESULTS AND DISCUSSION

**Measurement of genetic exchange in *B. subtilis* recombination-deficient strains.** The large number of *B. subtilis* genetic loci affecting the wild-type level of recombination may reflect the existence of several recombination pathways. To test this hypothesis, we first constructed an isogenic set of *rec* double mutant strains (Table 1) and then tested those mutant strains by measuring (i) the frequency of plasmid transformation, (ii) the frequency of chromosomal transformation, or (iii) plasmid DNA transfer by bacteriophage SPP1-mediated transduction. In the first two processes, the donor DNA consists of linear single-stranded molecules, while in the third process, the bacteriophage injects into the recipient cell double-stranded DNA that is a linear head-to-tail plasmid concatemer (21). Transformation with chromosomal DNA (chromosomal transformation) does not require replication for the integration of donor markers (14). In contrast, replication and recombination functions are thought to be required for the establishment of plasmid-derived donor DNA (plasmid transformation) (3, 10). Furthermore, chromosomal transformation is strictly dependent on RecA (15), whereas plasmid transformation is independent of RecA (5). By measuring chromosomal (intermolecular recombination) and plasmid (intramolecular recombination) transformation, we can examine both RecA-dependent and RecA-independent recombination events (see reference 21 for a review).

*B. subtilis* competent cells were transformed with 1 μg of homologous chromosomal DNA or plasmid DNA per ml to determine the transformation frequency of the Rec$^-$ mutant strains. When Rec$^-$ single mutant competent cells (with the exception of *recA* mutant competent cells) were analyzed, the chromosomal transformation frequencies did not change more than 10-fold relative to the wild-type value (3, 11, 13, 15, 23) (Table 2). On the basis of these data, we can infer that there is no single recombination pathway in *B. subtilis*.

TABLE 2. Relative transformation and transduction frequencies[a]

| Relevant genotype | Normalized chromosomal transformation[b] | Normalized plasmid transformation[b] | Normalized plasmid transduction[c] |
|---|---|---|---|
| *rec*$^+$ | 100 | 100 | 100 |
| *recB3*[d] | 71 | 97 | 95 |
| *recD41* | 73 | 99 | 95 |
| *recA4* | <0.01 | 97 | 77 |
| *recF15* | 72 | 98 | 91 |
| *recG40* | 68 | 97 | 81 |
| *recL16*[d] | 67 | 96 | 95 |
| *recM13*[d] | 74 | 103 | 90 |
| *recH342* | 63 | 91 | 45 |
| *recP149* | 80 | 100 | 86 |
| *addA5* | 60 | 72 | 89 |
| *addB72 addA5* | 53 | 69 | 90 |
| *recB3 addA5* | 5.9 | 11 | 81 |
| *recB3 recF15* | 3.2 | 19.6 | 59 |
| *recD41 addA5* | 4.8 | 11 | 109 |
| *recD41 recF15* | 1.9 | 24.5 | 68 |
| *recA4 addA5* | <0.01 | 7.2 | 29 |
| *recA4 recF15* | <0.01 | 7.6 | 19 |
| *recF15 addA5* | <0.01 | <0.01 | 10 |
| *recG40 addA5* | 5.5 | 3.6 | 86 |
| *recG40 recF15* | 3.4 | 13.0 | 28 |
| *recH342 addA5* | <0.01 | <0.01 | 45 |
| *recH342 recF15* | 0.09 | 0.04 | 17 |
| *recL16 addA5* | 5.8 | 21 | 77 |
| *recL16 recF15* | 3.3 | 12.3 | 40 |
| *recR13 addA5* | 3.2 | 16 | 31 |
| *recR13 recF15* | 5.0 | 23.0 | 28 |
| *recP149 addA5* | <0.01 | 0.04 | 80 |
| *recP149 recF15* | 0.03 | 0.01 | 26 |

[a] Data are reported as percentages and are the averages of at least three independent experiments.

[b] The yields of *met*$^+$ transformants (chromosomal transformation) and chloramphenicol-resistant (pBC30) transformants (plasmid transformation) were corrected for DNA uptake (the specific activity of the $^{32}$P-labeled DNA was $1.0 \times 10^6$ to $2.0 \times 10^6$ cpm/μg, and DNA uptake ranged from $1.0 \times 10^4$ to $8.0 \times 10^4$ cpm) and cell viability (see Materials and Methods). The values obtained were normalized to the YB886 (*rec*$^+$) value (taken as 100).

[c] The SPP1 phage amplified in wild-type cells bearing plasmid p1962 was used as a donor, and the Rec$^-$ single and double mutant strains were used as recipients. Bacteria at $2.0 \times 10^8$ cells per ml and phage SPP1 at $6.0 \times 10^8$ PFU/ml were mixed. The number of chloramphenicol-resistant transductants per surviving cell in the wild-type strain was $2.2 \times 10^{-3}$ and was assigned the value 100.

[d] In contrast to results previously described (14, 15), we showed that the transformation frequencies of the Rec$^-$ single mutants did not change more than threefold relative to the wild-type value. Since we used a prophage-free genetic background, we assumed that prophage induction may account for the discrepancy.

A severe block in chromosomal and plasmid transformation was observed when the *recF15* mutation was placed into the *addA5* genetic background (3) (Table 2). This result suggests that the *recF* and *addA* gene products act in alternative pathways.

The *addA5* and *addB72* mutant strains have impairments in different subunits of the AddBCD enzyme (3, 11, 12, 19). When the *addA5* mutation was introduced into the *addB72* genetic background, we observed that the double mutant strains were as deficient as the *addA5* parent strain (Table 2). The most economical assumption is that the *addA* and *addB*

mutations define an epistatic group. In the absence of the AddABC enzyme (*addA5* or *addA5 addB72* mutant strains), there was still a high residual level of recombination in *B. subtilis* (3, 11, 13) (Table 2). We have ruled out the possibility that the mutant enzymes retained their recombination activity, because many *addA5* double mutant strains (Table 2) were deficient in recombination.

The *recH* and *recP* single mutant strains had a high residual level of recombination (3) (Table 2). The presence of either the *addA5* or the *recF15* mutation in the *recH342* and *recP149* genetic backgrounds reduced intermolecular recombination (chromosomal transformation) 1,000- to 10,000-fold relative to the wild-type value. This result suggests that neither *addA5* nor *recF15* affects the same pathway as do *recH342* and *recP149*.

When either the *addA5* or the *recF15* mutation was placed in the *recB3*, *recD41*, *recG40*, *recL16*, or *recR13* background, chromosomal transformation was reduced 15- to 50-fold (Table 2).

The frequency of plasmid transformation was also analyzed. As shown in Table 2, the frequency at which plasmids were transformed was reduced more than 250-fold in the *recH recF*, *recP recF*, *recH addA*, and *recP addA* double mutant strains. In the remaining strains listed in Table 2, the plasmid transformation frequency was not reduced more than 14-fold relative to the wild-type frequency.

The *recA4* mutant strain has a block in chromosomal transformation but not in plasmid transformation (5, 14, 15) (Table 2). Furthermore, the presence of the *recF15*, the *recH342*, the *recP149*, or the *addA5 addB72* mutation (6) (Table 2) in the *recA4* background did not change plasmid transformation more than 10-fold relative to the wild-type value. On the basis of these results, we can confirm that the RecA protein is not required for intramolecular recombination (plasmid transformation).

In the case of SPP1-mediated plasmid transduction, the phage injects a linear double-stranded concatemeric plasmid DNA molecule which must be resolved to its monomeric circular form prior to establishment (8). To test whether the incoming linear double-stranded concatemeric plasmid DNA molecules can be resolved into monomeric circular molecules in Rec$^-$ strains, we used stock lysates of SPP1 amplified on wild-type cells bearing plasmid p1962 to transduce the mutant strains listed in Table 1. It was previously reported that plasmid establishment is independent of host *rec* gene products (8). Except for the *recF15 addA5* double mutant strain, in all tested Rec$^-$ double mutant strains p1962 transduction was only reduced by 1.2- to 5-fold relative to the *rec*$^+$ value (Table 2). These data indicate that the observed reduction in plasmid transformation in the Rec$^-$ double mutant strains was not due to a defect in the expression of the selectable marker or to a defect in plasmid replication (Table 2).

It was previously shown that lesions in the *recB*, *recD*, *recF*, *recG*, *recL*, and *recR* genes render *B. subtilis* cells very sensitive to DNA-damaging agents, whereas lesions in the *recH*, *recP*, *addA*, and *addB* genes render cells only moderately sensitive (3, 6, 17, 23). It was also shown that SOS induction, after irradiation with UV light or treatment with mitomycin, shows an indirect requirement for the *recB*, *recD*, *recF*, *recG*, *recL*, and *recR* genes, whereas there is no such requirement for the *recH*, *recP*, *addA*, and *addB* genes (3, 17, 23). Furthermore, it was shown that the *recF*, *recL*, and *recR* defects are partially suppressed either by the presence of the *recA73* mutation or by the presence of a plasmid-borne, heterologous *ssb* protein gene (1, 3). On the basis of published data and the above-described results, we conclude that *rec* mutations other than *recA* can be classified into at least three groups (α, β, and γ). Group α includes the *recB*, *recD*, *recF*, *recG*, *recL*, and *recR* mutations. Group β comprises the *addA5* and *addB72* mutations. Group γ includes the *recP* and *recH* mutations. Similarly, Lloyd and Buckman (22) examined DNA repair and recombination in *E. coli* strains carrying various mutation combinations in two or more RecF pathway genes and reported that the recombination genes in *E. coli* also define three epistatic groups.

In *E. coli*, the RecBCD and RecF functions define the RecBCD and RecF recombination pathways. The RecBCD pathway is the major pathway of conjugational recombination in Rec$^+$ *E. coli* cells (7, 30). It is thought that *E. coli* recombination systems with double-stranded DNA ends follow the RecBCD pathway, whereas those without double-stranded DNA ends follow the RecF pathway (see reference 30 for a review). On the basis of the data presented here, we infer that the β and α epistatic groups of *B. subtilis* could be analogous to the RecBCD and RecF recombination pathways of *E. coli*, respectively.

In cells in which the DNA taken up is in a linear single-stranded form, the AddABC enzyme does not appear to be required for transformational recombination (e.g., *B. subtilis*, *Streptococcus pneumoniae*, or *Haemophilus influenzae* [see reference 21 and citations therein]). Since a block in recombination was observed in the *recF addA* mutant strain (3) (Table 2), we must assume that a function(s) from either the α or the γ pathway is able to bypass the *addA* requirement.

In *B. subtilis* prophage-free Rec$^-$ single mutant strains (with the exception of *recA* mutant strains), chromosomal transformation frequencies did not change more than threefold relative to the wild-type value (3, 23). In contrast, in strains carrying combinations of mutations in α group genes, chromosomal transformation frequencies were reduced 20- to 50-fold (Table 2). This observation could be also explained by assuming that a redundant function within the same epistatic group was operative.

**Effect of *rec* mutations in a *recR1* mutant strain of *B. subtilis*.** We have shown that the *addA recB*, *addA recD*, *addA recG*, *addA recL*, *recF recR*, *recF recB*, *recF recD*, *recF recG*, *recF recL*, and *recF recR* double mutant strains have a high residual level of recombination (5 to 20%, relative to the wild-type value) compared with the *addA recF*, *addA recH*, *addA recP*, *recF recH*, and *recF recP* double mutant strains ($<2.5 \times 10^{-3}$). One way to explain this observation is to assume that the α, β, and γ groups (see above) define different recombination pathways and that the *recF* function is required for more than one pathway (17). To test this hypothesis, we transferred the *recR* mutation (classified into the α group) into Rec$^-$ single mutant strains and examined the efficiency of recombination of the Rec$^-$ double mutant strains.

As shown in Table 3, when the *recR* mutation was placed in the *recB*, *recF*, *recG*, or *recL* (group α) or *addA* (group β) background, a residual level of chromosomal recombination (1 to 5% relative to the wild-type value) was observed. On the other hand, when the *recR* mutation was placed in the *recH* or *recP* background (γ epistatic group), chromosomal transformation was reduced more than 1,000-fold.

The frequency at which plasmid pBG59 was established was not reduced more than six- to ninefold, relative to the wild-type value, when genes classified within groups α and β were inactivated. In the *recR recH* or *recR recP* (α plus γ) genetic backgrounds, the frequency of plasmid establishment was reduced 100- to 170-fold. Since a residual level of plasmid transformation was observed in the *recR* double

TABLE 3. Relative transformation frequencies[a]

| Relevant genotype | Normalized chromosomal transformation[b] | Normalized plasmid transformation[b] |
|---|---|---|
| *rec*$^+$ | 100 | 100 |
| *addA5 recR1* | 2 | 12 |
| *recB3 recR1* | 5 | 10 |
| *recF15 recR1* | 4 | 22 |
| *recG40 recR1* | 3 | 6 |
| *recL16 recR1* | 5 | 18 |
| *recH342 recR1* | 0.01 | 0.6 |
| *recP149 recR1* | 0.09 | 0.9 |

[a] Data are reported as percentages and are the averages of at last three independent experiments.

[b] The yields of *met*$^+$ transformants (chromosomal transformation) and neomycin-resistant (pBG59) transformants (plasmid transformation) were corrected for DNA uptake and cell viability, and the values obtained were normalized to the YB886 (*rec*$^+$) value (taken as 100).

mutant strains, we must assume that RecF is required for more than one recombination pathway (see above).

All of these data, taken together, allow us to conclude that *B. subtilis* has multiple pathways for recombination and that the *recH* and *recP* functions may define an as-yet-uncharacterized, third mechanism of recombination in *B. subtilis*.

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