GENE DISLINKAGE IN TRANSFECTION OF SP82G* PHAGE DNA

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COMPETENT *Bacillus subtilis* cells when infected with DNA isolated from bacteriophage SP82G give rise to infective phage particles (GREEN 1964). This reaction which has been observed with a number of different phages has been called *transfection* (FÖLDES and TRAUTNER 1964). There are two modes of transfection which depend on the bacteriophage from which the DNA was isolated. The first requires only single phage DNA molecules to carry out the infection. Representative types of *B. subtilis* phage that have been shown to act in this manner are ϕ 29 and SP02, (REILLEY and SPIZIZEN 1965, OKUBO and ROMIG 1965). The second form of transfection requires the interaction of several infective DNA molecules to establish an infective center. Among the *B. subtilis* bacteriophages whose transfection process is consistent with multiple DNA molecule requirements are SP3 (ROMIG 1962), SP50 (FÖLDES and TRAUTNER 1964), SP01 (OKUBO, STRAUSS and STODOLSKY 1964), SP82G (GREEN 1964) and ϕ 1 and ϕ 25 (REILLEY and SPIZIZEN 1965).

Marker rescue studies (GREEN 1966) indicate that the requirement for multiple molecular infection of *B. subtilis* with SP82G phage DNA results from a partial inactivation of the infecting DNA while in residence within the cell. The phage particles that are ultimately produced following this inactivation have been shown to result from an obligatory genetic interaction of at least two (and probably more) infective molecules (GREEN 1964; OKUBO, STRAUSS and STODOLSKY 1964). A consequence of this obligatory genetic interaction among transfecting DNA molecules is that linkage between phage markers decreases when compared to the linkage observed in standard phage crosses. These observations are consistent with a process that restores the integrity of the infective DNA by recombination of partially inactivated fragments.

This paper reports an analysis of genotypes arising from transfection with different genetically marked DNAs. DNA preparations of twelve different temperature sensitive markers of SP82G were used to infect competent *B. subtilis* cells in various pair-wise combinations. The cells were permitted to lyse and the genotypes of the output phage were determined. The results of these "transfection-crosses" show that the linkage characteristic of these markers in phage crosses is lost in transfection and that re-assortment of the input alleles is random

* In order to distinguish the bacteriophage of this and our previous studies from a different phage simultaneously given the designation "SP82" (FÖLDES and MOLNAR 1964), this and future references will use the designation SP82G.

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in the establishment of the infective center by the transfection process. The formation of infective phage in transfection involves random reassortment of fragments that are less than one fortieth of the known genetic map of SP82G.

MATERIALS AND METHODS

Strains. The bacterial strain *Bacillus subtilis* SB-1 (NESTER 1960), the bacteriophage SP82G (GREEN 1964), and the derived temperature mutants of SP82G (KAHAN 1966) have been described.

Techniques. The techniques and media used in this study are those described by GREEN (1966). Phage crosses were performed as previously described (GREEN 1966). Extraction of DNA from purified phage preparations was carried out by the phenol method of MANDELL and HERSHEY (1960). The map values in Table 1 and Figure 1 are based on the summation of the smallest map intervals between the two markers, (GREEN and COTE, unpublished). Where the smallest intervals exceeded 2.5%, a mapping function was used to determine the most likely map value for the interval.

The experimental scheme. Approximately equal amounts of DNA from two different temperature sensitive mutants of SP82G phage were mixed to make a total amount of 20 μg of DNA (in volume of 0.2 ml). One milliliter of competent cells at a titer of 2×10^8/ml was added to the DNA and incubated at 33°C for a one hour period. The cells were diluted fifty-fold in supplemented minimal medium (NM) and incubated until lysis (usually 2–3 hrs). The kinetics of appearance of phage following transfection under similar conditions has been previously described (GREEN 1964). The lysate was diluted, and plated by the agar overlay method. Plates were incubated at 33°C overnight, and about fifty well-isolated plaques were plugged out of the agar with Pasteur pipettes and placed in 0.5 ml of NM. Toothpicks were then used to transfer a small amount of each phage clone to the surface of plates that had been seeded with between 10^6 and 10^7 particles of one or the other parental phage in the top agar layer together with recipient bacteria. Both parental backcross plates, as well as a control plate with no phage and only bacteria in the top agar layer, were then incubated at 47°C. After a variable period (largely dependent on the leakiness of the parental testing phage on the plate) which was always in excess of 5 hrs, the plates were scored for recombination and/or complementation between the stabbed plaques and the testing parent. All of the expected parental and recombinant classes were typically seen to occur. As well, another class of bacteriophage was frequently observed which while recombinant for the wild-type alleles of both parents was unable to grow at 47°C. This class was considered in the overall analysis to be a typical recombinant class, although mutant at another locus.

The experiments reported here were the by-product of a search for various doubly mutant bacteriophage stocks and in most cases a single attempt was made to determine the distribution of genotypes in any single cross. Four of the transfection crosses reported were performed in duplicate. In certain crosses where one or the other marker was particularly leaky or had a high reversion rate there were undoubtedly scoring errors. No attempt has been made to select the data in regard to these properties.

RESULTS

The progeny of a total of twenty-nine "transfection crosses" were examined. The map distances between the genetic markers were established by phage crosses (Figure 1) and ranged between 1.3 and 39.5 map units. All regions of the known genetic map of SP82G (KAHAN 1966) were represented in the "transfection crosses." The results of the analysis of genotype are presented in Table 1 and lead to the following conclusions:

1.) Recombinants occur more frequently in transfection crosses than in typical

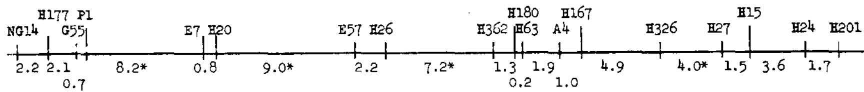

FIGURE 1.—A map of the temperature sensitive markers of SP82G used in this study. Six additional markers have been added to define the map more thoroughly. This map is the result of two and three factor bi-parental crosses and the inter-marker distances are derived generally from addition of map distances of 2.5% or less. The exceptions to this are the *P1–E7*, *H20–E57*, *H26–H362* and *H326–H27*, regions which presently have not been sub-divided. The values used for these intervals have been corrected to map distance by use of a mapping function (GREEN, unpublished).

phage crosses (the mean recombination frequency of all these crosses by phage infection is 12%; by transfection crosses 41%) and all genotypic classes can be recovered. 2.) Certain mutants show a marked allelic selection in the recovered progeny. 3.) Contingency testing by the FISHER method of exact probabilities indicates that the majority of the marker distributions in the progeny are not significantly different from that expected by random assortment of the surviving input alleles. Among the minority distributions which are definitely nonrandom there is no correlation with map distance between the markers. There is, however, a significant involvement of a single marker, *H177*, in these non-random distributions.

The first conclusion is evident from a casual examination of Table 1. The second conclusion is best emphasized by examining the allele ratios of the twelve markers in transfection progeny. In Table 2 the ratio in the transfection progeny for a given mutant gene to the wild-type gene carried by the same DNA molecule is given for each cross. This ratio is seen to vary from 0.99 to 0.32, and is always below 1.0. This suggests that there is selection against the temperature sensitive mutant allele at 33° in the presence of the wild-type allele. Both the delayed production of mature phage characteristic of transfection (GREEN 1964) and the approximately two cycles of phage growth which were permitted following transfection may have emphasized a slight selective difference in the alleles.

If the transfected markers were completely unlinked and randomly assorted by transfection we might expect equal size classes of all genotypes. The two factors that prevent this idealized result of random assortment are the aforementioned allele selection in favor of the wild-type allele of the temperature sensitive mutant and the apparent failure to achieve equal multiplicity of infection with the two types of DNA molecules present. This latter phenomenon has been previously reported (GREEN 1964) and may reflect the molecular weight, single strand scissions, and other molecular accidents of the various DNA preparations. Since the operation of these two factors does not permit the usual abbreviated method of scoring recombinants to be used, i.e. scoring only a single recombinant class and the total progeny, the genotype of each of the progeny phage in a cross was determined (Table 1).

In order to test whether the distribution of alleles in the progeny phage of a cross was random the data were analyzed by FISHER's exact probability for a

TABLE 1

Genotypes from transfection crosses

	H362+×+*H180* (1.3)	*H63*+×+*A4* (1.9)	*H177*+×+*G55* (2.1)	*H362*+×+*H167* (4.4)
− +	2	2	13	115
+ −	14	107	9	0
− −	0	7	2	6
+ +	4	7	14	7
+ + x^-	1	0	0	0
	1–P=0.100	1–P=0.155	1–P=0.087	1–P=1.0

	H362+×+*H167* (4.4)	*H167*+×+*H326* (4.9)	*H27*+×+*H24* (5.1)	*H362*+×+*H15* (5.5)
− +	3	35	25	20
+ −	88	2	6	2
− −	6	2	7	3
+ +	9	2	17	11
+ + x^-	6	0	1	0
	1–P=0.157	1–P=0.041*	1–P=0.515	1–P=0.605

	H63+×+*H326* (7.8)	*H362*+×+*H326* (9.3)	*G55*+×+*H20* (9.7)	*H177*+×+*H20* (11.8)
− +	2	1	87	9
+ −	106	33	0	0
− −	17	3	1	7
+ +	11	3	4	34
+ + x^-	5	0	0	0
	P=0.55	1–P=0.355	1–P=1.0	1–P=0.0001*

	H177+×+*H20* (11.8)	*H180*+×+*H27* (12.0)	*H180*+×+*H27* (12.0)	*H167*+×+*H24* (14.0)
− +	61	18	23	26
+ −	78	1	0	2
− −	7	3	13	1
+ +	21	19	9	28
+ + x^-	5	0	0	3
	1–P=0.005*	P=0.322	1–P=0.032*	1–P=0.576

	H362+×+*H15* (14.8)	*H63*+×+*H24* (16.9)	*H20*+×+*H362* (18.4)	*H20*+×+*H63* (19.9)
− +	16	2	44	20
+ −	102	21	10	1
− −	14	5	21	2
+ +	35	28	42	14
+ + x^-	9	1	0	10
	1–P=0.013*	P=0.145	P=0.083	P=0.45

	H20+×+*H167* (22.8)	*H20*+×+*H326* (27.7)	*G55*+×+*H326* (28.1)	*H177*+×+*H362* (30.2)
− +	5	3	3	4
+ −	17	33	64	36
− −	10	7	6	0
+ +	27	15	15	15
+ + x^-	5	4	3	6
	P=0.029*	P=0.496	1–P=0.34	1–P=0.024*

	H177+×+*H362* (30.2)	*H20*+×+*H15* (33.2)	*H177*+×+*H167* (34.6)	*H20*+×+*H24* (36.8)
− +	7	14	7	20
+ −	38	13	60	2
− −	2	6	1	3
+ +	3	26	4	38
+ + x⁻	3	1	15	2
	1–P=0.0005*	1–P=0.54	1–P=0.0001*	P=0.234

	H177+×+*H326* (39.5)
− +	6
+ −	38
− −	1
+ +	5
+ + x⁻	0
	1–P=0.0002*

Each set of genotypes represents a separate "transfection cross". For convenience the crosses have been listed as, for instance, "*H362*+×+*H180*" and in the presentation of genotypes the first entry refers to the nature of the *H362* allele, and the second, the *H180* allele. The parenthetical value to the right of the cross is the map distance between the markers as determined by phage crosses. More than forty progeny phage were examined in each transfection cross (the actual number of individuals tested was determined by the primary purpose of this study; the isolation of doubly mutant phage). Entered at the lower right of the genotype analysis is the cumulative probability for this or a more extreme configuration, P or 1–P (depending on which tail of the probability distribution the particular sample occupies) to occur. A significant departure from randomness is assumed when P or 1–P is 0.05 or less. One exceptional class (++x⁻) was observed in many of the crosses. This class carried both of the parental markers but also had a new temperature sensitive mutation. It was considered as part of the ++ class in the analysis. The frequency of this class of exceptional mutants among the phage where it could be scored (those particles which are recombinant for both wild-type markers) was 14.4%. Mutation of this level has been observed in transformation of *Bacillus subtilis* by YOSHIKAWA (1966) and may be a normal concomitant of the recombination process.

2 × 2 table. The allele distribution in nineteen of the twenty-nine crosses did not differ significantly from random (the 5% level of significance was used throughout). Among the ten transfection crosses in which the hypothesis of random distribution of progeny alleles was rejected, one marker *H177* was involved in six of the crosses. Only one cross involving marker *H177*, *H177* × *G55*, showed evidence for a random distribution of progeny alleles. If one assumes that the behavior of *H177* is a unique property of that marker and removes all crosses using *H177* from the analysis, then eighteen of twenty-two of the crosses (82%) show no significant differences from the expectation of random assortment of the surviving input alleles following transfection. Among the four remaining exceptional transfection crosses one, *H180* × *H27* has a non-significant duplicate and may represent a failure in the scoring of the genotypes. Such scoring failures may be related to high reversion of one of the markers in a cross, or to inability to select for the particular mutant phenotype (leakiness of the mutant). Among the exceptional four crosses there is no apparent correlation with any particular marker, map distance between the markers involved (they vary between 4.9 and 22.8 map units), or with the particular region of the genetic map.

TABLE 2

Output allele ratios

Cross	Ratios	Mean
$H180^{-}$/X+	0.736, 0.567, 1.437	*0.913*
$H362^{-}$/X+	0.286, 0.991, 0.50, 0.596, 1.0, 0.50, 0.840, 0.630, 0.909	*0.695*
$H177^{-}$/X+	0.555, 0.372, 0.977, 0.160, 0.692, 0.307, 0.636	*0.528*
$G55^{-}$/X+	0.478, 0.967, 0.428	*0.624*
$H326^{-}$/X+	0.742, 1.00, 0.932, 1.00, 0.909, 0.907	*0.915*
$H15^{-}$/X+	0.384, 0.794, 0.475	*0.551*
$H63^{-}$/X+	0.90, 1.055, 0.130, 0.225	*0.577*
$A4^{-}$/X+	0.991	*0.991*
$H167^{-}$/X+	0.857, 0.912, 1.0, 0.474, 0.551, 0.772	*0.761*
$H27^{-}$/X+	0.744, 0.20, 1.444	*0.794*
$H24^{-}$/X+	0.542, 0.091, 0.52, 0.119	*0.318*
$H20^{-}$/X+	0.250, 0.206, 0.817, 0.756, 0.555, 0.405, 0.464, 0.487, 0.383	*0.479*

The ratios of the genetic markers carried by a given DNA molecule in transfection. The ratio among transfection progeny of a given mutant marker to the wild-type allele of a second marker carried by the same DNA molecule is given in this table. A separate value is entered for each cross. In general, there is a marked selection against the temperature sensitive marker.

DISCUSSION

OKUBO, STRAUSS and STODOLSKY (1964) first reported the disturbance of linkage in the transfection of morphological markers of bacteriophage SPO-1. Their results clearly show a marked expansion of the genetic map of SPO-1 of between

three and five fold when transfection was compared to phage recombination.

Studies on the fate of infective phage DNA carried out by marker rescue techniques (GREEN 1964, 1966) suggest that partial inactivation of the infective DNA molecule takes place within the bacterial cell. Such partially inactivated molecules contribute genetically to superinfecting bacteriophage. The probability of simultaneous rescue of a pair of linked markers by a superinfecting phage is inversely related to both the time of residence of the molecule within the bacterium and the map distance between the marker pair. These observations together with the observations that infection of a competent cell with SP82G phage prior to the addition of infective DNA can "turn off" the inactivation process, and that foreign and UV-irradiated DNA can interfere with the intracellular inactivation (EPSTEIN and MAHLER 1968) support an hypothesis for the existence of an intracellular factor which randomly places damages on the infective DNA molecule and which can be controlled by the infecting phage particle. The present study suggests that the operation of this intracellular inactivation typically results in the isolation of the "rescuable" genes on *very* small regions of the phage genome from which they can only be rescued as independent units. Since the presently known genetic map comprises about fifty map units and the smallest interval examined and failing to maintain linkage was 1.3 map units in this study, the maximum size of the marker-bearing region which interacts in the restitution of the transfecting DNA molecules must be less than one-fortieth of the phage map. The question whether actual physical fregmentation of the phage DNA has taken place during the inactivation process or whether the fragmentation takes place in the rescue process that follows is not approached in this study. However, in other studies (GREEN, unpublished) involving recombination in the presence and absence of intracellular inactivation we conclude that recombination is not detectably affected by the intracellular inactivation process. These studies suggest that the damage introduced by the intracellular inactivation does not increase recombination, but that a relatively normal process of recombination is instrumental in the reconstruction of an undamaged (and now resistant) phage genome within the DNA infected cell.

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SUMMARY

The phage progeny resulting from mixed infection of competent *B. subtilis* cells by pairs of genetically distinct SP82G phage DNA molecules have been examined for the frequency of the genotype classes present. Although phage crosses show these marker pairs to have linkages ranging from 1.3 to 39.5 map units, random assortment of markers was found to be generally the case in the progeny derived from mixedly infecting DNA molecules. The infective center established by transfection is the result of the genetic reconstitution of independent portions of the infecting phage DNA that comprise less than 1/40 of the genetic map of the phage.

LITERATURE CITED

EPSTEIN, H. T., and I. MAHLER, 1968 Mechanisms of enhancement of SP82 transfection. J. Virology **2**: 710–715.

FÖLDES, J., and J. MOLNAR, 1964 Isolation and characterization of some *B. subtilis* phages with "transforming" activity. Acta Microbiol. Acad. Sci. Hungary **11**: 165–171.

FÖLDES, J., and T. A. TRAUTNER, 1964 Infectious DNA from a newly isolated *B. subtilis* phage. Z. Vererb. **95**: 57–65.

GREEN, D. M., 1964 Infectivity of DNA isolated from *Bacillus subtilis* bacteriophage, SP82. J. Mol. Biol. **10**: 438–451. —— 1966 Intracellular inactivation of infective SP82 bacteriophage DNA. J. Mol. Biol. **22**: 1–13.

KAHAN, E., 1966 A genetic study of temperature-sensitive mutants of the *subtilis* phage SP82. Virology **30**: 650–660.

MANDELL, J. D., and A. D. HERSHEY, 1960 A fractionating column for analysis of nucleic acids. Anal. Biochem. **1**: 66–77 .

NESTER, E. W., M. SCHAFER, and J. LEDERBERG, 1963 Gene linkage in DNA transfer: A cluster of genes concerned with aromatic biosynthesis in *Bacillus subtilis*. Genetics **48**: 529–551.

OKUBO, S., and W. R. ROMIG, 1965 Comparison of ultraviolet sensitivity of *Bacillus subtilis* bacteriophage SPO2 and its infectious DNA. J. Mol. Biol. **14**: 130–142.

OKUBO, S., B. STRAUSS, and M. STODOLSKY, 1964 The possible role of recombination in the infection of competent *Bacillus subtilis* by bacteriophage deoxyribonucleic acid. Virology **24**: 552–562.

REILLY, B. E., and J. SPIZIZEN, 1965 Bacteriophage deoxyribonucleate infection of competent *Bacillus subtilis*. J. Bacteriol. **89**: 782–790.

ROMIG, W. R., 1962 Infection of *Bacillus subtilis* with phenol-extracted bacteriophages. Virology **16**: 452–459.

YOSHIKAWA, H., 1966 Mutations resulting from the transformation of *Bacillus subtilis*. Genetics **54**: 1201–1214.