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Genetic Mapping of a Linked Cluster of Ribosomal Ribonucleic Acid Genes in *Bacillus subtilis*

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A ribosomal ribonucleic acid gene set consisting of genes for 16S, 23S, 5S, and 4S ribonucleic acid species has been genetically mapped to a position between the markers *recG13* and *abrB74* on the *Bacillus subtilis* chromosome and designated *rrnA*. A ribosomal mutation, *ksgA*, was found to be linked to *rrnA*. This places *rrnA* in a region of the chromosome where ribosome-related genes occur but that is not directly adjacent to the major cluster of ribosome-related markers.

The genes coding for 16S, 23S, and 5S ribosomal RNA species in *Bacillus subtilis* occur in multiple copies and are linked to each other in the order 16S, 23S, 5S (8, 9). These genes have been shown to be transcribed in the order 16S, 23S, 5S (24), and each set of genes is probably a distinct operon transcribed from a unique promoter, as are their cognate genes in *Escherichia coli* (17). The rRNA genes have been mapped genetically to an early-replicating region of the chromosome by using density transfer analysis (19). That at least some of the rRNA genes occur in the origin proximal region of the chromosome was confirmed by the heteroduplex analysis of Chow and Davidson (5). Their electron microscopic study demonstrated that there are 7 to 10 copies of the rRNA genes occurring in clusters on the chromosome and separated by DNA spacers which are not homologous to each other. At least two of the rRNA gene clusters are linked to the attachment site for the temperate phage SPO2 (5). We report here the genetic mapping of a set of rRNA genes, using a mapping strategy similar to that reported by Haldenwang et al. (12).

To accomplish genetic mapping of the rRNA genes, we constructed a plasmid containing these genes and a selectable chloramphenicol resistance determinant. When such plasmids are introduced into recombination-proficient *B. subtilis* strains, they integrate at the region of homology to the chromosome and form a stable insertion whose location can be determined by genetic crosses with the chloramphenicol resistance marker (12).

MATERIALS AND METHODS

Clones and subcloning strategy. The rRNA genes used in this study were isolated by W. Steinberg as *Bam*HI inserts of chromosomal DNA in the *E. coli* plasmid pBR313 and have been described previously (16). Plasmid p12E2 contains a portion of one rRNA linkage group consisting of part of a 23S gene and a 5S gene along with the immediately adjacent spacer region inserted into vector pBR313 (Fig. 1B). The chloramphenicol resistance gene was obtained from plasmid pCS540 (Fig. 1A), which is a chimera between the *E. coli* plasmid pSC101 and the *Staphylococcus aureus* plasmid pC194 (4). The chloramphenicol determinant of pC194 is functional in pCS540 and confers chloramphenicol resistance to both *E. coli* and *B. subtilis*, but the plasmid replicates only in *E. coli*.

*Eco*RI fragments of plasmid p12E2 were ligated to pCS540, which had been linearized by *Eco*RI digestion, using T4 ligase. Incubation was performed overnight at 14°C in a buffer of 0.1 M Tris (pH 7.8), 0.1 M NaCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 50 μg of bovine serum albumin per ml, 0.01 M dithiothreitol, and 0.1 mM ATP (11). The ligation products were transformed into calcium chloride-treated *E. coli* cells (7). Cells were incubated in 0.5% yeast extract–0.5% NaCl–1% tryptone to allow expression of plasmid resistance markers and then plated onto L-agar (1% tryptone–0.5% yeast extract–0.5% NaCl–0.1% glucose–1.5% agar) containing either 100 μg of ampicillin or 20 μg of chloramphenicol per ml. One of the transformants chosen for study contained a plasmid, designated pE2A, whose structure is shown in Fig. 1C.

Restriction endonucleases. *Bgl*I restriction enzyme was purified by R. Horton from *Bacillus globigii*, using the procedure of Bickle et al. (2). *Eco*RI was a gift from Mary Comer, and *Hin*dIII was purchased from Bethesda Research Laboratories, Inc. Digestion conditions were those described in the 1980 catalog, Bethesda Research Laboratories.

DNA purification, cleavage, and hybridization. *E. coli* plasmid DNA was prepared by a modified Clewell and Helinski (6) procedure. Chromosomal DNA from *B. subtilis* was prepared by the procedure of Saito and Muira (18). *Bgl*I fragments of chromosomal DNA were separated by electrophoresis in 0.35% agarose gels and transferred to nitrocellulose

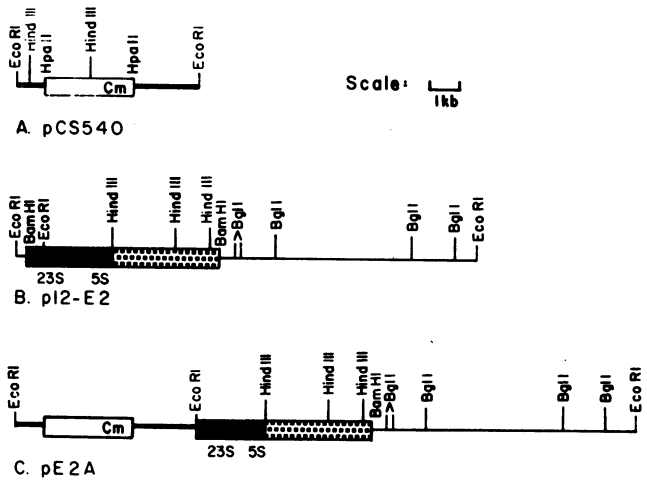

FIG. 1. *(A) Map of the plasmid vector pCS540. The circular DNA is shown in the linear form produced by EcoRI digestion. The HpaII fragment shown as a block represents the portion derived from pC194 and containing the Cm^r marker. The remainder of the plasmid DNA is from pSC101. (B) Restriction endonuclease map of p12E2. The circular plasmid molecule is drawn in linear form. The B. subtilis DNA insert is indicated by a block; pBR313 DNA is indicated by a line. Ribosomal RNA gene sequences within the insert are black; spacer DNA is stippled. (C) Restriction endonuclease map of pE2A. The plasmid is shown in linear form. This plasmid was generated by ligation of the EcoRI fragments of pCS540 and p12E2. Plasmid pCS540 sequences are shown as in A; pBR313 sequences, as a single line; and B. subtilis DNA, as in B.*

filters by the method of Southern (20). Filters were treated as described by Denhardt (10) and hybridized in 6× 0.15 M NaCl plus 0.015 M sodium citrate–0.5% sodium dodecyl sulfate–0.02% Ficoll–0.02% polyvinylpyrolidone overnight at 70°C to DNA probes labeled with ^{32}P by nick translation (15).

Genetic crosses. Phage PBS1-mediated transduction in *B. subtilis* was performed as described by Hoch et al. (14), and transformation was performed as described by Anagnostopoulos and Spizizen (1). Bacterial cultures and the appropriate genetically marked strains were as described by Trowsdale et al. (22).

RESULTS AND DISCUSSION

When plasmids pE2A and pCS540 were introduced into recombination-proficient *B. subtilis* 168 by protoplast transformation (4), 26 chloramphenicol-resistant transformants were obtained with pE2A, whereas no chloramphenicol-resistant transformants resulted from pCS540 vector alone. Repeated attempts to establish this vector plasmid as an autonomously replicating entity in either *rec*$^+$ or *rec* strains of *B. subtilis* have all failed. Eight of the pE2A transformants were tested for integration of the plasmid DNA into the chromosome by digesting total DNA from Cm^r (chloramphenicol-resistant) transformants and from the parent strain with the restriction endonuclease *BglI*. *BglI* cleavage yields seven fragments which hybridize to an rRNA probe (3). To reduce complexity of the patterns, only the *Hin*dIII spacer fragments from pE2A were labeled to probe the *BglI* digests that had been separated on an agarose gel and transferred to nitrocellulose filters by the method of Southern (20; Fig. 2).

This result reinforces the earlier assumption that spacer DNAs associated with rRNA gene clusters are unique to a particular cluster (5). In the transformant strains 1A and 2A (Fig. 2, lanes c and d), the 15-kilobase (kb) fragment (Fig. 2, lane b) disappeared, and three fragments of about 16, 12, and 11 kb appeared. The 12-kb band corresponded in size to a fragment derived from the free plasmid (Fig. 2, lane a) and suggests that plasmid pE2A is present in the transformants as free plasmid and integrated into the chromosome. This result was not observed in the studies of Haldenwang et al. (12), and experiments to explain the occurrence of free plasmid are in progress. pE2A was cut by *BglI* five times, but none of these sites occurred in the *B. subtilis* DNA insert. Therefore, when a transformant with a copy of pE2A integrated into its chromosome was digested with *BglI*, the disappearance of the 15-kb fragment and the appearance of two new spacer DNA reactive bands was consistent with the integration of the plasmid by a Campbell-type model at its region of homology to the chromosome. The fact that the 15-kb band disappeared eliminates a trivial possibility that the plasmid integrated at some region of plasmid homology and the location of the plasmid on the chromosome does not reflect the location of the rRNA genes and the spacer DNA.

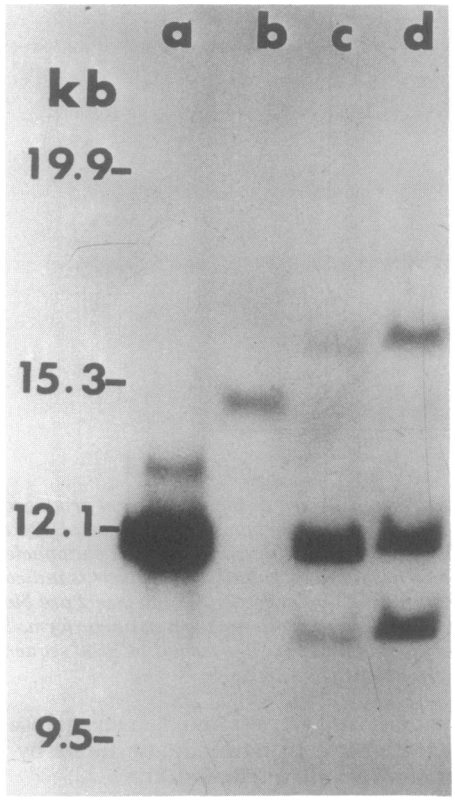

FIG. 2. *Autoradiograph of BglI-digested chromosomal DNA probed with spacer region DNA. BglI fragments of chromosomal DNA were separated by electrophoresis in 0.35% agarose gels to improve resolution of high-molecular-weight fragments. As a result, only DNA fragments of >10 kb are retained on the gel. The HindIII fragments from pE2A which contain only spacer DNA were labeled as probe. The DNAs used were plasmid pE2A (lane a), 168 parent (lane b), and Cm^r transformants 2A and 1A (lanes c and d, respectively).*

In this case, the chromosomal configuration of spacer DNA would have remained the same. From this analysis, we concluded that the Cm^r transformants contain pE2A integrated into the *B. subtilis* chromosome in the region of the specific ribosomal RNA genes which are contained in the insert of pE2A. Strain 2A was used in genetic mapping analysis. Since this is the first ribosomal RNA gene cluster characterized in *B. subtilis*, we designated it *rrn*A.

In earlier studies, two-factor PBS-1 transduction crosses suggested that the chloramphenicol resistance marker from the integrated plasmid pE2A in strain 2A mapped in the origin region of the chromosome near the *guaA* marker (3). To more precisely locate this locus, further two- and three-factor crosses were undertaken. PBS-1 transduction crosses with several strains gave informative results. By using strain 2A as a donor in these crosses, the order of markers was found to be *cysA-abrB*-Cm^r and *guaA-recG*-Cm^r (Table 1). This gives the order *guaA-recG-rrnA-abrB-cysA* across the origin region (Fig. 3). Two-factor transformation crosses between Cm^r and markers in this region were consistent with this location (Table 2). The Cm^r determinant was closest to its two flanking markers, *recG13* and *abrB74*.

Two-factor transformation crosses are grossly nonreciprocal, using the Cm^r marker and linked markers. For example, if a *guaA1* recipient is transformed with a Cm^r donor and Cm^r is selected, 32% cotransfer is observed for the two markers. However, if in the same cross *gua*$^+$ is selected, only 5% cotransfer of the two markers is observed. This discrepancy results from the fact that the Cm^r determinant is a large insertion of DNA in the region, and, therefore, a large piece of DNA must be integrated to obtain a Cm^r transformant. The length of the insertion is greater than 20 kb and, if the tandem duplication at both ends is taken into account, the total length across the insertion is on the order of 26 kb. This size DNA would have a cotransfer frequency in transformation of less than 10% (13). Thus, to obtain chloramphenicol-resistant transformants as a nonselected linked marker, a fairly rare class of transformants has to be selected.

In the course of these studies, we discovered that the position of the kasugamycin locus, *ksgA*, had been misinterpreted in previous crosses (21). Three-factor PBS-1 transduction crosses show that the *ksgA* locus is located between *guaA1* and *cysA14*, and not to the right of *cysA14*, as was previously thought (Table 3). Three-factor transformation crosses with markers in this region establish the order *abrB-ksgA-tms-26*. Since *ksgA* mutations result in high-level ribosomal resistance to kasugamycin (21) and *abrB* mutations cause altered mobilities

TABLE 1. *Three-factor PBS-1 transduction crosses with strain 2A as donor (111)*

Recipient (000)	Selection	Recombinant classes			No.	Order implied
		cys	*abr*	Cm^r		
cysA14 abrB74	*cys*$^+$	1	1	1	32	*cysA-abrB*-Cm^r
		1	1	0	19	
		1	0	1	0	
		1	0	0	68	
		gua	*rec*	Cm^r		
guaA1 recG13	*gua*$^+$	1	1	1	231	*guaA-recG*-Cm^r
		1	1	0	13	
		1	0	1	0	
		1	0	0	33	

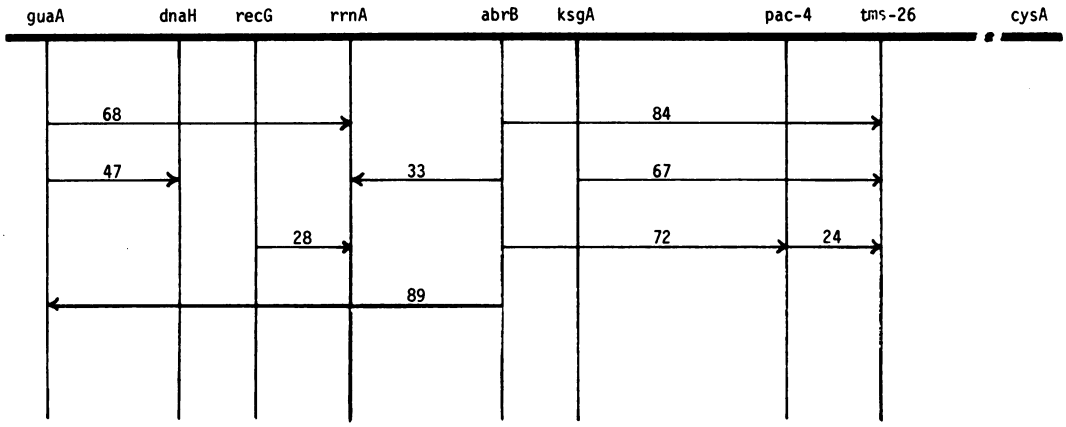

FIG. 3. *Genetic map of the origin region of the B. subtilis chromosome showing the location of the rrnA locus. Numbers are percent recombination in transformation.*

TABLE 2. *Two-factor transformation crosses between markers in the origin region*

Selected marker	Unselected marker[a]							
	guaA1	*abrB74*	*cysA14*	*recG13*	*pac-4*	Cm^r	*dnaH151*	*ksgA618*
Cm^r	136/427	395/587	1/160	69/96	0/124			
guaA1						6/104	54/104	
dnaH151	57/107					8/107		
tms-26		64/399						170/399

[a] Number of recombinants carrying the unselected marker/total number of recombinants tested.

TABLE 3. *Three-factor crosses to locate the ksg locus*

Recipient (000)	Donor (111)	Recombinant classes			No.	Order implied
		gua	*ksg*	*cys*		
guaA1 cysA14 (PBS-1 transduction)	*ksg-618*	1	1	1	25	*guaA-ksg-cysA*
		1	1	0	27	
		1	0	1	0	
		1	0	0	10	
		1	1	1	24	*guaA-ksg-cysA*
		0	1	1	6	
		1	0	1	0	
		0	0	1	31	
		tms	*ksg*	*abr*		
tms-26 abrB3 (transformation)	*ksg-618*	1	1	1	55	*tms-ksg-abrB*
		1	1	0	115	
		1	0	1	9	
		1	0	0	220	

of certain ribosomal proteins (23), it appears that a region of ribosomal proteins or ribosome-modifying enzymes occurs near the *rrnA* locus. A genetic map of the region of *B. subtilis* chromosome surrounding the *rrnA* locus is shown in Fig. 3.

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