GENETIC AND BASE SEQUENCE HOMOLOGIES IN BACILLI

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THE importance of map position in determining the rate of evolution of DNA base sequence has not been examined in detail. It is possible that map position influences are negligible, and that the changing environment and the nature of the gene product primarily determine those genes which evolve most rapidly. On the other hand, map position influences on evolutionary rate could be of such great importance that they provide selection pressure for map rearrangement.

Because the genome of *Bacillus subtilis* is replicated in sequential order from one end to the other (YOSHIKAWA and SUEOKA 1963 a, b), the replicating genome in growing cells has an average of two (or sometimes more (OISHI, YOSHIKAWA and SUEOKA 1964)) copies of early-replicating genes per copy of late-replicating ones. Unless genes mutate only at replication, this gene dosage effect could impose a pattern on the rate of evolution of *Bacillus* genes, since the target size for mutation of early genes is effectively doubled. Moreover, genes for which high dosage is advantageous might be localized in the early-replicating portion of the genome. DUBNAU *et al.* (1965) have pointed out that the position of rRNA and sRNA genes, the major cluster of which is early-replicating (OISHI and SUEOKA 1965; DUBNAU, SMITH and MARMUR 1965; DUBNAU *et al.* 1965; OISHI, OISHI and SUEOKA 1966; SMITH *et al.* 1968), can be rationalized in this way. Structural features such as membrane association of the origin and terminus of the *B. subtilis* genome (SUEOKA and QUINN 1968) might also influence the rate of evolution of genes near the origin and terminus.

The genus *Bacillus* offers unique advantages for a study of apparent evolution rate *vs.* map position because a transformation system and detailed genetic map are available. Heterologous transformation efficiency can be used as an index of genetic divergence. Further, DNA-DNA heteroduplexes may be prepared from strands originating from two different strains. This makes possible the measurement of the temperature of irreversible strand separation ($T_{m,i}$) for various markers in heteroduplex DNA. The melting point depression, $\Delta T_{m,i}$ (compared to homologous duplexes) is a measure of the percentage of mismatched bases in the most GC-rich portion of the heteroduplex. Since $\Delta T_{m,i}$ estimates correlate well with ΔT_m estimates (optical melting curves) of percentage of mismatched bases (LAIRD, MCCONAUGHY and MCCARTHY 1969), the $\Delta T_{m,i}$ values can be taken as a measure of genetic divergence for the entire DNA heteroduplex bearing the marker.

MATERIALS AND METHODS

Materials: CsCl Optical Grade Powder from the Harshaw Chemical Company and Polyallomer Spinco centrifuge tubes were used for all CsCl density gradients. Polyriboguanilic acid (Poly-G) was obtained from Biopolymers, Inc.

Bacterial strains: The bacterial strains used are described in Table 1. All strains except *B. subtilis* W23 and *B. globigii str^r* are derivatives of *B. subtilis* 168.

Map position of markers: Figure 1 shows a genetic map of the *B. subtilis* markers employed in these experiments. Markers used either were identical with those shown in the map of DUBNAU *et al.* (1968) or were shown to be linked to markers on that map by transformation. The designation of the *arg* marker employed as *arg A* was based on its strict requirement for arginine (MAHLER, NEUMANN and MARMUR 1963). The order of the cluster *ade-26* through *pur-6* was determined by two-factor transformation crosses as described by DUBNAU *et al.* (1967). The orientation of *ade-16* and *ade-17* is unknown, as is that of the *ade-26-pur-6* cluster.

Media: Minimal salts medium was that of SPIZIZEN (1958). For preparation of competent cells, first growth medium was minimal salts plus 0.5% glucose, 0.02% casamino acids, 20 µg/ml L-tryptophan and 50 µg/ml required growth factors. Second growth medium and reaction medium were the same: minimal salts plus 0.5% glucose, 0.01% casamino acids and 5 µg/ml L-tryptophan and required growth factors. Agar (2%) in minimal salts was supplemented with 0.5% glucose and 50 µg/ml of desired growth factors (each autoclaved separately).

TABLE 1
Bacterial strains

Strain*	Phenotype	Origin†
<i>B. globigii str^r</i>	Prototroph, str ^r	A spontaneous str ^r mutant of <i>B. globigii</i> SB512 (E. LEDERBERG)
<i>B. subtilis</i> W23	Prototroph, str ^r	N. SUEOKA
<i>B. subtilis</i> 746 str ^r	Prototroph, str ^r	A spontaneous str ^r mutant of WB746 (E. W. NESTER), which is a spontaneous revertant of 168 try-2
Mu8u5u16	ade-16 ⁻ , leu ⁻ , met B ⁻	N. SUEOKA
Mu8u5u17	ade-17 ⁻ , leu ⁻ , met B ⁻	N. SUEOKA
Mu5u11	ade-11 ⁻ , met B ⁻	WB2093 (<i>aro⁻, metB⁻</i>)‡ (D. NASSER) × Mu8u11 (<i>ade-11⁻, leu⁻</i>) (N. SUEOKA)
Mu5u12	ade-12 ⁻ , met B ⁻	WB2093 × Mu8u12 (<i>ade-12⁻, leu⁻</i>) (N. SUEOKA)
Mu5u26	ade-26 ⁻ , met B ⁻	WB2093 × Mu27u26 (<i>tyr⁻, ade-26⁻</i>) (N. SUEOKA)
Mu5u33	ade-33 ⁻ , met B ⁻	WB2093 × Mu33 (<i>ade-33⁻</i>) (N. SUEOKA)
Mu5u34	ade-34 ⁻ , met B ⁻	WB2093 × Mu34 (<i>ade-34⁻</i>) (N. SUEOKA)
WB2711	pur-6 ⁻ , met A3 ⁻	SB26 (<i>met A3⁻, try-2⁻</i>) (E. W. NESTER) × Mu8u5u6 (<i>pur-6⁻, leu⁻, met B⁻</i>) (N. SUEOKA)
WB2712	his A1 ⁻ , ura ⁻ , phe ⁻	SB5 (<i>his A1⁻, ura⁻, try-2⁻</i>) (E. W. NESTER) × SB133 (<i>try-2⁻, phe⁻</i>) (E. W. NESTER)
SB3	his A1 ⁻ , cys B3 ⁻ , try-2 ⁻	E. W. NESTER
SB323	ilv A ⁻ , met ⁻	E. W. NESTER
SB359	try-2 ⁻ , tyr-2 ⁻ , ilv ⁻	E. W. NESTER
BD47	phe ⁻ , ilv A1 ⁻	D. DUBNAU
SB32	try-2 ⁻ , arg-3 ⁻	E. W. NESTER
WB2713	leu ⁻ , ilv A1 ⁻	Mu8u5u16 × BD47

* All strains except *B. globigii str^r* are *B. subtilis* strains, and all *B. subtilis* strains except W23 are derivatives of 168 try-2⁻.

† Parental strains were crossed by transformation and are listed in the order: recipient × donor.

‡ The *met* marker in this strain originated from Mu8u5u6 (N. SUEOKA).

FIGURE 1.—Map of *B. subtilis* markers used. Markers shown above the line are identical to those on the map of DUBNAU *et al.*, (1967), from which this map is adapted. Markers shown below the line were assigned map positions as described in MATERIALS AND METHODS. The orientation of *ade-16*, *ade-17* and of the *ade-26*—*pur-6* cluster is unknown.

Competence regimen and transformation procedure: Competent cells were prepared by a previously described modification (CHILTON and HALL 1968) of the method of ANAGNOSTOPOULOS and SPIZIZEN (1961). Frozen competent cells were used for some experiments, but fresh cells were prepared where a high degree of competence was necessary. One ml aliquots of either frozen cells, thawed and diluted 1:10 into reaction medium, or fresh competent cells in second growth medium were mixed with DNA, shaken at 37°C for 30 min, and treated with 0.05 ml of 10 µg/ml DNase in 0.1 M MgSO₄ solution. Transformants selected on appropriately supplemented agar were scored after 36 hrs incubation at 37°C. Streptomycin resistance was scored by the method of DUBNAU *et al.* (1967).

Determination of homologous/heterologous transformation ratio: Because transforming efficiency of native DNA in *B. subtilis* is dependent upon its single-strand molecular weight (BODMER 1966), the donor DNA preparations used were selected to display similar single-strand molecular weight distributions in alkaline sucrose gradients. Transformation assays were performed using saturation levels of DNA (20 µg/ml, final concentration).

Strand separation with Poly-G: Poly-G reacts preferentially with one of the complementary strands of *B. subtilis* DNA fragments, apparently because of an asymmetric distribution of C-rich regions (OPARA-KUBINSKA, KUBINSKI and SZYBALSKI 1964). After the method described by KUBINSKI, OPARA-KUBINSKA and SZYBALSKI (1966), a solution of 25 µg/ml native DNA and 25 µg/ml poly-G in SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) was heated at 100°C for 6–8 min and quickly cooled in an ice bath. The resulting DNA/poly-G complex was centrifuged to equilibrium in CsCl (100 µg DNA per 5 ml gradient, Spinco SW39 rotor, 33,000 rpm, 20°C, 60–72 hrs). A typical profile of OD₂₆₀ from such a gradient is shown in Figure 2. Material at the bottom of the gradient is poly-G; when radioactively labeled DNA is used, no radioactivity appears in this region. The heavy peak appears larger than the light because of the higher concentration of poly-G in the heavy complex. Pooled Heavy and pooled Light fractions were dialyzed against an alkaline solution as previously described (CHILTON 1967) to free them from poly-G.

Formation of heteroduplex DNA: Dialyzed Heavy and Light fractions in 10⁻³ M Tris buffer, pH 7.3, were concentrated by evaporation and adjusted to 20 µg/ml DNA, 2× SSC. Equal volumes of the desired Heavy and Light samples were mixed and incubated at 60°C for 2 hrs. Control samples of Heavy alone and Light alone were similarly annealed; only negligible transforming activity could be detected in these samples. Figure 3 shows typical renaturation kinetics for Heavy 746 *str*^r and Light W23 alone and in 1:1 mixture as assayed by transforming activity for the *ade-16* marker.

The size of the DNA heteroduplex fragments used in this study was not determined but an upper limit can be estimated from the average molecular weight of the DNA strands annealed to form the heteroduplexes, *ca.* 1×10⁷. Neglecting degradation during annealing, the heteroduplexes should not exceed about 2×10⁷, i.e. of the order of 1% of the size of the *Bacillus* genome (MASSIE and ZIMM 1965; DENNIS and WAKE 1966).

FIGURE 2.—CsCl density gradient of poly-G complex from *B. globigii* DNA. The complex was prepared and centrifuged to equilibrium in CsCl as described in MATERIALS AND METHODS. Fractions (ca. 0.25 ml each) were diluted with 1 ml of distilled H₂O and OD₂₆₀ was measured. Fractions 7–9 and 11–13 were pooled and designated Heavy and Light strands, respectively.

Thermal dissociation of heteroduplex DNA: Renatured DNA prepared as described above was dialyzed against 0.1× SSC and 0.05 ml aliquots sealed in capillary tubes were heated for 5 min at various temperatures in a Haake ultrathermostat. Transforming activity remaining in the heated DNA samples was measured in the usual manner. A typical melting curve is shown in Figure 4. The temperature by which 50% of transforming activity is lost, $T_{m,i}$, for homoduplex DNA (Heavy 746 *str^r* × Light 746 *str^r*) was higher than that for either kind of heteroduplex DNA (Heavy 746 *str^r* × Light W23 or Heavy W23 × Light 746 *str^r*).

RESULTS

The efficiency with which two kinds of heterologous donor DNA can transform competent *B. subtilis* 168 strains was measured for a variety of genetic loci. To normalize for variations in competence of the recipient culture, homologous donor DNA (746 *str^r*, a 168 strain) was used as a standard. A number of *B. subtilis* 168 strains bearing various genetic markers were made competent and aliquots of competent cultures were transformed with saturating levels of DNA from homo-

FIGURE 3.—Renaturation kinetics for separated strands alone and in 1:1 mixture. Renaturation mixtures contained 10 $\mu\text{g/ml}$ of Heavy W23 DNA (●), 10 $\mu\text{g/ml}$ Light 746 *str*^r DNA (□) or 10 $\mu\text{g/ml}$ of each (○) in $2\times\text{SSC}$. Aliquots (0.01 ml) were sealed in capillaries and incubated at 60°C for various times. Recipient cells for transformation assay were strain Mu8u5u16. Data depicted are for the *ade-16* marker.

logous (746 *str*^r) and heterologous (W23 or *B. globigii str*^r) donors. The number of transformants using homologous DNA divided by that using heterologous DNA (homologous/heterologous ratio) for W23 donor DNA is shown in Table 2. For *B. globigii* donor DNA, homologous/heterologous ratio plotted against map position of the marker is shown in Figure 5. A semilogarithmic plot is used to display the wide range of ratios observed. A dramatic pattern of genetic relatedness is apparent between *B. subtilis* 168 and distantly related *B. globigii*; rudiments of this pattern can be seen in the case of W23, a close relative of strain 168, but differences of transformation efficiency are small and not very reproducible. Nevertheless, the two regions which appear most similar in 168 and *B. globigii* (*str* and *leu*) also appear closely related in W23 and 168. The map order of *ade-17* to *ade-16* and of the *ade-26* to *pur-6* group used in Figure 5 was chosen to give a smooth curve of transformation efficiency. If this assignment is in error, a slightly more complex pattern exists than that shown.

The apparent gradient of conservatism around *leu* and *str* is not due to co-transformation of nearby non-conservative markers linked to *leu* and *str*. None of the *ade* markers is co-transformed with *str* (CHILTON, unpublished results; DUBNAU *et al.* 1967), hence the *ade* clusters themselves must be conservative. The two *ilv A* markers studied, on the other hand, are co-transformed with *leu*. If the *ilv* markers were not conserved and merely entrained by the conservative *leu* gene, one would expect *ilv*⁺ transformants from *B. globigii* donor DNA always to be *leu*⁺ as well. However, *ilv A1* transformants arising from *B. globigii* donor

FIGURE 4.—Melting profiles for homoduplex and heteroduplex DNA. Curves shown are for homoduplex (Heavy 746 *str*^r × Light 746 *str*^r) renatured DNA (○) and two kinds of heteroduplex renatured DNA (Heavy 746 *str*^r × Light W23 [△] and Heavy W23 × Light 746 *str*^r [▲]). A sample of DNA in 0.1 × SSC was heated at each temperature for 5 min. Recipient for the transformation assay was strain SB3. The data shown are for the *try-2* marker.

DNA are no more frequently *leu*⁺ (Table 3) than are *ilv* *A1*⁺ transformants from homologous donor DNA. The *ilv* *A* locus itself must therefore be conserved. RAVIN and CHEN (1967) have demonstrated reduction of linkage in heterologous transformations between streptococci and pneumococci. Such linkage reduction is not seen in *Bacillus* transformation if transformants for the less conservative marker are selected and tested for co-transfer of the more conservative marker: homologous and heterologous DNA give a very similar percent co-transfer of

TABLE 2

Homologous/heterologous transformation ratios: W23 donor DNA

Marker	Assay 1	Assay 2	Homologous/heterologous ratio*		Assay 4	Assay 5
			Assay 3	Average†		
<i>ade-17</i>	..	..	..	..	..	5.5
<i>ade-16</i>	5.6	6.4	..	6.0	..	..
<i>str</i>	1.1	1.3	1.6	1.3	1.1	..
<i>ade-26</i>	..	..	..	..	..	1.7
<i>ade-11</i>	..	..	..	..	..	3.14
<i>ade-34</i>	..	..	..	..	..	1.9
<i>ade-12</i>	..	..	..	..	..	1.6
<i>ade-33</i>	..	..	..	..	..	1.7
<i>pur-6</i>	2.2	4.0	1.5	2.6	2.6	1.4
<i>cys B3</i>	2.9	3.7	2.4	3.0	..	..
<i>his A</i>	2.4	2.4	2.8	2.5	..	..
<i>met A3</i>	2.2	2.0	1.9	2.0	1.2	2.0
<i>ura</i>	5.1	4.4	5.0	4.8	..	..
<i>leu</i>	1.7	2.0	..	1.9	..	..
<i>phe</i>	1.7	2.0	1.2	1.6	..	..
<i>try-2</i>	4.2	2.2	1.7	2.7	..	..
<i>met B</i>	2.3	2.6	..	2.5	..	2.6

* Assays 1, 2 and 3 were done with the same pair of DNA preparations; assays 4 and 5 employed different pairs.

† Average of values in the first three columns.

TABLE 3

*Cotransformation of leu and ilv A1 by homologous and heterologous DNA**

Donor DNA	Fraction of <i>ilv</i> ⁺ which are <i>leu</i> ⁺	Fraction of <i>leu</i> ⁺ which are <i>ilv</i> ⁺
746 <i>str</i> ^r	32/56 = 57%	22/30 = 73%
<i>B. globigii str</i> ^r	24/45 = 53%	4/28 = 14%

* WB2713 competent cells were transformed with 20 γ /ml 746 *str*^r DNA or *B. globigii str*^r DNA and plated on agar supplemented with either leucine or isoleucine plus valine. Colonies were tested for cotransfer of *leu* and *ilv* markers by streaking on minimal agar.

leu⁺ with *ilv*⁺. The apparent linkage reduction when *leu*⁺ colonies are tested for co-transfer of *ilv A1* is ascribable to low efficiency of integration of the *ilv* marker (Figure 5).

The conservative nature of the *Bacillus str* marker and several other drug resistance markers closely linked to it (DUBNAU *et al.* 1967) has been observed by DUBNAU *et al.* (1965a) in a variety of heterologous transformations. These investigators also found *leu* and *pur-6* to be conservative compared to *met B* and *try-2* using *B. niger* donor DNA; thus the pattern we have observed appears to be present in other *Bacillus* species. *B. licheniformis* is too distantly related for this type of study; none of the numerous loci tested with *B. globigii* DNA could be detectably transformed by *B. licheniformis* DNA except *str*. This is consistent with the observation of DUBNAU *et al.* (1965) that, among the markers they

FIGURE 5.—Homologous/heterologous transformants: *B. globigii* donor. The ratio of transformants obtained with homologous (746 *str^r*) DNA to those with heterologous *B. globigii* DNA is plotted vs map position of the markers studied. Bars at the top of the graph indicate that too few transformants were obtained with heterologous DNA for accurate evaluation of the ratio.

studied, only *str* and micrococцин resistance markers of *B. subtilis* were transformable by *B. licheniformis* DNA.

In order to determine whether these variations in transformation efficiency accurately reflect patterns of DNA base sequence similarity (CHEN and RAVIN 1966 a, b), independent estimates of the latter were obtained for a number of markers. Heteroduplex DNA samples containing one strand of W23 DNA re-natured with a complementary strand of 746 *str^r* DNA were prepared and their melting points for various markers compared with those of homoduplex renatured DNA (746 *str^r* × 746 *str^r*). Separated complementary strands (KUBINSKI *et al.* 1966) were used to prepare heteroduplex DNA free from homoduplexes. Heteroduplexes (Heavy 746 *str^r* × Light W23 and Heavy W23 × Light 746 *str^r*) and homoduplexes (Heavy 746 *str^r* × Light 746 *str^r*) were prepared by renaturation, and melting curves such as that in Figure 4 were determined. The $T_{m,i}$ values found for ten genetic markers are listed in Table 4, together with the melting point depression ($\Delta T_{m,i}$). Figure 6 shows the pattern of $\Delta T_{m,i}$ plotted as a function of map position. A considerable resemblance to the pattern of transforming efficiency of Figure 5 is evident.

A similar experiment was attempted with heteroduplexes between *B. globigii str^r* DNA and 746 *str^r* DNA. Because the transforming activity of these heteroduplexes was, for many markers, quite low, it proved impossible to measure $\Delta T_{m,i}$ values accurately. The few reasonably reliable $\Delta T_{m,i}$ values obtained in this study (Table 5) were consistent with the patterns of Figures 5 and 6. The most conservative marker, *str*, has a $\Delta T_{m,i}$ of only 1°C whereas a very nonconservative marker, *met B*, has a $\Delta T_{m,i}$ of 9–12°C.

The $\Delta T_{m,i}$ values of Tables 4 and 5 can be compared with $\Delta T_{m,i}$ measurements obtained by another method in this laboratory. When heteroduplexes formed at 60°C in 1 × SSC between unlabeled filter-bound W23 DNA or *B. globigii* DNA and sheared radioactive 746 DNA are subjected to thermal denaturation (McCARTHY and McCONAUGHY 1968), they exhibit lower $T_{m,i}$ than homologous

TABLE 4

Mean thermal dissociation temperature of markers: homoduplexes and 746 × W23 heteroduplexes

Marker	Strain	$T_{m,i}$ for homoduplex DNA (°C)	$T_{m,i}$ for heteroduplex DNA (°C)		$\Delta T_{m,i}$
		H746 <i>str^r</i> × L746 <i>str^r</i>	H746 <i>str^r</i> × LW23	HW23 × L746 <i>str^r</i>	
<i>ade-16</i>	Mu8u5u16	79.5–80	77	77	2.5–3°C
<i>str</i>	WB2711	75.0–76	72	73	2–4°C
<i>pur-6</i>	WB2711	77	73	74	3–4°C
<i>his A</i>	SB3	78	72	71	6–7°C
<i>met A3</i>	WB2711	77.0–77.5	71.5	72	5–6°C
<i>ura</i>	WB2712	77	71–72	71–72	5–6°C
<i>leu</i>	Mu8u5u16	75.5	74	72	1.5–3.5°C
<i>phe</i>	WB2712	77	..	71	6°C
<i>try-2</i>	SB3	76	72	71	4–5°C
<i>met B</i>	Mu8u5u16	76–77	72	70	4–7°C

FIGURE 6.— $\Delta T_{m,i}$ values for W23-746 heteroduplexes. Heteroduplex and homoduplex DNA samples prepared as described in MATERIALS AND METHODS were subjected to thermal dissociation. The difference in $T_{m,i}$ for heteroduplex and homoduplex DNA, as measured by loss of transforming activity for various markers, is plotted *vs.* map position of the markers. The length of the bar indicates the range of estimates of $\Delta T_{m,i}$ for the two heteroduplex DNA samples ($H_{746} \times L_{W23}$ and $H_{W23} \times L_{746}$). Data are taken from Table 4.

duplexes by 4.5°C and 11.5°C, respectively (McCONAUGHY, unpublished data). The average of the $\Delta T_{m,i}$ values obtained for W23 $\times$ 746 heteroduplexes (Table 4) is also 4.5°C. The $\Delta T_{m,i}$ values for *B. globigii* $\times$ 746 heteroduplexes (Table 5) range up to 12°C. Their average is lower than that obtained by the filter method because only markers with high transforming activity could be measured, and most of these were in a conservative region of the genome.

The linear relationship between $\Delta T_{m,i}$ and percent mismatched bases (LAIRD

TABLE 5

Mean thermal dissociation temperature of markers: homoduplexes and 746 $\times$ B. globigii heteroduplexes

Marker	Strain	$T_{m,i}$ for	$T_{m,i}$ for	$\Delta T_{m,i}$
		homoduplex DNA (°C) <i>H746 str^r $\times$ L746 str^r</i>	heteroduplex DNA (°C) <i>H746 str^r $\times$ L(B. globigii)</i>	
<i>str^r</i>	WB2711	74	73	1°C
<i>ade-26</i>	Mu5u26	75	69	6°C
<i>pur-6</i>	WB2711	77	70	7°C
<i>met B*</i>	Mu8u5u16	75	65.5–67	9.5°C
		76	64–65	11–12°C

* Two values are given for duplicate experiments.

et al. 1969) allows one to calculate that *B. subtilis* 746 and W23 differ on the average in about 7% of their bases. This difference scarcely influences the efficiency of transformation: homologous/heterologous transformation ratios are only 2–3 for markers of average $\Delta T_{m,i}$.

The *met B* marker in *B. globigii* has a $\Delta T_{m,i}$ of 9–12°C (13–18% mismatched bases) and a homologous/heterologous ratio of 10^4 . However, even the *str* marker in *B. globigii* with a small $\Delta T_{m,i}$ (1°C) has a very large homologous/heterologous ratio (40) compared to that from W23 donor DNA. This suggests that some factor in addition to base sequence mismatching is in part responsible for the low transformation efficiency of *B. globigii* donor DNA. Since there is a correlation between $\Delta T_{m,i}$ and homologous/heterologous ratio, this unknown DNA modification factor appears to have a reasonably uniform effect on all map regions. An alternative possibility is that the *str* marker is tightly linked to an extremely conservative and abnormally high GC marker, that for ribosomal RNA. Its apparent small $\Delta T_{m,i}$ value would then reflect the properties of the rRNA gene while its integration efficiency would be governed by more proximal base sequence homology.

DISCUSSION

By two different measures, transformation efficiency of heterologous donor DNA and melting point of heteroduplex DNA, a pattern of differences can be discerned among the genomes of *B. subtilis* 746, *B. subtilis* W23 and *B. globigii*. While there are discrepancies in the details of the patterns, the clear feature of all is the relatively conservative nature of two regions of the genetic map: around the *str* marker and around the *leu* marker. Further, there appears to be a gradient of conservatism in the region of each of these markers. This result differs sharply from the left to right trend anticipated on the basis of gene dosage in the replicating genome, and the end *vs.* middle pattern anticipated from the membrane-bound nature of the origin and terminus.

The divergence observed at a given gene locus among various related species may reflect either the rate at which mutations occur or the efficiency with which the changes are fixed in the populations. While in general it is assumed that rates of mutation are much higher than rates of fixation, an exception may be provided by mutations occurring in the third position of the codon, which may be selectively neutral due to the existence of synonym codons. Thus, possible explanations of the observed pattern of gene divergence may rest either upon inherent differences in the sensitivity to mutation or in variable selection pressure dependent upon map position.

Conservative regions of the genome might have differential resistance to mutagenic agents by virtue of their location near rRNA genes, 60% of which map just to the right of *str* and 40% of which map near the *leu* marker (SMITH *et al.* 1968). The rRNA genes themselves seem to be conservative in base sequence in a variety of groups of organisms (DOI and IGARASHI 1965; DUBNAU, SMITH and MARMUR 1965; MOORE and MCCARTHY 1967; TAKAHASHI, SAITO and IKEDA 1967; MOORE and MCCARTHY 1968). The drug resistance markers of *Bacillus* and other genera

have also been found to be conservative (PAKULA 1961; RAVIN and DeSA 1964; NICKEL and GOODGAL 1964; CATLIN 1964; DUBNAU *et al.* 1965). DUBNAU, SMITH and MARMUR (1965) have suggested that this conservatism could be explained by supposing that drug resistance genes function in ribosome synthesis. Their conservatism would then be but another reflection of stringent evolutionary restrictions on ribosome structure.

If we suppose that both conservative regions of the *Bacillus* genome are centered about rRNA gene clusters, our results could indicate that the rate of evolution of neighboring genes is somehow influenced by them. The structure of the genome may be unusual in the region of rRNA cistrons in order to facilitate the very rapid transcription exhibited under conditions of high growth rate. Moreover, the accumulation of rRNA about the region of the genome where it is transcribed may exert a physical protection of the rRNA genes and on all DNA proximal to them by intercepting potential mutagenic agents. Either or both of these effects could explain the gradient of conservatism observed in the two conservative regions of the genome.

Variation in selection pressure might also be responsible for the patterns of conservatism we have observed. If transducing phages carrying long segments of DNA have played an important role in genetic interchange among bacilli in the course of evolution, there might well be selection pressure for homology around very important regions of the genome. It is plausible that drug resistance genes might constitute such an important region; another such area must then be postulated to map near the *leu* marker. Alternatively, the evolutionary influence of transducing phages may be responsible for the observed patterns of conservatism in quite a different manner, again supposing the conservative regions to be centered about genes coding for rRNA. Since rRNA cistrons are apparently tandem repeats, the probability of crossing over in this region of the genome is increased. A relatively frequent exchange of genetic material linked to rRNA loci could therefore be responsible for the homology now found among different species of Bacilli in these regions of the genome.

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SUMMARY

The extent of divergence of nucleotide sequence at various loci has been examined in three species of *Bacillus*. A correlation is obtained between the results of the two assays used: the relative efficiency of heterologous transformation and the decrease in the thermal stability of heteroduplex DNA formed by two strands originating from different species. The extent of difference between corresponding genes in a pair of organisms is quite variable and is a function of map position. Gradients of conservatism of base sequence occur on both sides of the loci for ribosomal RNA.

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