Restriction Site Polymorphism of Ribosomal Ribonucleic Acid Gene Sets in Members of the Genus *Bacillus*

PAUL GOTTLIEB† AND RIVKA RUDNER*

Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021

Hybridization of cloned ribosomal sequences to *Eco*RI-restricted genomic deoxyribonucleic acids of eight species and strains of the genus *Bacillus* produced multiband patterns consistent with the presence of 9 to 11 operons per genome. The basic structure of the repeating ribosomal gene set is highly conserved with the exception of one internal *Eco*RI site located near the abutment region between the 16S and 23S ribosomal ribonucleic acid determinants. In each of the *Bacillus* species studied, there are two abutment regions that differ in size by 0.2 kilobase; the larger region contains genes for isoleucine and alanine transfer ribonucleic acids at one-third the proportion of the smaller region. The occurrence of the *Eco*RI site in strains of *Bacillus subtilis* and *Bacillus licheniformis* gave rise upon cleavage to 1.2- and 1.4-kilobase abutment families. The absence of the *Eco*RI site in *Bacillus globigii*, *Bacillus pumilus*, and *Bacillus amyloliquefaciens* resulted in the emergence of 1.9- and 2.1-kilobase abutment families. Mixtures of the two types of families were not found in the *Bacillus* genomes studied.

Taxonomic relationships among various species of *Bacillus* have been studied by deoxyribonucleic acid (DNA)-DNA hybridization and by testing the transformability of auxotrophic and antibiotic resistance genetic markers (10, 21). We analyzed the degree of conservation and variation within ribosomal operons of members of the genus *Bacillus* by following the distribution of internal restriction sites in chromosome digests. The number and physical map of ribosomal ribonucleic acid (rRNA) gene sets in the genome of *Bacillus subtilis* strain 168M have been reported by workers from several laboratories (9, 15, 25; K. Bott, G. C. Stewart, and A. G. Anderson, in *Syntro Conference on Genetics and Biotechnology of Bacilli*, in press). There are 10 rRNA gene sets; each set comprises 16S, 23S, and 5S rRNA determinants, in that order, and two or three also contain genes for isoleucine and alanine transfer ribonucleic acids (tRNA^{Ile} and tRNA^{Ala}) (9). Unlike the situation in *Escherichia coli*, in which seven rRNA genes are scattered around the map (14), there is a unique clustering of the 10 gene sets in three groups in *B. subtilis*. The major cluster, which is composed of six or seven rRNA genes, is found in the chromosome segment delineated by *purA* and *purB* (Bott et al., in press; Rudner et al., unpublished data). Six gene sets have been mapped, *rrnO* and *rrnA* at the replication origin (15, 28), the closely situated repeats (*rrnI*, *rrnH*, and *rrnG*) near the attachment site of SPO2 (Bott et al., in press), and at least one (*rrnE*) between *dal*⁻ and *purB* (Rudner et al., unpublished data). The minor clusters containing three or four of the rRNA cistrons are located in the late replicating portion of the *Bacillus* genome. Operons *rrnB* and *rrnC* have been mapped in the region between *thr-5* and *aroG* (Bott et al., in press). At least one or two cistrons are believed to be located at the *ilvBC-leu* region (3, 23; P. Gottlieb, G. LaFauci, and R. Rudner, Gene, in press).

The basic physical map which is homologous in all gene sets of *B. subtilis* consists of three internal *Eco*RI and *Sma*I sites, two *Pst*I sites, and single *Bam*HI and *Hind*III sites (Fig. 1) (9, 25). The only restriction site which has been

found to be variable is the *Hind*III site distal to the 5S rRNA determinant (Fig. 1) (25). Chromosomal patterns after restriction with *Eco*RI and *Bam*HI have been used to calculate the number of gene sets in *B. subtilis* 168M (15, 25). The sizes of the various restriction fragments carrying ribosomal sequences vary considerably, indicating extensive heterogeneity in the inter-operon "spacer." To date, only one case of intragenic size heterogeneity has been reported, one which arises from the abutment region between the 3' terminus of 16S rRNA and the 5' terminus of 23S rRNA. There are two *Eco*RI-generated abutment fragments, measuring 1.1 and 1.3 kilobases (kb) according to Stewart et al. (25) or 1.2 and 1.4 kb according to our determinations and those of Loughney et al. (9). The larger spacer includes the genes for both tRNA^{Ile} and tRNA^{Ala} (9). We describe in this paper divergence among the *Bacillus* species involving sequences in the spacer region between the segment that transcribes the mature 16S and the 23S ribonucleic acid species.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The plasmids containing ribosomal insertions were gifts from K. Bott and were amplified in *Escherichia coli* strain HB101 (*pro leu thi lacY hsdR endA recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*). Figure 1 shows the sequences of genes coding for rRNA (rDNA) which were inserted into the plasmids used in this study. The cloned fragments originated as follows: (i) p21C4 contains a sheared 0.3-kb fragment around the *Eco*RI site in the 16S determinant cloned by adenine-thymine tailing into plasmid pMB9 (7); (ii) pBC194 contains a 1.1-kb *Eco*RI fragment with the abutment region of the 16S and 23S rDNAs and was subcloned from plasmid p14B1 into the *Eco*RI site of plasmid pBR322 (25); (iii) p12E2 contains a 6.3-kb *Bam*HI-generated insertion with homology to 23S and 5S rDNAs and the 3.5-kb spacer DNA of the *rrnA* operons (28) cloned into plasmid pBR313 by Zuber and Steinberg; (iv) pBC279 contains a 2.5-kb *Eco*RI-generated insertion with homology to 23S and 5S rDNAs cloned into plasmid pBR313 by Zuber and Steinberg; and (v) pMS102-B7 contains a 5.7-kb *Bam*HI insertion which originated from *rrnO* (15) containing all of

* Corresponding author.

† Present address: Public Health Research Institute, New York, NY 10016.

FIG. 1. Generalized restriction map of *B. subtilis* rRNA gene sets as proposed by Stewart et al. (25). The six rRNA fragments from *B. subtilis* inserted into plasmids are represented by solid lines; the dashed lines represent *B. subtilis* DNA sequences which carry no homology to rRNA. B, *Bam*HI; H, *Hind*III; S, *Sma*I; R, *Eco*RI. The internal *Eco*RI site marked with a circled R is variable in other *Bacillus* species. The cross-hatched area defines the spacer or abutment region between 16S and 23S rRNA determinants. Sizes are given in kilobases.

the 16S rDNA and one-third of the 23S rDNA cloned into bifunctional plasmid pMS102 (20).

Chromosomal and plasmid DNA isolation and restriction patterns. *Bacillus* DNAs were prepared from cells grown in VY medium (25 g of veal infusion broth [Difco Laboratories], 5 g of yeast extract [Difco], and 1,000 ml of water) and were purified by a modification (19) of the procedure of Marmur (13). Plasmid DNA was purified from 500-ml cultures of *Escherichia coli* cells grown in LB broth supplemented with either 100 µg of ampicillin per ml or 15 µg of tetracycline per ml, essentially by the procedure of Tanaka and Weisblum (27). Chromosomal DNA (5 to 10 µg) was digested with restriction endonucleases *Eco*RI, *Hind*III, *Sma*I, *Pst*I, and *Bam*HI (3 U of enzyme per µg of DNA for 12 h at 37°C) by using the conditions for digestion recommended by the supplier (New England BioLabs). DNA fragments were electrophoretically resolved in 0.75% agarose gels, stained with ethidium bromide, and transferred to nitrocellulose filters for hybridization (24).

Preparation of hybridization probes and densitometry. rRNA for use as a probe was isolated from *B. subtilis* 168T grown in VY medium and purified by the method of Margulies et al. (12); 5S, 16S, and 23S rRNA species were prepared in low-melting-point agarose (Bethesda Research Laboratories), and the rRNAs were 5' end labeled with [γ - 32 P]adenosine triphosphate and T4 polynucleotide kinase by the method of Maizels (11). Alternatively, cloned rDNA and *leu* probes were prepared by nick translation with [α - 32 P]adenosine triphosphate and [α - 32 P]cytidine triphosphate (18). The labeled DNA was freed from low-molecular-weight material by passage through a Sephadex G-50 column (Pharmacia Fine Chemicals, Inc.). DNA was routinely labeled to a specific activity of 0.5×10^8 to 2×10^8 cpm/µg. Filter hybridization was performed by using the conditions of Ostapchuk et al. (16). The sizes of the bands obtained by autoradiography were determined by reference to six lambda bands and were calculated from the relationship described by Bearden (2). Some autoradiogram films were scanned at 580 nm with a Gilford automatic spectrophotometer

TABLE 1. List of strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype ^a	Source
Bacterial strains		
<i>B. globigii</i> RUB562	Prototroph	C. Tackney
<i>B. pumilus</i> RUB502	bio Rif ^r Sm ^r Er ^r	A. Garro
<i>B. licheniformis</i> FDO1	Sm ^r	D. Dubnau
<i>B. licheniformis</i> ACTC 8480	Sm ^r	A. Garro
<i>B. amyloliquefaciens</i> H	Prototroph	A. Garro
<i>B. subtilis</i> W23	Prototroph SM ^r Er ^r Mic ^r	D. Dubnau
<i>B. subtilis</i> NCTC 3610	Prototroph	A. Sonenshein
<i>B. subtilis</i> 168T	<i>trpC2</i>	K. Bott
<i>B. subtilis</i> 166	<i>trpE26</i>	Bacillus Genetic Stock Center
<i>B. stearothermophilus</i> 2184	Sm ^r	M. S. Friedman
Recombinant plasmids^b		
p21C4	16S rRNA, Tc ^r	K. Bott
pBC194	16S-23S rRNA, Am ^r Tc ^r	K. Bott
pBC279	23S-5S rRNA, Am ^r	K. Bott
p12E2	23S-5S rRNA, Am ^r	K. Bott
pMS102-B7	16S-23S rRNA, Am ^r Km ^r	K. Bott
pRGR102	16S-23S rRNA, Tc ^r Cm ^r	Constructed in our laboratory

^a Er^r, Erythromycin resistance; Mic^r, micrococin resistance; Rif^r, rifampin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Am^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

^b See Fig. 1. The rDNA sequences were from *B. subtilis* 168 cloned in *Escherichia coli* plasmids. Plasmids pMS102 (20) and pJH101 (4) are hybrids containing, in addition to pBR322, pUB110-Km^r and pC194-Cm^r, respectively.

equipped with a model 6051 recorder. The relative areas of the peaks were determined by using a planometer (Noris Instruments).

RESULTS

Chromosomal patterns of rRNA homology in the genus *Bacillus*. Complete restriction of *Bacillus* DNAs with *Eco*RI, when they were probed with either 23S and 5S rRNAs or plasmids containing these sequences (pBC279 and p12E2), consistently produced 9 to 11 homologs on Southern blots. Figure 2 shows a composite of four autoradiograms obtained with *Eco*RI digests of *Bacillus* DNAs. Table 2 summarizes the molecular sizes and numbers of chromosomal restriction fragments which hybridized to the various probes found in each of the eight members of the genus *Bacillus* studied. As shown in Fig. 2, the multiple-band patterns were highly intense, indicating, as expected, that ribosomal sequences are extremely homologous in members of the genus *Bacillus* compared with other *Bacillus* sequences, such as the aromatic amino acid cluster (26) or the *thrA* gene (Rudner et al., unpublished data), which gave rise to hybrid bands of relatively low intensities. Similarly, other enzymes (data not shown), such as *Hind*III, *Bam*HI, *Sma*I, and *Pst*I, revealed that the probes hybridized with equal intensity to all *Bacillus* DNAs. The different multiple-band hybridization patterns with respect to homolog size clearly indicate variation not in the rDNA sequences but in the spacer regions. The lower bands resulted from multiple restriction sites internal to the repeating gene set (see below) (Table 3). The upper bands arose from one restriction site within the gene set and a second site beyond it in the intergenic spacer region, where evolutionary variation has produced fragments of different sizes.

The multiple-band patterns obtained were unique for each of the species and were highly reproducible between individual strains when data were available. For *B. subtilis*, many derivatives of strains 168M and NCTC 3610 were analyzed; only two are shown in Table 2 and Fig. 2, in which the band patterns are identical. Not counting the last three fragments, both 5S and 23S rRNAs or the cloned rDNA hybridized to nine distinguishable fragments in the *Eco*RI digests of genomic strain 168M and NCTC 3610 DNAs (Fig. 2 and Table 2). In each digest, at least one hybrid band appeared to be much darker than the others, and scanning of the autoradiogram revealed the presence of two comigrating rDNA homologs (see below). Thus, we agree with previous workers that there are at least 9, and probably 10, rRNA gene sets in *B. subtilis* strains 168M and NCTC 3610 (9, 25). Transformable *B. subtilis* strains 168M and NCTC 3610 had identical band sizes but shared less homology with strain W23. Similarly, the two *Bacillus licheniformis* strains studied showed close homology except for certain variations (namely, loss of a 6.5-kb hybrid band and a concomitant increase in size of a second band from 9.0 to 9.8 kb in strain ACTC 8480) (Table 2). Other variations are discussed below.

Number of *rrn* operons in the genus *Bacillus*, as determined by densitometry. Stewart et al. (25) determined from *Eco*RI restriction patterns that *B. subtilis* 168 has 10 rRNA gene sets per chromosome. This determination was made by counting 23S rRNA homologs observed on Southern blots. This approach is limited by the fortuitous migration of different fragments of the same molecular weight. Southern blots of *Bacillus* chromosomal digests probed with p12E2-23S,5S labeled DNA or 23S rRNA were subjected to densitometry measurements. *Eco*RI patterns were chosen

because the appearance of the internal small fragments clearly indicated that complete restriction had taken place and because the relative proportion of minimal intervals of gene sets that contained tRNA sequences could be calculated (see below). Finally, the intensity of each homolog band was independent of its molecular weight since all bands carried about 2.2 kb of 23S and 5S sequences from the central *Eco*RI site and rightward (Fig. 1). A densitometry tracing of an autoradiogram is shown in Fig. 3. The area of the smallest peak on each scan was designated as 1 and was used to estimate the multiplicity of the other bands. The sum of all band multiplicities provided the estimated total number of rRNA gene sets per genome. All of the *Bacillus* species appeared to have 9 to 11 gene sets (Table 3). As mentioned above, *B. licheniformis* strain ACTC 8480 was estimated to have 9 or 10 operons, whereas strain FDO1 produced a 6.5-kb band in addition to the 6.0-kb band (Table 2). Therefore, we concluded that the latter strain may possess 10 or 11 *rrn* operons.

Polymorphism of the abutment fragments in *Bacillus*. The spacer or abutment fragments which overlap the 3' end of the 16S rRNA sequences and the 5' end of the 23S rRNA sequences were identified by using the specific probes shown in Fig. 1. When *Eco*RI was used to cut *B. subtilis* DNA, only p21C4-16S, pBC194-16S,23S, and pMS102-16S,23S and 23S rRNAs hybridized to the 1.2- and 1.4-kb fragments (Fig. 2A and B) whereas 5S rRNA and pBC279-23S,5S and p12E2-23S,5S DNA probes did not hybridize to these fragments (Fig. 2C and D). The 0.9-kb fragment extended from the *Eco*RI site at the 5' end of the 23S coding sequence to the site near the 23S center. Therefore, it could hybridize with the comparable 23S determinants provided by plasmid probe p12E2-23S,5S (Fig. 2D). Table 3 shows the sizes of only these internal fragments and the hybridization probes that produced them. The two abutment fragments with similar hybridization patterns (that is, differing in size by 0.2 kb) were also obtained when *Sma*I was used (see below). Our findings, as well as those reported previously (9, 25), indicate that there are two families of rRNA gene sets in *B. subtilis*, which differ with respect to the size of the *Eco*RI fragments bridging the 16S and 23S rRNA determinants. The intensity of the 1.4-kb band shown in Fig. 2A and B indicates that this fragment, like its 1.2-kb counterpart, exists in multiple copies at a ratio of 3:7, as reported by Stewart et al. (25), and at a similar relative proportion of 0.31 according to our densitometry tracing of the autoradiograms (see below).

Among the five *Bacillus* species and their strains examined in this study, the same pattern of internal *Eco*RI fragments was observed in *B. subtilis* strains NCTC 3610, 168M, and W23 and *B. licheniformis* strain ACTC 8480 (Fig. 2 and Table 3). However, in the case of *Bacillus globigii* RUB562, *Bacillus pumilus* RUB502, and *Bacillus amyloliquefaciens* H, the *Eco*RI site (Fig. 1, circled R) within the 5' end of the 23S sequence was not present. In these three species, the absence of that *Eco*RI site resulted in larger abutment fragments (1.9 to 2.1 kb) and the loss of the 0.9-kb *Eco*RI fragment. The p12E2-23S,5S probe revealed the 2.1- and 1.9-kb abutment fragments in the species that lost the *Eco*RI site (*B. globigii*, *B. pumilus*, and *B. amyloliquefaciens*), but not in the species that retained the site (*B. subtilis* and *B. licheniformis*) (Fig. 2C). The loss of the internal *Eco*RI site resulted in extending the size of abutment fragments into the range of the p12E2-23S,5S probe (Fig. 1). Plasmid pBC279-23S,5S contains in its cloned sequence 23S rDNA extending only to the central *Eco*RI site (Fig. 1). As a result, neither the *B. subtilis* strains nor the *B. licheniformis* strains produced

FIG. 2. Hybridization of ^{32}P -labeled cloned rDNA to *Eco*RI digests of *Bacillus* DNAs after Southern (24) transfer. Each gel lane contained $2\text{ }\mu\text{g}$ of restricted chromosomal DNA. The probes were prepared by nick translation, and the resulting specific activities ranged from 0.5×10^8 to 2×10^8 cpm/ μg of DNA. The hybridization procedures of Anilionis and Riley (1) involved prehybridization of the filters in a solution containing 40% formamide, $4\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 250 μg of sonicated calf thymus DNA per ml, 1% glycine, and 0.05 volume of Denhardt solution at 42°C for at least 1 h. The hybridizations with the ^{32}P -labeled probes were for 18 to 22 h at 37°C in a similar solution in the presence of 10% dextran sulfate at a volume ratio of 40 ml/cm 2 of filter with digested chromosomal DNAs. (A) Autoradiogram of *Eco*RI-digested chromosomal DNAs probed with p21C4-16S. Lane 1, *B. subtilis* 168T; lane 2, *B. amyloliquefaciens* H; lane 3, *B. globigii* RUB562; lane 4, *B. pumilus* RUB502; lane 5, *B. licheniformis* FDO1; lane 6, *B. licheniformis* ACTC 8480. (B) Autoradiogram of *Eco*RI-digested chromosomal DNAs probed with pMS102-16S,23S. Lane 1, *B. licheniformis* FDO1; lane 2, *B. licheniformis* ACTC 8480; lane 3, *B. pumilus* RUB502; lane 4, *B. globigii* RUB562; lane 5, *B. subtilis* W23; lane 6, *B. subtilis* 168T; lane 7, *B. subtilis* NCTC 3610. (C) Autoradiogram of *Eco*RI-digested chromosomal DNAs probed with p12E2-23S,5S. Lane 1, *B. licheniformis* FDO1; lane 2, *B. licheniformis* ACTC 8480; lane 3, *B. pumilus* RUB502; lane 4, *B. globigii* RUB562; lane 5, *B. subtilis* W23; lane 6, *B. amyloliquefaciens* H; lane 7, *B. subtilis* GSY1269 *trpE26 ilvC1*. (D) Autoradiogram of *Eco*RI-digested chromosomal DNAs probed with pBC179-23S,5S. Lane 1, *B. licheniformis* FDO1; lane 2, *B. licheniformis* ACTC 8480; lane 3, *B. pumilus* RUB502; lane 4, *B. globigii* RUB562; lane 5, *B. subtilis* W23; lane 6, *B. subtilis* NCTC 3610; lane 7, *B. subtilis* 168T; lane 8, lambda DNA, *Hind*III digested. kbp, kilobase pairs.

TABLE 2. Molecular sizes of *EcoRI* chromosomal restriction fragments which hybridize to 23S and 5S rRNAs or plasmids pBC279 and p12E2 in the genus *Bacillus*

Fragment sizes (kb)						
<i>B. subtilis</i> NCTC 3610 and 168M	<i>B. subtilis</i> W23	<i>B. licheni- formis</i> FDO1	<i>B. licheni- formis</i> ACTC 8480	<i>B. globigii</i> RUB562	<i>B. pumilus</i> RUB502	<i>B. amylolique- faciens</i> H
		13.5		13.6 13.5 10.2	10.2m ^b	10.2
9.0 (1.09) ^c 7.7 (1.29)	9.9 7.2m	9.0m	9.8m	7.1m	6.5m	
6.1 (1.05)	6.1	6.5 6.0m	6.0m		5.6m 4.8m 3.8	5.6 3.8m
4.3 (2.02) 3.7m 3.5 3.4 (2.48) 2.9 (1.37)	4.3 3.7m 3.3	3.8m	3.8m	3.8m		
		2.5 2.3	2.5 2.3	2.6m		
2.1 (1.00)				2.1m ^{d,e} 1.9m ^{d,e}	2.1m ^{d,e} 1.9m ^{d,e}	2.1m ^{d,e} 1.9m ^{d,e}
1.4m ^c 1.2m ^c 0.9m ^{d,e}	1.4m ^c 1.2m ^c 0.9m ^{d,e}	1.4m ^c 1.2m ^c 0.9m ^{d,e}	1.4m ^c 1.2m ^c 0.9m ^{d,e}			

^a In *B. subtilis* NCTC 3610 and 168M, *B. licheniformis* ACTC 8480, and *B. pumilus* RUB502 there are 9 or 10 gene sets; in *B. subtilis* W23 and *B. globigii* RUB562 there are 9 gene sets; and in *B. licheniformis* FDO1 and *B. amyloliquefaciens* H there are 10 or 11 gene sets.

^b m, Multiple bands.

^c The numbers in parentheses are unit areas (see Fig. 3). Band multiplicity was calculated from the 1.00-U homolog of the 2.1-kb homolog.

^d Band visible with the p12E2-23S,5S probe.

^e Band visible with the 23S rRNA probe.

their abutment region as this probe shares no homology with it (Fig. 1 and 2D). Plasmid pMS102-16S,23S, as well as the end-labeled 23S rRNA probe, showed the three internal *EcoRI* fragments in the first group of *Bacillus* species, whereas the second group displayed the two large *EcoRI* pieces (Fig. 2B).

The relative proportions of the two families of rRNA gene sets (those containing tRNA sequences and those not containing tRNA sequences) were determined by densitometry. Figure 4 shows tracings of minimal *EcoRI* fragments from the two types of rDNA gene sets. The total area of both peaks was computed with the planimeter and the ratio was calculated. Type I *Bacillus* species gave ratios of 1.4-kb bands to 1.2-kb bands of 0.31, 0.31, and 0.28 (for *B. subtilis* strains 168M and W23 and *B. licheniformis* strain ACTC 8480, respectively). However, type II *Bacillus* species gave ratios of 2.1-kb bands to 1.9-kb bands of 0.34 and 0.42 (for *B. globigii* strain RUB562 and *B. pumilus* strain RUB502, respectively).

Restriction of chromosomal DNA with restriction enzyme *SmaI* confirmed the conservation of the *SmaI* sites (Table 3). The distinct 2.3-kb fragment was present in *B. subtilis*, *B. globigii*, and *B. pumilus*. When the chromosomal digests were probed with pMS102-16S,23S, they consistently gave rise to only two abutment fragments, the 0.5- and 0.7-kb fragments (Table 3). The size difference of 180 to 200 base pairs suggests that the abutment region was conserved with respect to size, sequence, and the relative proportions of the larger piece and the smaller piece.

Additional heterogeneity in internal fragment size was observed in two *Bacillus* strains. One of these was *B.*

licheniformis strain FDO1, which produced an extra abutment fragment when it was probed with plasmid pBC194-16S,23S or pMS102-16S,23S. As shown in Fig. 5, a 1.3-kb band was found in addition to the two abutment fragments (1.2 and 1.4 kb). The ratio of the 1.4-kb band to the sum of the 1.2- and 1.3-kb bands in strain FDO1 was 0.45, which was significantly higher than the value of 0.28 found for the 1.4- and 1.2-kb abutment fragments in strain ACTC 8480. As noted above, *B. licheniformis* strain FDO1 has 11 *rrn* gene sets (Table 2). The additional operon might contain an insertion of a single tRNA in its abutment region, giving rise to the *B. subtilis* DNA probes containing parts of tRNA^{Ile} or tRNA^{Ala} (9).

A similar situation was observed in the *EcoRI* digests of *B. amyloliquefaciens* strain H. Here the digests, when they were probed with pBC194, produced a doublet at the position of the 1.9-kb band and two minor bands (Fig. 5). The latter corresponded to the 1.4- and 1.2-kb bands found in the first group of *Bacillus* species (Table 3). Finally, *Bacillus stearothermophilus* strain 2184 gave rise to a single internal *EcoRI* fragment (1.9 kb) (Fig. 5).

DISCUSSION

In this paper we present data on the conserved nature of the ribosomal gene sets among *Bacillus* species. The basic *rrn* repeating unit is highly conserved, whereas extensive heterogeneity is found in the interoperon spacer regions. In chromosomal restrictions, *B. subtilis* strain NCTC 3610 is identical to strain 168M. In contrast, these two strains differ significantly from prototrophic strain W23, which was once used as a source of donor DNA for transformation. *B.*

TABLE 3. Molecular sizes of restriction fragments internal to the rRNA gene set in members of the genus *Bacillus*

Enzyme	Strain(s)	Fragment sizes (kb) with the following probes:					
		p21C4-16S or pBC194-16S,23S	23S	5S	pBC279- 23S,5S	p12E2- 23S,5S	pMS102- 16S,23S
<i>Eco</i> RI	<i>B. subtilis</i> 168T, NCTC 3610, and W23	1.4	1.4	— ^a	—	0.9	1.4
		1.2	1.2				1.2
			0.9				0.9
	<i>B. licheniformis</i> FDO1 and NCTC 8480 ^c	1.4	1.4	ND ^b		0.9	1.4
		1.2	1.2			0.9	1.2
			0.9				0.9
	<i>B. globigii</i> RUB562	2.1	2.1	—	—	2.1	2.1
		1.9	1.9			1.9	1.9
	<i>B. pumilus</i> RUB502	2.1	2.1	—	—	2.1	2.1
		1.9	1.9			1.9	1.9
<i>Sma</i> I	<i>B. subtilis</i> 168T, NCTC 3610, and W23	—	2.3	—	2.3	2.3	2.3
							0.7
							0.5
	<i>B. globigii</i> RUB562	—	2.3	—	2.3	2.3	2.3
							0.7
							0.5
<i>B. pumilus</i> RUB502		—	2.3	—	2.3	2.3	2.3
							0.7
							0.5

^a —, Hybrid band not present.^b ND, Not determined.^c *B. licheniformis* FDO1 exhibited three hybrid bands (1.4, 1.3, and 1.2 kb) (see Fig. 5).^d *B. amyloliquefaciens* H exhibited two additional faint hybrid bands (1.4 and 1.2 kb) (see Fig. 5).

FIG. 3. Densitometry tracing of an autoradiogram of *Eco*RI 23S rRNA homologs. Chromosomal DNA of *B. subtilis* NCTC 3610 was restricted to completion with endonuclease *Eco*RI (3 U of enzyme per μ g of DNA). Then 2 μ g of the digest was added to each well of a 0.75% agarose gel. Southern blots were made of these gels and hybridized to ³²P-labeled 23S rRNA (30 to 40 ng of probe per gel

subtilis W23 is known to differ from strains NCTC 3610 and 168M by its phage spectrum, cell wall components, and marker survival after restriction with *Eco*RI and *Hind*III (6, 26). Seiki et al. (20) reported DNA homologies between *B. subtilis* 168M and W23 of 70 and 67% in reciprocal hybridizations. Two *B. licheniformis* strains, strains FDO1 and ACTC 8480, show a close and expected homology based on the criteria used in this study. Some variation is manifest by an additional *rrn* homolog which is found in strain FDO1 but is not present in strain ACTC 8480. In addition, one or more rRNA gene sets of strain FDO1 may contain a different 16S,23S abutment region with only one tRNA sequence instead of the usual forms with two or none.

Previous estimates of the levels of homology between the DNA of *B. subtilis* 168M and the DNAs of *B. licheniformis* FDO1, *B. amyloliquefaciens* H, and *B. pumilus* NRRL B-3275 were to 24, 11, and 16%, respectively (10). The levels of DNA homology obtained from hybridization of ³H-labeled input *B. subtilis* 168 DNA and nitrocellulose disk-bound DNAs from 52 test strains of eight species were of 25,

lane). The hybridization reaction was for 12 h under the conditions described by Ostapchuk, et al. (16). The hybridized filter was filmed for 10 h at room temperature. The chart paper of the densitometer recorder was run at 20 cm/min, and the autoradiogram lane was scanned at 4 cm/min (580 nm). The numbers over the peaks are molecular sizes (in kilobases).

13, and 7% for *B. amyloliquefaciens* F, *B. licheniformis* (13 strains), and *B. pumilus* IFO 12110, respectively (21). Based on their DNA homology index data, Seki et al. divided the genus *Bacillus* into three groups. One of these groups had a homology index to strain 168M of more than 70% and transformed widely dispersed auxotrophic markers. The second group, with a level of DNA homology to strain 168 of 20 to 25%, did not transform auxotrophic markers but did transform antibiotic resistance. The third group had a level of DNA homology to strain 168 of 7 to 15% and showed some detectable transformation of antibiotic resistance markers (21).

The *Bacillus* strains examined in this study can be divided

FIG. 4. Densitometry tracings of autoradiograms of the two types of 16S and 23S abutment fragments. Chromosomal DNA was restricted with *Eco*RI as described in the legend to Fig. 3. Hybridization was with 32 P-end-labeled 23S rRNA. The region of the autoradiogram displaying the abutment fragments was scanned at 1 cm/min, and the chart recorder paper was run at 20 cm/min. (A) Type I rDNA abutment fragments (1.2 and 1.4 kilobase pairs) with the 23S determinant *Eco*RI site from *B. subtilis* NCTC 3610. (B) Type II rDNA abutment fragments (1.9 to 2.1 kilobase pairs) without the 23S determinant *Eco*RI site from *B. globigii* RUB562.

FIG. 5. Autoradiographic localization of the two types of abutment fragments in *Eco*RI digests of *Bacillus* DNAs probed with pBC194-16S,23S. For experimental details, see the legend to Fig. 2. Lane 1, *B. subtilis* NCTC 3610; lane 2, *B. subtilis* W23; lane 3, *B. licheniformis* FDO1; lane 4, *B. licheniformis* ACTC 8480; lane 5, *B. globigii* RUB562; lane 6, *B. pumilus* RUB502; lane 7, *B. amyloliquefaciens* H; lane 8, *B. stearothermophilus* 2184. kbp, kilobase pairs.

into two groups, those with the internal *Eco*RI site (type 1) and those without this site (type II). Upon digestion with restriction enzymes *Eco*RI and *Sma*I, the DNAs of these strains yielded abutment fragments differing in size by precisely 0.2 kb. With the exception of one strain, none of the strains gave rise to a mixture of both types of fragments (namely, a 1.4-kb fragment and a 1.9-kb fragment or a 1.2-kb fragment and a 2.1-kb fragment). In *B. amyloliquefaciens* H we observed small amounts of the lower bands (1.4 and 1.2 kb), yet more than 80% of the hybrids were the two larger abutment fragments (Fig. 4). Moreover, we predict no variation in the distribution of abutment fragments in *Bacillus* DNAs digested with restriction enzyme *Pst*I or in a double digest with *Pst*I and *Hind*III. The sizes expected for the conserved abutment regions are 3.1 and 3.3 kb for the former enzyme and 0.9 and 1.1 kb for the codigestion (9). The absence of additional heterogeneity in fragment size strongly suggests that the original loss of that *Eco*RI site which led to the appearance of the two larger abutment fragments (1.9 and 2.1 kb) could have occurred in a proto-*Bacillus* species before the main rRNA gene amplification. Interoperon spacer variations, genomic rearrangements, and gene duplication evolved later and led to the observed changes in the molecular sizes of the larger rDNA fragments. An evolution-

FIG. 6. Dendrogram analysis of the relationships among five species of *Bacillus* and their strains. The bacteria examined in this study were divided into two lines of descent based upon the structures of their rRNA gene sets. Type I *Bacillus* species have rRNA genes possessing the 5' 23S sequence *Eco*RI site (see Fig. 1), and the type II species have rRNA genes without the *Eco*RI site.

any dendrogram which illustrates a proposed derivation and relationship among these *Bacillus* species is shown in Fig. 6.

Alternatively, horizontal evolution via amplification of one variant *rrn* cistron could also produce this result. Unequal crossing-over can fix a given sequence type within a population of heterogeneous sequences (22). According to this model, an entire gene cluster is replaced by a different type of gene copy, an event that would occur after *rrn* gene multiplication. Smith (22) has presented a model of "cross-over fixation," which could effectively propagate a variant sequence with the elimination of another. This event also depends upon the occurrence of unequal crossing-over among tandem repeats of genes in a tandem array of a fixed size; through such unequal crossing-over the original genes are eventually eliminated and replaced by others. Such a model allows the fixation for type I or type II rDNA sequences to occur at any time in evolution and clearly explains how a gene variant originally in the minority can ultimately become the predominant or only type. In fact, we describe in another study mutants of *B. subtilis* displaying an rDNA deletion or duplication as evidence that such a mechanism might be occurring (Gottlieb et al., in press). The addition, alteration, or replacement of rDNA in transformants of these mutants lends further credence to the dynamic nature of the rDNA repeats (Gottlieb et al., in press; Rudner et al., unpublished data).

In summary, the physical organization of an rRNA gene set in the genus *Bacillus* is unique in size, as shown by the

positions of restriction site sequences which remained unchanged. It is intriguing that even the length of the spacer regions between 16S and 23S rRNA coding sequences, which potentially could have varied, are highly conserved. Only two sizes have evolved, differing by 0.2 kb; the larger abutment fragment contains the tRNA^{Ile} and tRNA^{Ala} genes (9). Similar tRNA sequences are located in the analogous region of the *Escherichia coli* rRNA operons (29), as well as in chloroplast rDNAs of *Euglena gracilis* (5), and *Zea mays* (8). This strong sequence homology between spacer regions of gram-positive bacteria and gram-negative bacteria, as well as the chloroplasts of eucaryotes, indicates functional significance and structural constraints. It is tempting to speculate that the spacer regions, particularly those with the tRNA genes, can form secondary structures which constitute part of a signal structure necessary for proper processing of the common precursors. On the other hand, the intergenic spacer region between adjacent rRNA operons seems to have suffered a higher genetic drift rate in the genus *Bacillus*.

ACKNOWLEDGMENTS

We thank the following students for assisting in some of these studies: D. Nembhard, R. Sepulveda, and W. Williamson.

This work was supported by Public Health Service grant GM16059 from the National Institute of General Medical Sciences, by City University of New York Faculty Research Awards 14024 and 663152, and by Public Health Service Minority Biomedical

Research Support Grant RRO81776-04 from the National Institutes of Health.

ADDENDUM IN PROOF

K. F. Bott (University of North Carolina Medical School, Chapel Hill) has made available to us the complete sequence of the *B. subtilis* *rrnB* operon which confirms the size of the abutment *Eco*RI fragments as 1.1 or 1.3 kb.

LITERATURE CITED

- Anilionis, A., and M. Riley. 1980. Conservation and variation of nucleotide sequences within related bacterial genomes: *Escherichia coli* strains. *J. Bacteriol.* **143**:355-365.
- Bearden, J. 1979. Electrophoretic mobility of high molecular weight double strand DNA on agarose gels. *Gene* **6**:221-234.
- Chilton, M. D., and B. J. McCarthy. 1969. Genetic and base sequence homologies in bacilli. *Genetics* **62**:697-710.
- Ferrari, F. A., A. Nguyen, D. Lang, and J. A. Hoch. 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. *J. Bacteriol.* **154**:1513-1515.
- Graf, L., H. Kussel, and E. Stutz. 1980. Sequencing of 16S-23S spacer in a ribosomal RNA operon of *Euglena gracilis* chloroplast DNA reveals two tRNA genes. *Nature (London)* **286**:908-910.
- Hemphill, H., and H. R. Whiteley. 1975. Bacteriophages of *Bacillus subtilis*. *Bacteriol. Rev.* **39**:257-315.
- Hutchison, K. W., and H. O. Halvorson. 1980. Cloning of randomly sheared DNA fragments from a ϕ 105 lysogen of *Bacillus subtilis*: identification of prophage-containing clones. *Gene* **8**:267-278.
- Koch, W., K. Edwards, and H. Kussel. 1981. Sequencing of the 16S-23S spacer in a ribosomal RNA operon of *Zea mays* chloroplast DNA reveals two split tRNA genes. *Cell* **25**:203-213.
- Loughney, K., E. Lund, and J. E. Dahlberg. 1982. tRNA genes are found between the 16S and 23S rRNA in *Bacillus subtilis*. *Nucleic Acids Res.* **10**:1607-1624.
- Lovett, P. S., and F. E. Young. 1969. Identification of *Bacillus subtilis* NRRL B-3275 as a strain of *Bacillus pumilus*. *J. Bacteriol.* **100**:658-661.
- Maizels, N. 1977. RNA labeling mediated by T4 polynucleotide kinase, p. 247-251. In G. Wulcox, J. Abelson, and C. F. Fox (ed.), ICN-UCLA Symposium on Molecular and Cellular Biology, vol. 8. Academic Press, Inc., New York.
- Marguiles, L., V. Remeza, and R. Rudner. 1971. Asymmetric template function of microbial deoxyribonucleic acids: transcription of messenger ribonucleic acid. *J. Bacteriol.* **107**:610-617.
- Marmur, J. 1961. A procedure for the isolation of DNA from microorganisms. *J. Mol. Biol.* **3**:208-218.
- Nomura, M., and L. E. Post. 1980. Organization of ribosomal genes and regulation of their expression in *Escherichia coli*, p. 671-691. In G. Chambliss, G. R. Craven, K. Davis, L. Cahan, and M. Nomura (ed.), Ribosomes: structure, function and genetics. University Park Press, Baltimore.
- Ogasawara, N., M. Seiki, and H. Yoshikawa. 1983. Replication origin region of *Bacillus subtilis* contains two rRNA operons. *J. Bacteriol.* **154**:50-57.
- Ostapchuk, P., A. Anilionis, and M. Riley. 1980. Conserved genes in enteric bacteria are not identical. *Mol. Gen. Genet.* **180**:475-477.
- Pace, N., M. Pato, J. McKibbin, and C. Radcliffe. 1973. Precursors of 5S ribosomal RNA in *Bacillus subtilis*. *J. Mol. Biol.* **75**:475-477.
- Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. I. *J. Mol. Biol.* **113**:237-251.
- Rudner, R., H. Lin, S. Hoffman, and E. Chargaff. 1967. Studies on the loss and restoration of transforming activity of the DNA of *B. subtilis*. *Biochim. Biophys. Acta* **144**:199-219.
- Seiki, M., N. Ogasawara, and H. Yoshikawa. 1981. Structure and function of the region of the replication origin of the *B. subtilis* chromosome. I. Isolation and characterization of plasmids containing the origin region. *Mol. Gen. Genet.* **183**:220-226.
- Seki, T., T. Oshima, and Y. Oshima. 1975. Taxonomic study of *Bacillus* by deoxyribonucleic acid-deoxyribonucleic acid hybridization and interspecific transformation. *Int. J. Syst. Bacteriol.* **25**:258-270.
- Smith, B. 1974. Unequal crossover and the evolution of multigene families. *Cold Spring Harbor Symp. Quant. Biol.* **38**:504-513.
- Smith, I., D. Dubnau, P. Morell, and J. Marmur. 1968. Chromosomal location of DNA base sequences complementary to transfer RNA and to 5S, 16S and 23S ribosomal RNA in *Bacillus subtilis*. *J. Mol. Biol.* **33**:123-140.
- Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
- Stewart, G., F. Wilson, and K. Bott. 1982. Detailed physical mapping of the ribosomal RNA genes of *Bacillus subtilis*. *Gene* **19**:153-162.
- Tackney, C., and R. Rudner. 1981. Genetic and nucleotide sequence homologies in *Bacillus* genomes. *Mol. Gen. Genet.* **183**:234-237.
- Tanaka, T., and B. Weisblum. 1975. Construction of a colicin E1-R factor composite plasmid *in vitro*: means for amplification of deoxyribonucleic acid. *J. Bacteriol.* **121**:354-362.
- Wilson, F., J. Hoch, and K. Bott. 1981. Genetic mapping of a linked cluster of ribosomal ribonucleic acid genes in *Bacillus subtilis*. *J. Bacteriol.* **148**:624-628.
- Young, R., R. Bram, and J. Steitz. 1980. rRNA and tRNA processing signals in the rRNA operons of *Escherichia coli*, p. 99-106. In D. Sull, J. A. Abelson, and R. R. Schimmel (ed.), Transfer RNA: biological aspects. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.