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THE GENETICS OF BACTERIOPHAGE SPO1

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SUMMARY Suppressor-sensitive mutants of *Bacillus subtilis* phage SPO1 were isolated. These mutants were classified into at least thirty-six cistrons by complementation tests. Among them, thirty-four cistrons were arranged in a linear chromosome map by two-factor crosses. Results of three-factor crosses confirmed the order of the cistrons.

Two cistrons gave maximal or near maximal recombination frequencies when crossed with any of the other cistrons tested. Therefore, these cistrons could not be located on the main map of the SPO1 chromosome.

The abilities of mutants from each cistron to synthesize DNA and to induce lysozyme activity in the restrictive host were determined. It appears that related functions tend to cluster together along the genetic map in SPO1 as in other phages.

INTRODUCTION

Many phages active on the transformable strain of *Bacillus subtilis* have been isolated and characterized by physical, chemical, and electron microscopic studies (Thomas and MacHattie, 1967; Szybalski, 1968). SPO1 is one of a number of independently isolated *B. subtilis* phages containing 5-hydroxymethyluracil (HMU) in place of thymine in their DNA (Okubo et al., 1964). DNA extracted from them with phenol can infect competent cells of *B. subtilis* (Spizizen et al., 1966). Genetic studies on these phages would facilitate experiments on the molecular events occurring during DNA replication and recombination and the mechanisms controlling viral growth. One example of such experiments is the characterization of a transcription program involving six classes of SPO1 messenger RNA (Wilson and Geiduschek, 1969; Gage and Geiduschek, 1971 a, b; Fujita et al., 1971).

We have isolated a suppressor strain of *B. subtilis*, which made it possible to isolate sup-

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pressor-sensitive phage mutants (Okubo and Yanagida, 1968). This paper reports the isolation of suppressor-sensitive mutants of SPO1 and mapping experiments with them. Kahan (1966), and more recently Green and Laman (1972) isolated temperature-sensitive mutants of SP82 (or SP82G), another phage containing HMU, and mapped them into a single, linear chromosome. In our experiments, most mutants of SPO1 could be arranged on a single non-circular chromosome map. However, two cistrons could not be located on the map, as these cistrons gave high recombination frequencies when crossed with any of the other cistrons.

MATERIALS AND METHODS

1. *Media*

Nutrient broth (NB) consisted of 1% peptone, 0.5% beef extract, 0.1% yeast extract and 2% NaCl (adjusted to pH 7.2 with NaOH). NB was also supplemented with 5×10^{-3} M $MgSO_4$ and 5×10^{-5} M $MnCl_2$. NB was used for all routine operations in Osaka (plating, crosses, complementation, and preparation of phage lysates). When growth in a defined medium was desired, the following medium (referred to as medium B) was used (Fujita, 1971): 1 mg of $MgSO_4$, 0.1 mg of $MnSO_4\cdot7H_2O$, 50 mg of L-tryptophan, 56 mg of $CaCl_2$, 5 g of glucose, 500 mg of NH_4Cl, 100 mg of NH_4NO_3, 100 mg of Na_2SO_4, 100 ml of 1 M Tris-HCl (pH 7.4), 5 g peptone and deionized water to 1 liter. A minimal medium was prepared as described by Spizizen (1958). It contained 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4\cdot7H_2O$, 0.1% sodium citrate and 0.5% glucose.

In Chicago, HML and L media were used for routine operations involving *su*$^-$ and *su*$^+$ bacterial strains, respectively (Fujita, 1971): HML consisted, per liter, of 5 g of glucose, 50 mg of L-histidine, 50 mg of L-methionine, 50 mg of L-leucine, 5 ml of nutrient broth (NBS), 56 mg of $CaCl_2$ and Spizizen's minimal medium salts. Medium L is HML medium without histidine and methionine. NBS is nutrient broth (DIFCO) supplemented with 5 g of NaCl, 96 mg of $MgSO_4$ and 11 mg of $CaCl_2$ per liter. Medium B was used to prepare lysates for electron microscopic studies.

2. *Bacteria and bacteriophage*

The properties of the phage SPO1 have been previously described (Okubo et al., 1964). The non-suppressor (*su*$^-$) strain of *B. subtilis* used in this work was HA101 (*leu*$^-$, *his*$^-$, *met*$^-$), a derivative of Marburg strain 168. A suppressor strain (*su*$^+$) derived from HA101, called HA 101-B, was used as a permissive host (Okubo and Yanagida, 1968). The genotype of HA101-B is *leu*$^-$, *met*$^-$, *his*$^-$, *su*$^+$. The *su*$^+$ gene suppresses both the *his*$^-$ and *met*$^-$ alleles, so that the phenotype of HA101-B is Leu$^-$. We noticed that the *su*$^+$ gene seems unstable. The strain has to be kept on a slant of minimal medium supplemented with leucine (20 μg/ml). In overnight cultures or when used as an indicator bacterium, HA 101-B was grown in minimal medium supplemented with leucine (20 μg/ml) and NB (0.004 ml/ml). If *su*$^+$ bacteria grown in nutrient broth are used as an indicator for *sus* mutant phages, very low plating efficiencies may be obtained. One must then make periodic reisolations of colonies of HA 101-B (*su*$^+$) from a plate of minimal medium supplemented with leucine, and test them for *su*$^+$ by their sensitivity to *sus* mutant phage or by their ability to suppress the *his*$^-$ or *met*$^-$ allele.

3. *Isolation of bacteriophage mutants*

Suppressor-sensitive (*sus*) mutants of SPO1 phage were independently isolated in Osaka and in Chicago (Fujita, 1971; Geiduschek, personal communication).

In Osaka, mutants of the *sus* O series (a capital "O" is omitted from the designation of this mutant series in this paper) were selected from phage grown in the presence of N-methyl-N'-nitro-N-nitrosoguanidine (NG). *B. subtilis* HA 101-B were grown to a cell density of about 2×10^8/ml in NB. NG was added to 50 μg/ml and wild type phages were infected at a multiplicity of infection of 3. Portions of the mixture were put into small tubes and shaken until lysis occurred. The lysates of each tube were plated on the *su*$^+$ host bacteria and incubated overnight at 37C. Individual plaques were picked out with sterile toothpicks and stabbed onto corresponding regions of two plates, one seeded with *su*$^-$ bacteria and the other with *su*$^+$ bacteria. Isolates producing a zone of lysis only on the *su*$^+$ lawn were resuspended in 1 ml of NB and purified by repeating the single plaque isolation procedure. The mutants were numbered sequentially in order of their isolation.

F-, N- and HA-series mutants were isolated in Chicago from populations treated with 10 to 20 μg/ml of NG (F- and N-series) (Fujita, 1971) and hydroxylamine (HA) (Freese et al., 1961, as used by Kahn, 1966), respectively. Suitable precautions were taken to minimize repeated isolation of the same mutant. For example, no more than three mutants of the HA- and N- series were ever isolated from one mutagenized stock.

4. *Preparation of mutant phage suspension*

To minimize wild type revertants in mutant phage preparations, we made the latter from either a single or only a few plaques. These plaques were suspended in 1 ml of NB and plated with 0.5 ml of su^+ bacteria by the soft agar method. The su^+ bacteria used were grown in minimal medium supplemented with leucine and NB. The plates were incubated at 37C overnight. Phage suspensions were made from confluent lysed plates. Phage titers were assayed on su^+ and su^- bacteria. Phage suspensions having su^-/su^+ plating efficiencies of 3×10^{-5} or lower were used in these experiments.

In Chicago, mutant phage stocks were prepared in medium L by infecting a log phase culture of su^+ with plate lysates grown from a single resuspended plaque. Cell debris was removed after lysis by centrifugation for 10 min at 8,000 rev/min (Sorvall, SS-34 rotor). Phage pellets were obtained by centrifuging the supernatant for 90 min at 17,000 rev/min (SS-34 rotor), and were allowed to stand overnight in 0.1 M Tris-HCl buffer (pH 7.2) containing $MgSO_4$ (600 mg/l) before resuspension.

5. *Complementation test*

About 10^6 plaque forming units (pfu) of a mutant were seeded with su^- bacteria by the soft agar method. A small drop of eacb mutant suspension (about 10^7 pfu/ml) to be tested was placed on the plates after the soft agar had hardened. Control plates were made using only the su^- bacteria in the lawn. Tests were repeated by interchanging the seeded and spotted phages. Mutants were scored as positive for complementation when confluent lysis of the bacterial lawn was observed in the spotted area. On the other hand, when the bacterial lawn developed normally in the spotted area, mutants were scored as negative. Sometimes, a few individual plaques were observed in a spot. These were scored as weakly positive for complementation, if the control spots showed no plaques due to revertants.

In Chicago, some complementation tests were performed on agar plates by mixing 5 μl spots containing 10^4 to 10^5 plaque forming units of each *sus* mutant on a plate seeded with su^- bacteria (Fujita, 1971).

Complementation was also measured by assaying the average burst size of su^- bacteria after mixed infection (liquid complementation) (Edgar et al., 1964). First the number of colony formers (CF) in a culture of su^- bacteria was counted, and then the culture was infected with two of the mutants to be examined, each at a multiplicity of infection of 5. After adsorption, anti-SPO1 serum was added to the infected culture to inactivate unadsorbed phages. Then, the culture was diluted 10^{-4} times with NB. The concentration of infective centers (IC) was counted by plating an appropriate dilution on su^+ indicator. The diluted culture was incubated for 2 hr at 37 C. Lysozyme (10 μg/ml) was added to complete lysis. The lysate was titrated on su^+ bacteria. The burst sizes were calculated as the titers of the lysate on su^+/CF or IC.

6. *Recombination*

Pair-wise *sus* × *sus* recombinations were performed in Osaka. HA 101-B (su^+) was grown in minimal medium supplemented with leucine and NB. One ml of an overnight culture was added to 5 ml of NB and shaken for 2 to 3 hr to obtain a cell density of 4×10^8/ml. To 0.5 ml of NB containing 1×10^9 phage particles of each of two *sus* mutants was added 0.5 ml of the su^+ culture. KCN (0.001 M) was added and the mixture was gently shaken for 10 min. Unadsorbed phages were inactivated by the addition of 0.1 ml of anti-SPO1 serum. The mixtures were diluted 10^{-4} fold with NB and shaken for 2 hr. The lysate was titrated on su^+ and su^- bacteria. The recombination frequencies were given as titer on su^- / titer on su^+ ×200. The factor 2 is used because recombinants that are double mutants are not detected by this assay but are expected to occur with the same frequency as wild type recombinants.

7. *DNA synthesis in infected bacteria*

Incorporation of ^{3}H-deoxyuridine (UdR) into an alkali-stable, acid-insoluble fraction was used as a measure of DNA synthesis, since UdR seems to be a precursor of the hydroxymethyluracil moiety of SPO1 phage DNA.

B. subtilis her-9 (su^-, UV-sensitive mutant) was grown in B-medium. Bacteria were centrifuged and

resuspended in B-medium lacking peptone. The suspension was irradiated with a 15 watt germicidal lamp for 15 sec at a distance of 50 cm to stop cellular DNA synthesis. Peptone was added to the UV-irradiated suspension. SPOl phage was infected at a multiplicity of infection of 5 and the mixtures were shaken at 37C. At time 0, ^{3}H-UdR was added to a final concentration of 1 μc/ml. At 10 min intervals, 1 ml of the infected culture was removed and chilled in an ice-water bath. Albumin (0.1 ml of 10 mg/ml) was added as a carrier. Each sample was then mixed with 0.1 ml of 5 N perchloric acid and kept for 30 min in the cold. Precipitates obtained by centrifugation were redissolved in 1 ml of 0.1 N KOH and incubated at 37 C for 18 hr. Each sample was again treated with 0.15 ml of 5 N perchloric acid and chilled for 30 min. The precipitates were collected on membrane filters, washed with cold 0.5 N perchloric acid and counted in a liquid scintillation counter.

In Chicago (Fujita, 1971), phage DNA synthesis was determined by continuous labelling of infected *su*$^{-}$ cells with ^{14}C-8-adenine from 9 to 30 min after phage infection. At these times, there is virtually no incorporation of label into host DNA, because of the phage-induced shut off of host DNA synthesis. Samples of 1 ml of labelled cells were added to 1 ml of 1 M KOH and incubated at 37 C for at least 12 hr. Each sample was treated with 0.5 ml of 50% trichloroacetic acid containing 1 mg of unlabelled adenine per ml, chilled, and diluted further with 2 ml of 5% trichloroacetic acid. Precipitates were collected on nitrocellulose membrane filters, washed with 5% trichloroacetic acid containing 100 μg of unlabelled adenine per ml, dried, and counted in toluene based scintillation fluid in a liquid scintillation counter.

8. *Preparation of extracts of infected bacteria and assay of enzyme activity*

Extracts were prepared as described by Roscoe and Tucker (1966). *B. subtilis su*$^{-}$ was grown in 50 ml of NB to a cell density of about 2×10^{8}/ml. SPOl phage was added at a multiplicity of infection of 5. At various times (30 or 40 min) after infection the cultures were rapidly chilled by pouring them onto crushed ice. The bacteria were collected by centrifugation, washed in 0.1 M Tris-HCl buffer, pH 7.5, and resuspended in 10 ml of the same buffer. The suspension was then sonicated at 20 kc for 15 min in an ice-water bath. Unbroken cells and debris were removed by centrifugation.

(a) Lysozyme: Lysozyme activity was determined essentially by the method of Sekiguchi and Cohen (1964). Three ml of chloroform-treated *B. subtilis* cells in 0.1 M Tris-HCl buffer were mixed with 0.2 ml of extract (10 mg/ml protein content) and the decrease of turbidity at 500 mμ was measured at room temperature. Lysozyme activity induced by SPOl phage seems to require a high ionic strength. So we added 0.1 M $MgCl_2$ to the chloroform-treated cell suspension. $MgCl_2$ could be replaced by NaCl. The activity of the extract is called " lysozyme " in this paper, but at present we do not know the mode of the action of the enzyme.

The turbidity of the chloroform-treated cell suspension decreased gradually without addition of extract. So a fresh cell suspension was prepared for each assay.

(b) Deoxyuridine hydroxymethylase: The method described by Roscoe and Tucker (1966) was used. The reaction mixture contained 50 μmoles of Tris-HCl buffer, pH 7.5; 10μmole of mercaptoethanol; 1 μmole of dUMP; 1 μmole of ^{14}C-HCHO (1×10^{6} count/min/μmole); 0.2 μmole of tetrahydrofolic acid, 0.4 ml of extract and water to give a final volume of 1.0. After 45 min incubation at 37 C, 0.2 ml of 3×10^{-2} M hydroxylamine hydrochloride was added and the tube was placed in boiling water for 2 min. Then it was centrifuged and a portion of the supernatant fluid was applied to Whatman No. 1 paper, and the chromatogram was developed overnight with a solvent composed of 20 ml n-propanol per liter of filtered solution containing 600 g $(NH_4)_2SO_4$ in 0.1 M potassium phosphate buffer, pH 6.8. The position of the radioactive CH_2OH-dUMP is the same as that of dUMP and the latter was easily detected under ultraviolet light. This region of the filter paper was cut out and radioactivity was assayed in a liquid scintillation counter.

(c) Deoxycytidylate deaminase: The enzyme activity was determined by measuring the conversion of radioactive ^{14}C-dCMP to dUMP (Warner and Lewis, 1966). The reaction mixture contained 3 μmole of $MgCl_2$, 4 μmole of 2-mercaptoethanol, 20 μmole of Tris, pH 8.0, 0.4 μmole of dCMP, 0.1 μmole of dCTP, 1 μmole of NaF, 2.6×10^{3} count/min of ^{14}C-dCMP (0.0015 μmole/ml) and water to 0.2 ml. This was mixed with 0.3 ml of extract (200 μg of protein) and incubated at 37 C for 30 min. Then it was boiled for 5 min and centrifuged and the supernatant was applied to a small column of Dowex-1 (0.6×3 cm, $HCOO^{-}$ type, 200–400 mesh, $\times8$) (Sekiguchi, unpublished method). dCMP was eluted with 12 ml of 0.4 N formic acid, and then dUMP

was eluted with 7 ml of 4 N formic acid-0.2 ammonium formate. The fractions containing dCMP and dUMP were put in vials and evaporated to dryness under an infrared lamp. The residues were dissolved in 0.5 ml of water and 10 ml of scintillation fluid were added.

RESULTS

1. *Classification of mutants by complementation*

Suppressor-sensitive (*sus*) mutants of SPO1 were classified into at least 36 cistrons by complementation tests (Table 1). The F series mutants were assigned to complementation groups in Chicago (Fujita, 1971) and representatives of each group were sent to Osaka where they were complemented with O-series mutants. N- and HA-series mutants were complemented in Chicago. The cistrons are numbered in order of their location on the genetic map, as shown in Fig. 1. It should be emphasized that the assignments presented in Table 1 represent the " best fitting " complementation map.

Independence of the function of each cistron was confirmed by burst size measurements in the complementation test. Table 2 shows some of the results of the liquid complementation test for adjacent cistrons.

Mutants, *sus* 87 and *sus* F12 (cistron 1), weakly complemented the mutants listed in cistron 2, as shown in Table 3a. In liquid complementation tests, bacteria infected with mixtures of mutants in these two cistrons liberated active phages, although the burst size was 3 (Table 3b). On this basis, we decided to divide them into two cistrons, 1 and 2.

Other pairs of mutants showing weak complementation in spot tests were also examined by liquid complementation. *Su*$^-$ cells infected with these pairs of mutants did not liberate active phages at levels higher than cells infected with either parent (Table 3b). From these results and results of recombination tests we subdivided these cistrons into 2a, 2b, 2c, etc. The same problem was observed with T7 amber mutants. Studier (1969) incorporated such mutants into a single cistron. The functions of these cistrons must be clari-

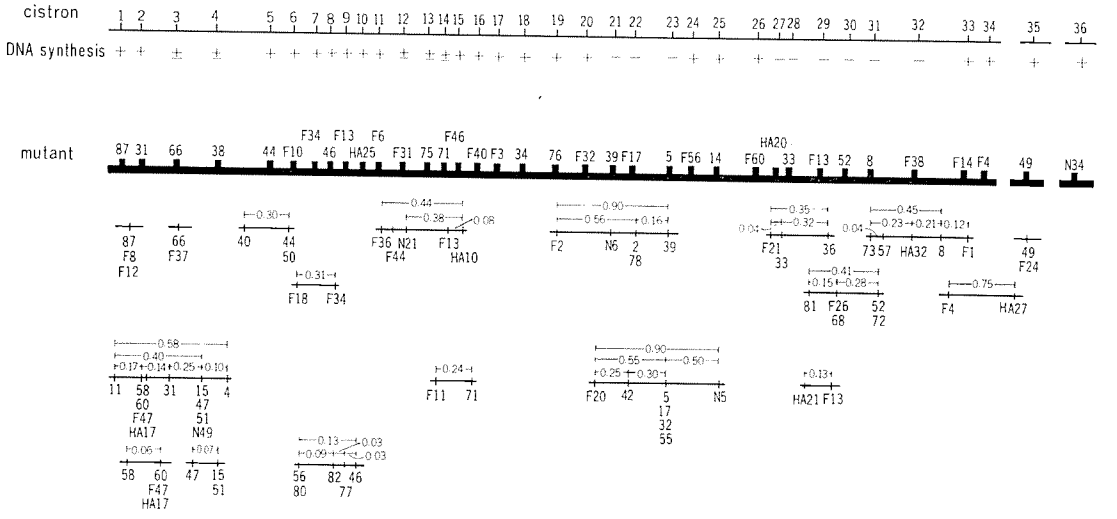

FIGURE 1. *A genetic map of SPO1 and intracistronic recombination. DNA synthesis was assayed in non-permissive condition. Sign + means that mutant synthesized DNA at rate comparable to that of wild type phage. Little or no phage DNA was synthesized in mutants signed −, as shown in Table 7.*

TABLE 1. *Classification of SPO1 sus mutants by complementation*

Cistron number	Representatives	Mutants isolated[e]
1	87	F8, F12, F15, F19, F29, F55
2 a	11	
b	60	58, F47, HA17
c	31	
d	47	15, 51, N49
e	4	
3	66	F37, F42, F45
4	38	
5	44	40, 50
6	F10	F7, F51
7 a	F34	
b	F18	
8	46	37, 56, 62, 70, 77, 80, 82
9	F43	F50
10	HA25	
11	F6	
12	(F31)[a]	F36, F44, (HA10), (N21)[b]
13	75	
14	71	F11
15	F46	(F48)[c]
16	F40	
17	F3	
18	34	
19	76	
20	F32	
21 a	39	2, 13, 78, F41, (N6, N69)[d]
b	F2	F58
22	F17	F23, F30, F33, F35, F52
23 a	5	17, 32, 42, 43, 55, F20, (F49)[c], (F59)
b	N5	
24	F56	
25	14	
26	F60	
27	HA20	
28	33	36, 45, 54, F21
29	F13	HA21
30 a	52	72
b	F26	F28, 68
c	81	
31	8	12, 57, 73, 86, F1, F5, F9, F22, F39, F54, F57, HA32
32	F38	
33	F14	
34	F4	HA27, F27
35	49	F24
36	N34	

[a] F31 is D-int, whereas F36 and F44 are D+. Thus F31 might represent another cistron (see Table 7), although F31 was used as a representative of cistron 12 in recombination experiments in Osaka. In Chicago, F36 and F44 were used as representatives of cistron 12.

[b] N21 did complemented F31 in Chicago, but not by liquid complementation in Osaka. HA10 also complemented F31 in Chicago, but not in Osaka. This group may have to be subdivided.

[c] F48 did not complement F49, but did complement other mutants in cistron 23. F48 and F49 may contain an additional mutation in common (Fujita, 1971).

[d] N6 and N69 clearly complemented F2 in Chicago.

[e] N-series mutants have not yet been classified. Some of them may fall into new cistrons (Geiduschek, personal communication).

TABLE 2. *Complementation between adjacent cistrons by burst size measurement*

Mutant	Cistron	Burst size
F 13	29	0.03
F 13 × 52		38.
52	30	0.03
52 × 8		51.
8	31	0.03
8 × F 38		9.4
F 38	32	0.01
F 38 × F 14		9.0
F 14	33	0.01
F 14 × F 4		13.
F 4	34	0.03
76	19	23.

TABLE 3. *Complementation between cistron 1 and cistron 2*

A) Spot test

Cistron	S \ B	F 12	11	60	HA17	F 47	31	47	N49	F 14[a]
1	87	−	(4)	(3)	(8)	(13)	(15)	(10)	(25)	+
1	F 12	−	(8)	(13)	(6)	(10)	(15)	−	(22)	+
2a	11	(3)	−	(4)	(2)	(3)	(7)		(5)	+
2b	60	(15)	(4)	−	−	−	(M)		(10)	+
2b	HA17	(5)	(1)	−	−	−	(3)	(3)	(5)	+
2b	F 47	(11)	(2)	−	−	−	(3)	(6)	(2)	+
2c	31	(30)	(11)	(2)	(6)	−	−	(14)	(4)	+
2d	47	(13)	(15)	(M)	(5)	(1)	(3)	−	−	+
2d	N49	(M)	(9)	(2)	(4)	(15)		−	−	+
2e	4	(M)	(15)	(15)	(11)	(20)	(M)	(17)	(M)	+
33	F 14	+	+	+	+	+	+	+	+	−

Symbols: B, phage seeded; S, phage spotted; −, no plaques in spot area; +, completely lysed in spot area; ( ), numbers of plaques in spot area; (M), many plaques in spot area.

[a] F14 was used as a control.

B) Liquid complementation

Mutants	Cistron	Burst size
87	(1)	0.76
11	(2a)	0.001
60	(2b)	0.001
31	(2c)	0.004
47	(2d)	<0.001
4	(2e)	<0.001
87 × 11	(1 × 2a)	3.0
87 × 31	(1 × 2c)	3.0
11 × 60	(2a × 2b)	0.03
31 × 47	(2c × 2d)	0.16
47 × 4	(2d × 2e)	0.08
31 × 66	(2c × 3)	33.

fied before any decision can be made on the independence of these subdivided cistrons. If the presently subdivided cistrons prove to be functionally independent, or if new cistrons are discovered, then these additional cistrons will be assigned fractional numbers, in accordance to the system of Studier (1969). If any of the presently subdivided cistrons prove to be identical in function, their subdivisions will be dropped. The subdivided cistrons are separated by less than one map unit, as seen later, and may represent adjacent genes, if their functions prove to be independent. It is conceivable that some of these mutants are polar mutants (Nakata and Stahl, 1967). However, we do not know whether they possess nonsense mutations, because the nature of the suppression mediated by the *su*$^+$ gene is un-

TABLE 4. *Recombination frequencies by two-factor crosses*

Cistron	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Mutant	87	31	66	38	44	F10	F34	46	F43	HA25	F6	F31	75	71	F46	F40	F3	34	76
87		0.6		5.5															
31			2.6	4.8	9.3	8.8	10.	9.0					14.	7.5				16.	22.
66				3.1	3.8		6.5	9.4					8.5	8.7				12.	11.
38					3.0	4.1	5.3	7.0		10.	7.3		8.3	4.6			9.7	7.9	9.8
44						1.4	3.3	4.4	3.0	2.5	5.0	4.0	7.1	8.4			6.0	6.7	11.
F10							1.2	3.0	2.7		5.6	3.4	11.						
F34								1.0	1.7	4.0	4.3	3.3	9.7						
46									0.3	1.0	2.0	2.4	3.6	2.6			7.3	7.6	6.2
F43										0.5	1.3	2.6							
HA25											1.0	2.8	5.5					7.0	
F6												1.8	3.5	2.4		3.5	5.0	5.8	5.9
F31													1.2	2.4	1.4	3.5	6.5	3.3	
75														1.0	0.7	1.5	4.1	3.5	8.7
71															0.2	1.0	3.2	4.5	6.1
F46																0.8	2.7		
F40																	1.2	3.0	
F3																		1.6	3.4
34																			2.1
76																			
F32																			
39																			
F17																			
5																			
F56																			
14																			
F60																			
HA20																			
33																			
F13																			
52																			
8																			
F38																			
F14																			
F4																			
49																			
N34																			

known.

2. *Two-factor crosses*

Pair-wise *sus*×*sus* crosses were carried out with one representative of each cistron. The recombination frequencies of given pairs of mutants are summarized in Table 4. The recombination frequencies of given pairs of mutants sometimes varied by as much as a factor of 2. We repeated the crosses several times and the average recombination frequencies were calculated. Values which deviated greatly from the average value for a given pair of mutants were excluded from the calculation. The most important factor affecting the recombination frequency seemed to be the multiplicity of infection. As the multiplicity decreased, the recombination frequency also

TABLE 4. Continued

20 F32	21 39	22 F17	23 5	24 F56	25 14	26 F60	27 HA20	28 33	29 F13	30 52	31 8	32 F38	33 F14	34 F4	35 49	36 N34
															29.	
	23.		22.		26.			22.	22.	16.	24.		40.	37.	31.	26.
	10.		20.		12.			20.		11.	25.				31.	
	17.		14.		13.			20.		22.	15.				34.	30.
	7.5		14.		10.			20.	14.	18.	16.		39.	30.	30.	
															10.	28.
															13.	
	7.8		11.		8.8			13.		13.	14.				33.	36.
															12.	
															14.	30.
10.	11.		12.		7.2			11.		9.0	12.				38.	
		5.6							13.					25.	16.	
6.7	9.1												12.	14.	29.	25.
5.6	4.0		6.7						12.		10.				40.	28.
															15.	
															22.	
4.6	5.0	7.2	7.1												26.	59.
2.8	6.1	7.0	8.6		8.2			12.	8.5	12.	12.				28.	
1.9	3.3	4.2	4.5		5 7			9.3		9.0	9.1		9.5	7.1	21.	23.
	1.5	3.4	4.4		6.8			9.0		9.5					32.	
		1.0	4.9	3.4	3.8			5.4	7.4	7.9	9.4		14.	10.	29.	29.
			2.2	4.6	3.2			7.4	5.0	9.3					24.	
				0.9	2.9	4.0		5.7	4.3	5.2	7.0		8.2	10.	28.	26.
					1.7	3.0	1.7		7.9	6.0					20.	
						2.4	2.1	2.5	3.8	4.7	5.9				37.	17.
							1.0	1.6	5.3	4.5					24.	
								0.1	2.0	2.6					23.	40.
									1.6	3.9	4.2		5.7	5.8	35.	30.
										1.5	2.0	6.0			13.	
											1.4	2.3	3.0	5.0	26.	15.
												2.5	4.0	4.2	25.	17.
													4.0	4.3	16.	
														0.5	36.	
															26.	23.
																22.

decreased. It is conceivable that other factors also affect the frequency. Some mutants may have a strong selective advantage over others. A slight variation in the ratio of one parent phage to another in a cross might influence the recombination frequency. We did not examine these factors systematically.

From Table 4, mutants could be linearly arranged in order of increasing recombination frequencies, although the additivity was not good. Fig. 1 shows the genetic map of SPO1.

Mutants, *sus* 49 and *sus* F24 in cistron 35 and *sus* N34 in cistron 36, gave high recombination frequencies in crosses with any of the other mutants tested, as shown in Table 4. Therefore, these two cistrons could not be located on the map shown in Fig. 1.

Crosses were also performed between mutants in the same complementation group. Fig. 1 shows results on intracistronic recombination. The order of mutants within given cistrons was determined, but their orientation relative to the main chromosomal map was not determined.

3. *Three-factor crosses*

Double *sus* mutants were crossed with single mutants to verify the arrangement of cistrons shown in Fig. 1. Later, newly isolated mutants were first crossed with double mutants, so that the locations of newly found cistrons could be deduced easily. Then, the mutants were crossed with single mutants in the same neighborhood. Table 5 summarizes the results of the three-factor crosses, and in general supports the order of the cistrons shown in Fig. 1. The precise orders of cistrons 6 to 9, 13 and 14, and 24 to 28 could not be determined from these crosses.

In most cases where single markers were presumed to be located between two markers, the frequency of wild type recombinants was much higher than the expected value, which is the product of the frequencies of the two single crossing over events. This phenomenon has been observed in crosses of many phages and is called high negative interference (Chase and Doermann, 1958; Mosig, 1970). The deviations seen in Table 5 were very large in most cases, but it was still possible to determine whether single mutants were located inside or outside the two markers.

4. *Mapping of a plaque morphology mutant*

Plaque morphology mutants were isolated previously (Okubo et al., 1964). One of the mutants, c_1, produces clear plaques with a distinct edge. Plaques of the wild type phage have a light halo, so the two types of plaques can be easily differentiated on HA 101 (su^-) bacteria. We crossed the c_1 mutant with the *sus* mutants and selected wild type plaques on su^- bacteria. As shown in Table 6, the c_1 locus seems to be located near *sus* F38, cistron 32.

5. *Function of cistrons*

The ability of mutants from each cistron to synthesize DNA in the restrictive condition was measured by the incorporation of ^{14}C-dUMP into the alkali-stable, acid-insoluble fraction. In Chicago, ^{14}C-adenine was used (Fujita, 1971). Most of the mutants could be easily classified as DNA-positive (D+) or DNA-negative (DO) as shown in Table 7. D+ means that mutants could synthesize DNA upon infection of su^- bacteria at the same or nearly equal rates as that of the wild type phage.

Certain other mutants were not clearly either D+ or DO (Table 7), but incorporated low or intermediate amounts of label into DNA. Several of these particular mutants (*sus* F—6, 11, 12, 31, 42, 45, and 47) were studied further, through an examination of their kinetics of DNA synthesis (Fig. 2). All of these mutants synthesized at least some viral DNA, in contrast to a control infection by the DO mutant *sus* F38. On the basis of this experiment, *sus* F42 and F45 (both mutants in cistron 3) were classified as DNA-arrested (DA).

Similarly, *sus* F47 (cistron 2b) was classified as DNA-delayed (DD), and *sus* F—6 (cistron 11), 11 (cistron 14), 12 (cistron 1) and 31 (cistron 12) were classified as DNA-int in

TABLE 5. *Recombination frequencies by three factor crosses*

Double × Single (cistron)	Frequency of wild type	Expected if			Possible order
		Outside		Inside	
31–38 × 87 (1)	0.23	2.6	0.33	0.008	31 38
(2c–4) × 47 (2d)	0.045	2.2	0.13	0.003	87 47 66
× 66 (3)	0.17	1.5	1.3	0.020	
31–44 × 66 (3)	0.37	1.9	1.3	0.003	
(2c–5) × 38 (4)	0.35	1.5	2.6	0.039	31 44
× F 10 (6)	0.52	0.5	5.0[a]	0.025	66 38 F 10 F 34 46
× F 34 (7a)	1.7	2.0	5.0		
× 46 (8)	1.8	2.2	4.5		
44–75 × F 10 (6)	0.19	3.0	0.5	0.015	
(5–13) × F 34 (7a)	0.48	2.5[a]	2.0	0.050	
× 46 (8)	0.23	1.8	2.2	0.040	
× F 43 (9)	0.50	1.5	1.5	0.023	44 75
× HA25 (10)	0.13	2.5	1.3	0.032	F 10 46 F 43 F 6 F 10 F 3
× F 6 (11)	0.01	1.7	2.5	0.043	F 34 HA25 (71)
× 71 (14)	0.05	0.5	4.2	0.021	(F 46)
× F 46 (15)	0.05	0.19	3.0[a]	0.005	
× F 40 (16)	0.28	0.7	3.0[a]	0.021	
× F 3 (17)	1.0	2.0	3.0		
F 6–F 3 × 46 (8)	0.80	3.4	1.0		
(11–17) × F 43 (9)	1.2	3.0[a]	0.6	0.018	
× HA25 (10)	0.88	3.0[a]	0.5	0.015	
× F 31 (12)	0.12	2.5	1.3	0.033	F 6 F 3
× 75 (13)	0.26	2.0	1.8	0.036	46 HA25 F 31 75 71 34 76
× 71 (14)	0.13	1.6	1.2	0.019	F 43 (F 40)
× F 40 (16)	0.38	0.6	1.8	0.011	
× 34 (18)	0.67	0.8	2.9	0.023	
× 76 (19)	2.3	1.7	3.0		
F 31–71 × 75 (13)	0.05	0.5	0.6	0.003	F 31 71
(12–14) × F 46 (15)	0.08	0.12	0.5	0.006	75 F 46 F 40
× F 40 (16)	0.25	0.36	1.8	0.007	
75–71 × F 46 (15)	0.08	0.12	0.19	0.0002	75 71
(13–14) × F 40 (16)	0.50	0.37	0.75		F 46 F 40 F 3
× F 3 (17)	1.5	1.6	2.0		
75–F 3 × 71 (14)	0.034	1.6	0.5	0.008	75 F 3
(13–17) × F 46 (15)	0.009	1.8	0.19	0.003	71 F 46 F 40
× F 40 (16)	0.065	0.6	0.8	0.005	
75–34 × HA25 (10)	1.0	3.5	2.5[a]		
(13–18) × F 6 (11)	1.2	2.9	1.8		
× F 31 (12)	1.1	1.7	0.60		75 34
× 71 (14)	0.10	2.3	0.50	0.012	HA25 F 31 F 46 71 F 40 F 3 76
× F 46 (15)	0.03	2.0[a]	0.19	0.004	F 6
× F 40 (16)	0.07	1.5	0.75	0.011	

Continued . . .

TABLE 5. *Continued.*

Double × Single (cistron)	Frequency of wild type	Expected if Outside		Expected if Inside	Possible order
× F 3 (17)	0.08	0.8	2.0	0.016	
× 76 (19)	1.7	1.0	4.4		
F 3–76 × 34 (18)	0.17	1.1	0.8	0.088	F 3 76
(17–19) × F 32 (20)	3.1	1.0	2.3		34 F 32
39–5 × F 32 (20)	0.30	2.2	0.75	0.018	
(21–23) × F 17 (22)	0.07	1.1	0.5	0.006	39 5
× F 56 (24)	0.31	0.45	2.1	0.010	F 32 F 17 F 56 F 60
× 14 (25)	0.33	1.4	1.9	0.027	(14)
× F 60 (26)	5.2	2.0	2.0[a]		
5–52 × 39 (21)	1.3	3.9	2.5		
(23–30) × F 56 (24)	0.12	3.0	0.18	0.054	
× 14 (25)	0.18	2.4	1.5	0.036	
× F 60 (26)	0.18	2.3	2.0	0.046	5 52
× HA20 (27)	0.15	1.3	4.5[a]	0.059	39 14 HA20 F 13 8 F 14
× 33 (28)	0.24	1.9	2.9	0.055	F 60 33 F 38
× F 13 (29)	0.16	0.8	2.2	0.018	(F 56)
× 8 (31)	0.6	0.7	3.5	0.025	
× F 38 (32)	1.5	1.2	3.7[a]		
× F 14 (33)	2.6	1.5	4.1		
33–8 × 14 (25)	0.56	3.0	1.3	0.039	
(28–31) × F 13 (29)	0.16	1.0	0.8	0.008	33 8
× 52 (30)	0.25	0.7	2.5		14 F 13 F 38 F 14
× F 38 (32)	1.2	1.3	2.5		(52)
× F 14 (33)	1.5	2.0	2.9		
52–F 4 × F 60 (26)	6.0	3.0[a]	2.3		
(30–34) × HA20 (27)	2.9	2.9[a]	0.8	0.023	
× 33 (28)	2.2	2.9	2.0		52 F 4
× F 13 (29)	0.47	2.9	0.8	0.023	F 60 33 F 13 8 F 38 (F 14)
× 8 (31)	0.18	2.1	0.7	0.015	HA20
× F 38 (32)	0.13	2.1	1.2	0.025	
× F 14 (33)	0.10	0.25	1.5	0.004	

[a] Estimated values. ( ) uncertain.

TABLE 6. *Mapping of a plaque morphology mutant, c_1*

Crosses (cistron)	Total plaques on su^+	Wild type plaques on su^-	RF[a]
C_1 × F 13 (29)	1200	40	6.7
× 52 (30)	1156	19	3.3
× 8 (31)	1130	20	3.5
× F 38 (32)	830	2	0.5
× F 14 (33)	1210	26	4.3
× F 4 (34)	1150	37	6.4

[a] Recombination frequency = wild type plaque on su^-/total plaque on su^+ × 200.

TABLE 7. *Biochemical natures of cistrons*

Cistron	Mutant	% of wild type level of ^{14}C-UdR incorporation in 40 min	DNA class	Lysozyme[j] activity
1	87	122	D+	+
2c	31	88	D+[a]	+
3	66	48[c]	D-int or DA[b]	±
4	38	25	D+ or DA	+
5	44	70	D+	+
6	F10	98	D+	+
7a	F34	80	D+[d]	+
8	46	93	D+	+
9	F43	78	D+	+
10	HA25	71	D+	+
11	F 6	92	D+[e]	±[k]
12	F31	12[e]	D-int	+
	HA10	52	D+ or D-int[f]	+
	F44	102	D+	+
13	75	42	D+ or D-int	+
14	71	42	D+ or D-int[g]	+
15	F46	88	D+	+
16	F40	73	D+	+
17	F 3	93	D+	+
18	34	83	D+	+
19	76	68	D+	−[k]
20	F32	62	D+	+
21a	39	0.9	DO[h]	−
22	F17	0.8	DO	−
23a	5	1.0	DO	−
24	F56	55	D+	+
25	14	60	D+	+
26	F60	70	D+	+
27	HA20	0.6	DO	−
28	33	0.8	DO	−[l,m]
29	F13	0.7	DO	−[n]
30a	52	0.7	DO[i]	−
31	8	0.8	DO	−
32	F38	0.9	DO	−
33	F14	77	D+	−[l]
34	F 4	51	D+	−[l]
35	49	60	D+	+
36	N34	65	D+	+

[a] F47 (cistron 2b) was classified as DD, as shown in Fig. 2 (Fujita, 1971).
[b] F37 and F42 were classified as DA, as shown in Fig. 2 (Fujita, 1971).
[c] Incorporation of label under permissive condition was also lower than that of wild type or D+ mutants.
[d] F18 (cistron 7b) was D+ (Fujita, 1971).
[e] F6 was classified as D-int, as shown in Fig. 2, in Chicago (Fujita, 1971).
[f] F36 and F44 were classified as D+ (Fujita, 1971).
[g] F11 was classified as D-int (Fujita, 1971).
[h] F2 (cistron 21b) was classified as DO (Fujita, 1971).
[i] F26 (cistron 30b) and N5 (cistron 30c) were also classified as DO (Fujita, 1971).
[j] +: Decrease of O. D./min/mg protein =0.025–0.030.
−: Decrease of O. D./min/mg protein =0.001–0.002.
[k] The enzyme activity of extracts prepared under permissive conditions was also low.
[l] Transcription control mutants (Fujita et al., 1971).
[m] dCMP deaminase and dUMP hydroxymethylase-negative.
[n] dUMP hydroxymethylase-negative.

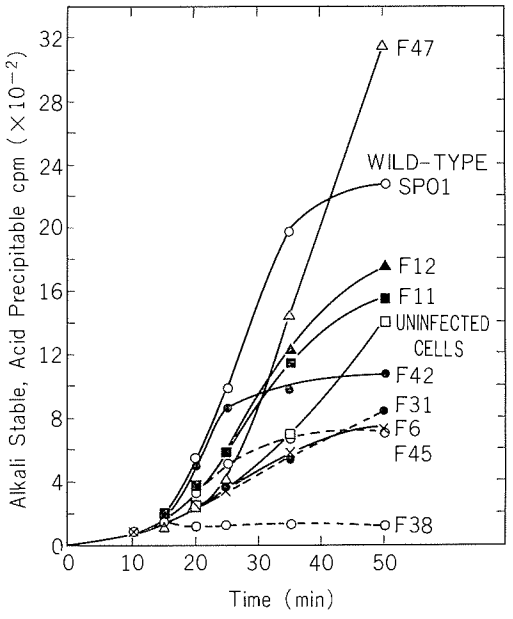

FIGURE 2. *Incorporation of adenine-8-^{14}C into the DNA of B. subtilis 168 T$^-$ (Su$^-$) cells infected with SPO1 mutants having low or intermediate levels of DNA synthesis. Adenine-8-^{14}C (0.05 μc/ml, 0.0037 μc/μg), and SPO1 were premixed and added at zero minutes. Other conditions are described in Materials and Methods. Sus F38, a DNA-negative (DO) mutant, was used as a control.*

Chicago (Fig. 2).

As expected, the different mutants in any one gene usually exhibited very similar patterns or levels of DNA synthesis. However, there were a few exceptions. For example, *sus* F31, which is D-int, maps into gene 12, which includes other mutants that are D+ (*sus* F—36, 44). *Sus* F12 (cistron 1) and *sus* F50 (cistron 9) are also D-int, whereas other mutants tested in their respective cistrons are D+. Cistron 2c was designated as D+, while cistron 2b (*sus* F47) was designated as DD (Fujita, 1971). This might suggest that these cistron are different and independent. A contradiction between the DNA-phenotype of cistron 11 exists between the Osaka and Chicago results, as is seen in the footnotes to Table 7. Unfortunately *sus* F6 is the only mutant presently in cistron 11. The *sus* F6 stock in Osaka should be retested with the original Chicago stock. We have not yet done this.

It became evident that DO mutants are clustered together in two regions. We assayed the activities of dCMP deaminase and dUMP hydroxymethylase which are induced upon wild type phage infection (Roscoe and Tucker, 1966). Preliminary results showed that dCMP hydroxymethylase was not induced in *su*$^-$ bacteria infected with *sus* F13 (cistron 29) and *sus* 33 (cistron 28). Moreover, *sus* 33 did not induce dCMP deaminase. The latter result is to be interpreted in terms of a defect in the normal temporal sequence of viral transcription, which leads mutants in cistron 28 to ceases viral transcription entirely, 8 to 10 min after infection of the nonpermissive host at 37 C (Fujita et al., 1971). Therefore, neither enzyme may be induced upon infection of *su*$^-$ bacteria by mutants of cistron 28.

Mutants, *sus* F14 and *sus* F4 (cistron 33 and 34) synthesize DNA under non-permissive conditions, but do not induce lysozyme activity. However, these genes do not seem to be structural genes for lysozyme, as active phage particles are not released by artificial lysis from phage-infected *su*$^-$ bacteria (Table 2). Electron microscopic studies in Chicago showed that those mutants lack the ability to synthesize a wide variety of late proteins (Fujita, 1971). These mutants resemble the maturation-defective mutants of T4 (cistron 33 and 55) and lambda (cistron Q) (Epstein et al., 1963; Thomas, 1968). Fujita et al. (1971) made extensive studies on the transcription patterns of these SPO1 mutants and reported that they are transcriptional control mutants.

Mutant *sus* 76 could not induce lysozyme activity under non-permissive conditions and under permissive conditions the activity was low. Active phage particles were liberated by artificial rupture of *su*$^-$ bacteria infected with the mutant (Table 2). Further experiments are needed, however, to determine whether or not this cistron is the structural gene for lysozyme. Stewart and Marmur (1970) stated that SP82, a similar HMU-containing phage,

might lack a lysozyme structural gene.

DISCUSSION

More than 120 suppressible (*sus*) SPO1 mutants were isolated independently in Osaka and Chicago, and classified by complementation tests into at least 36 cistrons. The existence of weakly complementing mutants within certain cistrons may indicate that additional cistrons might be represented by these mutants. A linear and non-permutated genetic map of SPO1 was constructed on the basis of two- and three-factor crosses. The molecular weight of SPO1 DNA is about 1×10^8, which provides the capacity to code for more than 100 proteins. Therefore, many genes probably remain unidentified at this time. Thus, one might argue that the map is actually circular. However, Kahan (1966), and more recently Green et al. (1971) reported that the map of SP82 (or SP 82G), a similar phage of *B. subtilis* containing HMU is linear, not circular, as judged by recombination data using temperature-sensitive mutants. The nucleotide sequence of SP82 DNA seems to have many similarities to that of SPO1 DNA (Truffaut et al., 1970) but no results of heteroduplex mapping have been reported yet.

Two cistrons, cistron 35 and 36, showed anomalies in recombination frequencies. They behave in genetic crosses as if they were unlinked to the main map. The reason for this strange mapping behavior is unknown at present. Green et al. (1972) also found one mutant which did not fall within the main genetic map of SP82G. They located it about 20 map units distant from one end of the main map, the total map distance being 54 map units. Cistron 35 of SPO1 might be located far from one end of the main chromosome map, and cistron 36 far from the other end. Both cistrons might be separated from the other cistrons by long regions that are not represented by existing mutants or which code for non-essential functions. Such a phenomenon exists in the map of phage P2 (Lindahl, 1969 a, b).

Alternatively, the two cistrons might be located near opposite ends of the main map.It could be postulated that there are weak regions between each of these cistrons and the nearest end of the chromosome, where recombination takes place with a higher frequency. Alternatively both cistrons could be located at one of the ends of the map, with a weak region between them. This postulated weak region might include single-strand breaks at defined positions in the DNA molecule, or might correspond to specific base sequences favored by the enzyme which catalyzes the primary breaks of recombination. In T5 DNA, there are single-strand breaks in defined positions (Abelson and Thomas, 1966; Bujard, 1969). Lanni (1968) reported that an amber mutant (*am* 15) of T5 phage gave maximal or near maximal recombination values with six scattered tester mutants. He suggested that *am* 15 was outside the known map of T5, perhaps in the first-step-transfer DNA. In phage T1, Michalke (1967) found that the recombination frequency is at least five times higher near the two ends of the DNA. Doermann and Parma (1967) and Mosig (1963, 1971) also showed in T4 that regions near ends of infecting bacteriophage chromosomes are susceptible to genetic recombination.

The function of each cistron was investigated. In general, the " early " mutants (DO) lie on the right half on the linear map shown in Fig. 1, and the " late " mutants (D+) on the left half. As in T4, lambda and other phages (Epstein et al., 1963; Mount et al., 1968; Studier, 1969), related functions appear to be clustered together on the genetic map. Green et al. (1972) also found that the organization of the SP82G genome is simple, consisting of three functional regions: genes involving DNA synthesis, tail synthesis, and head synthesis. Although they worked with a smaller number of cistrons, and mutants used were exclusively of temperature-sensitive type, in general, electron microscopic studies with SPO1 *sus* mutants seem to support such

an ordering (Fujita, 1971). We found two clusters of genes concerning with DNA synthesis. In Green's map, the region involved in DNA synthesis could also be separated into two parts by a D+ gene in the middle. Cistron 2 of Kahn's classification might correspond to the SPO1 cistron 29, as mutants of cistron 2 of SP82 are deficient in dUMP hydroxymethylase (cited by Green et al., 1972).

Maturation defective mutants (cistron 33 and 34) lie next to DO cistrons. Fujita et al. (1971) investigated the nature of these genes extensively and found that mutants in these cistrons are defective in the synthesis of middle and late species of messenger RNA. A similar proximity of the maturation defective cistrons to DO genes is seen in the lambda genome or even in T4, although the two maturation defective genes (33 and 55) are widely separated from each other (Mount et al., 1968; Thomas, 1968; Epstein et al., 1963). It seems that the SPO1 chromosome resembles that of the temperate phage lambda in the arrangement of genes, although SPO1 is virulent. It is interesting to add that recently we found a mutant of SPO1 which shows a carrier state in a strain of *B. subtilis* (unpublished data).

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