JOURNAL OF BACTERIOLOGY, June 1969, p. 1087-1097
Vol. 98, No. 3
Printed in U.S.A.
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Chromosomal Location of Genes Regulating Resistance to Bacteriophage in *Bacillus subtilis*[1]

F. E. YOUNG,[2] C. SMITH, AND B. E. REILLY[3]

Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received for publication 10 March 1969

Many of the viruses which infect *Bacillus subtilis* require glucosylated polyglycerol teichoic acid for adsorption. These mutants can be divided into three classes on the basis of enzymatic defects and growth on galactose-minimal medium. Transduction with phage PBS1 reveals that two of these, *gtaA* and *gtaB*, are linked to *hisA1*, whereas the *gtaC* locus is linked to *argC*. Analysis by deoxyribonucleic acid-mediated transformation indicates that these loci exist in a cluster between the *hisA1* and *argC4* loci. Anomalies in mapping in the group II region of the chromosome exist. The basis of these anomalies is discussed.

In the decade following the discovery of transformation in *Bacillus subtilis* by Spizizen (15), many investigators have used this method to study the chromosomal location of mutations (for reviews, see 5 and 16). Because this technique could be used only in studies of closely linked markers, other techniques such as the analysis of the number of copies of genes during growth and replication (11, 21, 22) and PBS1 transduction have been widely employed in mapping experiments (5). With the exception of occasional anomalies in the map distances observed in reciprocal crosses, a high degree of correlation exists among these methods (1, 5). Thus, it has been possible to localize the gross position of the markers by transduction with PBS1 and to perform the fine structure analysis by deoxyribonucleic acid (DNA)-mediated transformation and transduction with SP10.

The observation that resistance to many viruses that infect *B. subtilis* was frequently associated with deletion of glucose from teichoic acid provided a simple method for isolation of a large number of mutants defective in carbohydrate metabolism (23). Thus, it was possible to ascertain whether the loci governing glucosylation of teichoic acid were confined to one region of the chromosome. Although we were not surprised to learn that the genes regulating glucosylation of teichoic acid (F. E. Young, C. Brown, and B. E. Reilly, Bacteriol Proc., p. 56, 1968) and the structure and function of flagella (G. Grant and M. I. Simon, Bacteriol. Proc., p. 29, 1968) were in one region of the chromosome, we were amazed to discover that the recombination between two linked markers (*hisA1* and *argC*) was anomalous. The experiments presented below clearly demonstrate that two of the genes governing phage resistance (*gtaA* and *gtaB*) are linked to the *hisA* locus, and one of the genes, *gtaC*, is linked to *argC*. Despite the problems of mapping with PBS1 in this portion of the chromosome, the data demonstrate that the genes governing glucosylation of teichoic acid exist in a cluster in the group II region of the chromosome.

MATERIALS AND METHODS

Strains. To minimize genetic incompatibilities among variants of *B. subtilis*, all mutant loci were transferred to *B. subtilis* 168 by DNA-mediated transformation with high concentrations of DNA (congression; 12). These mutants are designated in Table 1 according to the convention of Demerec et al. (4). We have utilized the classification of C. Anagnostopoulos (*personal communication*) for methionine-requiring mutants. A mutant which grows only on methionine is called *metC*. Therefore, the mutant *metA3* of Dubnau et al. (5) is designated *metC3*. Mutants which grow on cystathionine, homocysteine, and methionine are designated *metA*. Mutants which respond to homocysteine or methionine are classified as *metB*. Because of an increasing interest in structural components of cells, we have elected to use a genotypic designation for phage resistance. Therefore, the symbol *gta* has been adopted for the pathway involved in glucosylation of teichoic acid in *B. subtilis* 168.

[1] Presented at the Annual Meeting of the American Society for Microbiology, Detroit, Mich., May 1968.

[2] Faculty Research Associate, American Cancer Society.

[3] Present address: Department of Biology, New Mexico State University, Las Cruces, N.M.

TABLE 1. *Summary of strains*

Strain	Genotype designation	Origin	Parent strain	Source
BR5	*trp-2 ade-1*	Congression[a]	SB22	E. Nester
BR13	*trp-2 ura-1*		SB5	E. Nester
BR19	*trp-2 hisA1*		SB1	E. Nester
BR27	*trp-2 purA16*		Mu8u5u16	N. Sueoka
BR31	*trp-2 metB5*		Mu8u5u16	N. Sueoka
BR51	*trp-2 metC7*		SB26	E. Nester
BR62	*trp-2 purB6*		Mu8u5u6	N. Sueoka
BR70	*trp-2 ile-2*		GSY111	C. Anagnostopoulos
BR75	*trp-2 cys-1*		168	B. Strauss
BR77	*trp-2 thr-1*		Mu8u5u5	N. Sueoka
BR82	*trp-2 pro-1*	NA[b]	168	M. Canner
BR85	*trp-2 argC4*	Congression	168	J. Marmur
BR123	*trp-2 argO1*		279	M. Kelly
BR141	*trp-2 metA1*		GSY402	C. Anagnostopoulos
BR151	*trp-2 lys-3 metB10*		279	M. Kelly
BR290	*trp-2 gtaB290*		168/29	
BC7	*trp-2 gtaC51 hisA1 cysB3*	Congression	BD92/BY51[e]	
BC8	*gtaA12 hisA1 cysB3*		BD92	
BC9	*gtaA12 argC4*	Congression	BR85/BY12[e]	
BC19	*trp-2 argC20*	NGT[c]	168	
BC21	*trp-2 argC21*	NGT	168	
BC137	*hisA1 argC4*	Congression	BR19/BR85[e]	
BC141	*hisA1 argC4*	Congression	BR19/BR85[e]	
BC246	*gtaC38*	PBS1[d]	BY38/BC141[e]	
BC369	*hisA1 argC4 metD1*	NA[b]	BC141	
BY1	*trp-2 gtaA1*	Ultraviolet	168	
BY9	*trp-2 gtaB9*			
BY12	*trp-2 gtaA12*			
BY38	*trp-2 gtaC38*			
BY44	*trp-2 gtaA44*			
BY47	*trp-2 gtaA47*	NG		
BY50	*trp-2 gtaC50*	Ultraviolet		
BY51	*trp-2 gtaC51*			
BY61	*trp-2 gtaB61*			
BY70	*trp-2 gtaC70*		Mu8u5u5(SPO$_2$)Spo$_2$	L. B. Boyce
BY71	*gtaB71*		Cb1-1	J. Szulmajster
BM642	*hisA1 spoC1*	Congression	BR19	M. Rogolsky
BP17	*metA1 argO1*		BR123	
BD45	*hisA1 argC4 leu-8 trp-2*		SB26	D. Dubnau
BD71	*hisA1 argC4 ura-1*		SB3	
BD92	*hisA1 cysB3 trp-2*			

[a] Markers introduced by transformation (12).
[b] Transformation with DNA treated with nitrous acid (10).
[c] Transduction with PBS1 lysates obtained from cells grown on nitrosoguanidine.
[d] Transduction with PBS1.
[e] Donor/recipient.

Mutants defective in glucosylation have been grouped into three classes: *gtaA*, defective in uridine diphosphate (UDP) glucose:polyglycerol teichoic acid glucosyl transferase (TAG transferase); *gtaB*, which behave physiologically as if they were deficient in UDP:glucose pyrophosphorylase (UDPG-PPase), but which do not have a significant enzymatic defect; and *gtaC*, which are defective in phosphoglucomutase. The three classes can be distinguished by their growth in minimal-citrate media supplemented with galactose and casein hydrolysate (23). The growth of *gtaA* strains is stimulated by galactose, *gtaB* strains are not affected by galactose, and *gtaC* strains are inhibited by galactose. The presence of citrate in Spizizen's minimal medium (15) is necessary for the galactose effect. Phage-resistant mutants were obtained by direct

selection of spontaneous mutants with the use of phage φ25 or φ29, or after ultraviolet irradiation of the parent strain and challenge of the surviving population with phage φ25 or φ29 (23).

Additional mutants were sought by transformation of *B. subtilis* 168 with DNA isolated from phage-resistant strains that were treated in vitro with nitrous acid (10). In these experiments, the transformed population was incubated for 2 hr in Difco Antibiotic Medium No. 3 to permit expression of phage resistance, and then was incubated with phage (multiplicity of infection = 0.5) for 18 hr at 37 C. A sample of washed cells was incubated at 37 C in minimal-glucose medium containing the auxotrophic requirements of the strain for 2 hr, treated with penicillin (10,000 units/ml) for 1 hr, incubated with penicillinase (10,000 units/ml) for 15 min, and then plated on Tryptose Blood Agar Base (TBAB, Difco). The surviving colonies were examined for auxotrophic requirements.

Propagation of bacteriophage. The media and methods for preparing and assaying phage were similar to those described previously (23).

Transduction. A modification of the procedures of Takahashi (18) and Hoch et al. (8) was used to prepare lysates and obtain transduction. Unless specified otherwise, all incubation was done at 37 C in a G25 incubator (New Brunswick Scientific Co., New Brunswick, N.J.) at 110 rev/min. Highly motile variants of each donor strain were grown in Antibiotic Medium No. 3 for 2 to 3 hr to produce a final turbidity of 125 to 150 Klett units (Klett-Summerson colorimeter, filter no. 66). A sample of a stock suspension of phage PBS1 was added to a diluted culture of the donor strain (25 Klett units, 5×10^7 cells/ml) to produce a final multiplicity of infection of 0.1. The culture was incubated for 1 hr, then for an additional 2 hr with chloramphenicol (5 μg/ml), and finally for 18 hr without aeration. After centrifugation, the supernatant liquid was incubated with deoxyribonuclease (50 μg/ml) for 15 min at 37 C, and filtered through a type HA filter (0.45 μm; Millipore Corp., Bedford, Mass.). To determine whether the phage in the lysate contained large fragments of host DNA (9), the cotransduction frequencies of the *lys-3*, *trp-2*, and *metB* loci were examined. Only lysates which gave 20 to 30% cotransduction of *lys-3* and *metB* were used.

The recipient strain was grown on TBAB for 18 hr, transferred into 2.5 ml of Antibiotic Medium No. 3 (25 to 40 Klett units), incubated for 5 hr, and then infected with PBS1 at a multiplicity of infection of 1 in a final volume of 1.0 ml. After 15 min, the culture was diluted with 3.0 ml of Spizizen's minimal medium (15) and centrifuged at 8,000 rev/min for 5 min; the pellet was suspended in minimal medium (1.0 ml), and samples (0.1 to 0.2 ml) were plated on minimal-glucose agar (15) supplemented with the amino acids required to meet the auxotrophic requirements for all of the mutant loci except the one used for selection. The transduced clones were subcultured again and examined for nonselected auxotrophic requirements.

Late logarithmic cultures of *B. subtilis* contain two to four spontaneous phage-resistant mutants per 10^6 viable cells. Therefore, one can readily distinguish between phage-sensitive and phage-resistant clones merely by replicating the subcultured transductants on TBAB in the presence and absence of phage. Only rare colonies will be noted in the replicated streak of phage-sensitive transductants. This technique only shows the phenotype. Enzymatic analysis and growth in minimal galactose medium distinguish *gtaA*, *gtaB*, and *gtaC* (23). This number of spontaneous phage-resistant mutants in the late logarithmic cultures is only two- to fivefold lower than the usual number of transductants obtained in our experiments (2×10^{-6} to 1×10^{-5} transductants per plaque-forming unit). Consequently, direct selection for phage resistance following PBS1 transduction proved unsatisfactory.

Transduced clones were examined for the production of spores by replica plating on AK agar (BBL). After 2 days at 37 C and 3 days at room temperature, the brown clones which contained spores were readily distinguished from the pale asporogenic clones.

Transformation. The procedures for preparation of DNA, development of competence, and genetic transformation were similar to those described previously (24), with the exception that cells were grown for 5 hr in medium 1 and 65 min in medium 2. To determine the frequency of transformation to phage resistance, samples of transformed cells were plated on TBAB in 2.0 ml of semisolid medium and incubated at 37 C. At various times, these cultures were covered with a fine film of phage φ25.

Enzyme assays. Mutants were assayed for phosphoglucomutase, UDPG-PPase, and TAG transferase as described previously (23).

RESULTS

Localization of mutants by transduction. Initially, we scanned the chromosome of *B. subtilis* 168 to determine the location of the phage-resistant loci by transducing the BR mutants shown in Table 1 with lysates of PBS1 made on phage-resistant wild-type strains. These experiments (Tables 3, 5, and 6) demonstrate that all of the phage-resistant mutants studied to date are linked either to the *hisA* or the *argC* loci. To

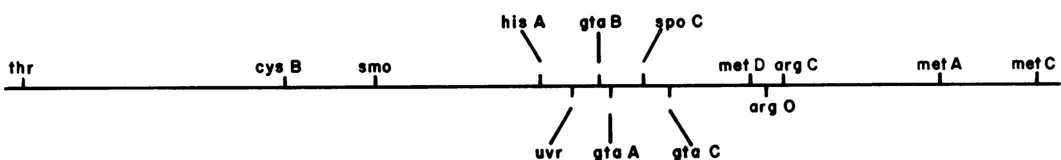

FIG. 1. *Partial map of group II region of the chromosome compiled from data in Tables 3–6 and 8–11.*

facilitate analysis of the data, a composite map of the region is shown in Fig. 1. In double mutants in which one of the defects was in the pathway for arginine biosynthesis, we consistently encountered feeding problems with ornithine, citrulline, or arginine. For instance, in crosses between Arg^- His^- strains with lysates of PBS1 grown on wild-type strains, we frequently observed two to three times more Arg^+ than His^+ transductants. In other experiments in which BC369 (carrying *hisA1*, *argC4*, and *metD1*) was transduced with lysates of PBS1 grown on wild-type, the cotransfer of Met^+ in Arg^+ transductants was 90%, whereas the cotransfer of Arg^+ in Met^+ transductants was greater than 97%. Thus, it appeared as if Arg^- clones were not surviving at the expected frequency. To test this possibility, BC369 (carrying *hisA1*, *argC4*, and *metD1*) was transformed with DNA (0.01 μg/ml) isolated from BR123 (carrying *argO*, *trp-2*). Samples were plated on minimal glucose-agar supplemented with histidine and arginine or with histidine, ornithine, and citrulline to select for Met^+ transformants and on minimal glucose-agar supplemented with histidine and methionine to select for Arg^+ transformants. As shown in Table 2, the number of Met^+ transformants was less than 10% of the Arg^+ transformants. Furthermore, all the Met^+ transformants which survived were also Arg^+. In other experiments, some Arg^- clones were identified. Therefore, in all subsequent experiments, greater reliability was placed on crosses in which Arg^+ transductants were selected and examined for unselected markers rather than crosses in which other markers, such as His^+, were selected and Arg^+ was examined as the unselected trait.

TABLE 2. *Survival of Met^+ and Arg^+ transformants of BC369*

Selection	Transformants per ml	Unselected marker	
		Arg^+	Met^+
		%	%
Arg^+	1,930	—	<1
Met^+	190	100	—

Lysates of PBS1 made on wild-type phage-resistant and wild-type phage-sensitive strains were used to transduce phage-sensitive and phage-resistant auxotrophs, respectively. The percentage of cotransfer of the *gtaB* locus was independent of whether phage sensitivity (Pha^s) or phage resistance (Pha^r) was the unselected marker. The average cotransfer of *gtaB* with *hisA1* is 47% (Table 3). There is some variation in the frequency of cotransfer in different recipients (Table 3, line 1) and with different *gtaB* mutants. A weak but definite linkage exists between the *cysB* locus and the *gtaB* locus (Table 3). No linkage was observed between *gtaB* and *argC* (Table 3). Two additional crosses permitted the positioning of the *gtaB* locus with respect to other known mutants. Rogolsky and Spizizen have reported a linkage between *spoC1* and *hisA1* (M. Rogolsky and J. Spizizen, Bacteriol. Proc., p. 22, 1967). We confirmed this linkage in crosses between phage-resistant mutants and BM642 carrying *hisA1* and *spoC1*. In these experiments, lysates of PBS1 grown on wild-type class A, B, and C mutants were used to transduce BM642. The His^+ transductants were examined for Pha^s and the production of spores (Spo^+) on AK agar. The low number of Pha^s, Spo^+ recombinants (Table 4, line 4) is evidence in favor of the order *hisA1*, *gtaB290*, *spoC1*. The relative position of *gtaB290* with respect to *uvr-1* was determined by a cross between a strain carrying *uvr-1*, *hisA1*, *cysB3*, and *trp-2* and a PBS1 lysate made on BR290 (carrying *gtaB290*). Analysis of this cross indicated the order *hisA1*, *uvr-1*, *gtaB290* (J. Hoch, *in preparation*).

Lysates of PBS1 prepared on different class A phage-resistant mutants were used to transduce various auxotrophs. As shown in Table 5, the *gtaA* locus is linked to *hisA1* with an average cotransfer of 44%. As observed with *gtaB* mutants, the frequency of cotransfer varies when the

TABLE 3. *Linkage of gtaB mutants to the hisA1 locus*

Donor strain	Proportion of phage-resistant recombinants in prototrophic transductants of						
	Histidine auxotrophs			Cysteine auxotrophs		Arginine auxotrophs	
	BR19	BD92	BD71	BR75	BD92	BR85	BD71
gtaB290	267/600	115/400	115/200	5/500	2/200	0/200	1/150
gtaB9	108/200	—[a]	—	6/400	—	0/200	—
gtaB61	189/400	—	—	1/400	—	0/400	—
gtaB71	201/400	80/200	—	2/400	—	0/200	—

[a] Not determined.

same lysate is used to transduce different auxotrophs (Table 5, line 2). There is no significant linkage to the *cysB* or *argC* loci. As shown in Table 4, the presumed order of *hisA1* and *gtaA12* with respect to *spoC1* is *hisA1*, *gtaA12*, *spoC1*.

The *gtaC* mutants are linked to *argC* with an average cotransfer of 9% (Table 6). As noted with the *gtaA* and *gtaB* mutants, those *gta* loci which are linked to *hisA* do not contransduce with *argC* and conversely. The failure to obtain linkage with *metA1* indicates that the *gtaC* locus is located to the left of *argC4* (Fig. 1). Transduction of BM642 carrying *hisA1*, *spoC1* by lysates of PBS1 grown on *gtaC* mutants (Table 4c) demonstrate that there is no linkage between *hisA1 spoC1* and *gtaC51* (Table 4c). In other experiments with lysates made on phage-sensitive strains, it was also not possible to demonstrate linkage of *spoC1* with *argC4*.

TABLE 4. *Analysis of three-factor crosses with hisA, gta, and spoC1 [donor: (a) BY12, (b) BR290, (c) BY51; recipient: BM642 (hisA1, spoC1); selected phenotype: His+ (1)[a]]*

(a) Donor genotype: *gtaA12*
Presumed order: *hisA1*, *gtaA12*, *spoC1*

hisA1	*gtaA12*	*spoC1*	No. of recombinants
1[a]	1	1	65
1	1	0	66
1	0	0	69
1	0	1	0

(b) Donor genotype: *gtaB290*
Presumed order: *hisA1*, *gtaB*, *spoC1*

hisA1	*gtaB290*	*spoC1*	No. of recombinants
1	1	1	40
1	1	0	65
1	0	0	93
1	0	1	2

(c) Donor genotype: *gtaC51*
Presumed order: *hisA1*, *spoC1*, *gtaC51*

hisA1	*spoC1*	*gtaC51*	No. of recombinants
1	1	1	0
1	1	0	51
1	0	0	148
1	0	1	1

[a] The designations 1 and 0 refer to donor and recipient genotypes, respectively.

Transfer of phage resistance by DNA-mediated transformation. To determine the time required for expression of phage resistance, competent populations of *B. subtilis* 168 were incubated with DNA (1 μg/ml) from the three classes of phage-resistant mutants for 30 min at 37 C, and the reaction was terminated with deoxyribonuclease (5 μg/ml). Samples of cells were diluted in 2.0 ml of semisolid medium (47 C), spread on petri dishes containing 25 ml of TBAB, and incubated at 37 C. At various intervals, the plates were spread with phage ϕ25. As shown in Fig. 2, there is a linear increase in phage-resistant clones which reaches a plateau after 120 min at 37 C on TBAB. No differences in expression time were observed among the preparations of DNA from the different classes of phage-resistant strains. The transfer of phage resistance was just as efficient as transformation of any other trait in *B. subtilis*.

Because the *gtaC* mutants lyse on galactose-minimal medium and the growth of *gtaA* mutants is stimulated on galactose, it is possible to determine whether the *gtaA* and *gtaC* loci are linked by transformation. In these experiments, BC7 (carrying *gtaC51*, *hisA1*, *cysB3* and *trp-2*) was transformed with limiting concentrations of DNA (0.3 μg/ml) isolated from BC9 (carrying *gtaA12* and *argC4*). In each of the experiments shown in Table 7, the Gal+ transformants were examined for the unselected traits His+ and Pha_r. The distance between the loci *hisA1*, *gtaC*, and *trp-2* is too great to have any two of these traits in one fragment of transforming DNA;

TABLE 5. *Linkage of gtaA mutants to hisA1*

Donor strain	Proportion of phage resistant recombinants in prototrophic transductants of						
	Histidine auxotrophs			Cysteine auxotrophs		Arginine auxotrophs	
	BR19	BD92	BD71	BR75	BD92	BR85	BD71
gtaA1	105/200	—[a]	—	0/152	—	0/150	—
gtaA12	245/400	145/400	89/180	1/800	0/200	0/200	0/200
gtaA47	139/400	—	—	0/400	—	0/200	—

[a] Not determined.

TABLE 6. *Linkage of gtaC mutants to argC4 and argO1*

Donor strain	Proportion of phage resistant recombinants in prototrophic transductants of						
	Arginine auxotrophs			Methionine auxotrophs		Histidine auxotrophs	
	argC4		*argO1* BR123	*metA1* BR141	*metC7* BR51	*hisA1* BR19	*hisA1* BD92
	BR85	BD71					
gtaC38	23/200	—[a]	—	—	—	—	—
gtaC50	7/200	—	—	—	—	—	—
gtaC51	71/700	11/200	25/200	0/300	0/200	0/500	0/400
gtaC70	85/800	—	—	0/200	—	2/600	0/200

[a] Not determined.

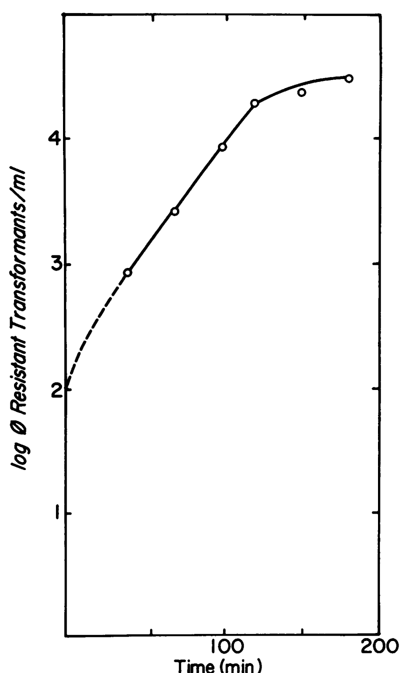

FIG. 2. *Expression of phage resistance in transformed clones. A competent culture of B. subtilis 168 was incubated with DNA (1 μg/ml) isolated from wild-type B. subtilis carrying gtaA12. After 30 min at 37 C with shaking, the reaction was terminated with deoxyribonuclease (5 μg/ml), and samples were plated on Tryptose Blood Agar Base in semisolid agar. After various intervals of incubation at 37 C, the preparation was challenged with phage φ25.*

therefore, the degree of congression (pseudodoubles) was determined by examination of the His+ transformants for the unselected traits Pha^s and Try+ and of the Gal+ transformants for His+. As shown in Table 7, 20% of the Gal+ colonies are still Pha^r. Theoretically, this could occur only if these clones were *gtaA12*, not *gtaC51* (Fig. 3). Therefore, 12 of these clones were examined for

TABLE 7. *Cotransformation of phage resistance loci gtaA and gtaC*

Expt	Selected marker[a]	Unselected markers[b]		Selected marker[a]	Unselected markers[b]	
	Gal+	Pha^r	His+	His+	Pha^s	Try+
1	97	20	ND[c]	72	0	ND
2	68	16	1	67	1	4
3	47	8	1	64	1	2

[a] Number of colonies examined for selected marker Gal+ or His+.

[b] Number of colonies containing the unselected markers in clones selected for the Gal+ or His+ phenotype.

[c] Not determined.

phosphoglucomutase. Eight of these had wild-type levels of phosphoglucomutase and four had levels of phosphoglucomutase which were 2- to 10-fold lower than wild-type. All four of the Gal+ Pha^s clones tested had wild-type levels of phosphoglucomutase. Thus, *gtaA12* and *gtaC51* appear to be linked by transformation; however, it is impossible consistently to link *gtaA12* to *argC4* by transduction.

Anomalies in the group II region. The faiure to obtain a cotransfer of the *gtaA* and *gtaB* loci with *argC4* was indeed surprising, especially in view of the linkage of *gtaA* and *gtaC* by transformation. Therefore, we examined the group II region by use of a series of two point crosses. In all experiments, at least two different recipients were studied. In general, the results (Table 8) were similar to those obtained by Dubnau and coworkers (5). For instance, we obtained an average cotransfer of 32 and 29% for the *thr-5* and *cysB3*, *cysB3*, and *hisA1* loci respectively, as compared with 31 and 22% for the same markers in Dubnau's experiments (5). Two discrepancies were noted. First, the cotransfer of *metC7* with *argC4* (*metA* of Dubnau et al.) was 28%, whereas

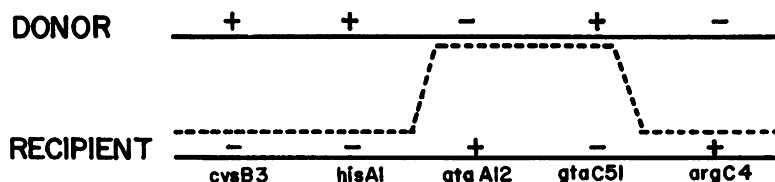

FIG. 3. *Diagram of transformation experiment shown in Table 7. Donor: DNA isolated from BC9 carrying argC4 and gtaA12. Recipient: BC7 carrying cysB3, hisA1, trp-2, and gtaC51.*

TABLE 8. *Linkage values derived from two-factor crosses with PBS1 transduction*[a]

Selected marker	Cotransfer of unselected markers						
	thr-5	*cysB3*	*hisA1*	*argO1*	*argC4*	*metA1*	*metC7*
thr-5	—	31 (93/300)	0 (0/200)		0 (0/200)		
cysB3	33 (198/600)	—	24 (170/725)		0 (0/200)		
hisA1	0 (0/190)	33 (160/491)	—		0 (0/1200)	0 (0/200)	
argO1			0 (0/200)	—		62 (143/231)	
argC4			0 (0/691)		—	60 (168/281)	28 (28/100)
metA1			0 (0/200)	23 (102/449)	27 (116/429)		

[a] The frequency of linkage of unselected donor marker found in selected markers in various recipients. The data are presented as percentage cotransfer. The ratios in the parentheses show the actual number of donor-type recombinants (numerator) and the total number of transductants examined (denominator). These data are a summary of many experiments with the strains listed in Table 1.

Dubnau and co-workers obtained a cotransfer of 10% when Arg^+ clones were examined for cotransfer of a different *metC* mutant. More significantly, although Dubnau et al. reported a 20% cotransfer of *hisA1* with *argC4*, we were not able to demonstrate any linkage between these loci in 13 different experiments in which a total of four recipients were utilized, including those studied by Dubnau and co-workers (5). In aggregate, greater than 3,000 transductants were examined. Therefore, additional *argC* mutants were sought by use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) and ultraviolet. Four mutants were isolated which required arginine or citrulline for growth (*argC*). No linkage was obtained between these mutants and *hisA1*. In other experiments, BR19 (carrying *hisA1*, *trp-2*) was transduced with lysates prepared on wild-type which had been grown in NG. The His^+ transductants were examined for Arg^-. Neither of the two *argC* mutants isolated by this procedure (Table 1) displayed linkage between *hisA1* and *argC*. Six *hisA1*, *argC4* mutants were created by congression in experiments in which BR85 was transformed with DNA isolated from BR19. In one of these, BC141, linkage between *hisA1* and *argC4* was routinely demonstrable by PBS1 transduction. With the use of 11 different lysates, 198 of 2,175 Arg^+ transductants were also His^+. On the other hand, only 8 of the 1,454 His^+ transductants examined were also Arg^+. If the selection was made for His^+ Arg^+ directly by plating the tranductants on minimal-glucose-agar, no transductants were obtained. We attributed this to the problems encountered in feeding Arg^- clones or Arg^- heterogenotes. Consequently, in all subsequent experiments, His^+ and Arg^+ transductants were selected separately and examined for the nonselected marker. Unexpectedly, in most of the crosses, the donor loci determining either phage resistance or phage sensitivity were not identifiable in the recombinants. Representative data from some of the experiments in which BC141 was transduced with lysates made on various phage-resistant strains are shown in Table 9. In all cases, Arg^+ was the selected trait. The possible genotypes of the Arg^+ transductants are shown below the table. Class A, in which only the *argC4* locus is transferred, is the largest class. One would expect most of the His^+ Arg^+ recombinants to contain the donor *gta* locus (class C) on the basis of the map shown in Fig. 1 and the transformation data (Table 7). If multiple exchanges occurred, one would find some class D recombinants also. Surprisingly, in only one of the crosses (Table 9, line 4) were most of the His^+ Arg^+ recombinants also phage-resistant. A lysate of PBS1 was prepared on one of the His^+, Arg^+ Pha^r recombinants (BC246) obtained in the experiment summarized in Table 9, line 4. As shown in line 5 of Table 9, a backcross with BC141 carrying *hisA1* and *argC4* did not yield an

TABLE 9. *Classes of recombinants of BC141 obtained by transduction with lysates of PBS1 prepared on different phage-resistant mutants*[a]

Mutant	Recombinant class[b]			
	A	B	C	D
BY12 (*gta*A12)	193	0	0	7
BY44 (*gta*A44)	506	0	0	69
BR290 (*gta*B290)	124	0	0	26
BY38 (*gta*C38)	165	26	8	1
BC246 (*gta*C38)	188	11	0	1

[a] Donor: wild type carrying *gtaA* or *gtaB* or *gtaC*. Recipient: BC141 carrying *hisA1*, *argC4* (His⁻ Arg⁻ Pha^s). Selection: Arg⁺.

[b] Class A recombinants contain the donor *argC* locus and the recipient *gta* and *hisA* loci; class B recombinants contain the donor *argC* and *gta* loci and the recipient *hisA* locus; class C recombinants contain the donor *argC*, *gta*, and *hisA* loci; class D recombinants contain donor *argC* and *hisA* loci and the recipient *gta* locus. Consistent with data from Tables 3, 5, and 6, only the *gtaC* locus is linked to *argC*.

increased number of His⁺ Arg⁺ clones. In fact, the only His⁺ Arg⁺ clone was Pha^s. Similarly, when the His⁺ clones were examined, seven were found to be Arg⁺. All of these were Pha^s. The only Pha^r, His⁺ recombinant was Arg⁻. Thus, there was no greater transfer of the *hisA1*, *gtaC51*, and *argC4* loci in this backcross.

The introduction of a suppressor for His⁻ linked to *argC* could produce His⁺ Arg⁺ recombinants in BC141. To test for this, lysates of PBS1 made on five His⁺ Arg⁺ recombinants were used to transduce BD92 carrying *cysB3* and *hisA1*. Since the expected frequency of Cys⁺ His⁺ transductants was obtained (29%), a suppressor was unlikely. Furthermore, His⁻ Cys⁺ recombinants were not detected in crosses between these lysates and strains carrying *cysB3*. Because *cysB3* is linked to *hisA* (Table 8), we should have isolated His⁻ clones if the His⁺ phenotype in the donor was due to a suppressor linked to *argC4*.

Additional markers in the group II region. To obtain other mutants linked to *gtaC*, DNA from BY51 was treated with nitrous acid (10), and the Pha^r transformants were examined for additional amino acid requirements. A methionine auxotroph which would not grow on any of the intermediates of the methionine pathway was isolated. Because DNA isolated from this strain, BC369, will transform mutants carrying *metA1*, *metB10*, and *metC7* at the same frequency as *trp-2*, it has been designated *metD1*. As shown in Table 10, this locus is cotransferred by PBS1 with *argC4* at a frequency of 90%. Analysis of the

TABLE 10. *Analysis of crosses with mutant BC369 [donor lysate 168; recipient BC369 (hisA1 gtaC51 metD1 argC4)]*

(a) Selection Arg⁺

Recombinants				Expt no.	
hisA1	*gtaC*	*metD1*	*argC4*	1	2
1[a]	1	1	1	0	0
0	1	1	1	3	0
1	0	1	1	21	11
0	0	1	1	160	164
0	1	0	1	2	0
0	0	0	1	15	25
				201	200

(b) Selection Met⁺

Recombinants				Expt no.	
hisA1	*gtaC51*	*metD1*	*argC4*	1	2
1	1	1	1	0	0
0	1	1	1	1	1
1	0	1	1	30	23
0	0	1	1	169	164
0	0	1	0	0	11
0	1	1	0	0	1
				200	200

[a] The designations 1 and 0 refer to donor and recipient genotypes, respectively.

crosses also revealed an anomalous recombination with *gtaC*. Although 8% of the *Arg⁺* clones were also His⁺, none of these was Pha^s. Similarly, none of the Met⁺ His⁺ transductants was Pha^s. Therefore, it was not possible to transfer the entire *hisA1*, *gtaC51*, *metD1*, *argC4* region of the chromosome.

The presumed order of *metD1*, *argO1*, *argC4*, and *metA1* was established by three-point crosses. A lysate of PBS1 prepared on BP17 (carrying *metA1* and *argO1*) was used to transduce BR85 (carrying *argC4*, *trp-2*). The Arg⁺ clones were examined for *metA1*. If the number of Met⁻ Arg⁺ transductants was negligible, the order would be *argC4*, *argO1*, *metA1*, whereas the normal frequency of cotransfer of *metA1* (30 to 60%, Table 8) would indicate the order *argO1*, *argC4*, *metA1*. Because 26 of 73 Arg⁺ transductants were Met⁻, the probable order is *argO1*, *argC4*, *metA1*.

The distribution of unselected markers in Arg⁺ transformants was also used to establish the position of *metD1* with respect to *argC4* and *argO1*. Congression was minimized by using concentrations of DNA which were less than 0.1 μg/ml.

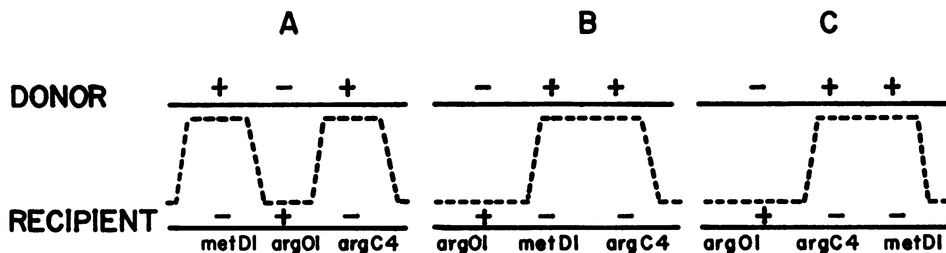

FIG. 4. *Possible position of loci metD1, argO1, and argC4 in the experiments summarized in Table 11.*

TABLE 11. *Location of metD1 by DNA-mediated transformation*

Genotype of		Phenotype of Arg+ recombinants		Cotransfer[a]
DNA	Recipient	Met−/Arg+	His−/Arg+	
				%
argO1	*hisA1, metD1, argC4*	193/193	0/193	0
Wild type	*hisA1, metD1, argC4*	177/206	199/206	14
hisA1, metD1	*argO1*	19/104	0/104	18

[a] Transfer of donor *metD* locus to Arg^+ recombinants.

The distance between *hisA1* and *argC4* is greater than can be transferred on one fragment of DNA; therefore, the number of His^+ Arg^+ transformants serves as a control for congression. Thus, as shown in Fig. 4, only the position of the loci shown in cross A would result in the rare Arg^+, Met^+ transformants obtained in this experiment (Table 11, line 1). The presumed order *metD1*, *argO1*, *argC4* is further substantiated by a greater cotransfer of *metD1* with *argO1* than with *argC4* (Table 11, lines 2 and 3). Correcting for the number of Met^+ Arg^+ psuedodoubles produced by congression (reavealed by the number of His^+ Arg^+ transformants), the cotransfer of *metD1 argC4* is approximately 10%.

A linkage between the rough marker in SB26 and *hisA1* was observed by G. Grant (*personal communication*). We confirmed this in a cross using a lysate made on BP17, carrying the smooth colonial morphology (*smo-1*) *argO1* and *metA1*, and BC8 carrying *cysB3*, *hisA1*, and *gtaA12*. Analysis of the transductants revealed that 49% of the Cys^+ transductants were smooth, 29% of the His^+ transductants were smooth, and 96% of the Cys^+ His^+ transductants were smooth. Thus, the *smo-1* marker is most probably located as shown in Fig. 1.

DISCUSSION

Although this study has clearly demonstrated that the *gtaA* and *gtaB* loci are linked to *hisA1* and that the *gtaC* locus is linked to *argC*, it has revealed limitations in mapping with phage PBS1. The anomalies encountered in mapping loci adjacent to the *argC* locus have produced a formidable barrier. The nonreciprocal cotransduction of the *hisA*, *metD*, and *metA* loci with the *argC* locus is most probably due to the inability to feed Arg^- clones. A similar nonreciprocal cotransduction was observed by Dubnau (5) between *argC4* and *metA3*. The inability to select directly for His^+ Arg^+ recombinants is also probably due to lack of survival of the Arg^+/Arg^- heterogenote.

The investigation of Dubnau and co-workers revealed a linkage between *hisA1* and *argC4* by two techniques, PBS1 transduction and analysis of marker frequency following density shift (5). We were unable to obtain linkage between the *hisA1* and the *argC4* loci in our study with either our reference strains or those of Dubnau. The only strain in which linkage could be consistently established between the *hisA1* and *argC4* regions was BC141, a strain derived by transformation of BR85 with DNA isolated from BR19. Yoshikawa observed a mutation frequency of 20% in competent cells that were transformed by DNA (20). Obviously, further studies are required to determine whether there is a deletion or inversion in BC141. Recently, G. Grant (Ph.D. Thesis, Univ. of California, San Diego, 1968) observed that some of the asporogenic mutants isolated by treatment of BD71 with acridine orange displayed a linkage between *hisA1* and *argC4*. Experiments are in progress to determine whether this is due to an additional mutation induced by acridine (17) or due to curing of an episome (13).

In BC141, one does not usually obtain the expected recombination of donor loci controlling phage sensitivity with most lysates (Table 9). We have also observed this in crosses with strains carrying only *hisA1* and *metD1*. Therefore, the anomaly cannot be attributed to problems of feeding arginine auxotrophs. Hayes (7) suggested that differences observed between recombination of linked markers, *thr ara leu*, by transduction with P1 and conjugation may be due to areas of nonhomology. As with the *hisA1*, *argC4* segment of the chromosome of *B. subtilis*, the *thr*, *leu* genes approach the maximal length of the transducible fragment (7). Therefore, the selected genes could fall within separate but effectively paired regions. With double recombination events, it is possible for the middle marker, *ara*, to be excluded. With transfer of a longer segment in conjugation, there is a higher probability of terminal pairing on either side of the entire segment *thr ara leu*. In other strains of *E. coli*, this exclusion of the *ara* locus was not observed (19). By analogy, the nonreciprocal cotransduction in the *hisA1*, *argC4* region suggests that the fragments of DNA introduced by PBS1 transduction may not be completely homologous. M. D. Chilton (*personal communication*) demonstrated that the DNA-DNA homology between *B. subtilis* 168 and *B. subtilis* W23 in the region of the chromosome around the *hisA* locus is very low. Repeated mutation and reversion of the *gta* loci and the genes governing the structure of flagella, which are unessential for the life of the cell but critical for virus adsorption, may have produced areas of nonhomology.

Alternatively, the problems encountered in mapping in the *hisA*, *argC* loci could be related to a selective breakage of the chromosome by PBS1, induction of a defective virus (14), or the presence of an episome. Two observations indicate that PBS1 is pseudolysogenic in *B. subtilis*. Csiszár and Ivanovics (2) were able to distinguish small glistening lysogenic colonies from the nonlysogenic clones on yeast extract-peptone-agar after transduction with phage 3NT. This phage is similar to or identical with PBS1 (8). Secondly, most PBS1 transductants are initially resistant to phage ϕ1. After four serial subcultures, less than 20% of the clones are resistant. Thus, the discrepancy between the absence of His$^+$ Arg$^+$ transductants on direct selection and the identification of 10% His$^+$ Arg$^+$ transductants when His$^+$ is the unselected trait may be related to pseudolysogeny with PBS1 or an unstable heterogenote. Recently, Eisenstark et al. observed heterogeneity of transducing particles of P22 (6). In these experiments, the transducing fragments that covered the *argB*, *thy lys* region are not homologous in regard to the beginnings and ends. The establishment of linkage between *gtaA* and *gtaC* by DNA-mediated transformation suggests that there may be artifacts of chromosomal breakage encountered in transduction in this region or differences in recombination in PBS1 transduction and transformation. The latter is supported by the observation that PBS1 transduction is not effected by the *rec-1* locus to the same extent as SP10 transduction or DNA-mediated transformation (8). In view of the anomalies encountered in the *hisA1 argC* region, a detailed genetic analysis must await the isolation of more auxotrophic mutants and the identification of a better phage for general transduction.

ACKNOWLEDGMENTS

This study was aided by grant E 288 E from the American Cancer Society and Public Health Service grant GM12956-03 from the National Institute of General Medical Sciences.

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