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Genetic Analysis of Fusion Recombinants in *Bacillus subtilis*: Function of the *recE* Gene

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ABSTRACT

Bacillus subtilis protoplast fusion allows the study of the genetic recombination of an entire procaryotic genome. Protoplasts from bacterial strains marked genetically by chromosomal mutations were fused using polyethylene glycol and the regenerated cells analyzed. Recombinants represent 19.3% of heterozygotic cells; they are haploids. Individual characterization of clones show a unique particular phenotype in each colony suggesting that recombination takes place immediately after fusion, probably before the first cellular division. Recombination occurs in the whole chromosome; in one-third of the cases both reciprocal recombinants could be shown in the colony. The genetic interval that includes the chromosome replication origin shows the highest recombination level. Our results suggest that the RecE protein accounts for most of the fused protoplast recombination; however, some "replication origin-specific" recombination events were independent of the *recE* gene product.

THREE different approaches can be used to achieve genetic recombination in *Bacillus subtilis*. (1) The natural transformation system requires the *recE* product gene activity and depends upon the development of a state of competence which involves the elaboration of gene products necessary for the binding and uptake of transforming DNA (DUBNAU *et al.* 1973; HAHN, ALBANO and DUBNAU 1987; DUBNAU 1989). It appears that the recombinant donor DNA molecule involved in the genetic exchange is converted to a single strand DNA intermediate before the establishment of the heterologous recombinant DNA complex (DUBNAU and DAVIDOFF-ABELSON 1971). (2) Transduction mediated by phages is also a *recE* dependent recombination pathway (DUBNAU *et al.* 1973). The recombinogenic DNA molecules are double stranded and homogeneity in the DNA modification pattern is necessary to accomplish the transduction process (TRAUTNER *et al.* 1974). (3) The fusion of *B. subtilis* protoplasts allows the formation of cellular associations containing both complete parental genomes (SCHAEFFER, CAMI and HOTCHKISS 1976); these segregate recombination products (reviewed by HOTCHKISS and GABOR 1985).

A complex genetic situation arises when protoplasts fuse and the resulting fusion bodies start to regenerate as a diploid bacteria. This diploid cell can survive to produce in some cases a genetic recombinant bacterium. The physiological factors implicated in the genetic exchange produced by fusion are completely unknown. In this paper, we present an analysis of this type of recombination. Using appropriate genetically marked strains, genetic exchanges were found in the whole chromosome. We show that *recE* gene product activity is involved in most of the genetic exchanges observed. However, genetic intervals that included the *B. subtilis* replication origin showed higher recombination frequencies than other similar sized regions. At least part of this excess in recombinogenic activity may be determined by *recE*-independent components.

MATERIALS AND METHODS

Culture media: Bacteria were generally grown in Schaeffer's medium (SCHAEFFER, MILLET and AUBERT 1965). Phage stocks and transduction experiments were grown in Pennassay Broth. Competence development during transformation and selection for auxotrophic markers were accomplished in Spizizen minimal media supplemented as required (ANAGNOSTOPOULOS and SPIZIZEN 1961). The protoplast wall regeneration media were as described by WYRICK and ROGERS (1973) and by SANCHEZ-RIVAS (1982). Recombinants were selected by replica-plating on Spizizen minimal base medium supplemented with all the metabolites necessary for the growth of each particular recombinant; amino acids and bases were used at 40 μg/ml; rifampin and erythromycin were utilized at 5 μg/ml.

Bacterial strains and phages: Table 1 gives the characteristics of the strains used in this work. Figure 1 shows the map locations of relevant loci. In MO507 and MO508 the mutated *recE* allele is also present. These *recE* deficient strains were constructed using the Zahler method: PBS1-phage transduction of spectinomycin resistance marker into wild type recipient cells gave 45% coheritance with the *recE* mutation (unpublished results). The bacteriophage used was

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TABLE 1

Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Origin or reference
Bacillus subtilis		
CU3497	*trpC2 ilva2 recE4 spcB1*	DR. ZAHLER
BD108	*argA3 lys21 metB5 pheA12 purA16 rplV1*	D. DUBNAU
BD224	*thr-5 trpC2 recE4*	D. DUBNAU
QB944	*cysA14 purA26 trpC2*	DEDONDER *et al.* (1977)
S6	*thrA pyrA trpF7 rfm486*	SCHAEFFER, CAMI and HOTCHKISS (1976)
MO501	*argA3 metB5 pheA2 purA16 trpC2 rplV1*	This work
MO507	*argA3 metB5 pheB12 purA16 trpC2 recE4 rplV1 spcB1*	This work
MO508	*thrA pyrA trpF7 recE4 rfm486 spcB1*	This work
Plasmids		
pAK1	pJH101 vector with the *Sph*I-*Eco*RI fragment of 8 kbp which contains the *B. subtilis gyrA*, *gyrB* and *recF* genes	LAMPE and BOTT (1984)
pHV438	pHV32 vector with a 4.4-kbp fragment contains the *B. subtilis thyB* gene and the *X* region	NIAUDET, GOZE and EHRLICH (1982)
pHV33	pBR322 and pC194 ligated in the *Hin*dIII site. Replicates in *B. subtilis* and in *E. coli*	PRIMROSE and EHRLICH (1981)

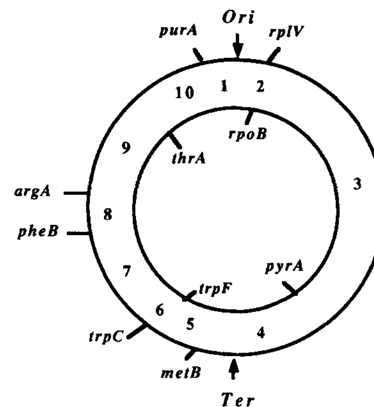
The strain MO501 was constructed by the transformation-congresion method, using QB944 DNA as donor and BD108 cells as recipient. Trypthophan deficient mutants were screened among the selected lysine proficients recombinants. The RecE$^-$ strains were constructed by PBS1 transduction using the CU3497 strain as donor and the MO501 and S6 cells as recipients. The Rec deficient phenotype is coherited at 45% with the Sp^R marker.

the wild-type strain of PBS1 (TAKAHACHI 1961).

Genetic transformation and transduction: Genetic transformation and transduction were carried out as described by DUBNAU, DAVIDOFF-ABELSON and SMITH (1969) and by DUBNAU and DAVIDOFF-ABELSON (1971).

Fusion experiments and recombinant selection: Bacterial protoplast fusions were performed according to SCHAEFFER, CAMI and HOTCHKISS (1976). Well isolated colonies growing in the wall regeneration medium were transferred with toothpicks to a rich medium (SCHAEFFER, MILLET and AUBERT 1965) and screened for recombinants by replica plating onto 16 different selective media. The same treatment was realized on protoplasts obtained from separate parental strains, no reversion of the genetic markers was observed. The phenotype of each recombinant was verified after purification of single cells which were screened on the same selective media. One pair of media was designed in order to visualize the reciprocal phenotypes formed by the genetic exchange into the intervals 1 and 10. These phenotypes are: Rifamycin®-Adenine$^-$ and Erythromicin®-Adenine$^+$.

For each recombinant, we were able to score the number



INTERVAL	GENETIC MARKERS	DISTANCE (Degrees)	SIZE (kbp)	PERCENTAGE OF CO-TRANSDUCTION
1	*pur*A - *rpo*B	24	239	1.7
2	*rpo*B - *rpl*V	0.01	1	99
3	*rpl*V - *pyr*A	127	1265	ND
4	*pyr*A - *met*B	58	578	ND
5	*met*B - *trp*F	6	60	31.5
6	*trp*F - *trp*C	0,01	1	99
7	*trp*C - *phe*A	37	369	ND
8	*phe*A - *arg*A	20	199	6.8
9	*arg*A - *thr*A	24	239	1.7
10	*thr*A - *pur*A	64	638	ND

FIGURE 1.—Genetic intervals used in this work. Ten intervals are defined by genetic markers. Their size was calculated taking into account the recombinant frequencies obtained by transduction and utilized to propose the genetic map of *B. subtilis* (HENNER and HOCK 1980; PIGGOT 1989). The *B. subtilis* chromosome has an estimated size of 5500 kbp organized in 360°. Using the conversion graph established by HENNER and HOCK (1980); one degree of the map is equivalent to 9.96 kbp. Employing this factor; we converted the relative genetic distance in degrees to kbp. Taking into account the imprecision of linkage values derived from PBS1 transduction these data represent an approximate physical distance. The two chromosomes (one of each parental strain fused) are represented by two circles (the sizes of which are drawn unequal for convenience). Inner circle corresponds to S6 strain and outside circle to MO501 strain. The origin of DNA replication is located in the interval 1 and the terminus in the interval 4. *argA*, arginine; *metB*, methionine; *pheB*, phenylalanine; *pyrA*, uracil; *purA*, adenine; *rplV*, erythromycin resistance; *rpoB*, rifampin resistance; *thrA*, threonine; *trpC* and *trpF*, tryptophan; NL, markers nonlinked.

of genetic exchanges promoting a specific phenotype to each interval by detection of different growth on specific selective media. In this manner, the number of genetic exchanges in each recombinant and subsequently in the population were totalled. The frequency of recombination in a particular interval is calculated from the ratio of exchanges in a given interval to the total exchanges in the recombinant population. The observed recombination frequencies from different experiments were pooled after verifying that they satisfied a standard χ^2 test with a 5% confidence limit and 12 degrees of freedom; theoretical = 21,06.

Chromosomal and plasmid DNA purification: Chro-

mosomal DNA purification from *B. subtilis* cells was realized according to SAUNDERS *et al.* (1984). Plasmid DNA was purified: (1) from *Escherichia coli* according to MANIATIS, FRITSCH and SAMBROOK (1982) and (2) from *B. subtilis* according to GUERRY, LEBLANC and FALKOW (1973).

Southern blots and hybridization: After endonuclease DNA digestions, the different DNA fragments were separated in a 0.8% agarose gel then transferred onto Hybond N-Nylon (Amersham catalog No. RPN2020N) according to SOUTHERN (1975). A probe was made from DNA of pAK1 plasmid ^{32}P-radiolabeled by the Multiprimer kit (Amersham catalog No. RPN1601Z). Hybridization and washing conditions were done as described previously (GUILLÉN, AMAR and HIRSCHBEIN 1985).

RESULTS

Fusion product analysis: Diploids created by polyethylene glycol (PEG) treatment of *B. subtilis* protoplasts produce recombinants which are found among the primary regenerant cells (SCHAEFFER, CAMI and HOTCHKISS 1976) and among the segregation progeny produced during the growth of the unstable noncomplementing diploids (Ncd) (HOTCHKISS and GABOR 1980; GABOR and HOTCHKISS 1983). However, no extensive analysis had yet been made of primary recombinants resulting from PEG protoplast fusion. Nevertheless, in previous work the phenotypic analysis of the recombinant progeny from Ncd or prototrophic diploid clones suggest a stimulation of genetic exchange into the intervals that include the origin or the terminus of DNA replication (SANCHEZ-RIVAS *et al.* 1982; GABOR and HOTCHKISS 1983; LÉVI-MEYRUEIS and SANCHEZ-RIVAS 1984).

We constructed an appropriate strain (MO501) to evaluate in the same protoplast fusion experiment with the S6 strain, the genetic recombination frequencies in different regions of the bacterial chromosome in primary recombinants. The position and size of the ten genetic intervals that we tested is shown in Figure 1. The smallest of these corresponds to 1 kb (interval 6) and the largest to 1265 kb (interval 3). Protoplasts of strains MO501 and S6 were fused and the recombinant phenotypes of the regenerated cells determined. The values in Table 2 summarize the results of 1244 exfusant (3 independent crosses). These show that colonies with a recombinant phenotype appear with a percentage of 19.3%. In these fusion crosses, the bacteria with a biparental phenotype represent 5.7, 4.9, and 2.7 percent of the total exfusant population. They generate noncomplementing diploids (Ncd) in which only one chromosome is still expressed after several generations of growth (HOTCHKISS and GABOR 1980; GUILLÉN, AMAR and HIRSCHBEIN 1985). In one cross (II), we found that after two passages on selective media (as described in MATERIALS AND METHODS), 8% of exfusant colonies conserved the diploid state, producing progeny with a prototrophic phenotype. Such clones interpreted as complementing diploids were not further studied. The remaining exfusant population corresponded to the parental phenotypes in similar proportions. The addition of different diploid and recombinant phenotypes revealed that at least 30% of regenerated cells corresponded to heterozygotic clones.

TABLE 2

Fusion crosses of strains MO 501 and S6

Fusion cross	No. of colonies analyzed	Observed phenotypes			
		Recombinants	Biparental	Parentals	Prototrophic
I	520	114 21.9%	30 5.7%	376 72.3%	0
II	364	71 19.5%	18 4.9%	243 66.7%	32 8.7%
III	360	60 16.6%	10 2.7%	290 80.5%	0
Σ	1244	245		909	

The fusion crosses between S6 and MO501 strains were performed according to SCHAEFFER, CAMI and HOTCHKISS (1976). Isolated wall regenerated colonies were grown in rich medium and subsequently replica plated on the different selective media. In a similar manner 100 of wall-regenerated colonies obtained from treatment of parental strains were analyzed; nonreversion of genetic markers was observed.

Genetic recombinant in the whole *B. subtilis* chromosome: We scored each type of genetic exchange occurring in a particular recombinant class according to their nutritional requirements. Among 245 stable recombinants recovered in the three independent experiments a total of 694 genetic exchanges were scored, which produced 48 different phenotypes.

Even if the minimal number of exchanges necessary to produce a stable recombinant phenotype is assumed; we are forced to conclude that in order to obtain certain complex phenotypes multiple exchanges were necessary. Therefore, our data below suggest that multiple exchanges are frequent in the diploid cells. These are shown in Table 3. We found 156 recombinant colonies with a minimum of 2 crossovers, 81 with a minimum of 4, 3 with 6, and 5 with 8 genetic exchanges. The stability of the recombinant phenotype was verified by growth in nonselective conditions followed by a supplementary phenotype analysis of these colonies on the different selective media.

The frequency of recombination in each genetic interval was calculated. The results show that recombination takes place along the whole *B. subtilis* chromosome (Table 3).

The statistical χ^2 treatment of the individual recombinant frequency values observed in each interval permit them to be considered as belonging to the same normal distribution. Thus, the *B. subtilis* chromosome genetic recombination values measured by fusion of protoplasts are quite reproducible (Table 3 and Figure 2). Any interpretation of the above data

TABLE 3

Genetic exchanges distribution on the *B. subtilis* chromosome

	Fusion I + II + III		
Interval	No. of genetic exchanges	Percentage	Percent crossover by kbp
1	246	35.4	0.14
2	108	15.5	1.55
3	34	4.8	0.003
4	96	13.8	0.02
5	5	0.7	0.011
6	4	0.57	0.57
7	6	0.86	0.002
8	13	1.8	0.009
9	86	12.39	0.05
10	96	13.8	0.02
Crossovers		694	
Recombinants		245	
Colonies with			
2 crossovers		156	
4 crossovers		81	
6 crossovers		3	
8 crossovers		5	
Total phenotypes		48	
Analyzed colonies		1244	

The results represent the values of three independent experiments. From each experiment the number of recombinants was scored and the different crossovers were determinated in each recombinant. The total number of crossovers among the recombinant population corresponds to 100% recombination. According to MATERIALS AND METHODS, a χ^2 statistical analysis of the data permits grouping of recombination frequencies values in each interval. The practical χ^2 found was 13.5.

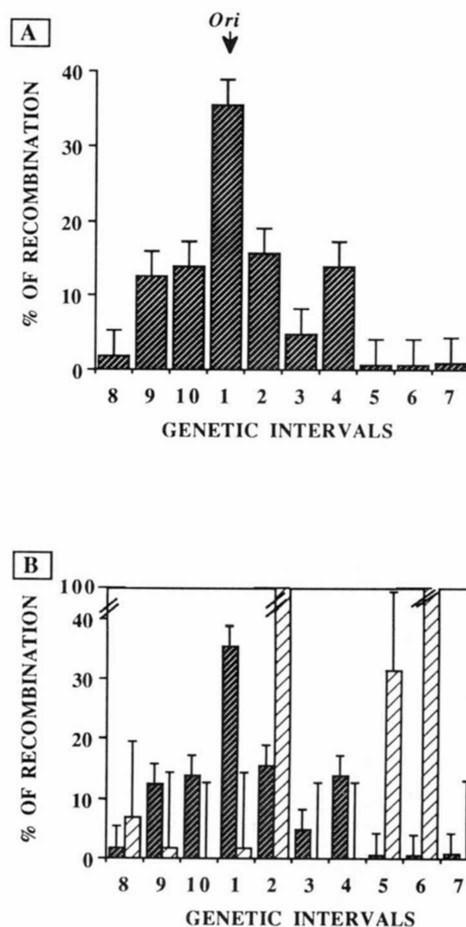


FIGURE 2.—Mode of chromosomal recombination during protoplasts fusion crosses. (A) The percentage of recombination in each interval was calculated from the number of exchanges in the interval divided by the total exchanges among the recombinant population. *B. subtilis* chromosome is represented in a linear form arbitrarily interrupted in interval 7. (B) The recombination frequencies obtained by protoplast fusion (Table 3, dark bars) and by transduction (Figure 1, light bars) are compared.

should take into account the physical size of each genetic interval since a deformation of the results should be created by the fact that for long intervals all even and all odd numbers of genetic exchanges will give the same phenotype, thus resulting in an underestimate of the recombinant frequency in such an interval. Moreover, comparison of the rates of recombination in DNA fragments that have similar sizes, confirms that the highest recombination frequency appears in intervals 1, 2, 9 and 10 (1.7×10^3 kbp of DNA with 8 genetic markers tested) which show 77% of the total recombination events.

Equivalent sized region within intervals 4, 5, 6, and 7 (1.71×10^3 kbp, 8 genetic markers tested) show a recombination rate of 16%. By this analysis, we can conclude that the genetic recombination obtained in diploids cells of *B. subtilis* in the absence of any selective growth pressure is enhanced in the regions that include the replication origin of the chromosome.

Comparison of recombination frequencies obtained by protoplast fusion with those obtained by transduction: A comparison of recombination frequencies obtained by the two genetic systems that involve exchange between two double stranded DNA molecules was made. No extensive analysis is available to elaborate a genetic map by fusion of protoplasts, so the conversion of cell-fusion recombination frequency between independent markers to genetic distance is not possible. However, genetic distances obtained by transduction can be transformed into recombination frequencies since the relationship used to constructed the *B. subtilis* map was previously determined (HENNER and HOCH 1980). In Figure 1, the position and size of each genetic interval in the *B. subtilis* chromosome is shown. In addition, following the rules of construction of the *B. subtilis* genetic map, the transduction recombination frequency in intervals 1, 2, 5, 6, 8 and 9 was calculated. The other genetic intervals used in this work are not suited to this kind of treatment; the sizes of these DNA regions are higher than the maximal size transduced by PBS1 phage (275 kbp) utilized to construct the map and, by definition, no genetic linkage is possible between markers separated more than 275 kbp.

The recombination pattern visualized by protoplast

fusion is quite different from that observed by transduction, as shown in Figure 2B. The two sets of values do not match; in transduction, the recombination between close markers is significantly higher (intervals 2, 5 and 6), while in fused protoplasts the frequency of these phenomena seems to be influenced by other factors than the genetic distance between two particular markers. Hence the hypothesis of hotspots of cell-fusion recombination is proposed.

Reciprocity of recombination by protoplast fusion: The phenotypic analysis of fusion products by subculturing of diploid cells had shown the presence of recombinants with reciprocal phenotype (GABOR and HOTCHKISS 1983).

During the performance of the experiments reported above, two media were used to evaluate the eventual reciprocity of genetic recombination concerning intervals 1 and 10. In this analysis, it was observed that only 15 colonies out of 1244 generate a mixture of two recombinant phenotypes and grow on the two media that allow visualizing the reciprocal phenotype. These are Ade$^-$, Thr$^-$, Uri$^-$, Trp$^-$, Rif® and Met$^-$, Trp$^-$, Phe$^-$, Arg$^-$ Ery®. They correspond to only one-third of the recombinants produced by the genetic exchange within these intervals. These results suggest that the reciprocality of recombination take place in one part of genetic exchanges observed in the origin of replication area. In addition, they support the first observation reported by GABOR and HOTCHKISS (1983) in which reciprocal recombination was observed in several regions of the chromosome.

Genetic exchanges in fused protoplasts after a selective pressure: In order to confirm our earlier results and to exclude the possibility of preferential growth of recombinants caused by exchanges in the origin area, an analysis of genetic recombination in exfusant clones growing in a selective medium was done. The desired type of genetic exchange giving the selected phenotype is imposed by the selection. According to the earlier results, the simplest hypothesis is that the second genetic exchange necessary for the formation of a stable recombinant occurs, for most recombinants, in the region of the replication origin. Tryptophan$^+$ recombinants were consequently selected after fusion of MO501 and S6 strains in a minimal regeneration medium (this phenotype involves an exchange into interval 6).

The frequency of Trp$^+$ recombinants is 2.5×10^{-5} among the regenerated protoplasts. From the phenotypic analysis of the 75 Trp$^+$ recombinants obtained, 417 genetic exchanges were scored. The comparison of the recombination rates in different intervals show that 70% of the recombinational events appear in intervals 1, 2, 9 and 10 (Figure 3). Therefore, 54.9% of genetic exchanges in the Trp$^+$ recombinants occurred in the origin region (intervals 1, 2 and 10). A

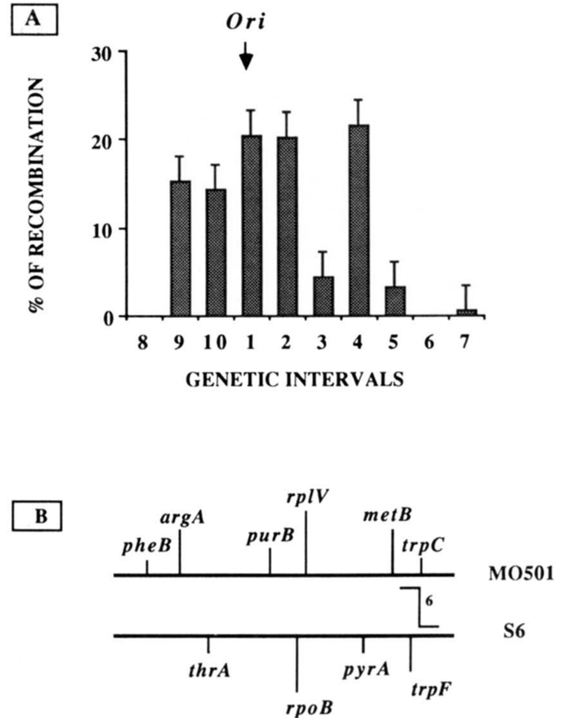


FIGURE 3.—Mode of chromosome recombination after selection of Trp$^+$ recombinants. PEG-treated protoplasts (SCHAEFFER, CAMI and HOTCHKISS 1976) were plated on selective regeneration medium (SANCHEZ-RIVAS 1982) without tryptophane. The Trp$^+$ phenotype was verified in 75 recombinants; these were replicated on selective media to determine their complete phenotype. In these experiments recombinants with 11 different phenotypes were recovered (a total of 342 events of recombination). No reversion of Trp$^-$ phenotype was observed when 2.5×10^7 protoplasts of each parental strain were plated separate on the regeneration medium without tryptophan. (A) The percentage of recombination in each interval was calculated as described in MATERIALS AND METHODS. (B) Scheme of the cross. Only the mutant alleles are represented. The cross line points out the desired genetic exchange.

stimulation was also observed in interval 4, close to the selected exchange: 21.6% of recombination was localized in this DNA region which contains the terminus of chromosome replication. Thus, the conclusion reached is that the second exchange necessary for the production of these Trp$^+$ recombinants occurred in the most part of them in the origin area. In this manner, we can exclude the possibility of trivial artifacts entering the phenotypic analysis in fusion crosses.

Protoplast fusions of *recE* mutant strains: The *recE* mutants of *B. subtilis* are inhibited in all known homologous recombination pathways. In order to determine the influence of the *recE* gene product in the recombination obtained by protoplast fusion, the *recE4* mutation was introduced into the same strains used for the recombination experiments. The resulting strains (MO507 and MO508) were, as expected, recombination deficient, (both transduction and transformation were decreased by at least 10^7, data not shown). The PEG treatment and regeneration of pro-

TABLE 4

Fusion crosses of strains MO507 and MO508

Cross	Colonies analyzed	Observed phenotypes			
			Parentals		
		Recombinants	MO507	MO508	Biparentals
I	409	201 49.1%	0	206 50.9%	2 0.5%
II	200	4 2%	40 20%	148 74%	8 4%

The fusion crosses were carried out according to SCHAEFFER, CAMI and Hotchkiss (1976). In the first experiments, protoplasts of two parents were mixed in a proportion of 1:1. In the second series of experiments, three times more protoplasts of the MO507 strain were used. Wall regenerated colonies were transferred with toothpicks to a rich medium. After growth, the plates were replica plated on the different selective media.

TABLE 5

Protoplast transformation of Rec deficient and Rec proficient strains by plasmids pAK1, pHV438 and pHV33

Plasmids	Frequency of Cm^R		
	MO507	MO508	S6
pAK1	3.8×10^{-3}	1.2×10^{-4}	2×10^{-4}
PHV438	$<5 \times 10^{-7}$	$<10^{-7}$	2×10^{-4}
pHV33	0.2	0.12	0.2
Protoplast regeneration (cells/ml)	2.10^6	10^7	2.5×10^8

The protoplasts of MO507, MO508 and S6 strains (2×10^8) were transformed with 1 μg of DNA of each plasmid following the method of LÉVI-MEYRUEIS, FODOR and SCHAEFFER (1980). The transformation frequencies were determined by the number of Cm^R clones scored among the regenerated colonies.

toplasts made from strains showed lower regeneration frequencies of 0.12% (MO507), and 1% (MO508), instead 20–30% of regenerants classically obtained with Rec$^+$ strains, confirming previous findings of our laboratory (GUILLÉN *et al.* 1986).

Analysis of fusion products of *recE* strains: MO507 and MO508 were fused. In some experiments both strains were present in a ratio 1:1 in the protoplast mixture, while in others, three times more MO507 was used, since this strain proved to be more sensitive to the PEG treatment than MO508. The results of the phenotypical analysis of regenerants are shown in Table 4. Large variations of the data were observed between different experiments which preclude their combination after statistical analysis. Thus, the results were not pooled for discussion purposes. In the first experiments, recombinants were obtained at a proportion of 49%, all having a phenotype consistent with one exchange in interval 1 and another in interval 10 (Ade$^-$ Uri$^-$ Trp$^-$ Thr$^-$ Rif®).

A similar phenotype would have been obtained if the MO508 parent had acquired an Ade$^-$ mutation. This possibility was excluded by analyzing, in parallel, a self-fusion experiment of parental strains. One hundred wall regenerated colonies were analyzed as described before. Phenotypic changes in the parents, such as reversion of the auxotrophic alleles, were not revealed by the analysis of colonies from independent self fusions of MO507 or MO508 protoplasts.

In the second type of fusion experiment, in which three time more MO507 protoplasts were used, recombinants appear at 2% of wall regenerated colonies. Two phenotypes were observed between the recombinant colonies: Ade$^-$ Uri$^-$ Trp$^-$ Thr$^-$ Rif® and Ade$^-$ Phe$^-$ Arg$^-$ Trp$^-$ Met$^-$ Ery® Rif®; both of them needed at least one genetic exchange in interval 1.

These results, when compared with those obtained with Rec$^+$ strains, indicate that most cell-fusion recombinational events are dependent on RecE. Nevertheless, a residual recombinational activity takes place in the absence of RecE in intervals that include the replication origin. Counter-selection of a parent enhances the frequency of these recombinants. Results that express the same effect were obtained in RecE$^+$ crosses; in which six times more recombinants were observed for a ratio of viable regenerated protoplasts of 1.5 (GABOR and HOTCHKISS 1979).

Transformation of protoplasts of Rec deficient strains with integrative plasmids: The following experiments were devised to generalize and extend the finding that recombination near the origin can occur in the absence of RecE wild-type activity. Protoplasts of the Rec$^-$ strains MO507 and MO508 were PEG-transformed with three different plasmids: (1) pAK1 which contains 8 kbp of DNA corresponding to the replication origin of the *B. subtilis* chromosome (LAMPE and BOLT 1984); (2) pHV438 which is a vector similar to pAK1 but contains 4 kbp of *B. subtilis* chromosome from the *ThyB* and *X* regions (NIAUDET, GOZE and EHRLICH 1982), (3) the pHV33 *E. coli-B-subtilis* bifunctional plasmid (PRIMROSE and EHRLICH 1981).

The pAK1 and pHV438 plasmids do not replicate autonomously in *B. subtilis*, they are potentially able to integrate into the bacterial chromosome of a Rec$^+$ strain via homologous recombination of the DNA insert. The Cm® marker carried by these plasmids was used for selecting the transformants. In parallel experiments the same plasmids were used to transform an otherwise isogenic Rec$^+$ strain (S6). The results are shown in Table 5: pAK1 which contains the *Ori* region transforms S6 (Rec$^+$), MO507 and MO508 (Rec$^-$) at the frequencies expected for an integrative plasmid. This is unexpected, since the Rec$^-$ strains should be not transformable under these conditions. For instance, when pHV438 is utilized as a donor, no transformants were obtained in the Rec$^-$ recipients cells, whereas 0.02% of Rec$^+$ regenerated protoplasts were Cm® transformants. Parallel transformation with pHV33, an autonomously replicating plasmid yielded a similar number of transformants in the three strains.

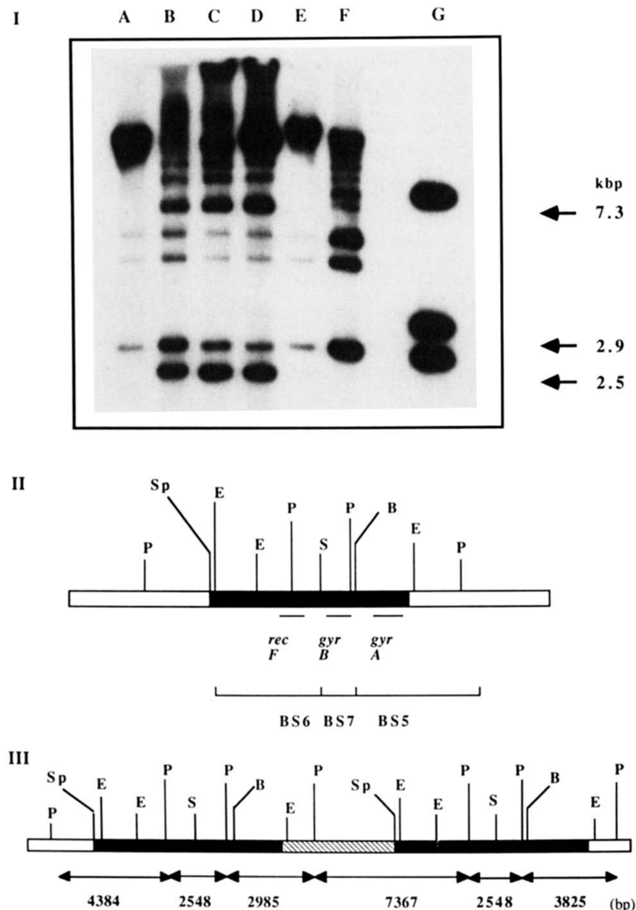


FIGURE 4.—Southern blot analysis of DNA from RecE-independent *B. subtilis* origin recombinant strains. Two micrograms of each DNA were digested by the *Pst*I endonuclease, electrophoresed in an agarose gel, blotted and probed with pAK1 DNA plasmid. (I) A, MO508 (Rec$^-$); B, S6pAK1 (Rec$^+$); C, recombinant 1; D, recombinant 2; E, recombinant 3; F, recombinant 4; G, pAK1 plasmid. (II) Restriction map of the *Ori* region on the *B. subtilis* chromosome. (III) Physical map of the *Ori* region after integration of the pAK1 plasmid. B, *Bam*HI; E, *Eco*RI, P, *Pst*I; S, *Sal*I; Sp, *Sph*I.

Genomic blot analysis of the Rec$^-$ Cm$^®$ recombinants: Studying the replication origin region of the *B. subtilis* chromosome an 8-kbp *Ori*-specific DNA fragment was cloned producing the pAK1 plasmid (LAMPE and BOTT 1984). Since no autonomous replicative capacities had been shown for pAK1 plasmid, the results described above indicate the chromosomal integration of pAK1 by a *recE* independent mechanism. In order to test this hypothesis, DNA purified from the Rec$^-$ recombinants strains and from a Rec$^+$ recombinant was analyzed by Southern blot. The hybridization pattern of the *Pst*I restricted DNA from the Cm$^®$ recombinant cells with the pAK1 labeled plasmid is shown in Figure 4.

The *Pst*I restriction pattern of DNA obtained from Rec$^-$ Cm$^®$ recombinants 1 and 2 is equivalent to those of the DNA from a Rec$^+$ Cm$^®$ recombinant; some differences could be relevant to the hybridization intensity of 4.4- and 3.8-kbp fragments, as a less intense signal is observed with the Rec$^-$ DNA preparations. Nevertheless, the Cm$^®$ phenotype seems to occur by integration into the chromosome of pAK1, which is conducive to a duplication of the specific cloned DNA origin. A similar mode of recombination in these three strains seems to have occurred. For instance, two DNA deletions have appeared, in all of them the expected 7.3-kbp fragment appears as 6.5-kbp and the expected 2.9-kbp fragment as a 2.3-kbp DNA fragment. These DNA fragments contain the *recF* or the *gyrA* gene previously identified in the BS5 and BS6 DNA fragments that are the first to be replicated in *B. subtilis* (LEVINE *et al.* 1987). Conversely, DNA patterns from recombinant 4 (Rec$^-$ Cm$^®$) and from Rec$^-$ parental strain are the same. Finally, the DNA profile from recombinant 3 (Rec$^-$ Cm$^®$) is heterogeneous; probably a mixture of cells containing integrated plasmid with cells without plasmid has been recovered.

The general view of the performed genomic analysis suggests that in some cases pAK1 is able to be incorporated into the chromosome by a *recE* independent mechanism. However, the persistent Cm$^®$ phenotype even in cells that do not show DNA integration supports the existence of some free plasmid. Moreover, a phenotypic instability of the Cm$^®$ marker carried by pAK1 was observed when the recombinants were grown over 4 cellular generations without chloramphenicol. The loss of the Cm$^®$ marker seems to coincide with the excision of DNA sequences carrying the *cat* gene, since a replicative plasmid can be recovered in competent *E. coli* cells by use of DNA minipreparations from cultures grown in presence of chloramphenicol of the four Rec$^-$ recombinants studied above (results not shown).

DISCUSSION

Homologous recombinant by protoplasts fusion: When *B. subtilis* protoplasts were fused by PEG treatment, heterozygous cells formed. This raised the possibility of using fusion as a bidirectional way to exchange genetic material in bacteria. Among the exfusants, haploid recombinants appeared at a frequency of 19.3%. Most of the recombinants were produced by two genetic exchanges between the parental chromosomes. The proportion of recombinants produced by four crossovers was high (3.3%); those with six or more crossovers were rare. Nevertheless, the majority of colonies consisted of a single recombinant genotype, although some contained both reciprocal phenotypes. Thus a single recombination event, presumably before any genome replication or/and cellular division, is the most common occurrence in fused protoplasts.

In fusion crosses the existence of diploid clones in which an entire chromosome is unexpressed (Ncd)

was previously demonstrated. Unstable Ncd clones segregate during growth in nonselective medium in a phenotypically mixed population (HOTCHKISS and GABOR 1980). In addition, stabilization of these clones led to a homogeneous phenotype of bacterial population (GUILLÉN, AMAR and HIRSCHBEIN 1985). The influence of these phenomena on the analysis in this study appears to be minor. In fact, all the recombinants subject to our study are phenotypically stable as judged by the absence of segregation of new phenotypes during their growth in rich medium. Nevertheless, it is possible that among recombinants some stable Ncds were present. In this case, the chromosome inactivation had occured after genetic recombination since the silent chromosome in Ncds is devoid of recombination capacities (FTOUHI 1989).

Reciprocal recombination: Pairs of reciprocal recombinants were found in a third of the population of recombinants having crossovers in intervals 1 and 10. They were the only reciprocal phenotypes searched for in these experiments. The production of reciprocal recombinants was first observed by GABOR and HOTCHKISS (1983). Nevertheless, in other independent crosses, reciprocal recombinants were not recovered (SANCHEZ-RIVAS *et al.* 1982). The finding implying that bacteria can display reciprocal crossovers, was confirmed in our experiments. The reciprocal recombinants are the products of genetic exchange between two replicons that are entirely conserved after recombination and independently segregated into daughter cells. However, this pathway is not the usual fate of the exfusant bacteria. In fact, in most of the recombinant population, reciprocal recombination was not observed, indicating that other recombination pathways function in fused protoplasts.

High frequency of recombination in the chromosomal replication origin: The recombinants analyzed in this work showed multiple genetic exchanges in different areas of the bacterial chromosome (Fig 2). Comparison of recombination frequencies in chromosomal regions equivalent in size show that the most important recombinogenic activity appears in the intervals 1, 2, 9 and 10. More frequent exchanges were found near the origin of chromosome replication (interval 1). This observation permits a clarification of a controversial point raised by previous work in which recombinants obtained after selection or during the growth of noncomplementing diploids were analyzed. In independent crosses, preferential occurrence of crossovers at sites close to the replication terminus was sometimes noted, while at other times preference was for sites near the origin of replication (SANCHEZ-RIVAS *et al.* 1982; GABOR and HOTCHKISS 1983). The scarceness of markers, the heterogeneity of the crosses or the small number of recombinants recovered could explain this variability. More homogeneous results were obtained in this work in which we studied a large number of recombinants from the same cross. Our results reveal that the genetic interval bearing the replication origin of the chromosome has a high recombinogenic activity and that a second area with an elevated recombination rate corresponds to a genetic interval containing the terminus of replication.

Diverse biological activities are classically rationalized by the physical association of the DNA with cell envelope components. For example, a transformation assay demonstrated that genetic markers near the replication origin and terminus are stably associated with membrane fragments and wall material (YAMAGUCHI and YOSHIKAWA 1973; HOROWITZ *et al.* 1979). It may be supposed that during protoplast fusion, these attachments enhance genetic recombination. In addition, it is plausible to imagine that the DNA replication activity by itself stimulates recombination in these chromosome areas. Generation of single stranded DNA at the replication origin and resolution of replicated molecules at the terminus involves reactions like DNA cutting, helix destabilization and DNA ligation; these enzymatic activities could enhance chromosome recombination. The possibility that DNA replication has a particular role in promoting recombination has been raised in the case of *B. subtilis* strains carrying an integrated copy of plasmid pE194 between directly repeated DNA sequences (NOIROT, PETIT and EHRLICH 1987). When plasmid replication is induced, recombination between the repeated sequences is stimulated by up to 450 times.

In the protoplast fusion system, the influence of the DNA replication activity was also invoked to explain the nonreciprocality observed in the majority of genetic exchanges. This hypothesis proposes an asynchronous DNA replication of chromosomes during the diploid state followed by a non-asymmetric chromosomes segregation. Thus, the reciprocal recombinant chromosome replicated with delay should by lost during segregation (HOTCHKISS and GABOR 1985).

RecE protein activity in recombination by protoplast fusion: The RecA protein accounts for most of the homologous recombination in *E. coli*. One known activity of this protein is that it makes heteroduplexes between DNA molecules carrying long patches of sequence homology. This ATP dependent enzymatic reaction can form Holliday junctions which are the putative structural intermediate complexes leading to homologous recombination (for a review; SMITH 1988). Although the mechanism of recombination in bacteria other than *E. coli* are poorly understood, counterparts of the RecA protein have been found in several species. Much evidence indicates that the *recE* gene in *B. subtilis* encodes a protein equivalent to *E. coli* RecA.

In vitro, the RecE protein enhances DNA strand

exchange in the presence of ATP (LOVETT and ROBERTS 1985). *In vivo*, RecE protein is required for the formation of the DNA donor-recipient complex during genetic recombination (DUBNAU *et al.* 1973). The cloned *B. subtilis recE* gene restores the SOS response and homologous recombination in the *recE* mutant cells; its product is a 45-kD protein (MARRERO and YASBIN 1988).

One of the main aims of this work was to determine the role of the RecE protein in recombination using the protoplast fusion system. These findings show that RecE is essential to accomplish recombination through most of the *B. subtilis* chromosome. By crossing mutants affected in this protein activity, a total inhibition of genetic exchange was observed in most genetic intervals. One exception was found; *recE*-independent recombinational activity was observed in chromosomal areas encompassing the origin of replication. This observation was reinforced by results obtained from experiments using an origin-region-specific plasmid to transform Rec deficient strains by the protoplast fusion method. The integration of the *Ori* sequences into the chromosome by a similar pathway to that used in Rec proficient strains was observed. A unique recombinant DNA structure similar to those found with the RecE proficient strains was obtained.

The origin region is particularly sensitive to recombination during protoplast fusion of *B. subtilis*. Work conducted by other groups had show that DNA amplification in the chromosome origin area occurs when Rec^+ cells were protoplasted; these chromosome rearrangements generate a tetracycline resistant phenotype (WILSON and MORGAN 1985; SHISHIDO *et al.* 1988). Recently, it has been shown that the amplification of a DNA fragment close to the replication origin is required for the expression of tetracycline resistance (IVES and BOTT 1989).

The general overview of these results is that by protoplastization of *B. subtilis*, diverse genetic exchanges can be produced. When the fusion is stimulated by treatment with PEG, pairs of replicons carrying the DNA at or near the replication origin can recombine even in the absence of a functional RecE protein. The role of DNA replication (during or after wall regeneration) in such recombination remains to be discovered. Nevertheless, it is reasonable to suppose that it could be mediated by one or more enzymes normally involved in the initiation of DNA replication.

The ability of the *Ori* sequences to recombine independently of the RecE gene product seems to be a more general fact in bacteria. This is also the case of plasmids carrying *E. coli OriC*, which in the absence of the RecA activity are able to integrate unstably into the chromosome (MASTERS, ANDRESDOTTIR and WOLF-WATZ 1978). It seems that the RecE activity is necessary for the stable maintenance of the pAK1 integrated sequences. This observation raises the questions of the role of the RecE activity as a regulator of the *Ori* specific recombination or rather as a part of the control of the initiation of chromosomal replication. There are some observations indicating that in *recA* mutants of *E. coli* chromosomal replication is perturbed. It was suggested that the absence of RecA activity in *E. coli* causes initiation of newly formed replication forks to stall. DNA is accumulated in such a way that RecA deficient cells contain more DNA than wild-type strains growing at the same rate (SKARSTAD and BOYE 1988). Genetic recombination could result in this case from the overproduction of replicating DNA. This hypothesis needs to be tested.

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