Sequential Replication of the Chromosome of
Bacillus licheniformis[1]

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A sequential replication map of the chromosome of *Bacillus licheniformis* was constructed by employing the method of gene-frequency analysis presented by Yoshikawa and Sueoka. Our analysis of 11 genetic markers was based on the hypothesis that the chromosome initiated replication at a fixed origin and proceeded in a linear fashion to the terminus. The proposed locations of markers were validated by cotransformation and cotransduction analyses. Bacteriophage SP-15 cotransduced markers that failed to show linkage by transformation.

A unique method, employing genetic transformation for demonstrating sequential replication of the bacterial chromosome, was presented by Yoshikawa and Sueoka (27). According to their model, the chromosome replicates in a single sequential order and the frequency of a marker in replicating cells is a function of its location on the chromosome. Using the *Bacillus subtilis* transformation system, they constructed a map of the *B. subtilis* W23 chromosome by comparing the frequencies of markers in deoxyribonucleic acid (DNA) extracted from exponentially growing cells to those in DNA extracted from stationary-phase cells. The stationary cells contain non-replicating chromosomes and give uniform frequencies of all markers. The proposed map was then confirmed by the use of density-transfer experiments (28).

When gene-frequency analysis experiments were first performed with *B. subtilis* W168, replication polarity was not demonstrable (21), but Yoshikawa, O'Sullivan, and Sueoka (26) showed that the apparent lack of polarity of replication was an artifact. Unlike *B. subtilis* W23, *B. subtilis* W168 lacks rigid regulation of initiation of DNA synthesis, and cells from the stationary phase of growth do not contain completed chromosomes. When marker frequencies from exponentially growing cells were compared to spore DNA, it was found that W168, like W23, initiates replication at a defined point and proceeds in a sequential order to the terminus.

The development of a bacterial transformation

system for *B. licheniformis* (10, 15, 25) permitted us to extend the use of gene-frequency analysis to a study of the replication of the chromosome of *B. licheniformis*.

MATERIALS AND METHODS

Bacterial strains. The various cultures of *B. licheniformis* employed during this investigation are shown in Table 1. Multiply marked auxotrophic mutants were obtained by ultraviolet irradiation, employing the method of Iyer (12), or were constructed by bacterial transformation by the method of Thorne and Stull (25).

Mutants have been assigned tentative genotypic designations in accordance with the recommendations of Demerec et al. (4).

Bacteriophage. The generalized transducing bacteriophage SP-15 was supplied by M. J. Taylor Stedman; it was propagated on *B. licheniformis* 9945A and FD01 by the methods of Taylor and Thorne (23). We are also indebted to Mrs. Stedman for donating SP-15 phage antiserum and preimmune serum.

Media. Minimal 1, NBSG, NBSG-X, NBY, and TM have been described by Thorne and Stull (25). Potato-extract medium (24) was solidified by the addition of agar (20 g/liter).

Preparation of spores. Spores for inocula in transformation and transduction experiments were prepared by inoculating 25 ml of Trypticase Soy Broth (BBL) in a 250-ml Erlenmeyer flask with a loopful of growth from a potato-extract medium slant. The cultures were incubated at 37 C on a rotary shaker (250 rev/min) until most of the cells had sporulated. The spores and cells were sedimented by centrifugation at 12,000 × g for 20 min, and each pellet was suspended in 5 ml of sterile distilled water. The suspensions were heated at 65 C for 45 min and stored at 4 C. These preparations usually contained 10^9 spores/ml.

Spores from which DNA was to be isolated were produced by inoculating FD01 spores into forty

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TABLE 1. *Description of Bacillus licheniformis cultures*

Strain	Previous name[a]	Genotypic designation[b]	Origin or reference
9945A		Wild type	Gwinn and Thorne (10)
FD01	9945AS[r]	*str-1*	Goldberg, Gwinn, and Thorne (9)
FD03	M28-RT-M20-RT-T10	*pep-1 pur-1*	Thorne and Stull (25)
FD07	M28-RT-M20-RT-T10-D3	*pep-1 pur-1 met-1*	Fort Detrick, Frederick, Md.
FD08	M28-RT-M16	*pep-1 ura-1*	Fort Detrick, Frederick, Md.
FD09	M28-RT-M19	*pep-1 trp-1*	Thorne and Stull (25)
FD010	M28-RT-M13	*pep-1 tyr-1*	Thorne and Stull (25)
FD6		*pep-1 pur-1 leu-2*	Ultraviolet (UV) irradiation of FD03
FD19		*pep-1 pur-1 leu-2 his-3*	UV irradiation of FD6
FD21		*pep-1 pur-1 leu-2 lys-3*	UV irradiation of FD6
FD28		*pep-1 pur-1 leu-2 arg-3*	UV irradiation of FD6
FD31		*pep-1 pur-1 leu-2 gly-2*	UV irradiation of FD6
FD50		*pep-1 pur-1 ura-1*	FD08 →[c] FD03
FD51		*pep-1 pur-1 trp-1*	FD09 → FD03
FD52		*pep-1 pur-1 trp-1 tyr-1*	FD010 → FD51
FD54		*pep-1 pur-1 leu-2 gly-2 tyr-1*	FD010 → FD31
FD55		*pep-1 pur-1 met-1 trp-1*	FD09 → FD07
FD56		*pep-1 pur-1 met-1 ura-1*	FD08 → FD07
FD57		*pep-1 pur-1 leu-2 gly-2 arg-3*	FD28 → FD31
FD58		*pep-1 pur-1 leu-2 his-3 arg-3*	FD28 → FD19
FD59		*pep-1 pur-1 leu-2 lys-3 tyr-1*	FD010 → FD21

[a] Several of the strains listed have previously been designated with different names. These are indicated, where applicable, with pertinent literature citations.

[b] Abbreviations used: *pep*, inability to synthesize glutamyl polypeptide; for nutritional requirements: *pur*, adenine, guanine, hypoxanthine, or xanthine; *arg*, arginine; *gly*, glycine; *his*, histidine; *leu*, leucine; *lys*, lysine; *met*, methionine; *trp*, tryptophan; *tyr*, tyrosine; *ura*, uracil; for resistance marker: *str*, resistance to streptomycin.

[c] Constructed by transformation. The head of the arrow indicates the recipient strain.

250-ml flasks, each containing 50 ml of Schaeffer's sporulation medium (20). The cultures were incubated on a rotary shaker at 37 C for 24 hr and then placed at 4 C for 3 days to allow lysis of vegetative cells. The spores were collected by centrifugation at 11,700 × *g* for 30 min and suspended in 200 ml of sterile distilled water. The suspension again was centrifuged for 20 min, and the pellet was then suspended in 100 ml of 0.15 M NaCl-0.1 M ethylenediaminetetraacetate (*p*H 8.0); 10 ml of lysozyme (40 mg/ml) was added, and the suspension was incubated at 37 C for 30 min with occasional shaking. Additional lysozyme (5 ml) was added, and incubation was continued for an additional 30 min; 4 ml of sodium lauryl sulfate (saturated in 45% ethyl alcohol) was added, and the mixture was incubated with occasional shaking for 20 min at 37 C. The suspension was centrifuged at 12,000 × *g* for 20 min, and the viscous supernatant fluid was discarded. The pellet was resuspended in 200 ml of sterile distilled water and then centrifuged again for 20 min. The spores were washed with 200-ml portions of water five times. After the last washing, they were resuspended in 10 ml of distilled water and stored at 4 C until used. No intact cells were observed in the final suspension.

DNA preparations. For routine transformation studies, FD01 cells were grown and DNA was isolated as described by Marmur (16) and employing modifications of Gwinn and Thorne (10). DNA concentrations were usually determined by the method of Burton (3). DNA used for gene-frequency analysis was extracted from FD01 cells grown in Difco Penassay Broth (12.2 g/liter). Batch cultures (300 ml/Fernbach flask) were grown on a reciprocal shaker (5-cm stroke, 100 excursions/min) at 37 C. The optical density of the cultures was followed (Bausch & Lomb Spectronic-20 colorimeter), and, at various times during the growth period, cultures were removed and heated at 65 C for 10 min (Fig. 1) and DNA was then isolated as described. All preparations except no. 1 were contaminated with spores. Samples 2, 3, 4, and 5 were subsequently sterilized with hot phenol (10).

For cotransformation experiments, DNA was isolated from FD01 cells grown in NBY broth (10) by the benzoate-phenol method of Kelly and Pritchard (14).

To isolate DNA from spores, 2.8 g (wet weight) of clean spores was suspended in 5 ml of buffer [10 mM tris(hydroxymethyl)aminomethane, 0.1 mM MgCl₂, and 10⁻⁴ M spermine, *p*H 8.0]; 0.2 ml of saturated sodium lauryl sulfate in 45% ethyl alcohol was added to the buffer just before use. Cold alumina (5 g; Alcoa
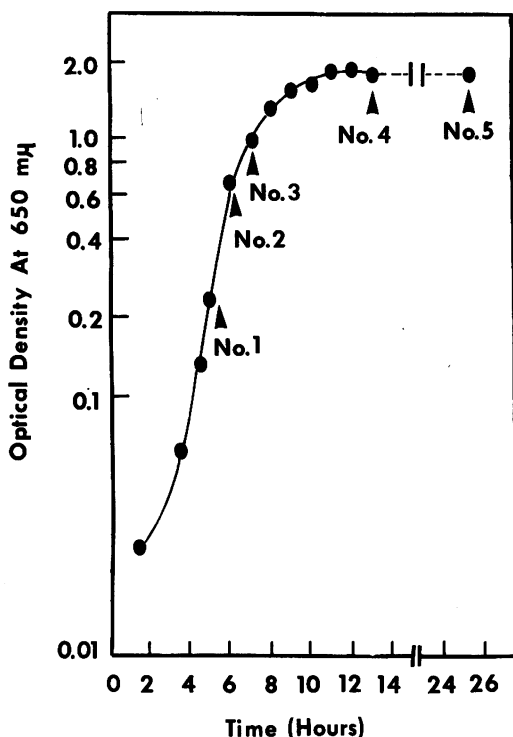
FIG. 1. *DNA samples from various growth phases of Bacillus licheniformis in Penassay Broth. Cells were grown in batch cultures at 37 C with shaking.*

A-301) was added to a precooled mortar. The spores were added, mixed with a glass rod, and ground thoroughly with a pestle for 4 min. Cold buffer (10 ml) was used to transfer the mixture to a sterile centrifuge tube, and the contents were centrifuged at 30,900 × g for 30 min to remove unbroken spores and alumina. The supernatant fluid was transferred to a sterile ground-glass-stoppered 250-ml flask; 2.6 ml of 5 M NaClO₄ was added and mixed (with wrist action). Then 6 ml of chloroform and 0.6 ml of isoamyl alcohol were added, and the tube was shaken vigorously for 10 min. The mixture was centrifuged at 30,900 × g for 20 min, and the aqueous phase (ca. 20 ml) was transferred to two test tubes; 20 ml of 95% ethyl alcohol was added to each tube, and the contents were mixed and then allowed to stand at 4 C for 4 hr. The slightly cloudy mixtures were centrifuged at 30,900 × g for 30 min, and the minuscule pellets from both tubes were dissolved in 1 ml of 0.15 M NaCl–0.015 M sodium citrate. The preparation was brought to 2 M NaCl and filter-sterilized (HA membrane, 0.45 μ pore size; Millipore Corp., Bedford, Mass.). The final product contained approximately 100 μg of DNA/ml as estimated by its transforming activity.

Transformation procedures. Transformations were performed by employing the methods of Thorne and Stull (25). Most auxotrophs were highly transformable

(1 to 3%) after 23 to 24 hr of incubation in NBSG-X. Two of the auxotrophs required unusual conditions to attain competence for transformation. FD50 and FD52 were grown for 19.5 hr in NBSG-X supplemented with uracil (30 μg/ml) and L-tryptophan (30 μg/ml), respectively. Recipient cells were incubated in the presence of three different nonsaturating levels of DNA for 3 hr, and the reactions were terminated by the addition of 50 μg (per ml) of deoxyribonuclease (once crystallized; Worthington Biochemical Corp., Freehold, N.J.). Cells untreated with DNA were run as controls in all experiments. Revertants were rarely observed.

Transduction procedures. Cells for transduction were grown by the method of Taylor and Thorne (23). A mixture containing 1 ml of phage (ca. 5.5 × 10⁹ plaque-forming units/ml) and 0.1 ml of deoxyribonuclease (2 mg/ml in 0.1 M MgSO₄·7H₂O) was preincubated at 37 C for 15 min to ensure that results were not complicated by transformation from bacterial DNA. Recipient cells (2.7 × 10⁹ to 5.5 × 10⁹ contained in 0.9 ml broth) were added, and the complete mixture was incubated at 37 C on the reciprocal shaker for 45 min. A cell control was always run simultaneously [0.9 ml of cells, 0.1 ml of deoxyribonuclease, and 1.0 ml of peptone diluent with 15% glycerol (v/v)]. A control containing phage inactivated with SP-15 antiserum was not run with each experiment, but was performed several times to ensure that results were due to SP-15-mediated transduction.

Scoring of transformants and transductants. The cultures were plated on minimal 1 agar plus thiamine (10 μg/ml) supplemented with the specific growth requirements for the various classes of recombinants (50 μg/ml for amino acids and 30 μg/ml for purines or pyrimidines). Many classes of recombinants grew more rapidly when thiamine was incorporated into the minimal medium. Thiamine was omitted from the medium when scoring for *pep⁺* transformants because its presence permitted the *pep* population to overgrow and mask the *pep⁺* colonies. The numbers of transductants obtained when plated in the presence and absence of SP-15 antiserum were identical. All plates were incubated at 37 C. Transformants were usually scored between 48 and 72 hr. Transductants were usually scored at 48 hr.

Mapping procedures. Relative frequencies of markers in DNA samples from exponentially growing cells, stationary-phase cells, and spores were compared. For each marker and for each DNA sample, the number of transformants obtained was normalized to the number obtained for the *pur* marker, which was common to all of the recipient auxotrophs. The normalized values were again normalized to the value for the spore DNA sample. The frequency distribution of markers as a function of their position on the chromosome was calculated by the methods of Yoshikawa and Sueoka (27) and Sueoka and Yoshikawa (22).

RESULTS

Establishment of the mapping system. Initial experiments showed that a significant difference

TABLE 2. Calculation of relative frequencies of markers pur and met in DNA from exponential-growth phase and spores in Bacillus licheniformis

DNA concn	Exponential phase[a]			Spore			Relative ratio, exponential/spore	
	No. of transformants[b]		Ratio, pur/met	No. of transformants[b]		Ratio, pur/met	Observed	Avg
	pur⁺	met⁺		pur⁺	met⁺			
μg/ml								
0.1	754	691	1.09	313	536	0.58	1.88	
0.05	367	288	1.27	166	289	0.57	2.23	2.04
0.01	832	712	1.17	324	558	0.58	2.02	

[a] Sample no. 1, Fig. 1.
[b] The recipient strain was FD07. Numbers given are total transformants scored for each marker from five plate counts. For plating, the recipient populations were diluted 10⁻² for 0.1 μg and 0.05 μg (per ml) of DNA, and 10⁻¹ for 0.01 μg (per ml) of DNA.

TABLE 3. Relative frequencies of pur to various markers in DNA from Bacillus licheniformis FD01[a]

Recipient strain	Markers	DNA extracted from				
		Exponential cells			Stationary cells	
		No. 1	No. 2	No. 3	No. 4	No. 5
FD31	pur/gly	0.99	1.22			
FD50	pur/pep	1.04	1.41			
FD19	pur/his	1.09	1.10	1.10*	1.24	1.15*
FD28	pur/arg	1.16	1.12			
FD19	pur/leu	1.40	1.35	1.32*	1.29	1.19*
FD50	pur/ura	1.93	1.88			
FD52	pur/trp	2.01	2.02			
FD52	pur/tyr	1.86	2.30			
FD21	pur/lys	2.03	2.05			
FD07	pur/met	2.06	2.20	1.50*	1.56	1.57*

[a] The numbers of the DNA samples are the same as in Fig. 1. The ratios (pur/marker) for spore DNA are taken as 1.00 in this set of data. These values are the averages of several repeated experiments (except those marked with an asterisk, which are based on one experiment), in which more than 1,000 colonies of each class were scored.

existed between frequencies of pur and met for exponential-phase DNA and spore DNA, and that the ratios of markers were independent of the DNA concentrations employed (Table 2). Table 3 presents the compiled ratios of pur to various markers from the different growth phases normalized to spore DNA. All values, except where noted, were based on counts of more than 2,000 colonies for each transformant class.

The ratios from exponential DNA samples between pur and the 10 markers tested, when normalized to spore DNA, did not significantly exceed the value of 2.0. This maximal value was predicted if the chromosome replicated in a one-end oriented fashion as envisaged by Yoshikawa and Sueoka (27). Therefore, we have assumed from our data that cells of B. licheniformis,

when grown in Penassay Broth, possess a single chromosomal replication point.

The data in Table 3 are interpreted as follows. (i) The ratios of individual pairs of markers (e.g., pur/his) were quite similar in DNA isolated from exponential cell samples no. 1 and no. 2. (ii) Provided that spore DNA did indeed represent a state of equal-gene frequency, it is evident that cells in the stationary phase of growth (no. 4 and no. 5) did not represent a condition in which all chromosomes were completely replicated. Marker ratios in the DNA sample from late-exponential-phase cells (no. 3) and stationary-phase cells did not attain the value observed for spore DNA. (iii) Since pur was approximately twice as frequent as several of the markers employed, the mapping

system implied that the *pur* locus was near the origin of the chromosome.

The genomic map of *B. licheniformis* presented in Fig. 2 was constructed by arbitrarily assigning *pur* a value of 0 and mapping the other markers in relation to it. The confidence limits were derived from the theoretical considerations presented by Sueoka and Yoshikawa (22).

Linkage analysis. The considerable overlap exhibited by the confidence limits of the various marker positions on the chromosome indicated
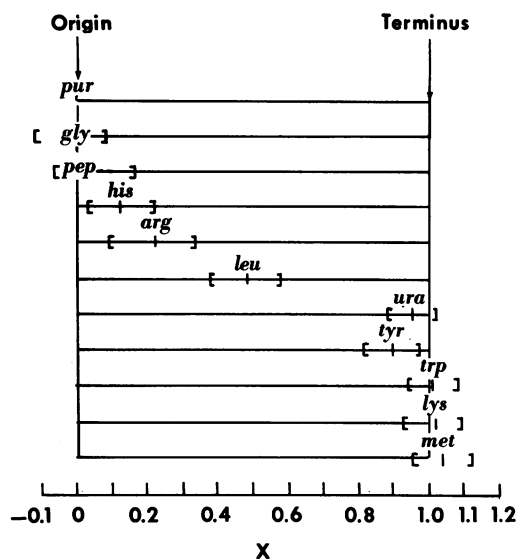

FIG. 2. *Sequential replication map of Bacillus licheniformis. Marker positions, X, were calculated from the frequency data presented in Table 3; pur was arbitrarily assigned a value of 0, and the other markers were mapped in relation to it by using the formula:* $R(pur/X_1) = 2^{X_1 - 0}$.

that it would be difficult to propose an exact sequence for marker replication. Cotransformation experiments showed that trp^+ tyr^+ were strongly linked (Table 4), and met^+ trp^+ were weakly linked (Table 5). Owing to our inability to produce highly competent populations of FD59, we were unable to adequately test lys^+ tyr^+ for cotransformation. Linkage was not observed for other combinations of markers when tested by this method.

SP-10 and SP-15 are generalized transducing bacteriophages for *B. subtilis* and *B. licheniformis* (23). It appeared that transductional analysis of the proposed map with SP-10 would be unsatisfactory because linked markers in *B. subtilis* are cotransduced by SP-10 with the same frequency as observed by cotransformation (8, 11). Although no information was available on the size of SP-15, we elected to use it with hopes that it might transfer DNA segments longer than the transforming fragments, as had been shown in *B. subtilis* with phage PBS-1 (1, 11). Results (Table 6) show that trp^+ tyr^+ were cotransduced with approximately 100% frequency. Strong linkages were also observed for trp^+ met^+, arg^+ leu^+, gly^+ pep^+, his^+ pep^+, and lys^+ tyr^+. The frequencies of cotransduction of various linked markers by phage propagated on 9945A and FD01 were comparable.

The linkage data confirmed the relative positions of the markers on the proposed map. It is apparent that *pur*, *gly*, *pep*, and *his* loci are in the first segment of the chromosome, *arg* and *leu* are in the middle, and *ura*, *trp*, *tyr*, *lys*, and *met* are in the terminal region. The transduction data indicate that *arg* may be located closer to *leu* than presented on the map, and it may not be as close to the *gly-pep-his* region as shown.

TABLE 4. *Cotransformation of* trp^+ *and* tyr^+ *in Bacillus licheniformis*

Concn of DNA[a]	No. of transformed cells/ml[b]					Percentage cotransfer index[c]	
	pur^+	trp^+	tyr^+	pur^+ trp^+	trp^+ tyr^+	pur^+ trp^+	trp^+ tyr^+
μg/ml							
0.1	1.79 × 10⁵	1.54 × 10⁵	1.32 × 10⁵	3.50 × 10²	8.45 × 10⁴	0.15	41.9
0.05	3.93 × 10⁴	3.29 × 10⁴	2.72 × 10⁴	<10	1.89 × 10⁴		45.9
0.025	1.29 × 10⁴	1.08 × 10⁴	1.00 × 10⁴	0	6.60 × 10³		46.5
0.00625	2.90 × 10³	7.50 × 10²	7.25 × 10²	0	4.50 × 10²		43.9
0	0	0	0	0	0		

[a] DNA was isolated by the method of Kelly and Pritchard (14) and was diluted in the presence of *Escherichia coli* carrier DNA (100 μg/ml).

[b] The number of recipient FD52 cells was 2.70 × 10⁸/ml, based on the average of three plate counts. The number of transformed cells was based on the average of two plate counts.

[c] The cotransfer index was calculated according to Nester and Lederberg (18).

TABLE 5. *Cotransformation of trp⁺ and met⁺ in Bacillus licheniformis*

Concn of DNA[a]	No. of transformed cells/ml[b]					Percentage cotransfer index[c]	
	pur⁺	*trp⁺*	*met⁺*	*pur⁺ trp⁺*	*trp⁺ met⁺*	*pur⁺ trp⁺*	*trp⁺ met⁺*
µg/ml							
0.1	2.58×10^4	1.98×10^4	2.48×10^4	8.0×10^1	7.23×10^2	0.18	1.65
0.05	7.40×10^3	8.25×10^3	9.70×10^3	0	2.20×10^2		1.24
0.01	1.83×10^3	1.52×10^3	1.85×10^3	0	8.67×10^1		2.62
0	0	0	0	0	0		

[a] DNA was isolated by the method of Kelly and Pritchard (14) and was diluted in the presence of *E. coli* carrier DNA (100 µg/ml).

[b] The number of recipient FD55 cells was 2.11×10^8/ml, based on the average of three plate counts. The number of transformed cells was based on the average of three plate counts.

[c] The cotransfer index was calculated according to Nester and Lederberg (18).

TABLE 6. *Cotransduction of markers in Bacillus licheniformis with bacteriophage SP-15[a]*

Recipient	Transductants		Cotransduction[c]
	Class[b]	Colonies/ml	
FD52	*trp⁺*	2,550	%
	tyr⁺	2,530	
	trp⁺ tyr⁺	2,520	99.2
FD54	*pur⁺*	8,460	
	leu⁺	3,250	
	gly⁺	2,680	
	tyr⁺	2,390	
	gly⁺ pep⁺	845	31.5
FD55	*trp⁺*	4,450	
	met⁺	4,550	
	met⁺ trp⁺	2,850	63.3
FD58	*pur⁺*	17,400	
	leu⁺	7,000	
	his⁺	13,350	
	arg⁺	6,200	
	leu⁺ arg⁺	2,925	44.3
	his⁺ pep⁺	8,400	62.9
FD59	*lys⁺*	4,150	
	tyr⁺	3,610	
	lys⁺ tyr⁺	2,470	63.7

[a] Donor strain, 9945A or FD01.

[b] Only groups showing positive linkage have been included. All other combinations of markers possible with the mutants presented in Table 1 were not cotransduced.

[c] Calculated according to Barat et al. (1).

DISCUSSION

We have based our analysis of the data presented on the replication of the *B. licheniformis* chromosome on the chromosomal replication model presented by Yoshikawa and Sueoka (27);

i.e., the replication proceeds from a single point and continues in an oriented sequential fashion. Models consisting of separate replicating subunits cannot be ruled out, and they may invalidate the mapping scheme if they exist. The placement of markers by gene-frequency analysis has occasionally been questioned. For example, the *trp* region on the *B. subtilis* W23 chromosome, whose original location was proposed by Yoshikawa and Sueoka (27), has since been assigned a map position nearer the terminus of the chromosome in accord with other experimental observations (1, 14, 17) and with more recent marker-frequency analysis (19). Nevertheless, the basic tenet of gene-frequency analysis has been supported by other experimental procedures during the past 4 years, notably by genetic mapping by means of density-transfer methodology (28), transformation, and transduction (6). A map constructed by gene-frequency analysis shows the probable replication order of markers and approximately defines the replication origin and terminus.

In constructing a map of *B. licheniformis*, we were aware of limitations in the method. The map rests, for example, on the validity of our assumption that the DNA from spores does contain an equal frequency of genes. The precision with which the location of individual markers can be assigned is restricted to relatively large segments of chromosomal length; for closely linked markers, even the order may be in doubt, since wide overlaps in positions result when the theoretical errors are calculated (22). However, the method has allowed us to construct a rough topography of the chromosome with an apparently well-defined origin. The precise order of most of the markers will have to be determined by a combination of the techniques to which we have previously referred.

The apparent shift of *gly* and *pep* markers in samples no. 1 and no. 2 of DNA isolated from

logarithmic cells (Table 3) is not readily explainable. The DNA governing the biosynthesis of these two products could have been denatured by the extended period of heat used in the sterilization of the DNA by hot phenol. However, the cotransduction found for his^+ pep^+, as well as the apparent lack of linkage between gly^+ arg^+, gly^+ leu^+, and leu^+ pep^+, clearly indicate that gly and pep are located on the first segment of the chromosome. The inability to demonstrate linkage between pur^+ gly^+, pur^+ his^+, and pur^+ pep^+ suggested that gly was further from pur than the map shows.

From the high frequencies of cotransduction of various combinations of markers observed, we concluded that either bacteriophage SP-15 carries larger pieces of DNA than are present in any of our transforming DNA preparations, or transforming DNA carrying widely separated linked loci may be fragmented during uptake by competent cells. The latter possibility cannot be ruled out, as Kelly (13) presented evidence that linkage values can be decreased by two separate recipient cells attempting to take up a single DNA molecule. Electron microscope photographs (Tyeryar and Vaituzis, *unpublished data*) show that SP-15, like PBS-1, is a relatively large bacteriophage. It appears that SP-15 will be an extremely useful vector for fine mapping of the *B. licheniformis* genome.

B. licheniformis and *B. subtilis* are very closely related, yet separate and distinct species. *Bergey's Manual* (2) differentiates between the two species by the ability of *B. licheniformis* to reduce nitrates to nitrites with the production of gas under alkaline, anaerobic conditions and to yield good growth under anaerobic conditions in glucose broth. Genetic differences are indicated by differences in guanine plus cytosine base compositions, i.e., 46% versus 43% for *B. licheniformis* and *B. subtilis*, respectively (7, 9), and the lack of interspecific bacterial transformation for auxotrophic markers (9). Employing techniques of DNA-ribonucleic acid (RNA) hybridizations, Doi and Igarashi (5) presented evidence suggesting that many members of the genus *Bacillus* possess a small number of identical nucleotide sequences. Genetic similarities between *B. subtilis* and *B. licheniformis* have been demonstrated by DNA-RNA, DNA-DNA hybridization studies and by interspecific transformation for several antibiotic resistance markers (7, 9). Dubnau et al. (7) postulated that there is a "core" of conserved genetic material among members of the genus *Bacillus*. The "core" region is composed of complementary nucleotide sequences, thus permitting the hybridizations cited above.

Inasmuch as markers of *B. licheniformis* have not been biochemically compared with those of *B. subtilis* W23 or W168, only the most tentative comparisons between the genetic maps of these species of *Bacillus* are warranted. However, both species have *trp*, *tyr*, *lys*, and *met* loci clustered in the terminal region and a *pur* marker near the origin (6, 19).

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