Genetic Study of Suppressor-Sensitive Mutants of the *Bacillus subtilis* Bacteriophage $\phi 29$

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With bacteriophage $\phi 29$ of *Bacillus subtilis* 133, suppressor-sensitive (*sus*) hydroxylamine mutants have been isolated. Intracistronic and intercistronic quantitative complementation placed the mutants in 13 cistrons, and three-factor crosses have been used to assign an unambiguous order for 10 cistrons. Recombination frequencies have been presented for several regions of the genome to facilitate comparison of the *sus* system with the previously published temperature-sensitive mapping systems.

Bacillus subtilis bacteriophage $\phi 29$ is a small, virulent, morphologically unique virus with highly infectious DNA (3, 10). Its DNA is a linear, nonpermuted duplex, and the intact single polynucleotide strands can be isolated by polyuridylic-guanylic acid (poly [UG]) binding (2, 8). However, in the virus, the DNA is a supercoiled circle held by a protein linker (9).

In the present study, a collection of suppressor-sensitive (*sus*) mutants has been grouped by quantitative complementation, and 10 cistrons have been placed in an unambiguous order by three-factor crosses. This map is being expanded and will be used in an effort to combine the *sus* and temperature-sensitive (*ts*) collections as they become available (6, 11).

Our *sus* mutant collection is currently being used to analyze transcription and gene function and as an adjunct in our morphological investigations.

MATERIALS AND METHODS

Phage and bacteria. Phage $\phi 29$ (10) was the object of this study. The properties of the nonpermissive host strain, *B. subtilis* SPOA₁₂, once named 12A, have been described (7).

The permissive host, *B. subtilis* L15, contains the *su*⁺ suppressor isolated by Georgopolous (5). L15 (*su*⁺, *met*⁻ *thr*⁻) has a smooth colonial morphology and is asporogenous.

Bacteriophage assay. Standard phage techniques were used (1), and the media and methods of viral preparation and purification have been published (2, 10). Unsupplemented Difco Antibiotic Medium 3 (PB) was used for complementation, burst size, and recombination experiments.

Mutagenesis with hydroxylamine. We have employed three methods of mutagenesis and segregation of the mutated DNA.

In method A (13), our modification employed purified $\phi 29$ suspended at a concentration of 10¹² phage/ml in a solution containing 0.2 M potassium phosphate (pH 6.0), 0.02 M MgSO₄, and 1 M NH₂OH·HCl. The mixture was incubated at 37 C, and the reaction was terminated by a 1,000-fold dilution in PB. Mutants were isolated from samples giving 1 to 5% viral survival. The virus suspension was plated with the permissive host, *B. subtilis* L15, to give confluent lysis, and after 18 h at 37 C the phage were harvested in PB. A single mutant was isolated from a given plate lysate (see below).

Alternatively, with method B (13), mutagenized stocks were used to infect the permissive host (10⁷ cells/ml) in PB at a multiplicity of infection (MOI) of 5. After 30 min at ambient temperature for adsorption, samples were diluted in PB to give about 100 infected cells per tube and were incubated at 37 C for 2 h until lysis occurred.

The third method (C) of mutagenesis was that of Freese et al. (4). The ice-cold stopping mixture was 2 ml of acetone in 100 ml of PB, and, after 10 min of quenching, the virus was diluted 20-fold into PB and stored at 4 C. Samples with 0.5 to 5% viral survival were grown for one cycle in the permissive host as in method B.

Isolation of mutants. Single mutant phage were adsorbed to the permissive host for 20 min, and following addition of *B. subtilis* SPOA₁₂ the mixture was plated to yield isolated single plaques. The ratio of the permissive to the nonpermissive host was 10:1 with 2 × 10⁷ strain L15 cells per plate.

Clones with unusual morphology were transferred by sterile toothpicks to indicator lawns of both hosts. Mutant clones were suspended in PB and plated to give isolated plaques on the permissive host. This process was repeated and the progeny of a single clone were used to prepare a lysate. These *sus* mutants were assigned a number and stored at 4 C.

Complementation. Provisional assignment to a complementation group employed a qualitative method. Representative mutants of each cistron were used to infect the nonpermissive host at an MOI

greater than 2, and 2×10^7 infected cells were used per plate. Mutant clones were transferred by toothpicks from the permissive host to the test set.

Verification used the quantitative or burst size method described previously (6). Results were expressed as the ratio of the burst size of mixedly infected bacteria to the wild-type burst size in the nonpermissive host, multiplied by 100. For each mutant, both an intracistronic and an intercistronic burst size were obtained.

Burst sizes of suppressor-sensitive mutants.

Cells of the permissive host L15 were grown in PB at 37 C to 10^8 cells/ml and infected with phage at an MOI of 10. After 15 min at 37 C for adsorption, $\phi 29$ antiserum ($K > 2,500$) was added, and at 5 min the cells were diluted 1,000-fold in PB and incubated for 2 h at 37 C. Lysozyme (10 μ g/ml, A grade, Calbiochem, Los Angeles, Calif.) was added, and at 15 min the lysates were plated. Infectious centers were plated immediately following the 1,000-fold dilution in PB.

Recombination. In a two-factor cross, cells of strain L15 grown in PB at 37 C to 2×10^8 cells/ml were infected at an input MOI of 10 with each virus. At 20 min, unadsorbed phage were neutralized by $\phi 29$ antiserum ($K > 2,500$) for 5 min, and the cells were diluted 1,000-fold in PB and incubated for 2 h at 37 C. Lysozyme (10 μ g/ml) was added and at 15 min the lysates were plated.

The recombination frequency in two-factor crosses is expressed as twice the plaque count on the nonpermissive host times 100, divided by the plaque count on the permissive host.

The three-factor cross for cistron localization employed three double mutants. These were obtained by testing the progeny of the two-factor cross by qualitative complementation and verifying the results with quantitative complementation.

In the three-factor cross, an input MOI of 20 of each virus was used. The high MOI was used to minimize potential adsorption or injection problems inherent in the use of multiple *sus* mutants. However, a high MOI can increase the recombination frequency in some

cases because reversion can accumulate at one site in double mutants. We have attempted to minimize this by preparing and testing several lysates of each double mutant.

RESULTS

Complementation. Our set of 133 hydroxylamine mutants comprises 13 cistrons by complementation (Table 1). Although we have varied the pH conditions of mutagenesis (see Materials and Methods) to make this set of mutants more representative, one undesirable feature of this single mutagen approach is that 51 mutants cluster in cistron K.

The results of intercistronic burst-size complementation of *sus* mutants presented in Table 2 are representative of values obtained when the

TABLE 1. Assignment of mutants to cistrons based on quantitative complementation tests

Cistron	Reference mutant	Frequency of occurrence by method of mutagenesis		
		A	B	C
A	628		1	5
B	629		1	
C	603		1	6
D	369	5	3	6
E	626		1	5
F	710		1	1
G	614		1	2
H	756		3	6
I	302	6	5	6
J	305	1		
K	330	29	12	10
L	300	1	3	11
M	741			1

TABLE 2. Intercistronic burst-size complementation of *sus* mutants^a

Mutants	A628	B629	C603	D369	E626	F710	F769	G614	H704	H756	I302	J305	K330	L300	M741
A628	0.28 ²	13 ³	21 ²	28 ²	15 ²	9 ²	19 ¹	13 ²	26 ²	39 ¹	18 ²	22 ²	19 ²	23 ²	15 ²
B629		1.7 ⁴	21 ²	15 ²	14 ²	13 ²	13 ¹	14 ²	32 ²	25 ¹	12 ²	13 ³	15 ²	12 ²	23 ⁴
C603			0.23 ²	21 ²	28 ²	18 ²	23 ¹	28 ²	33 ²	29 ¹	24 ²	26 ²	28 ²	28 ²	15 ³
D369				0.45 ⁵	16 ²	7 ²	21 ¹	19 ²	25 ²	19 ¹	21 ²	21 ²	29 ⁴	17 ²	12 ³
E626					0.64 ³	13 ²	29 ¹	21 ²	27 ²	39 ¹	15 ²	17 ²	12 ²	18 ²	16 ²
F710						0.18 ²	0.82 ¹	9 ²	12 ²	11 ¹	6 ²	12 ²	10 ²	13 ²	11 ²
F769							0.54 ³	12 ¹	11 ¹	29 ¹	17 ¹	12 ¹	34 ¹	24 ¹	16 ²
G614								0.09 ²	30 ²	30 ¹	10 ²	12 ²	12 ²	12 ²	11 ²
H704									0.18 ²	0.23 ¹	8 ²	32 ²	32 ²	44 ²	10 ²
H756										1.0 ²	18 ¹	19 ¹	39 ¹	24 ¹	20 ³
I302											0.11 ²	12 ²	16 ²	12 ²	14 ²
J305												0.27 ³	15 ²	6 ²	10 ²
K330													0.17 ³	20 ²	11 ²
L300														0.11 ²	11 ²
M741															2.7 ⁴

^a Burst-size measurements are expressed as the percentage of the wild-type control. Superscripts represent the numbers of experiments used to compute the average values.

entire set was tested in a single experiment. Mutants were always assigned to a cistron by this method, thus the superscripts listed represent a minimal number of tests. To illustrate the reproducibility of this method, in 17 intracistronic tests with mutant L300 as one member, the average burst size was 0.17. The burst-size value in 40 intercistronic tests including L300 was 16 ± 6.8 . This differential value of 50- to 100-fold between intracistronic and intercistronic bursts is obtained consistently.

Mutants B629 and M741 have given high base-line values, but on occasion have given control bursts of less than 1%. The degree of complementation in intercistronic bursts indicates that these mutants can be assigned to new cistrons.

Burst sizes of suppressor-sensitive mutants. Most *sus* mutants have a burst size comparable to wild-type virus in the permissive host L15 (Table 3). We have included data for F769 to demonstrate that some mutants of the F group can give a reasonable burst. However, both H704 and H756 have a reduced burst size, and this may be a property of H group mutants.

Recombination. Results of three-factor crosses presented in Table 4 establish the unambiguous sequence of *sus* cistrons shown in Fig. 1.

Our interpretation of the results presented in Table 4 is based in part on a more detailed analysis of a series of three-factor crosses em-

TABLE 4. Results of three-factor crosses

<i>sus</i> Markers			Tests n	Recombination frequency ^a		
1	2	3		1 × 23	2 × 13	3 × 12
A628	G614	I302	3	1.1	0.11	1.2
A628	D369	I302	1	1.6	0.08	1.5
A628	C603	G614	2	1.8	0.20	2.0
A628	C603	D369	1	1.6	0.22	0.80
C603	D369	G614	1	0.7	0.06	0.87
C603	E626	G614	2	1.6	0.28	0.71
D369	E626	G614	2	1.4	0.38	1.4
G614	H756	I302	2	2.5	0.17	2.5
A628	I302	J305	1	2.7	0.36	3.4
I302	J305	K330	3	2.6	0.30	1.5
J305	K330	L300	2	2.1	0.27	2.1

^a The central mutant is listed in column 2, thus 1-2-3 order is the left to right cistron order.

FIG. 1. This genetic map of $\phi 29$ *sus* mutants merely reflects cistron order. Cistron letters appear above the solid line; mutants used are below. Recombination frequencies are not implied.

ploying qualitative complementation to identify each recombinant class. In one set of three three-factor crosses using the mutants A628, I302, and K330 as one parent and the appropriate double mutants as the other parent, 3,834 clones were examined. The 216 recombinant viruses had the distribution of genotypes expected for the three-factor cross. For example, we obtained 39 wild-type recombinants and 40 triple mutants.

A control A628 × I302 cross and the three-factor crosses were run simultaneously; in nine control two-factor crosses, the recombination frequency was 5.3 ± 0.83 with a range of 4.3 to 6.6.

Table 5 contains data obtained with two-factor crosses using markers near both ends of the map. The two-factor crosses employ an MOI of 10, and the recombination percentage for the control interval A628 to I302 in 11 experiments was 5.4 ± 0.88 with a range of 3.8 to 5.9%. With the adjacent cistrons J, K, and L, the results in Table 5 indicate reasonable additivity and the standard deviations' good reproducibility. The results presented for cistrons A, C, and E are not as extensive but are comparable. The highest value for reversion in a control infection was 0.02% for E626 and in most instances was less than 0.001%.

Mutants of cistrons B, F, and M have not been assigned a position in Fig. 1 because we

TABLE 3. Burst sizes of wild-type and suppressor-sensitive mutant phage^a

Mutant	Phage/cell	<i>sus</i> ⁺ burst (%)
<i>sus</i> ⁺	317	100
A628	97	31
B629	329	104
C603	205	65
D369	929	293
E626	130	41
F710	15	5
F769	144	45
G614	707	223
H704	38	12
H756	81	26
I302	657	207
J305	321	101
K330	537	169
L300	143	45
M741	68	21

^a Three experiments were used to compute these burst sizes. The permissive host L15 was infected at a multiplicity of 10, and $\phi 29$ antiserum was added 15 min later. In 5 min the infected cells were diluted 1,000-fold into PB, incubated for 2 h at 37 C, and lysed with lysozyme.

TABLE 5. *Intercistronic recombination of sus mutants^a*

Mutant	A628	C603	Mutant	J305	K330
C603	1.5 ± 0.10^7		K330	1.1 ± 0.34^8	
E626	2.5^3	1.3 ± 0.26^7	L300	2.2 ± 0.5^6	0.91 ± 0.1^7

^a Values presented are recombination frequencies. Superscripts represent the number of experiments used to compute the means and the standard deviations.

have not been able to construct the three double mutants. In the F710 $\times$ G614 cross, we observed more than 1% of wild-type clones but could not detect the double mutant in a sample of more than 7,000 clones.

With F710 we have employed two- and three-factor crosses to position the marker. F710 gave 0.13% recombination with the G614-H756 double mutant and 2.9% with the H756-I302 double, suggesting the order G-F-H. However, the results in Table 6 fail to substantiate this conclusion unequivocally. These data indicate that F710 and G614 are equidistant from H756, and F710 and E748 are equidistant from G614. With typical markers we would predict better additivity, and therefore we might suspect an odd phenotype from F710. We have observed a reduced burst size.

We now use three-factor crosses with double mutants in adjacent cistrons as a preliminary step in mapping. But B629 and M741 present technical problems in that both give relatively high and variable base values in complementation (Table 2), and B629 on occasion gives minute plaques on the nonpermissive host. The two-factor crosses presented in Table 7 with *sus* mutant B629 have low recombination values for this system. The map position of mutant B629 must be close to that of A628.

DISCUSSION

Two genetic studies of $\phi 29$ have been published, and both have employed *ts* mutants and two-factor crosses to determine gene order. The map of Hagen et al. (6) was constructed with the asporogenous *B. subtilis* strain SPOA₁₂ and contains thirteen complementation groups. The genetic study of Talavera et al. (12) used *B. amyloliquefaciens* H_M, and the map contains 11 cistrons with order implied for nine cistrons. In the *B. subtilis* system, recombination frequencies varied by a factor of two from day to day, and recombination values did not always show good additivity (6).

We constructed a *sus* system to determine whether it might be more useful than the *ts* system to investigate the $\phi 29$ genome and to facilitate the identification of proteins as specific

TABLE 6. *Intercistronic recombination of sus mutants^a*

Mutant	E748	G614	F710
G614	0.77		
F710	0.92	0.72	
H756		0.95	
		3.3	3.3
		3.8	3.8

^a Values presented are recombination frequencies.

TABLE 7. *Intercistronic recombination of sus mutants^a*

Mutant	A628	C603	G614	H756	L300
B629	0.06^3	0.29^3	0.77^3	1.1^3	6.0^1

^a Values presented are recombination frequencies. Superscripts represent the numbers of experiments used to compute the average values.

gene products by polyacrylamide gel electrophoresis.

The burst sizes in control complementation experiments have been lower and more reproducible in the *sus* system than in the *ts* system (6). Moreover, recombination frequencies are less variable in the *sus* system. In an analysis of 10 experiments with *ts* mutants 204 and 219 of cistrons 3 and 4, we calculate a mean recombination percentage of 0.54 ± 0.45 with a range of 0.1 to 1.6%. With *ts* mutants 142 and 224 of cistrons 10 and 11, eight experiments gave 1.7 ± 0.92 with a range of 0.35 to 3.4% (6; and analysis of unpublished *ts* data). By comparison, five mean recombination percentages from the *sus* system with sample sizes of 6 or more gave values of 1.5 ± 0.1 , 1.3 ± 0.26 , 1.1 ± 0.34 , 2.2 ± 0.5 , and 0.91 ± 0.1 . These results, plus the values obtained over the longer A628-I302 interval used as a control, suggest that the *sus* system is more reliable for quantitative studies in *B. subtilis*.

Ten *sus* cistrons (Table 3 and Fig. 1) have been placed in an unambiguous order by a series of three-factor crosses. Analysis of these three-factor crosses has identified a set of double mutants in cistrons known to occupy adjacent

loci on our map. These and additional multiple mutants will facilitate the positioning of new cistrons. Therefore, this system will be useful in integrating the *ts* systems under investigation (6, 11).

The problems that occur with mutants F710, M741, and B629 are inherent in any viral genetic study. For instance, the inability to construct the relevant double mutant with F710 may reflect the gene functions, the mechanism of the suppression, and the positions of the point mutations. Therefore, we have included data with F769, for any new mutant may permit the construction of the pertinent double mutant. F769 falls in the same interval as F710 (data not shown).

The map derived for *B. amyloliquefaciens* has twice as many map units as that for *B. subtilis* (6, 12). This suggests that these systems may be quite different and that it may be wise to defer additional quantitative mapping with $\phi 29$ and *B. subtilis*.

We are adding to our collection of hydroxylamine mutants and have begun to isolate mutants with bromodeoxyuridine. These *sus* mutants have been used for investigation of protein synthesis during infection and for analysis of gene function and morphological development.

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