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Electrophoretic Separation of *Bacillus subtilis* Genes

(*Eco*R$_I$/agarose gel electrophoresis/genetic transformation/DNA/restriction nuclease)

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ABSTRACT **The cleavage of *Bacillus subtilis* DNA by *Eco*R$_I$ restriction endonuclease produces segments which retain various degrees of genetic transforming activity. The active segments analyzed thus far, range in size from 23 to 3 kilobases and can be partially separated by agarose gel electrophoresis. Various markers can thus be enriched from 30- to 60-fold.**

The discovery of site-specific endonucleases (DNA-restriction enzymes) has furnished a major technical advance in separating genes. Site-specific cleavage‡ of a uniform population of DNA molecules has yielded an ensemble of segments,‡ unique in size and genetic composition. The utility of these for physical and chemical methods of separation has already been demonstrated in several laboratories (1, 2) by the separation of DNA segments of small viruses and plasmids.

The separation of genetically specific DNA from bacteria and higher organisms is complicated by the greater size of the genome and by the vulnerability of the chromosome to random shear during isolation; thus, the molecules subsequently exposed to cleavage are not altogether homogeneous. Nonetheless, if these molecules are larger than the produced segments, effective assortment of genes into unique segments should still be possible, with only minor contamination due to shear. Cleavage of *Hemophilus influenzae* DNA by the *H. parainfluenzae* endodeoxyribonuclease, followed by size-separation of segments by neutral sucrose gradient centrifugation, has been reported (3). However, the enrichment of specific activity by this method is not significantly better than was previously achieved (4).

We have found that enrichment and separation of specific genetic activity of bacterial DNA can be improved with cleavage by the *Eco*R$_I$ endonuclease followed by low-voltage agarose gel electrophoresis. We used transforming DNA from *Bacillus subtilis* for biological assay. The reduction of biological activity seen after cleavage, is comparable to that caused by shear-paring of the DNA molecules. Cleaved DNA, after size-sorting by electrophoresis in agarose gels, gives a reproducible banding pattern. When the DNA in these gels is assayed for biological activity, peaks of activity for different genetic markers are observed in positions that correlate with the DNA banding pattern. Almost complete separation of several genes, and enrichments in specific transforming activity of 30- to 60-fold, have been attained.

Abbreviation: Kb, kilobase.

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‡ Cleavage and segment will be used to describe interval-specific-DNA products, in contrast to fragments caused by breakage or shear).

MATERIALS AND METHODS

B. subtilis Strains used are described in Table 1. They are all derivatives of SB168, widely used in different laboratories.

DNA Preparation. The method described by Klotz and Zimm (5) was modified as follows. *B. subtilis* cells were grown in minimal Spizizen medium supplemented with 0.1% of hydrolyzed casein, 50 μg/ml of tryptophan, 200 μg/ml of deoxyadenosine, and 1 μCi/ml of [^{3}H]thymidine (NEN, diluted to a specific activity of 100 mCi/mmol). Cells were harvested in mid-exponential phase, washed with standard saline–citrate solution (0.15 M sodium chloride–0.015 M sodium citrate, pH 7), and resuspended in 1/100 volume of 20% sucrose in 0.05 M Tris·HCl—1 mM EDTA at pH 7.6; 10 mg/ml of lysozyme was added, and the suspension incubated at 37° for 10 min. Two volumes of 1% lauroyl sarcosylate in 0.1 M EDTA at pH 9.6 were added, followed by addition of 10 mg/ml of Pronase. The solution was incubated at 50° until clarified, and 1.23 times (w/w) of CsCl was added. This solution was centrifuged in a Spinco rotor 40 for 48 hr at 36000 rpm. Centrifuge tubes were pierced, DNA-containing fractions pooled and dialyzed against 10 mM Tris·HCl at pH 8, 10 mM NaCl, 1 mM EDTA at pH 8 (TEN). The yield of DNA was approximately 0.5 mg/100 ml of culture, with a specific activity of about 20,000 cpm/μg.

*Eco*R$_I$ *Enzyme Digestions.* DNA (20–400 μg/ml) was incubated with the enzyme (a generous gift of Drs. S. Cohen and P. Wensink) at 37° for 10–60 min in a solution containing 100 mM Tris·HCl at pH 7.6, 5 mM $MgCl_2$, and 20–100 mM NaCl (gelatin was sometimes added to 0.01%). Completeness of degradation was verified by sucrose gradient centrifugations.

Electrophoresis was performed in tubes (7, 1); the buffer contained 0.5 μg/ml of ethidium bromide. DNA was visualized and photographed under long-wavelength UV light (UVL-21, Ultraviolet Products, Inc., San Gabriel, Calif.). DNA was recovered from gels for transformation by finely mashing 1–5 mm thick slices and incubating the slurry in 0.2–1 ml of Spizizens minimal medium supplemented with 0.5% glucose and 0.02 M $MgCl_2$ at 37° for 2–3 hr. Close to 60% of DNA is found in the supernatant by that time. In some cases, a slice of gel containing DNA of interest was placed on top of a new gel, and the DNA was electrophoresed into the new gel for 10 min at 150 V, followed by 12–24 hr at 20 V (2 V/cm of gel).

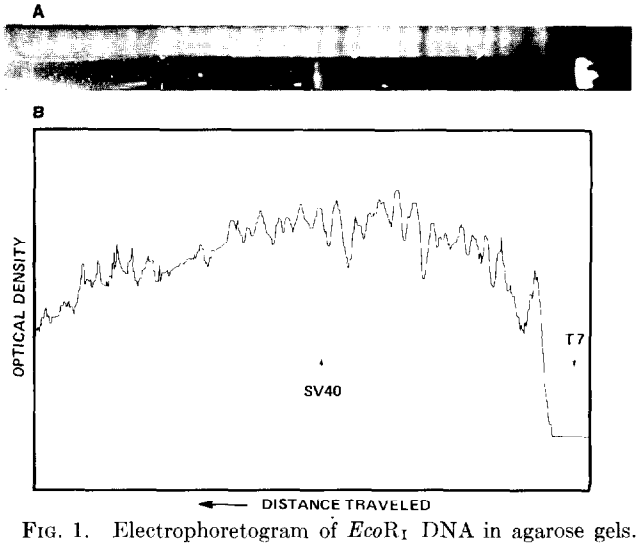

FIG. 1. Electrophoretogram of *Eco*R_I DNA in agarose gels. *B. subtilis* DNA (300 μg/ml) was cleaved with *Eco*R_I endonuclease (see *Materials and Methods*). A sample containing 10 μl of digestion mixture and 5 μl 0.05% bromphenol blue in 30% sucrose was layered on a 5 × 100 mm gel of 0.74% agarose in 0.089 M Tris, 0.002 M EDTA, 0.089 M borate buffer (TEB). The sample was run into the gel for 3 min at 150 V and thereafter at 2 V/cm of gel (20 V) for 24 hr. Photographs were made under long wavelength ultraviolet light, and these were scanned by the Joyce-Loebel Densitometer. The locations of DNA from bacteriophage T7 and simian virus 40 (SV40) are indicated for comparison.

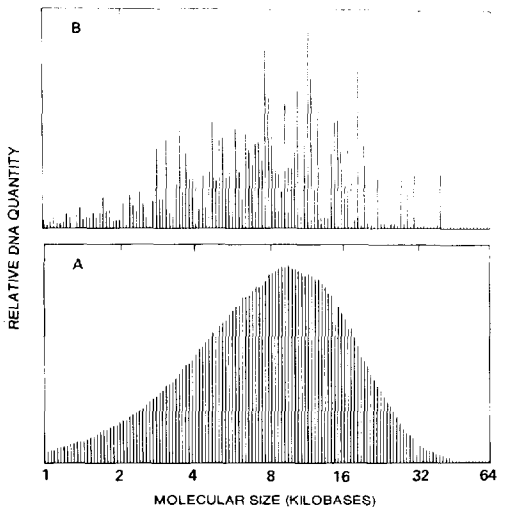

FIG. 2. DNA patterns obtained by computer simulation of electrophoretic separation of *B. subtilis* DNA fragments (A) or segments (B).

RESULTS

Sedimentation and Electrophoresis of EcoR_I Degraded DNA. Sucrose gradient centrifugation of *B. subtilis* DNA before and after degradation with *Eco*R_I restriction endonuclease showed a reduction of weight-average molecular weight from 80 to close to 8 million. The final value is close to that reported for other DNAs exposed to the same treatment (8).

Electrophoresis of cleaved *B. subtilis* DNA in agarose gels results in the appearance of a number of bands in a reproducible pattern (Fig. 1A). A similar finding was reported for *Eco*R_I-cleaved *E. coli* DNA (1). Densitometric measurement of gel fluorescence is shown in Fig. 1B. The bands observed in Fig. 1A do not correspond to unique chromosome segments since the sum of their sizes is only about one tenth that of the entire *B. subtilis* chromosomes, (2500 kilobase), (5); this finding is corroborated by the high background fluorescence displayed in Fig. 1B. It would indeed be surprising if the several hundreds of segments produced by the cleavage of *B. subtilis* genome were resolvable by our electrophoretic procedures and the observed banding was unexpected. Either a patterned distribution of *Eco*R_I cleavage sites along the chromosome, or a random clustering of sizes would lead to appearance of bands. To distinguish between these possibilities, a computer simulation of the cleavage was performed.

TABLE 1. *Strains used: Recipient strains of donor strain SB1070 (genotype thyA thyB)*

Recipient Strains	Genotype
SB130	*aroE hisB*
SB194	*trypE*
SB326	*gly met*
SB420	*trypB inh-1*
SB564	*aroB trypE tyrA*
SB626	*aroBtyrA hisA ura-1*
SB863	*aroB trypC tyrA hisA cys-1 leu str*
SB1023	*aroB trypC hisB tyrA cys-1 lys*
SB1035	*ura-1 arg tryp leu*
SB1067	*purA16 metB5 argC4 leu8*

All strains have been deposited in the *B. subtilis* stock collection of the Department of Genetics, Stanford University. Pedigrees will be provided upon request.

It was postulated that shearing occurs at random during isolation, and that the cleavage sites are randomly located on the chromosome; i.e., the size of segments follows a discrete exponential distribution. Fig. 2 shows the distribution of fragments obtained by simulating the response of a 2500 kb molecule: (*a*) for random shear breakage to pieces of 10 kb (average size); (B) for random shear breakage to 120 kb (the size of *B. subtilis* DNA after isolation) followed by cleavage at fixed points, distributed randomly along the chromosome, to the 10 kb size. The first mode of breaking gives rise to a continuous distribution (Fig. 2A), the second to a pattern resembling the banding observed in the electrophoretogram of the *Eco*R_I degraded DNA (Fig. 2B). Consequently, the electrophoretic pattern of the cleavage products of *B. subtilis* (and, very likely *Escherichia coli*) chromosome can be explained without postulating non-random intervals of *Eco*R_I sites within the chromosome.

The qualitative aspects of the simulation pattern do not change with assumed further shear of the chromosome as long as the weight-average molecular weight of the fragments before cleavage exceeds that expected for a 30 kb molecule; more extensive breakage progressively blurs the pattern.

Transforming Activity of EcoR_I Degraded (Cleaved) B. subtilis DNA. The survival of different markers after *Eco*R_I cleavage varies from close to 60% (*metB5*) to below 0.1% (*lys, gly*) (Table 2). Residual activity does not change with either prolonged incubation of DNA with the enzyme, or further addition of enzyme. This finding is difficult to relate to the simplest all-or-none model of marker inactivation due to cleavage sites within the corresponding gene. An alternative model better fits our data: the survival of a marker is postulated to be a function of two variables, the length of the fragment carrying

TABLE 2. *Survival of biological activity of genetic markers after Eco*R_I *digestion*

Marker	Recipient	Survival (%)	Range of survival (%)
metB5	SB1067	60	59–60.5
leu	SB1023	10	7–13
trypC-tyrA	SB863	8.2	6–13
trypE	SB194	9	
trypC	SB1023	11	5–15
hisB	SB1023	11	5–15
tyrA	SB1023	14	5–15
inh-1	SB420	19	
hisA	SB863	6.7	4.3–11.3
cys	SB1023	2.2	0.7–3.6
ura-1	SB1035	4.5	2.7–6.9
ade16	SB1067	4.3	0.5–8
aroE	SB130	10	
aroB	SB1023	0.36	0.25–0.47
lys	SB1023	0.02	0–0.1
gly	SB326	0	

DNA from SB1070 was degraded with *Eco*R_I endonuclease as described in *Materials and Methods*. Completion of reaction was tested by determination of molecular size by neutral sucrose gradient centrifugation. Biological activity was measured by transformation at limiting DNA concentrations. Survival is expressed as percentage of transforming activity remaining after *Eco*R_I digestion. Assays were conducted as in (6).

the marker, and the distance of the marker from the site of action of the endonuclease; however, survival is independent of the configuration of the DNA termini. From it we can predict that:

(*a*) transforming activity of DNA will be reduced by both shear and by cleavage to about the same level, provided that: (*1*) the average size of fragments produced by either shearing or enzymatic digestion is similar; (*2*) the number of cuts introduced by shearing into the marker studied is low; (*3*) the marker is not so close to the cleavage site as to be completely inactivated by the *Eco*R_I cut. To test this prediction, we sheared *B. subtilis* DNA to a (weight-average) size of 13 kb

TABLE 3. *Separation of genetic markers by agarose gel electrophoresis*

Peak	*trypC-tyrA*	Other markers		
		metB5	*hisA*	*ura-1*
trypC-tyrA	—	<0.001	0.005	0.06
metB5	0.004	—	0.001	0.02
hisA	<0.0003	0.04	—	0.014
ura-1	<0.0003	0.01	0.002	—

DNA from SB1070 was digested by *Eco*R_I and 3 μg of DNA loaded on 0.73% agarose gels in TEB buffer. After an initial electrophoresis of 150 V for 3 min, the gels were electrophoresed at 20 V for 24 hr. Slices (1 mm) were cut and DNA extracted as described in *Materials and Methods*. Biological activity was determined by transformation and peaks of activity for four markers were found (Fig. 4); in each case, the activity of other markers was determined for each peak. Separation of markers was calculated as the ratio R_A/R_B, where R_B and R_A are ratios of activities of peak to contaminating marker before and after electrophoresis, respectively.

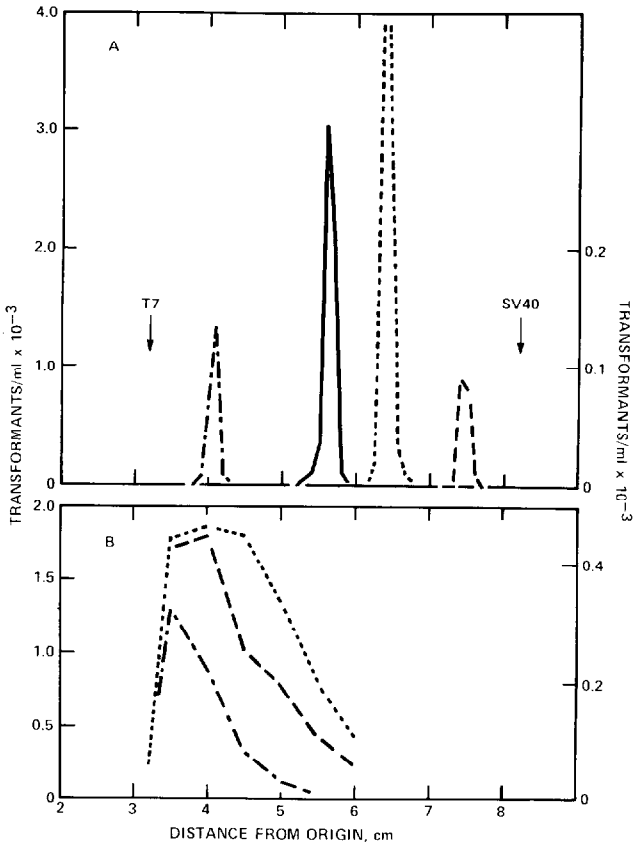

FIG. 3. Separation of genetic markers by agarose gel electrophoresis. (A) DNA from SB1070 was treated with *Eco*R_I endonuclease and electrophoresed in agarose gels as in Fig. 1. Transforming activity of DNA after electrophoresis was measured as described in *Materials and Methods;* different regions of the gel were screened for different genetic markers. Peaks from left to right correspond to *trypC-tyrA metB5*, *hisA* (left-hand scale), and *ura-1* (right-hand scale). (B) DNA was sheared to a weight average molecular weight, corresponding to that expected for a 10 kb molecule, by passage 10 times through a 30 × 1 hypodermic needle, then electrophoresed and tested for biological activity as above. Top, middle, and bottom line correspond to *hisA*, *ura-1* (left-hand scale), and *trypC-tyrA* (right-hand scale), respectively.

(close to that observed after cleavage). Assuming a Poisson distribution of cuts, one can show that 90% of targets of size 1.5 kb (the length of a gene coding for a polypeptide of 500 amino acids) are intact following shear. Activity of *trypC*, *hisB*, *aroB*, and *lys* were 11%, 6%, 6%, and 7%, respectively, of values before shearing. Cleavage reduces activity of the first two markers to a comparable level, while almost completely inactivating the last two (Table 2). These data indicate that the decrease of transforming activity of *trypC* and *hisB* resulting from cleavage of the DNA can be explained by reduction in DNA size. The inactivation of *aroB* and *lys* by *Eco*R_I, by cleavage but not by shear, could be a consequence of their close proximity to a site of action of the enzyme and subsequent destruction at some stage during recombination.

(*b*) Reduction of transforming activity by *Eco*R_I will be correlated with the molecular size of the DNA before degradation; however, markers close to the *Eco*R_I cleavage site will be inactivated regardless of the starting DNA size. Two experiments were performed to verify this prediction: (*1*) DNA was separated by preparative sucrose gradient cen-

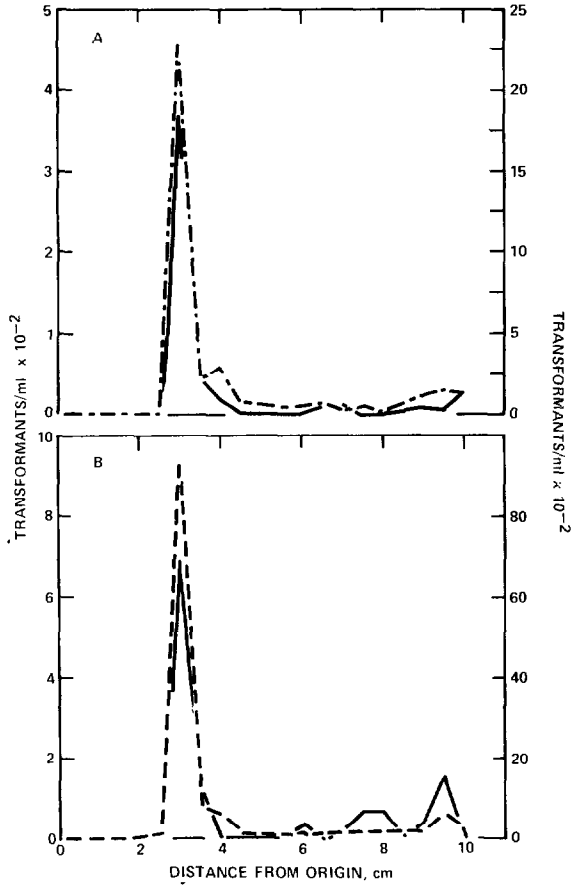

FIG. 4. Coincident electrophoretic mobility of markers in the *trypE-aroE* region. DNA from SB1070 was cleaved and electrophoresed for 18 hr as in Fig. 1; 5 mm slices were assayed for biological activity of four markers (shown on A and B for clarity): (A) ——, *trypC-tyrA* coselected (scale in left ordinate); –·–·–, *trypC* (scale on right ordinate). (B) —— *hisB* (scale on right); – – –, *tyrA* (scales on left ordinate). In other experiments, the region of gel corresponding to the peak was sliced into 1 mm slices, and the following markers were shown to migrate together as a single peak: *trypE, trypC, trypA, hisB, tyrA, inh,* and *aroE*.

trifugation into fractions larger and smaller than 35 kb; both were subsequently cleaved and the decrease in *trypC* biological activity measured. As expected, the activity of the heavier fraction of DNA fell 10-fold; the lighter fraction only 2-fold. (*2*) DNA was sheared to a size of 15 kb; this DNA was subsequently cleaved and the drop in biological activity for the *trypC* and *hisB* markers measured. The same experiment was performed with DNA of an initial size of 120 kb. The survival of *trypC* and *hisB* activity was 8 to 10 times greater with the 15 kb DNA. The *lys* marker was completely inactivated regardless of the DNA size before cleavage.

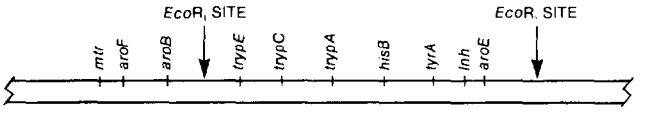

FIG. 5. Genetic composition of one $Eco R_I$ segment. Map position of the markers indicated are those of Nester *et al.* (9). Localization of the $Eco R_I$ site between *aroB* and *trypE* is described in the *text*. The position of the site to the right of *aroE* is uncertain because of a lack of genetic markers for mapping in that region; however, the size of the segment (23 kb, see Table 4) assures that the site is near *aroE*.

(*c*) The activity of markers far from a cleavage site depends on the size of DNA segment on which they are located. Some degree of correlation is found between survival of markers and the molecular size of segments bearing them, as determined by gel electrophoresis (see Tables 2, 5, and text below). However, the survival of *metB5* is anomalously high, indicating the importance for survival of other factors besides size.

Partial Purification of Genes by Agarose Gel Electrophoresis. The reproducible banding after electrophoresis of cleaved DNA impelled efforts to map surviving markers on the banding pattern in an attempt to purify specific genes. The cleaved DNA was electrophoresed in agarose, and the gels were sliced and assayed for transforming activity as described in *Materials and Methods*. Fig. 3A shows the pattern of transforming activity for four markers. It is evident that an excellent separation of markers is achieved; only trace activities of any marker are observed in the peak of another (Table 3) or outside of its own peak (Fig. 4). In a control experiment, DNA was sheared to a size similar to that of the cleaved DNA and then electrophoresed and assayed in the same way. No separation of markers was obtained, all the activities being spread in a very broad peak (Fig. 3B). The molecular sizes of DNA fragments carrying the markers shown in Fig. 3A was calibrated against the six fragments obtained after cleavage of λ-DNA (1) and are shown in Table 4. In addition, molecular sizes of fragments containing *cys, leu, ura-1* and *ade16* markers were shown to be 10.5, 9, 6, and 3.2 kb, respectively.

$Eco R_I$-produced segments of *B. subtilis* chromosomes are purified 30- to 60-fold by gel electrophoresis, as estimated by the increase in specific activity of genetic markers (Table 4), and by the fact that almost all of the biological activity could be recovered in a band that comprises only 2–3% of the total

TABLE 4. *Partial purification of genes by gel electrophoresis*

Marker	Size (kb)	Time of electrophoresis	Specific activity		Enrichment factor	Theoretical enrichment	Purity %
			Before	After			
trypC-tyrA	23	24 hr	1.17×10^3	3.07×10^4	26.2	109	24
metB5	15	14 hr + 18 hr	8.03×10^2	4.24×10^4	52.8	167	32
hisA	11.4	24 hr + 18 hr	5.78×10^3	3.31×10^5	57.3	219	26
ura-1	8.4	14 hr + 18 hr	6.9×10^1	3.13×10^3	45.2	298	15

Cleavage, electrophoretic separation, and determination of specific biological activity of SB1070 DNA was done as described in *Materials and Methods*. Enrichment factor is calculated as the ratio of specific activities of $Eco R_I$-cleaved DNA before and after fractionation. Theoretical enrichment is equal to the ratio of the molecular sizes of *B. subtilis* chromosome and to that of the segment. The purity of each segment is calculated as ratio of enrichment factor achieved to the theoretical enrichment for the segment. Specific activity is expressed as transformants per μg of DNA.

TABLE 5. *Linkage between B. subtilis genes transformed by Eco*R_I*-restricted DNA*

	Cotransfer of transformant (r)											
	aroB		*trypC*		*hisB*		*tyrA*		*inh*		*aroE*	
Recipient (marker)	1	2	1	2	1	2	1	2	1	2	1	2
SB564(*trypE*)	0	0.83					0.18	0.40				
SB420(*trypC*)									0.13	0.29		
SB1023(*hisB*)	0	0.25	0.33	0.38			0.33	0.38				
SB130(*hisB*)											0.38	0.34
SB130(*aroE*)					0.24	0.46						

EcoR_I-degraded DNA from SB1070 was used to transform several strains for single markers. Cotransfer of unselected markers was determined by replica-plating. The cotransfer index (r) was calculated as in Nester *et al.* (9). For each cotransfer, values given are: (1) transformation by *Ece*R_I-treated DNA, (2) transformation by untreated DNA.

DNA. The purity of different fragments can be estimated by comparing the achieved enrichment factor (Table 4) with that theoretically possible; the latter corresponds to the ratio of the molecular size of the *B. subtilis* chromosome to that of a cleavage-produced segment. By this criterion, as many as three to six different segments may be located in peaks corresponding to different genetic markers (Table 4). These results are consistent with the conclusion drawn earlier that the visually observed bands are not composed of unique DNA segments.

Peaks of biological activity were correlated with the banding pattern after electrophoretic separation of segments, by visualizing the pattern under the UV light, cutting a particular region into 1 mm slices, and determining the biological activity of DNA. This simple procedure was very reliable in our hands; data presented in Fig. 3A were obtained in such a way.

Genetic Composition of One EcoR_I *Segment.* A number of genes coding for proteins involved in the synthesis of aromatic amino acids are clustered in the *B. subtilis* genome (9). Several of these (*trypE, trypC, hisB, tyrA, inh, aroE*) survive cleavage to a comparable degree; the gene to the left (*aroB*) of that sequence suffers extensive loss (Table 2). These data indicate that the left cleavage site adjoins the latter gene (*aroB*), leaving intact the rest of the gene sequence (Fig. 5). This is corroborated by the following results: (*a*) linkage of *aroB* to either *trypE* or *hisB* is completely destroyed by cleavage whereas the linkage between the other markers is only slightly reduced (Table 5); (*b*) when cleaved DNA is electrophoresed into agarose gels, the linked markers between *trypE* and *aroE* migrate with identical mobility (Fig. 4); (*c*) when electrophoretically separated DNA from this peak is assayed for biological activity at limiting concentrations of 0.03–0.1 μg/ml, normal linkage between the markers in the region *trypE* to *aroE*, but *not* to *aroB*, can be demonstrated. These data do not strictly exclude the possibility that *aroB* remains on the *trypE-aroE* segment following cleavage; if so, however, it must be so close to the *Eco*R_I cleavage site as to be inactive in the transformation assay.

DISCUSSION

This paper is directed at easy and simplified approaches to the purification of genetic material from bacterial sources. For analytical purposes, the resolution of very small amounts of cleaved DNA by gel electrophoresis in tubes is feasible.

As for future extensions: at a preparative level, larger amounts of DNA should be amenable to separation in slabs. The coupling of this method with purification depending on parameters other than size (such as buoyant density or differential melting) could lead to even higher purity. Treatment of the separated segments with other restriction enzymes followed by a second round of electrophoresis could lead to further dissection of these segments. Alternative efforts to link the electrophoretically enriched DNA segments to independent replicons such as pSC101 (10, 11), ColE1 (12), or lambda (13) and subsequent cloning (work in progress) are directed to the amplification of unique DNA segments.

The partial characterization of the segment containing the *trypE-aroE* gene sequence demonstrates the potential of uniquely defined, partially purified DNA segments in mapping. The long term goal of such endeavors is the complete mapping of the chromosome on to a set of amplified clones of DNA.

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