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A recombination test to classify mutants of *Bacillus subtilis* of identical phenotype

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SUMMARY

We have developed a recombination test in *Bacillus subtilis* that provides a tool for rapid genetic classification of mutants of identical phenotype. The test has been used to classify 25 ts mutants in nine recombination classes that have been proved by independent evidence to correspond to nine genetic loci.

1. INTRODUCTION

The genome of *Bacillus subtilis*, like that of other bacterial species used in microbial genetics, e.g. *Escherichia coli* and *Salmonella typhimurium*, is a single circular DNA molecule consisting of several thousand genes (3000–6000). Hong & Ames (1971) have estimated that in *Salmonella typhimurium*, of the postulated 4500 genes present, only 1000 are *remediable essential* – that is, genes whose function can be substituted by the presence in the medium of suitable growth factors; of the others, 1500 are *non-remediable essential* genes and 2000 are *silent* (that is, coding for functions that are not expressed or not essential in normal growth conditions). Mutants defective in non-remediable essential functions, such as macromolecular syntheses, cell division and differentiation, are by definition *lethal*.

Horowitz & Leupold (1951) and Epstein *et al.* (1963) have introduced in microbial genetics the use of temperature-sensitive (ts) conditional lethal mutants able to grow at a permissive temperature and unable to yield progeny at another temperature, defined as *non-permissive*. The ts phenotype is due to a single aminoacid substitution which restricts the thermal stability of the mutant protein to a narrower temperature range compared to the wild-type protein.

By definition, all ts mutants have the same phenotype: their isolation (by replica-plating) is easy, but a preliminary classification prior to the finer physiological characterization is rather laborious. The problem is to identify the identical mutations and those affecting the same function.

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In *B. subtilis* the most reliable method for such screening is the recombination index method (Lacks & Hotchkiss, 1960; Ephrati-Elizur, Srinivason & Zamenhof, 1961) that makes it possible to recognize those mutations that map close enough to limit the frequency of double recombinants in reciprocal crosses. This is achieved by evaluating the recombination frequency between two markers of identical phenotype and comparing it with the recombination frequency for outside reference markers.

The present work describes a recombination test in *B. subtilis* which is a simplified, qualitative version of the recombination index method. It is a plate assay of genetic transformation which attributes markers of identical phenotype to *recombination classes*. We also report the description (both genetic and physiological) of the classes of mutants so identified.

Table 1. *List of bacterial strains*

Strain	Original denomination	Genotype	Source
PB 19	SB 19	Prototroph	J. Lederberg
PB 566/2	.	*thyAB arg*	M. Polsinelli
PB 1666	.	*pheA12 leu-8 ilv A64*	PB 3376 plus DNA PB 3351
PB 1676	BD 70	*metC3 trpC2*	J. A. Lepésant
PB 1681	GSY 292	*trpC2 gltA292*	J. A. Hoch
PB 1682	SB 120	*aroD120 trpC168*	E. W. Nester
PB 1683	60935	*dal metC trpC2*	E. Freese
PB 1698	WB 932	*aroG932*	E. W. Nester
PB 1711	WB 906	*aroI906*	J. A. Hoch
PB 1721	CU 479	*trpC2 ctrA1*	S. A. Zahler
PB 3046	.	*furA*	M. Polsinelli
PB 3350	C14	*cysA14*	I. Takahashi
PB 3351	H12	*pheA12*	I. Takahashi
PB 3357	Mu8u5u16	*purA16 leu-8 metB5*	N. Sueoka
PB 3361	HLL 39	*purB6 leu-8 metB5 trpC2 hisA1 lys-21 thr-5 ery-101*	P. Schaeffer
PB 3372	BC 26	*phe A12 argA3 ery*	J. Copeland
PB 3373	BC 34	*pyrA26*	J. Copeland
PB 3374	BC 39	*trpC2 leu-2 argC4*	J. Copeland

2. MATERIALS AND METHODS

(*a*) *Bacterial and phage strains*. The strains of *Bacillus subtilis* used in this study are listed in Table 1. Phage PBS-1 (Takahashi, 1963), a general transducer, was used in all transduction crosses.

(*b*) *Culture media*. Media *NB* (Bacto-Nutrient Broth, Difco), *PY* (Bacto-Antibiotic Medium N. 3, Difco), *MM* (Minimal Medium, Davis & Mingioli, 1950), *TM* (Transformation Medium, Spizizen, 1958), *TBAB* (Tryptose Blood Agar Base, Difco) and *Y* (Yamagishi & Takahashi, 1968) were used as indicated, supplemented where necessary with 0·2% Casamino Acids (Difco), 20 μg/ml aminoacids, or 20 μg/ml nucleosides. The same media were solidified with 2% Bacto-Agar (Difco).

(*c*) *Nitrosoguanidine* (*NTG*) *mutagenesis*. The procedure of Adelberg, Mandel &

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Chein Ching Chen (1965) was followed with minor modifications. An exponential culture of PB 566/2 in NB medium plus thymine (20 μg/ml) was harvested by centrifugation, resuspended in Maleate Buffer (pH 8·0) and treated with 100 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chem. Co., USA) for 30 min at 30 °C with agitation. The cells were washed, resuspended in NB medium, grown for 4 h at 35 °C with agitation and frozen in liquid nitrogen in 10% glycerol. Aliquots were plated on NB agar and ts mutants were isolated by replica-plating (Lederberg & Lederberg, 1952) on NB agar plates at 35 and 46 °C.

(*d*) *DNA transformation in liquid medium.* Competent cell cultures were obtained as in Spizizen (1958). Transforming DNA was extracted as described by Marmur (1961). The DNA concentration was determined by the diphenylamine method (Dische, 1955). Standard transformation assays were performed at a DNA concentration of 1 μg/ml. Recombination indices were calculated as described in Ephrati-Elizur *et al.* (1961).

(*e*) *Macromolecular syntheses.* DNA and protein synthesis were evaluated by the cumulative incorporation into TCA-insoluble material of [^{3}H]thymidine (324 mCi/mmole) and of [^{14}C]arginine (1 Ci/mmole) present in NB medium at the concentrations of 0·4 and 0·1 μCi/ml, respectively. Precursor incorporation was followed at 35 °C and for 90 min after a shift to 46 °C. The radioactive compounds were purchased from the Radiochemical Centre, Amersham, U.K.

(*f*) *Transduction mapping.* PBS-1 lysates of ts mutant strains were used in transduction crosses with strains (Table 1) carrying suitable biochemical markers, following the procedure of Hoch, Barat & Anagnostopoulos (1967). The map position of the relevant markers used in these experiments is shown in Fig. 2. After selection on the appropriate medium (MM plus supplements), recombinants were picked and tested for their ts phenotype in patches on the same selective medium or on PY medium after single colony isolation.

Mapping results are expressed as percentage of recombination according to the convention of Barat, Anagnostopoulos & Schneider (1965): map distance = 100 − % co-transfer.

3. RESULTS

(i) *Isolation of ts mutants*

Seventy heat-sensitive (ts at 46 °C) mutants have been isolated from strain PB 566/2 mutagenized by NTG. Forty ts mutants that had retained the biochemical markers of the parental strain (*Arg, Thy*) and that reverted to ts$^+$ at a frequency of at least 10^{-7} were used for further studies.

(ii) *The recombination test*

The recombination test consists of a DNA-mediated transformation assay carried out directly on agar plates. The competence state of recipient cells is obtained by growth on PY agar plates. The period of incubation at 35 °C that gives maximal competence has been experimentally established to be 15–18 h.

For each recipient strain at least two streaks on PY plates are replicated on to

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selective plates on which 30–50 μg of donor DNA are spread. NB agar plates were used to select for ts+ recombinants. The replicas were incubated at 35 °C for 3–4 h to allow for the phenotypic expression of the ts+ markers, and then shifted to 46 °C for 20 h. Selection for Thy+ recombinants is obtained by replica on thymineless plates (spread with DNA) and incubation at 35 °C. False-negative results might depend upon the state of integrity of the DNA or the degree of competence of the

Table 2. *The five possible responses of a cross; DNA (Thy+ ts) × cell (thyAB ts)*

Possible response	Cross (+DNA)*		Control (−DNA)		Conclusions†		
	Thy+	ts+	Thy+	ts+	C	HR	RT
1	+	+	−	−	+	−	1
2	+	+	−	+	+	+	*ns*
3	+	−	−	−	+	−	0
4	−	−	−	−	−	−	*ns*
5	−	+	−	+	−	+	*ns*

* Thy+ (ts+) = selection for Thy+ (ts+) recombinants or revertants; +(−) = presence (absence) of recombinants or revertants.

† C = (+ or −) competence; HR = (+ or −) high reversion rate to ts+ phenotypes; RT (recombinants test): 1 = the two mutations belong to different *recombination classes;* 0 = the two mutations belong to the same *recombination class; ns* = result not significant.

Table 3. *Results of the recombination test*

Recipient strains	Donor DNA (from Thy+ derivatives)									
	E1	E12	E14	E19	E23	E40	E48	E49	E50	E69
E1	0	1	.	1	0	1	1	1	1	1
E2	0	1	.	1	0	1	1	1	1	1
E3	1	1	.	1	1	1	1	0	1	1
E11	.	0	1	1	1	1	1	1	1	1
E12	.	0	1	1	1	1	1	.	.	.
E13	.	0	1	1	1	1	1	.	.	.
E14	.	1	0	1	1	1	1	1	1	1
E19	.	1	1	0	1	1	1	1	1	1
E23	.	1	1	1	0	1	1	1	1	1
E40	.	1	.	1	1	0	1	.	1	1
E44	.	0	1	1	1	1	1	1	1	1
E45	.	0	1	1	1	1	1	1	1	.
E47	.	1	1	1	1	1	1	1	1	1
E48	.	1	1	1	1	1	0	1	1	.
E49	1	1	.	1	1	1	1	1	0	.
E50	1	1	.	1	1	1	1	1	0	0
E57	.	1	1	1	1	1	1	1	0	0
E58	.	1	1	1	1	1	1	1	0	0
E59	1	1	1	1	1	1	1	1	0	0
E63	.	0	1	1	1	1	1	1	1	.
E64	.	0	1	1	1	1	1	1	1	.
E66	.	0	1	1	1	1	1	1	1	.
E67	.	0	1	1	1	1	1	1	.	.
E69	.	1	1	1	1	1	1	1	0	0
E70	0	1	.	1	0	1	1	1	1	1

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recipient, and false-positive results might depend upon high reversion rates to ts+ phenotypes; thus every cross must have suitable controls. With this aim we constructed by transformation the Thy+ derivates of some of the original mutants. The Thy+ ts strains have been used as the source of the donor DNAs for the recombination test, allowing us to use the same DNA preparation to obtain both Thy+ and ts+ recombinants. Table 2 displays the five possible results obtainable in a typical cross and their most likely interpretation.

Table 4. *Rearrangement of the data in Table 3 in a two-entry matrix*

	1	2	23	70	3	49	11	12	13	44	45	63	64	66	67	14	19	40	47	48	50	57	58	59	69
1	0																								
2	0																								
23	0	0	0																						
70	0		0																						
3	1		1																						
49	1	1	1	1	0	0																			
11			1			1																			
12	1	1	1	1	1	1	0	0																	
13			1					0																	
44			1			1		0																	
45			1			1		0																	
63			1			1		0																	
64			1			1		0																	
66			1			1		0																	
67			1			1		0																	
14			1			1	1	1	1	1	1	1	1	1	1	0									
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0								
40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0							
47			1			1		1								1	1	1	0						
48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0					
50	1	1	1	1	1	1	1	1		1	1	1	1	1		1	1	1	1	1	0				
57			1			1		1								1	1	1		1	0				
58			1			1		1								1	1	1		1	0				
59	1		1			1		1								1	1	1		1	0				
69	1	1	1	1	1	1	1	1								1	1	1	1	1	0	0	0	0	0

(iii) *Classification of ts mutants in recombination classes*

Preliminary experiments showed that seven strains had high ts+ reversion rates and eight strains did not become competent for DNA transformation. These strains were not used in recombination tests (they are listed in Table 8 in groups 10–14). Table 3 is a summary of the data obtained by crossing the remaining 25 ts

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strains. It can be seen that only ten strains have been used as donors and that not all the possible crosses have been carried out. The data are nevertheless sufficient to classify the mutants into nine *recombination classes*. This can be easily visualized by redistributing the data in a two-entry matrix (Benzer, 1957) where all the mutants that give a '0' result (see Table 2) in reciprocal crosses are grouped together. Such a recombination matrix is shown in Table 4 where the 25 strains are represented on both axes irrespectively of the role performed in the crosses.

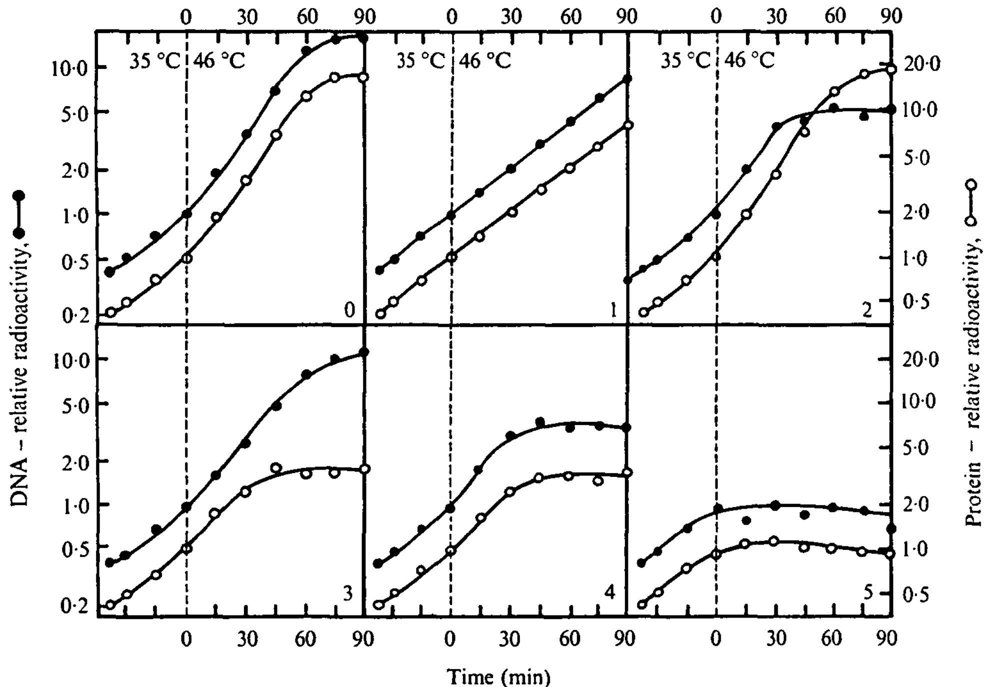

Fig. 1. Protein and DNA synthesis monitored by radioactive precursor cumulative incorporation (see Methods for details). The six graphs show the behaviour of the parental strain PB 566/2 and of five ts mutants, one for each physiological class listed in Table 6. To allow comparison, the incorporation data ([^{3}H]thymidine or [^{14}C]-arginine cpm incorporated/ml) have been normalized by the value (between 1 and 2×10^3) attained at the time of the shift to the higher temperature; 0 = PB 566/2, ts$^+$ control; 1 = E14; 2 = E39; 3 = E40; 4 = E1; 5 = E9; the numbers correspond to those assigned to the physiological classes, as listed in Table 6.

(iv) *Physiological characterization of ts mutants*

To obtain a preliminary physiological characterization of the 40 ts mutants, protein and DNA synthesis at non-permissive temperature have been evaluated. Protein and DNA synthesis have been followed by radioactive precursor cumulative incorporation for 90 min after shifting cultures growing exponentially in NB medium at 35–46 °C; the data (representative curves are shown in Fig. 1) have been compared with those obtained with ts$^+$ controls.

Eighteen ts mutants are defective in one or both the macromolecular syntheses,

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some mutants stop the incorporation of the radioactive precursor at once after the temperature shift, others show a delayed-stop phenotype. Overall, five kinds of response have been found and are listed in Table 5. As can be seen in Table 8 the

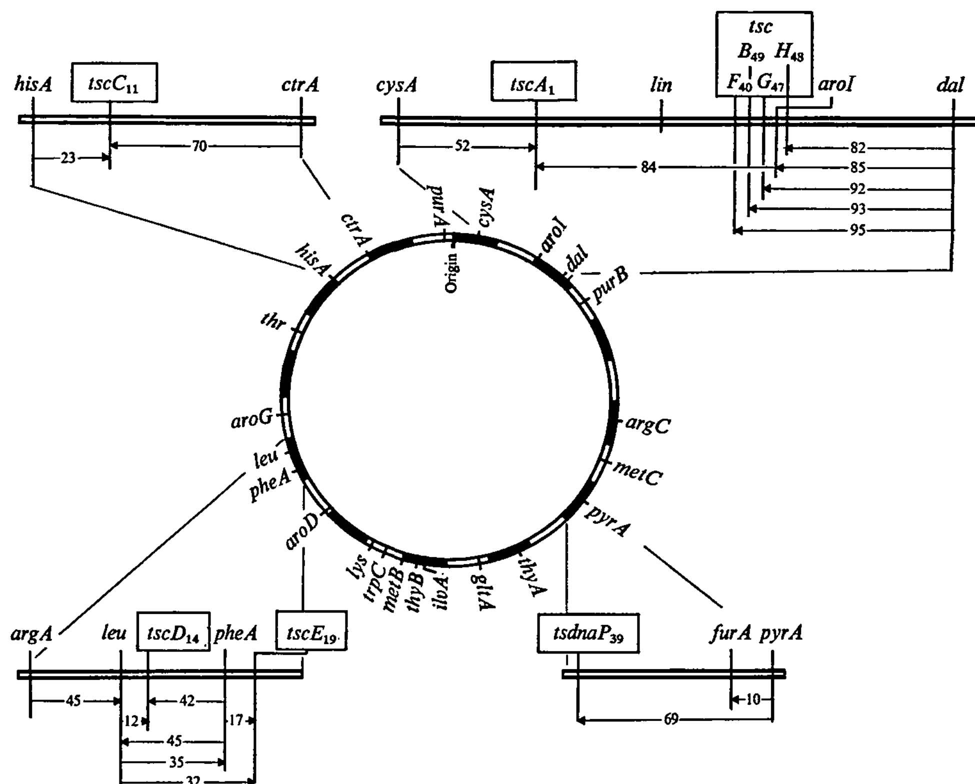

Fig. 2. Genetic map of *Bacillus subtilis* 168 (drawn according to Lepésant-Kejzlarova *et al.* (1975) and subdivided in 24 segments of about 90 PBS-1 map units each). All the markers on the map have been tested for cotransduction with the ts markers. The order of markers *tscB*, *F*, *G* and *H* relative to *aroI* is only tentative. Mapping of *ts-dnaP* is from Riva *et al.* (1975).

Table 5. *Physiological classification of ts mutants*

Physiological class	DNA	Protein	Strains	No. of strains
1	N*	N	E4, E11, E12, E13, E14, E18, E19, E27, E44, E45, E47, E48, E50, E57, E58, E59, E63, E64, E65, E66, E67, E69	22
2	DS	N	E37, E39, E43	3
3	N	DS	E34, E40, E42	3
4	DS	DS	E1, E2, E3, E23, E33, E41, E49, E70	8
5	IS	IS	E8, E9, E32, E35	4

* N = normal synthesis at 46 °C (same as *wt* control); DS = delayed stop; IS = immediate stop.

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classifications derived from the recombination test and from the physiological characterization show no discrepancy: all mutants assigned to a recombination class also belong to the same physiological class.

(v) *Genetic mapping of the ts mutations*

The ts markers of mutants assigned to various recombination classes by the recombination test have been mapped by PBS-1 transduction.

In a preliminary screening, lysates from one ts strain of each recombination class of mutants were used to transduce different recipient strains carrying auxotrophic markers: 50–100 recombinants from each cross were scored for their ts phenotype. The strains used for transduction crosses are listed in Table 1 and the relevant markers indicated on the circular map in Fig. 2 (their location is such as to 'cover' most of the *B. subtilis* chromosome). Once the linkage of a ts marker with a biochemical marker of known location is established, the map order is determined by checking the linkage with other proximal biochemical markers. Table 6 shows the relevant crosses performed and the actual number of recombinants tested for each cross.

Table 6. *Linkage of ts markers determined from two-factor transduction crosses*

Recombination class	Donor strain	Selected markers in recipient strains										
		purA	*cysA*	*aroI*	*dal*	*purB*	*metC*	*pheA*	*leu-8*	*thr-5*	*hisA*	*ctrA*
tscA	E1	0/72	28/61	8/50	.	.	.	.	0/60	.	.	.
	E23	.	35/60	7/50	.	.	.	.	.	.	.	.
	E70	.	56/100	17/100	.	.	.	.	.	.	.	.
tscB	E3	.	.	67/68	5/80	0/95	0/80	.	.	.	.	.
	E49	.	.	51/52	16/242	0/80	0/75	.	.	0/80	0/80	.
tscF	E40	0/70	0/65	56/58	10/189	0/58	0/80	.	0/65	0/65	0/65	.
tscG	E47	0/65	0/65	51/53	15/193	0/65	0/65	.	0/60	0/60	0/60	.
tscH	E48	0/70	0/70	99/100	18/100	0/70	0/70	.	.	0/70	0/70	.
tscC	E11	0/60	0/65	0/70	0/70	0/100	0/70	0/100	0/100	0/100	116/151	32/106
tscD	E14	0/70	0/70	.	0/70	0/95	.	30/52	45/51	0/100	0/100	.
tscF	E19	0/100	0/80	.	0/65	0/100	.	54/65	48/72	.	0/100	.

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For recombination classes containing more than one mutant, several or all members of the class were tested and in all cases their ts markers were located in the same map position. This represents a further check on the validity of the recombination test.

The genetic loci corresponding to the recombination classes A to G have been named *tscA* to *tscG* (*t*hermo*s*ensitive re*c*ombination class). The *tscD* and *tscE* markers showed linkage with both *leu* and *pheA* but the calculated map distances were not additive (as sometimes occurs in PBS-1 transduction mapping). Their location relative to *pheA* and *leu* was then established by selecting for Phe^+ Leu^+ recombinants and scoring for ts and ts^+ clones; in one case (*tscD*) no such double recombinants were ts^+, indicating that the *tscD* locus should be located between the selected markers. In the other case, 8 out of 42 Phe^+ Leu^+ recombinants were ts^+, indicating that the *tscE* locus is probably located outside the *leu-pheA* region. The order *leu-pheA-tscE* was then established by three-point crosses (Table 7).

Loci *tscA*, *B*, *F*, *G* and *H* are scattered in the *cysA-dal* region and are all linked to *aroI* as shown in Fig. 2.

Table 7. *Three-point crosses to determine the order of the markers* leu-8, pheA *and* tscE (*donor strain E19*)

Recipient strain	Recombinants: Selection	Recombinants: Classes	Recombinants: Number
PB 1666	Leu^+	Leu^+ Phe^+ ts	47
		Leu^+ Phe^+ ts^+	8
		Leu^+ Phe^- ts	1
		Leu^+ Phe^- ts^+	16
		Total examined	72
	Phe^+	Phe^+ Leu^+ ts	40
		Phe^+ Leu^+ ts^+	8
		Phe^+ Leu^- ts	14
		Phe^+ Leu^- ts^+	3
		Total examined	65

Of these the only two classes the mutants of which belong to the same physiological class are *tscG* and *tscH* (see Tables 5, 8). They could then be the same locus since both are closely linked to *aroI*. However, their respective distances from *dal* suggest that they are on opposite sides of the *aroI* marker. The locus *tscC* was mapped between *hisA* and *ctrA*.

The mutations of the five strains belonging to the recombination class *K* were not mapped; the mutations allow a considerable amount of residual growth at 46 °C and the ts phenotype is visible only on single-colony isolates (such as in the recombination test) and not on the patch test used to check the ts character of transductants.

Among the ts strains unclassifiable by the recombination test, mutant E39 has been described by Riva *et al.* (1975), named *dnaP* and mapped linked to *pyrA*.

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(vi) *Comparison of the recombination test with quantitative data*

The two markers *tscB49* and *tscG47* map very close to each other (see Table 6 and Fig. 2) and belong to two different physiological classes (Table 5): they provide a good comparison of the qualitative results of the recombination test with the quantitative results obtained by the recombination index method. The results are shown in Table 9 and indicate that the recombination test is very sensitive since it is still positive ('1') for a recombination index of the order of 0·02. The other

Table 8. *Summary of the characterization of ts mutants*

Group	Recombination class*	Physiological class†	Genetic locus‡	Competence	Mutants	No.
1	A	4	*tsc* A	+	E1, E2, E23, E70	4
2	B	4	*tsc* B	+	E3, E49	2
3	C	1	*tsc* C	+	E11, E12, E13, E44, E45, E63, E64, E66, E67	9
4	D	1	*tsc* D	+	E14	1
5	E	1	*tsc* E	+	E19	1
6	F	3	*tsc* F	+	E40	1
7	G	1	*tsc* G	+	E47	1
8	H	1	*tsc* H	+	E48	1
9	K	1	*nd*	+	E50, E57, E58, E59, E69	5
10	*nd*§	2	*dna* P	−	E39	1
11	*nd*	2	*nd*	−	E37, E43	2
12	*nd*	1	*nd*	−	E4, E27	2
13	*nd*	5	*nd*	−	E8, E9, E35	3
14	*nd*	*nc*§	*nd*	*nc*	E18, E32, E33, E34, E41, E42, E65	7
					Total examined	40

* See Table 4.
† See Table 5.
‡ See Table 6 and Fig. 1.
§ *nd* = not determined; *nc* = not characteristic. This group contains all strains that show a very high ts+ reversion rate.

Table 9. *Comparison of Recombination Index (RI) and Recombination Test (RT) results*

Recipient strain*	Donor DNA†	Recombinants/ml × 10^{-4} ts+	thy+	Recombination index‡	Recombination test§
*tsc C*13	wt	5·0	1·8	0·0004	0
	*tsc C*12	5×10^{-3}	4·0		
*tsc A*23	wt	5·0	2·5	1·25	1
	*tsc E*19	6·0	2·4		
*tsc B*49	wt	4·7	3·6	0·0215	1
	*tsc G*47	$2·8 \times 10^{-2}$	1·0		

* *thyAB*.
† Thy+.
‡ Calculated as in Ephrati-Elizur *et al.* (1961).
§ As defined in Table 2.

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results reported in the table for comparison refer to the more obvious cases of *tsc* markers located in the same *tsc* recombination classes or in *tsc* loci mapped far apart from each other.

4. DISCUSSION

We have developed a simple technique that permits a rapid genetic classification of ts mutants of *Bacillus subtilis* by means of a *recombination test* consisting of a transformation assay on agar plates spread with DNA on to which competent cells obtained on Penassay Agar are placed by replica-plating.

We have isolated 70 ts mutants after nitrosoguanidine mutagenesis; 40 of such mutants were due to revertible, possibly single-point ts mutations and 25 of these were suitable for the recombination test.

The results of the test have been compared with transformation frequencies obtained by standard procedures. No discrepancy has been observed between the two types of results.

A preliminary physiological characterization of the mutants at 46 °C revealed five physiological classes with respect to protein and DNA syntheses. As is shown in Table 8, all mutants attributed to a recombination class by the recombination test also belong to the same physiological class.

Transduction mapping of ts mutations belonging to recombination classes *A* to *H* has defined the loci *tscA* to *tscH*.

All this independent evidence shows the recombination test to be a simple, rapid and efficient technique for preliminary genetic classification of mutants of identical phenotype in *Bacillus subtilis*.

A comparison of the recombination test all or none (1/0) results with the recombination indices has been made and has confirmed the sensitivity of the test which is still positive for recombination indices as low as 0·02. This means that mutations fail to be included in the same recombination class even if they are so closely linked as to reduce the recombination index to 2%. Therefore mutations assigned by the test to the same recombination class are very closely linked and the test has proved to be a powerful tool for fine genetic mapping that can be used in addition to the recombination index method and not just a simplified qualitative version of it.

The distribution of the ts mutations in nine loci is very unequal, indicating that genes coding for different proteins mutate to thermosensitivity at very different rates. Analogous conclusions were reached by Karamata & Gross (1970), who found an unequal distribution of 55 ts mutants altered in DNA synthesis in 9 *dna* loci. They also found that *dna* mutants represent less than 10% of all ts mutants (55/600) which corresponds roughly to our finding (3/40).

Although the number of ts mutants examined in this work is not very high, it is remarkable that all the loci described, far from being scattered all around the chromosome, are grouped in four rather limited chromosome sections. Asato & Folsome (1967) and Nukushina & Ikeda (1969) had shown that genes located all around the genetic map (of *E. coli* and *B. subtilis*, respectively) can mutate giving rise to ts mutants. The two different findings are compatible considering that they

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were examining all kinds of ts mutations, while we have selected for mutations in non-remediable essential genes whose distribution on the genetic map might not be ubiquitous. The existence on the map of 'hot spots' of ts mutability suggests the existence of clusters of numerous non-remediable genes in limited chromosomal sectors.

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