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Mapping of ribosomal RNA gene sets with integrable plasmids in *Bacillus subtilis*

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MAPPING OF RIBOSOMAL RNA GENE SETS WITH INTEGRABLE PLASMIDS IN *BACILLUS SUBTILIS*

by
Giuseppe LaFauci

A dissertation submitted to the Graduate faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, City University of New York

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Abstract

MAPPING OF RIBOSOMAL RNA GENE SETS WITH INTEGRABLE PLASMIDS IN BACILLUS SUBTILIS

by

Giuseppe LaFauci

Advisor: Rivka Rudner

Integrable plasmids, containing different cloned rDNA fragments from within the transcriptional unit of the rRNA gene set of Bacillus subtilis, were constructed using the bifunctional vector plasmid pJH101 (Ferrari et al., 1983). All constructs were able to transform B. subtilis strains to chloramphenicol resistance (Cm^r) at low efficiencies. Plasmids pGR102 and pWR103, both containing cloned 16S, abutment, and 23S sequences, had the highest transforming efficiencies. Southern hybridization of BclI restricted chromosomal DNAs revealed that each Cm^r-transformant lost a parent-type rDNA band and gained one or more larger band(s), indicating that one or more copies of a plasmid integrated in a given rRNA gene set. Southern hybridization analysis of DNA double restricted with BclI and SalI proved that in all strains the entire plasmid (either a monomer or a multimer) is inserted into a rRNA gene set. Plasmid insertion occurred by homologous recombination between cloned rDNA sequences and a given rRNA gene set (Campbell-like integration). The comparison of the rRNA BclI patterns obtained with DNAs of parental and Cm^r-transformant strains proved that nine of the ten gene sets present in the chromosome of the bacterium were

involved in plasmid integration. PBS1 transduction crosses with the nine mapping kit strains of Dedonder et al., 1977, revealed that the plasmids can integrate in five different regions of the chromosome: 1) purA-cysA; 2) cysA-aroI; 3) dal-1-purB33; 4) tre-12-glyB133; 5)aroG-thr-5. Additional transduction crosses allowed the mapping of three unassigned rRNA gene sets: rrnK (4.8Kb BclI rrn-homolog) between cysA and amyE; rrnE (6.6Kb BclI rrn-homolog) between pha-1 and furB; and rrnD (5.4Kb BclI rrn-homolog) between glyB133 and tre-12. Nine mapped rRNA gene sets were assigned to individual BclI rrn-homologs. The physical and genetic data indicate that four gene sets (rrnH, rrnI, rrnG, and rrnK) are located in the region of the chromosome between cysA and aroI. These genes form two distinct clusters each containing two rRNA gene sets: rrnH-rrnI (4.9 and 5.8Kb BclI rRNA homologs, respectively), and rrnG-rrnK (5.5 and 4.8Kb BclI rRNA homologs, respectively). Laboratory strains of B. subtilis having only nine rRNA gene sets are described. The deleted gene sets are either rrnK (BD170, trpC2 thr-5) or rrnI (CU420, trpC2 leuB6, ilvC4). In six out of twenty-seven transformants, plasmid integration into a clustered rRNA gene set was associated with the deletion of the adjacent gene set. It is postulated that deletions could result from two simultaneous crossover events between a multimer plasmid and two clustered rRNA gene sets (rrnH-rrnI or rrnG-rrnK). Models showing the possible mechanism are presented.

This thesis is dedicated to the memory of my father

Santi Carmelo LaFauci (1921-1966)

To my wife Madeline, my love and gratitude for her understanding and support throughout the course of my graduate study. In order that my scientific interests might be fulfilled, her sacrifices have been great. For her support and that of my mother, Maria, I am truly grateful. To my daughters Michelle and Vanessa, who had to cope with a very busy father during the evenings spent writing this dissertation, my love.

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Invaluable contributions to my spirit and knowledge were made by fellow members of the Rudner Laboratory. Most notable in this regard were Russell Widom, Paul Gottlieb, Erich Jarvis, Miriam Rivas, Robin Eisner, Yoshiko Setoguchi, and Inga Richter. Special thanks to Russell Widom for providing plasmid pWR103, and to Erich Jarvis and Robin Eisner for contributing transduction data.

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INTRODUCTION

Three ribosomal RNA species and more than fifty ribosomal proteins constitute the ribosome of Escherichia coli. In exponentially growing cells, almost all the ribosomes are engaged in protein synthesis, and little or no free rRNAs and free r-proteins are present (Nomura et al., 1984). The number of ribosomes present in the cell is proportional to the growth rate (Gausing, 1977) and the total amount of protein synthesis, necessary at a certain stage of the cell cycle is obtained by controlling the number of ribosomes present in the cell (Nomura et al., 1984). According to Nomura and Post, 1980: "...the bacterial cells have some regulatory mechanisms to insure that the amount of each ribosomal component synthesized is balanced in the same stoichiometric relation as found in the ribosome." Seven rRNA operons, each containing a 16S, 23S, and 5S cistron, are found in the chromosome of this organism (Kiss et al., 1977; Ellwood and Nomura, 1982; Kenerly et al., 1977). These operons have been mapped (Nomura et al., 1976; Nomura et al., 1977), and the DNA sequence of six of the seven rRNA promoter regions have been determined (Nomura et al., 1984). The r-proteins are coded by genes that are organized into at least twenty different operons (Nomura et al., 1984). The regulation of r-protein synthesis has been demonstrated to occur at post-transcriptional level (Fallon et al., 1979; Dennis

and Fiil, 1979; Olsson and Gausing, 1980; Parson and MacKie, 1983). Fallon et al., 1979, on the basis of gene dosage experiments, proposed a model for transcriptional feedback regulation of r-proteins. A r-protein in each r-protein operon acts as feedback inhibitor of the translation of its own mRNA, so that the synthesis of r-proteins is coupled with the process of ribosome assemblage. The regulatory r-proteins are able to bind strongly and specifically to rRNAs during the early steps of ribosome assembly (Nierhaus, 1980) and the regulation of r-protein synthesis results of a competition between similar binding sites on rRNAs and mRNAs for repressor r-protein binding. In presence of free rRNAs the repressor proteins are used to assemble the ribosome and cannot bind to their mRNAs. When free rRNAs are not present the repressor r-proteins bind to their mRNAs and inhibit translation by a translation coupling mechanism (Oppenheimer and Yanofsky, 1980; Schumperli et al., 1982; Yates and Nomura, 1981). Since the balanced production of r-proteins is regulated by the presence of free rRNAs, the control of mRNA gene sets play an important role in determining the amount of ribosomes present in the cell. To explain the growth rate-dependent regulation of rRNA synthesis, Jinks-Robertson et al., 1983 proposed a ribosome feedback regulation model. Cells always have a higher capacity for making all ribosomal components, but the synthesis rate of rRNA is feedback inhibited by free,

nonfunctioning ribosomes when produced in excess of the amount needed for protein synthesis. In comparison to E. coli, little is known of chromosomal organization and regulation of rRNAs and r-proteins genes in Bacillus subtilis (Smith, 1982). This organism is a gram positive bacterium that under conditions unfavorable for vegetative propagation can form refractile endospores significantly more resistant than vegetative cells to heat, dessication and other destructive agents. The endospores, in turn, can be activated by nutrients (germination) to form new vegetative cells and start a new growth cycle. During sporulation as well as during proliferation the cell must undergo specific regulation of macromolecular synthesis. Since ribosome synthesis is involved in cell proliferation and probably in the differentiation of this bacterium the elucidation of the structure, organization, and function of rRNA genes is an important aspect of the molecular biology of B. subtilis.

GENOMIC ORGANIZATION OF rRNA GENES IN Bacillus Subtilis

The genes coding for tRNAs and for 16S, 23S, and 5S rRNAs are present in multiple copies in the genome of Bacillus subtilis as demonstrated by Smith et al., 1968. Their experiments showed that 60-80% of rRNA genes are located in the region of the chromosome proximal to the origin of replication and the remaining 40-20% are located

in the terminus. It was believed that in each of these two regions of the chromosome the genes coding for 16S, 23S, and 5S rRNAs are interspersed with each other.

The first clue on the organization of rRNA genes was produced by Colli and Oishi, 1969, who showed a physical linkage between 16S and 23S genes. Single strand DNA was isolated by using the methylated albumin Kieselguhr technique developed by Rudner et al., 1968. The isolated strands were then shared to reach an average single strand molecular weight of two million and hybridized to either tritium labeled 16S or 23S rRNAs. The RNA-DNA hybrids were isolated by Cs2S04 density gradient centrifugation in presence of mercuric ion, and treated with alkali to hydrolize the RNA. The remaining ssDNA was then hybridized with the other rRNA species. Fragments having an average molecular weight of two million hybridized to both 16S and 23S rRNA genes. Margulies et al., 1970 also showed that 5S, 16S, and 23S rRNAs hybridize exclusively to the H strand.

Chow and Davidson, 1973, demonstrated by electron microscopy of heteroduplex formed by denaturing and renaturing B. subtilis DNA in presence and in absence of a large excess of rRNAs, that 16S and 23S genes are closely linked and that the spacer between the two genes must be either homologous in all gene sets, or shorter than 50 base pairs. The length of each set is 4830 +/-250 bp and all the sets are homologous as suggested by the absence of

internal loops in the heteroduplex structures formed when rDNAs renature out-of-register. According to the authors, there are 7-9 gene sets and some of them are clustered. The spacer between different sets is unique and its length varies from 0.1 to 55Kb. Two gene sets separated by a spacer 0.6Kb long, are found 6.2Kb away from the attachment site of phage SP02.

Transcriptional mapping analysis using actinomycin D to inhibit RNA synthesis was performed by Bleyman et al., 1969 to demonstrate that each 5S rRNA cistron is part of a transcriptional unit containing a 23S rRNA cistron. For the 16S and 23S rRNA genes the authors' data suggested an independent transcription. However, the experiments conducted by Zingales and Colli, 1977, who studied the transcription pattern of 16S and 23S rRNA genes using rifampicin to inhibit transcription initiation, favored the hypothesis of cotranscription of the two genes with the 16S being transcribed first.

Mature rRNA species were estimated to be of the size 1.67Kb (16S), 3.33Kb (23S) and 0.12Kb (5S) by Attardi and Amaldi, 1970. Precursors of the individual rRNA have been described (Loughney et al., 1983a). The precursor to 23S rRNA (p23S) is 75-150 nucleotides larger than the mature rRNA (m23S); p16S contains 200 nucleotides more than m16S. Pace et al., 1973 described two precursors of 5S rRNA; p5A

is approximately 50% longer than the mature molecule, while p5B is 25% longer than m5S. Other p5S molecules have been described by Sogin et al., 1976. All contain at their 5' end 66 or more nucleotides which are not found in m5S and they vary in the number of additional nucleotides found at their 3' end. In E. coli similar precursors of 23S, 16S, and 5S have been described. Kindler et al., 1973 discovered an E. coli strain lacking RNase III activity which was incapable of efficient rRNA processing and accumulated large amounts of a 30S precursor species (Dunn and Studier, 1973; Nikolaev et al., 1973). Gegenheimer et al., 1977 showed that the individual precursors of rRNAs were intermediates in the processing of the 30S primary transcript to produce mature RNAs. Even though it has not been possible to find large primary transcripts in B. subtilis, it is thought that each rRNA gene set is cotranscribed and that the primary transcript is processed to give p16S, p23S, and p5S and finally mature RNAs (Loughney et al., 1983a).

With the advent of restriction enzyme analysis and the Southern hybridization technique, the organization of the B. subtilis rRNA gene set was better resolved. Edgell, Hutchison and Bott, 1975, observed that rRNAs derived from vegetative or sporulating cells hybridized to the same HaeIII restricted chromosome fragments. They concluded that rRNAs transcribed during both phases of the cell cycle are from the same cistrons or from cistrons

with identical HaeIII site distribution.

Moran and Bott, 1979, analyzed Southern blots containing B. subtilis genomic DNA restricted with BamHI. The same eight BamHI fragments hybridized to 23S and 5S labeled rRNAs. Since the pattern observed by using 16S rRNA as probe was completely different from the one obtained with 23S and 5S probes, the 5S rRNA cistron must be more closely linked to the 23S cistron than to the 16S cistron. The authors confirmed these results by Southern blot hybridization of plasmid p12E12 which carries a BamHI insert containing rRNA genes.

Southern blot hybridization analysis of SmaI restricted chromosomal DNA (Moran and Bott, 1979) revealed that tRNA genes are interspersed between and closely linked to rRNA gene sets. The early reports showed that in B. subtilis tRNA genes are clustered in groups of large transcriptional units (Bleyman et al., 1969) which are found together with rRNA genes close to the origin of replication and near the terminus (Smith et al., 1968). Hybridization of Southern blots of EcoRI restricted chromosomal DNA with either labeled rRNAs or tRNAs showed that rRNA and tRNA genes are closely associated. Several tRNA gene clusters are localized immediately downstream to some of the 5S rRNA genes; others are found between tandem rRNA gene sets (Wawrousek and Hansen, 1983; Wawrousek et al., 1984; Vold, 1985).

Loughney, Lund, and Dahlberg, 1982, found that two

chromosomal EcoRI fragments 1.2Kb and 1.4Kb long hybridize to both 16S and 23S rRNAs. Two rDNA fragments with similar hybridization characteristics are obtained also with PstI (3.1 and 3.3Kb) and with SmaI (0.5 and 0.7Kb), suggesting that all these fragments contain the spacer region between 16S and 23S rRNA genes (abutment) and that this spacer region in some gene sets is 200 bp larger than the one found in other gene sets. Sequence analysis revealed that the large abutment contains an extra 175 bp. Two regions of the transcript of this sequence could be folded into the cloverleaf structure of $tRNA^{Ile}$ and $tRNA^{Ala}$. At least two of the 9 or 10 rRNA gene sets of B. subtilis, like all the gene sets of E. coli, have tRNAs in the abutment.

Bott, Wilson and Stewart, 1981, and Stewart, Wilson and Bott, 1982, proposed a restriction map of the rRNA gene set. All gene sets are homologous with regard to the restriction sites within the coding sequences for 16S and 23S rRNAs (Fig.1).

THE STRUCTURE OF THE rRNA GENE SETS

Stewart, Wilson, and Bott, 1982, and Wawrousek and Hansen, 1983, reported the upstream flanking sequences of one of the rRNA gene sets found in the cluster near att SPO2. In contrast with the E. coli rRNA operons in which two promoters have been described, only a -35 (TTGACT) and

a -10 (TATACT) are found in this gene set. The transcription start site is immediately downstream from TTG sequence which is found in the heptanucleotide sequence GTTGCTT. According to Wawrousek and Hansen, 1983, a 116 nucleotide long leader sequence could be present if the 16S structural gene starts at sequence homologous to the E. coli 16S gene.

The structure and organization of rrnA and rrnO was described by Ogasawara et al., 1983a, who sequenced the 5' ends and the 3' ends of these gene sets, which are found to be transcriptional units (operons). The complete sequence of rrnB, which contains 21 tRNA genes immediately downstream to the 5S cistron, was reported by Green et al., 1985. Two tandem promoters P1 and P2 are present in rrnA, rrnB, and rrnO from which transcription is initiated in vitro by both E. coli and B. subtilis RNA polymerase. P2, the promoter proximal to the structural genes, is preferred by both enzymes. P2 is also preferred in vivo for transcription from rrnO, while P1 is preferred in vivo for transcription from rrnA and rrnB. Consensus sequences for -35 and Pribnow boxes are: and respectively. Repeating poly A and poly T stretches upstream from the -35 boxes are found in all rRNA operons and tRNA operons analyzed. A 7 bp long sequence whose consensus is is present in rRNA operons and starts from the transcription initiation site. A leader sequence 111 bp long is well conserved in rrnA, rrnO, and

rrnB. In both rrnO and rrnB, however, the 3' terminal nucleotide of the leader sequence is replaced by a 65 bp long sequence which is the same in both operons. The abutment between 16S and 23S cistrons of rrnA and rrnO is 347 bp long and contains two genes for $tRNA^{Ile}$ and $tRNA^{Ala}$. The 167 bp long abutment of rrnB does not contain tRNA genes. The sequence of another abutment not containing tRNA genes has been reported by Bott and Hollis, 1982. It is 170 bp long and it is homologous to the sequence of the abutment of rrnB except for 6 bases. Not considering the tRNA genes the sequences of large and small abutments are highly homologous. The sequence of the intercistronic spacer between 23S and 5S cistrons has been determined from 5 different gene sets: rrnO,rrnA (Ogasawara et al., 1983), rrnB (Green et al., 1985), the rRNA gene set (rrnI) upstream of trnB (Wawrousek et al., 1984), and the rRNA gene set associated with trnD (Wawrousek et al., 1984). This sequence is well conserved except for an extra 78 bp found inserted at the level of the sixth nucleotide upstream from the starting of the 5S cistron in rrnA. The intercistronic spacer between 23S and 5S of the rRNA gene set associated with trnD is 110 bp long and contains sequences which correspond to the consensus promoter of B. subtilis with both -35 and -10 regions (Wawrousek et al., 1984). The 5S cistron of this gene set corresponds to the 5S rRNA minor species described by Raue and Planta, 1977, and it is part of the

same transcriptional unit to which 15 of the 16 tRNA genes of trnD belongs. Following the 5S rRNA gene the transcripts of rrnO and rrnA can form a stable hair-pin structure with a tail of long poly U stretch, typical for a transcription terminator signal. The same structure has been isolated as a 3' terminal portion of p5A, one of the precursors of 5S rRNA (Sogin et al., 1976). No termination structure is found immediately downstream of the 5S gene of rrnB. This gene set, in fact, is followed by trnE with which it forms an operon. At the end of tRNA genes terminator structures are found. It is though that, like in E. coli, the rRNA gene sets of B. subtilis upon transcription produce polycistronic transcripts that are processed to form the mature rRNAs. DNA sequence analysis of cloned rRNA genes reveals that regions of the primary transcript may form two long RNA stem structures, each surrounding respectively the 16S and 23S rRNAs (Ogasawara et al., 1983a). In both stems an almost identical cleavage site for a putative processing enzyme, analogous to the E. coli RNaseIII, exists. About 50% of the bases present in the leader sequences of rrnO and rrnA can form a stable double helical structure with 80 bases of the 16S/23S abutment. This structure would surround the 16S rRNA. In the same operons 83 bp of the abutment, located immediately downstream of the two tRNA genes, can form a similar double helical structure with 56 bp found in the 23S/5S spacer (Ogasawara et al., 1983a).

MAPPING OF CLONED *Bacillus subtilis* GENES

Integrative mapping was first introduced in *B. subtilis* by Haldenwang et al., 1980, who determined the chromosomal location of a cloned *B. subtilis* developmentally regulated gene (0.4Kb gene) by using a plasmid capable of site directed insertion into the chromosome. Their strategy involved the use of the bifunctional vector plasmid p1949, which carries a Cm^r determinant capable of expression in *B. subtilis* and *E. coli*. While the vector can replicate in *E. coli*, it cannot replicate in *B. subtilis*. The authors inserted a DNA fragment containing the 0.4Kb gene into p1949, and used the resultant plasmid p1949-2 to transform a Rec^+ strain of *B. subtilis* to antibiotic resistance. Plasmid p1949-2 was able to insert into the chromosome by homologous recombination at a site of homology with the cloned DNA insert (Campbell-like integration). The examination of the pattern of endonuclease restriction sites within and near the integrated vector confirmed that the entire plasmid was inserted into the resident 0.4Kb gene. Transductional crosses with phage PBS1 allowed the authors to map the Cm^r determinant (0.4Kb gene) into the *purA-cysA* region of the chromosome.

MAPPING rRNA GENE SETS

The heteroduplex analysis of Chow and Davidson, 1973, localized two tandem rRNA gene sets 6.2Kb apart from the SPO2 attachment site. The structures observed allowed the authors to propose four simple (A-D) and three larger linkage groups (E, F1, and F2) (Appendix).

Wilson et al., 1981, used the integrative mapping technique first decribed by Haldenwang et al., 1980, to map rrnA. A region of the B. subtilis chromosome immediately adjacent to a rRNA operon is cloned in a bifunctional plasmid vector which cannot replicate in B. subtilis. Using the region of homology the cloned plasmid inserts via a Campbell-like integration into the chromosome leaving the entire plasmid, with its antibiotic marker(s), adjacent to the homologous region. PBS1 transducing mapping is then used to localize the genomic position of the antibiotic marker which coincides with the location of the rRNA operon. The authors used the integrable plasmid pE24, which contains a chloramphenicol resistance marker and a portion of the rRNA gene set consisting in part of the 23S gene, the 5S gene and the immediately adjacent spacer 3' of the 5S cistron, to transform B. subtilis. Integration of pE24 into the homologous region of the chromosome was proved by Southern blot hybridization of BglI restricted chromosomal DNA extracted from chloranphenicol resistant transformants.

transducing crosses localized the chloranphenicol resistance marker between recG and abrB.

The early replicating region of B. subtilis chromosome can be density labeled with a limited amount of BrD(^{3}H)Urd by using a synchronous initiation of chromosomal replication in germinating spores. Ogasawara et al., 1979, and Seiki et al., 1979 demonstrated that replication starts within a BamHI fragment (B7) and proceeds bidirectionally. Among the three EcoRI fragments (E19, E22, and E6) which constitute the B7 fragment, the middle E19 is the first to replicate followed by E22 in one direction and E6 in the other. Henkes et al., 1982, reported that when B. subtilis DNA is restricted with BamHI or SmaI, separated electrophoretically, blotted and hybridized to ^{32}P labeled E19 or E22, at least 7 different bands are produced. The same 7 bands are produced when blots are probed with either 23S or 16S rRNAs. Consequently E19 and E22 must contain rRNA gene sequences, and a rRNA gene set (rrnO) must be present at the origin of replication. Ogasawara et al., 1983b, using similar experiments found that rrnO is situated in the junction between BamHI fragments B7 and B3. Another rRNA operon rrnA, already mapped by Wilson et al., 1981, is found in the junction of the early replicating BamHI fragments B5 and B6. Both rrnO and rrnA are localized in the purA, guaA region of the chromosome.

Bott et al., 1984, mapped rrnB and rrnC between

thr-5 and aroG. The 5' end of the first gene set is present together with unique upstream sequences in the Charon 28 phage J25 (Ferrari et al., 1981). An EcoRI fragment containing sequences immediately upstream of the rRNA sequences present in phage J25 was cloned by the authors in the bifunctional vector pJH101 to produce the integrable plasmid pGS322 which was used to map rrnB. The authors used the integrable plasmid pAC701, containing a unique fragment 5' to the rDNA sequences present in Rapaport cosmid clone cos7 to map rrnC. The genetic locus of rrnO between guaA and purA was confirmed by subcloning the EcoRI fragment E6' (a fragment to the left of the 5' end of rrnO) from plasmid pMS102 (containing the BamHI rDNA fragment B7) into pJH101 to obtain the integrable plasmid pE6'. The same group performed Southern hybridization analysis using rDNA probes to confirm that some rRNA gene sets are located close to attSPO2. In one of such experiments the largeset SalI restriction fragment in digest of chromosomal DNA was found to be larger in a SPO2 lysogen than a non-lysogen.

Zuber (Ph.D. dissertation, University of Virginia, 1982, and Wawrousek and Hansen, 1983) have determined the nucleotide sequence of the spacer between two rRNA gene sets that are partially cloned in plasmid p14B1. Six tRNA genes (asn, thr, gly, arg, pro, ala) are present in this spacer, which is 0.8Kb long and corresponds to the spacer that Chow and Davidson, 1973, refer to as the 1Kb spacer.

Zuber determined the sequence of part of the spacer between the two rRNA gene sets represented in plasmid p14B8. This spacer is 216 bp long and could correspond to either the 0.1Kb or the 0.3Kb spacers of Chow and David, 1973. Phage p11 isolated from the Hoch collection (Ferrari et al., 1981), contains an entire rRNA gene set and the 0.8Kb spacer present in plasmid p14B8. Bott et al., 1984, subcloned on EcoRI fragment containing unique sequences immediately downstream of the rRNA gene set present in phage p11 in the integrable plasmid pBC278 which integrates near the cysA gene of B. subtilis (45% cotransduction).

According to the linkage D of Chow and Davidson, 1973, three rRNA gene sets clustered one after the other, and spaced 1Kb and 0.3Kb are found 6.2Kb apart from attSPO2 (Appendix). Plasmids p14B8 and p14B1 could each represent two adjacent rRNA gene sets of the cluster. Bott et al., 1984, tentatively designed the three gene sets of the cluster as rrnG, rrnH and rrnI. The order of these sets could be either rrnG-rrnH-rrnI or vice versa.

THEORETICAL CONSIDERATIONS

The integrative mapping technique of Haldenwang et al., 1980, has been used to localize rRNA gene sets in the chromosome of Bacillus subtilis (Wilson et al., 1981; Bott et al., 1984). In all cases reported, a chromosomal DNA fragment situated either immediately downstream or upstream to a given rRNA gene set, was cloned in a bifunctional plasmid and used to transform the bacterium to the vector's antibiotic resistance. This strategy is laborious in as much as it allows to map only one rRNA gene set with a given integrable plasmid. Theoretically, integrable plasmids containing cloned DNA sequences from within the transcriptional unit of the rRNA gene set should be able to insert, via a Campbell-like integration, into any given rRNA gene set. Integration of such constructs would allow the mapping of all ten rRNA gene sets present in the genome of the bacterium. Bott et al., 1984, reported that bifunctional plasmids containing pure 16S or 23S rDNA sequences are not able to transform B. subtilis. The absence of transformants is attributed, by the authors, to the inability of these plasmids to recombine with the bacterial chromosome. This interpretation is supported by the results of Iglesias et al., 1983, who reported that cloned rDNA sequences do not produce "plasmid facilitation" (e.g., the uptake of momomeric forms of plasmid DNA by competent B. subtilis is

not enhanced by cloned rDNA sequences from homology with the chromosome as it is with other cloned chromosomal fragments).

The lack of recombination between rDNA sequences postulated by Bott et al., 1984, could be caused by the particular features (sequences; secondary structure) of the cloned rDNA fragment. If this hypothesis is correct, it should be possible to find other rDNA fragments that, upon insertion into an integrable plasmid, would synapse and recombine with one of the resident homologous sequences and mediate the Campbell-like integration of the plasmid. Integration of such plasmids should interrupt the transcriptional continuity of a given rRNA gene set. Since ten rRNA gene sets are present in the genome, the inactivation of a gene set could be tolerated by the bacterium if other rRNA gene sets are contemporaneously transcribed. However, if a given rRNA gene set is the only one being transcribed in a particular stage of the cell cycle of the bacterium, transformants having a plasmid insertion in such gene set would not be found.

OBJECTIVES

The aims of this project are:

a) To construct integrable plasmids containing different cloned fragments of the rRNA gene set of *Bacillus subtilis*;

b) To establish whether one or more of such constructs are capable of Campbell-like integration into the chromosome of the bacterium;

c) To complete the mapping of rRNA gene sets in *B. subtilis*;

d) To ascertain whether plasmid integration can occur into all rRNA gene sets.

MATERIALS AND METHODS

BACTERIAL STRAINS

Transformable mutant derivatives of B. subtilis 168M were strains obtained from different researchers. Table 1 presents relevant genotype information on them. Transformant strains constructed in this study are also listed in Table 1. E. coli strain HB101 (hsdm$^-$, hsdr$^-$, recA$^-$, gal$^-$, pro$^-$, leu$^-$, rpsl) was used to maintain and amplify the plasmids used in this study.

PLASMIDS

Plasmids p12E2-23S, 55 (Moran et al., 1979a), pBC194-16S, 23S (Stewart et al., 1982) and pMS102-B7 (Seiki et al., 1981) were used as sources of rDNA sequences (Fig. 1). The bifunctional plasmid pJH101 (Ferrari et al., 1983) was used as vector to construct integrable plasmids containing rDNA sequences. All plasmids show "relaxed control" of replication and could be amplified to high copy number in E. coli strain HB101 by stoppage of protein synthesis with chloramphenicol (Clewell and Helinski, 1972).

CULTURE MEDIA

Liquid B. subtilis cultures were grown in VY (2.5% veal infusion, Difco Laboratories, Detroit, MI; 0.5% yeast extract, Difco); solid medium was LB-agar (1.0% Bactotryptone, 0.5% Bacto-yeast extract, 0.5% NaCl, 0.1% glucose, 1.5% agar, adjusted to pH 7.0). To maintain the transformant strains containing integrated plasmids (chloramphenicol resistance - Cm^r), media were supplemented with 5-10 ug of chloramphenicol (Cm) per ml. Minimal medium consisted of Spizizen salts (0.44 M KH2PO4, 0.08M K2HPO4, 0.003M sodium citrate, 0.001M MgSO4) supplemented with 0.5% glucose, 1% sodium glutamate, and 100 ug/ml (final concentration) of the appropriate amino acid or nucleotide (Anagnostopoulos and Spizizen, 1961). Plating was performed in the same media containing 1.5% agar. Modified minimal medium containing 0.2% trehalose (filter sterilized) as carbon source was used to select tre-12 transductants (Dedonder et al., 1977; Lepesant-Kejzlarova et al., 1975). Plates containing minimal medium supplemented with 40 ug of 5-fluorouracil and 40 ug of uracil per ml, were used for the detection of $FurB^r$ and $FurB^s$ transductants. TBAB (tryptose blood agar base - Difco) plates containing 1% of potato starch (Sigma) were used to discriminate between $AmyE^+$ and $AmyE^-$ transductants (Nicholson et al., 1985). E. coli cells containing plasmids were grown in LB broth (1.0% Bacto-tryptone, 0.5%

Bacto-yeast extract, 0.5% NaCl, 0.1% glucose adjusted to pH 7.0) supplemented either with 100 ug of ampicillin per ml or 15 ug of tetracycline per ml, depending on the antibiotic resistance marker present in the plasmid. Solid medium was LB-agar (1.5% agar) supplemented either with 100 ug/ml of ampicillin (Ap), 15 ug/ml of tetracycline (Tc), or 5-10 ug/ml of chloramphenicol (Cm).

ENZYMES

Restriction emzymes were purchased either from New England Biolabs (NEB) or International Biotechnology, Inc. (IBI). B. subtilis chromosomal DNA (5 to 10 ug, 2 O.D. solution in TE buffer: 10mM Tris-HCl pH 8.0, 1 mM EDTA) was digested for 12 hours individually or doubly with the appropriate restriction endonuclease (3 units of enzyme per ug of DNA) by using the conditions recommended by the suppliers. Plasmid DNA was restricted for 3-4 hours using 5-6 units of enzyme per ug of plasmid. E. coli DNA polymerase I and the Klenow fragment of polymerase I were purchased from Boehringer Mannhein. Polynucleotide Kinase-T4 was from NEB. T4 DNA ligase was either from IBI or NEB. Sticky end and blunt end ligation was performed as recommended by IBI. The ratio between target and vector DNAs was 2:1. DNAse I type DP dissolved in 0.2M MgS04 to 100 ug/ml was from Worthington. RNAase A and RNAase T1 were also purchased from Worthington. Stock

solutions of these enzymes were 2 mg/ml and 10,000 units/ml respectively. Pronase was from Calbiochem. It was brought to 5 mg/ml stock solution, adjusted to pH5 and heated at 80°C for ten minutes. The pH was then brought to neutrality and NaCl added to 1M. RNAase A was heated at 80°C for 10 minutes to inactivate contaminating DNAase.

MISCELLANEOUS COMPOUNDS USED

Tris base, bovine serum albumin, ethidium bromide, and agarose were purchased from Sigma. Ribonucleoside triphosphates and deoxy-ribonucleoside triphosphates were from P.L. Biochemicals. Deoxyadenosine 5'-triphosphate (α-^{32}P) and deoxycytidine-5'-triphosphate (800 Ci/mmole) were obtained from New England Nuclear (NEN). Adenosine-5'-Triphosphate (α-^{32}P) 2900 Ci/mmole was also purchased from NEN. Phenol (purified crystals) was obtained from ANALA-R. Amino acids (L) were purchased from Celbiochem. Purified bacteriophage lambda CI857 S7 DNA (500 ug/ml) was from NEB. Sephadex G-50 was from Pharmacia Fine Chemicals, Inc. Calf thymus DNA was from Worthington.

TRANSFORMATION OF COMPETENT B. SUBTILIS CELLS

The method of Anagnostopoulos and Spizizen, 1961, as modified by Rudner et al., 1967 was used. A 5ml veal infusion broth supplemented with 0.5% yeast extract (VY) culture was inoculated with bacteria isolated from a single colony. After incubation at 37°C for 6-8 hours, the culture was diluted 1:100. A VY culture (20 ml) was then started using 0.1 ml of the diluted cell suspension. After overnight incubation at 37°C in a shaking water bath (New Brunswick Scientific), 10 ml of the culture was centrifuged in a clinical centrifuge. The pellet was suspended in 2.5 ml of Spizizen minimal medium I (Spizizen salts, 0.5% glucose, 0.02% vitamin-free casamino acids, 0.1% yeast extract, 0.8% L-arginine, 100 ug/ml of each amino acid required, depending on the strain being transformed. An aliquot of these cells able to produce a Klett of 25-35 units (red filter-660 nm) was then used to start a 20 ml culture in medium I which was shaken at 37°C for 4-5 hours (stationary phase). The stationary culture was then diluted 1:10 in minimal medium II (Spizizen salts, 0.5% glucose, 0.01% vitamin-free casamino acids, 0.05% yeast extract, 2.5mM $MgCl_2$, 1mM $CaCl_2$, 0.5mM spermine tetrahydrochloride and 5 ug/ml of each required amino acid. Incubation of this culture at 37°C for 90 minutes produced the maximum amount of competent cells. When frozen competent cells were prepared, the procedure

was performed at 10-fold greater volume. After the last step, the competent cells were rapidly pelleted, resuspended in minimal medium II containing 5% of glycerol (1/10 of the initial volume), frozen in a dry ice-ethanol slurry and stored at -70°C. The cells could be thawed and reconstituted with Spizizen II for transformation. The transforming DNA (0.1-10 ug in 1 X SSC) was transferred to a sterile 12 X 100mm Wasserman tube, to which was added 0.9ml of the competent cell suspension. After incubation on a roller for 30 minutes at 37°C, 0.1ml of 100 ug/ml solution of DNAase I was added to hydrolize the remaining DNA. The samples were diluted using dilution saline (0.15M NaCl, 0.02 M K_2HPO_4, pH 7.0). Aliquotes of 0.1 ml, 0.2 ml of the appropriate dilution were plated on LB-agar supplemented with 10 ug/ml of Cm or other selective agar medium. Plates were incubated for 48 hours at 37°C. A transformation control tube containing 0.1ml of 1 X SSC with no DNA added was always included in each transformation experiment. Plating on TBAB (tryptose blood agar base - Difco) was used to determine viable cells and transformation efficiencies. An aliquot of competent cells were always spotted on the appropriate plates to test for the strain requirements.

TRANSFORMATION OF *ESCHERICHIA COLI* WITH PLASMID DNA

The procedure used is a modification of that of Mandel and Higa, 1970. Three-tenths of one ml of an overnight culture in LB broth of *E. coli* strains HB101 was used to inoculate a 25 ml culture in the same medium, which was incubated at 37°C and grown to a Klett reading of 100 units (2-3 hours). The culture was then chilled on ice for 10 minutes, 10 ml were transferred in a sterile capped glass tube and centrifuged for 5 minutes in a clinical centrifuge. The pelleted cells were resuspended in 10 ml of 0.01M CaCl2, recentrifuged and resuspended in 5 ml of cold 0.03M CaCl2. The suspension was kept on ice for 20 minutes, centrifuged, and the resulting pellet resuspended in 1 ml of cold 0.03M CaCl2. To an aliquot of 0.2ml of these calcium treated competent cells plasmid DNA was added (0.1 to 1.0 ug) and mixed gently. After incubation on ice for 30 minutes, the tubes were transferred to a 42°C water bath for 2 minutes (heat stock). LB broth (1.5ml) was then added to each transformation tube, which was incubated on a roller for 90 minutes at 37°C, to allow phenotypic expression of the antibiotic resistance markers. The cells were plated on LB plates containing the appropriate antibiotic. After incubation for 24 hours at 37°C the colonies were replica plated on petri dishes containing the other antibiotics to ascertain the presence of the wanted plasmid.

PBS1 TRANSDUCTION

Motile bacteria, isolated using swarmer plates (TBAB diluted 1:1 with VY medium), were used to innoculate a 2ml VY culture which was grown overnight with shaking at 37°C. An aliquot of this culture (about 0.2ml) was used to start a 10ml culture in VY (initial Klett reading of 15 units) which was grown to 100 Klett units. Two-tenths of an ml of PBS1 lysate (10^8 PFU/ml) was then added to the cells. Incubation continued until the Klett reading dropped indicating that lysis had occurred. One mg of DNAase I was added to the lysate which was kept at 37°C for 12-16 hours. The lysate was centrifuged to remove debris and sterilized by filtration (millipore-45 um).

Cultures in VY of motile B. subtilis recipient strains (Kit strains) were grown overnight at 37°C. Each culture was diluted (1.0 ml of cells into 9.0 ml of VY medium) and grown to 250-300 Klett units. One ml of bacteria suspension was added to a test tube containing 0.1 ml of transducing lysate. The tube was then incubated for 30 minutes on a roller at 37°C. After centrifugation the cells were resuspended in 1 ml dilution salts. Samples of 0.05 ml of undiluted and 0.1 ml of a 10^{-1} dilution were then plated on LB plates containing 5-10 ug/ml of Cm and on the appropriated minimal plates.

PREPARATION OF BACILLUS SUBTILIS CHROMOSOMAL DNA

An isolated colony of B. subtilis was used to innoculate a 2 ml veal infusion broth supplemented with 0.5% yeast extract. In case of Cm^r transformant strains Cm (5-10 ug/ml) was also added. The culture was incubated on a roller at 37°C for 6 hours. An aliquot of the culture was used to inoculate two or more 500 ml cultures in the same medium that were then incubated overnight at 37°C in a shaker. Cells were harvested by centrifugation in 250 ml centrifuge bottles (7,000 rpm, 5 minutes, washed and resuspended in 0.15M NaCl, 0.1M EDTA pH 8.0. Cells were lysed by using lysozyme and SDS. DNA isolation was according to the procedure of Marmur, 1961 as modified by Rudner et al., 1967. Phenol was equilibrated with 1M Tris-HCl pH 8.0. Dialysis was done in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Stock DNA solutions had routinely an optical density (260 nm), between 30 and 50 O.D. They were stored in TE buffer over a drop of chloroform. The O.D. ratio 280 nm/260 nm was about 0.5. Aliquot of stock solutions were diluted to 100 ug/ml (2 O.D. 260 nm) and kept at 4°C.

PLASMID ISOLATION FROM ESCHERICHIA COLI

An aliquot (10-20 ml) of an overnight culture in LB of E. coli strain HB101, carrying the plasmid to be

isolated was used to inoculate a 500 ml culture in LB broth supplemented either with ampicillin (100 ug/ml) or tetracycline (25 ug/ml). The culture was grown at 37°C in a shaker until a Klett reading (red filter) of 180-200 units was obtained; then solid chloramphenicol was added to reach a final concentration of 180 ug/ml. Incubation at 37°C with shaking was continued to amplify the ColE1 type plasmids (Clewell and Helinski, 1972). Cells were harvested by centrifugation in a Sorvall RC2B, resuspended in a 8 ml of Tris-sucrose buffer (50 mM Tris-Hl pH 8.0, 25% sucrose) and cooled on ice. Solid lysozyme (7 mg) and RNAase (to a final concentration of 20 ug/ml) was added to the resuspended cells which were then kept on ice for 15 minutes. Following the addition of 0.8 ml of a 250 mM solution of EDTA pH 8.0, the suspension was kept on ice for an additional 15 minutes; 8 ml of cold 3X Triton lytic mix (0.3% Triton-X100, 187 mM EDTA pH 8.0, 150 mM Tris-HCl pH 8.0) was added and the viscous suspension kept on ice for 15-30 minutes. Centrifugation at 17,000 rpm (SS-34 rotor) for 90 minutes was used to pellet down large molecular weight species; the supernatant was extracted with phenol buffered with Tris-HCl pH8. After adding sodium acetate to 0.3M, the plasmid DNA was precipitated with 3 volumes of 95% ethanol at -20°C for several hours. The plasmid was collected by centrifugation for 20 minutes at 17,000 rpm, vacuum dried and dissolved in 3ml of TE buffer pH 8.0. An aliquot of plasmid preparation (1.5 ml)

was then purified by CsCl-ethidium bromide density gradient.

CsCl-ETHIDIUM BROMIDE DENSITY GRADIENTS

The volume of the plasmid lysate (1.5 ml) was brought to 6 ml by adding TE buffer pH 8.0 and poured onto 7.35g CsCl (Biological grade, IBI) in a beaker. Two ml of a stock solution of ethidium bromide (1 mg/ml) was then added. The solution was poured into 8 ml polyallome tubes (Beckman) which were previously boiled in 1 mM EDTA pH 8.0. The tubes were then centrifuged at 38,000 rpm for 48 hours in a T150 fixed-angle rotor at 15°C. The fluorescent band containing the closed circular plasmid DNA was visualized by exposure to short-wavelength ultraviolet light and extracted by using a Pasteur pipette. To remove the ethidium bromide the sample was extracted three times with isopropanol, saturated with water and CsCl. The sample was then dialyzed overnight against TE buffer pH 8.0, which was changed several times.

ENZYMATIC REACTIONS AND DNA MANIPULATION

<u>B. subtilis</u> chromosomal DNA (2 O.D.260 nm solution) was routinely restricted in 1.5 ml polypropylene centrifuge tubes (Eppendorf) in 100 ul final volume. Restriction buffer (10x) was the one supplied by IBI.

Three units of enzyme were used per each ug of DNA. Incubation temperature was 55°C for BclI, 25°C for SmaI, and 37°C for all the other restriction enzymes used. To perform double restriction with enzymes requiring different ionic strength (BclI and SalI) the DNA was first restricted overnight with the enzyme requiring lower ionic strength (BclI). Before adding the second restriction enzyme, 20 ul of the appropriate 10X restriction buffer was added and the final volume of the reaction was brought to 200 ul. Plasmid restriction was performed using 5-6 units of enzyme per ug of DNA. About 2-3 ug of plasmid were digested per reaction. The reaction volume was usually 50 ul.

The recessed 3' end fragments of double stranded DNA were filled by using the Klenow fragment of DNA polymerase I (Maniatis et al., 1982). The restriction fragments were ethanol precipitated and resuspended in 1 X nick translation buffer, 2 ul of each dNTP, 1 unit of Klenow and 1 X nick translation buffer to a final volume of 25 ul were then added. Incubation was at room temperature for 30 minutes. The reaction was stopped by adding 1 ul of 0.5M EDTA. The fragments were extracted once with phenol equilibrated with 1M Tris-HCl pH 8, once with phenol-chloroform (1:1) and once with chloroform-isoamylalcohol (24:1). After precipitation with 2 x volumes of 95% ethanol, the fragments were resuspended in 1 x Ligase buffer.

Sticky-end ligation and blunt-end ligation were performed according to IBI protocols. For sticky end ligation the ratio between the amount of the vector and insert was 2:1 and the final DNA concentration was 20 ug/ml. The reaction conditions were 25 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 1 mM DTT, 0.4 mM ATP. T4 DNA ligase was added to a final concentration of 0.5 units/ml; incubation was at 4°C for 16 hours. For blunt-end ligation the final concentration of DNA was 50 ug/ml and the final concentration of T4 ligase was 100 units/ml. Incubation was at 20°C for 2 hours.

GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed in horizontal configuration. For chromosomal DNA the gel apparatus used was 22 X 13 cm. The comb had twelve teeth and under the condition used formed wells able to contain 50-60 ul of sample. Running buffer was 89 mM Tris-HCl pH 8.0, 89 mM boric acid, 2.5 mM EDTA; containing 0.5 ug/ml of Ethidium bromide (Maniatis et al., 1982). Agarose type II low endo-osmotic (sigma) was added to 150 ml of running buffer to form either 0.75% or 0.85% solutions after melting on a magnetic stirrer with heating element. The loading buffer was 50% glycerol containing 0.25% of both brophenol blue and xylene cyanol. It was used to adjust the DNA samples (20-30 ul containing 2-3 ug/DNA) to 5%

glycerol, 0.025% bromphenol, 0.025% xylene cyanol. Gels were run either at 35 mA and 100 volts for 14-16 hours or 5mA and 10 volts for 4-5 days. A lane in each gel always containined bacteriaphage lambda DNA restricted with HindIII. The known molecular weight of the 7 fragments produced from lambda (21.9, 9.3, 6.2, 4.3, 2.3, 2.0, and 0.5Kb) was used to calculate the molecular weight of rDNA homologs. Plots of mobility expressed in mm vs. molecular weight expressed in dalton and raised to the (-) 0.666 power were constructed according to Bearden 1979. Gels loaded with BclI restricted chromosomal DNA were run until the fourth band of HindIII (4.3Kb) reached the bottom of the gel. Since ethidium bromide was present in the running buffer, the gel could be examined immediately over an u.v. transilluminator (C-63 Ultraviolet Products). Gels were photographed by a Polaroid HP-4 system onto Polaroid Type 57 film (ASA 3000) through Wrattan 25A and 8 gelatin filters (Kodak).

Restrictions of plasmid DNA were run either in minigel apparatus or in 22 x 13 cm apparatus. The minigel apparatus was 9 x 5 cm. A ten teeth comb was used to form wells able to contain 15 ul samples. The gel was formed by using 25 ml of agarose solution in running buffer (see above). After loading samples, gels were run at 80 mA and 150 volts for 1 hour. Minigels were also run to analyze completion of restriction reactions.

Restriction fragments were recovered from agarose

gels using the electrolution into dialysis bags procedure described by (Maniatis et al., 1982). The gel slice containing the restriction fragment was collected into a dialysis bag filled with running buffer. Most of the buffer was removed from the bag which was tied and put in an electrophoresis tank. Electrolution was at 100 volts for 3 hours. The polarity of the current was then reversed for 2 minutes, the dialysis bag opened and the buffer surrounding the gel slice recovered. The eluate was extracted once with phenol equilibrated with Tris-HCl pH 8.0, once with phenol/chloroform and twice with chloroform-isoamyl alcohol. The DNA was then twice ethanol precipitated and dissolved in TE buffer pH 8.0.

SOUTHERN BLOT TRANSFER

The electrophoreticaly separated DNA fragments were transferred to nitrocellulose membranes type BA85 (Schleicher & Schuell, Inc.) according to the procedure of Southern, 1975. The HindIII fragments of bacteriaphage lambda DNA, were left on the gel for nitrocellulose transfer. The DNA fragments were denatured by soaking the gel slab in 500 ml of denaturing solution (1.5 M NaCl, 0.5M NaOH). After shaking for 1 hour in this solution, the gel was soaked in neutralizing buffer (1 M Tris-HCl pH 7.5, 1.5 M NaCl) for one hour with constant shaking. The blotting apparatus consisted of a glass sheet on top of

which a strip of Whatman 3M paper was collocated. The 3M paper made contact with 20 X SSC (Maniatis et al., 1982) in two glass containers which were placed under the glass sheet. A nitrocellular sheet, cut to the size of the gel, was soaked for five minutes in 2 X SSC and then placed on top of the gel. Perfect contact between the nitrocellulose filter and the gel was insured by squeezing out with a 5ml glass pipet the air spaces between the gel and the filter. A sheet of 3M paper having the size of the filter was soaked in 2 X SSC and placed on top of the nitrocellulose sheet. A stack of paper towels (about 7 cm thick) was placed on top of the 3M sheet. The stack was compressed by collocating on top of the paper towel glass sheets (200-400 g). After 12 to 24 hours the nitrocellulose membrane was peeled away from the gel, soaked for 5 minutes in 6 X SSC, dried, and baked in a vacuum oven for two hours at 80°C.

HYBRIDIZATION

The baked filters were hybridized at 42°C in 40% formamide, 4 X SSC, 20 mM sodium phosphate pH 7.5, 100 ug/ml sonicated single strand calf thymus DNA, 1X Denhardt's solution (0.02%, Ficoll, 0.02% polyvinylpyrolidone, 0.02% BSA), 0.1% SDS (Ostapchuk and Riley, 1980). Prehybridization solution was 40% formamide, 4 X SSC, 50 mM sodium phosphate buffer pH 6.5,

250 ug/ml sonicated single strand calf thymus DNA, 1% glycine, 5X Denhardt's solution. Each filter was placed in a heat-sealable plastic bag (Daisy "Seal-a-Meal") and 2-8 ml of prehybridization solution was added. After removing air bubbles the bag was sealed and then incubated at 42°C for 3-12 hours. The fluid was then removed after opening the bag at one corner. Hybridization solution (4 ml per each 100 cm^2 of filter) and ^{32}P labeled ss DNA probe were then added. Nick translated lambda DNA was also added. The air bubbles were removed and the bag corner resealed. Hybridization was at 42°C for 12-24 hours. Depending on the size of the filter 2-6 X 10^6 cpm of probe was added. The hybridized filter was first washed in 4 X SSC, 0.1% SDS for 15 minutes at room temperature, and then in 2 X SSC, 0.1% SDS at 68°C for 2 hours. During this time the washing solution was changed three times. The same procedure was used when the probe was end labeled RNA. The filter was then left to dry over a sheet of 3M paper end wrapped in Saran wrap. Autoradiography was done at -70°C in X-ray cassette boxes (Picker) with top and bottom Cronex lightening plus intensifying screens. Time of exposure was usually 12-24 hours.

NICK TRANSLATION

The procedure of Rigby et al., 1977 was used. The reaction buffer was 50 mM Tris-HCl pH 7.5, 10 mM MgS04, 1 mM DTT, and 50 ug/ml BSA. The reaction volume was 20-50 ul. The stock solution of pancreatic DNAase I was 1 mg/ml in 50 mM Tris-HCl pH 7.5, 10 mM Mg SO4, 1 mM DDT, 50% glycerol. It was stored at -20° and diluted 1:40,000 in dilution buffer before using. The DNase I dilution buffer was: 50mM Tris-Hcl pH 7.5, 10 mM Mg SO_4, 0.1 mM DTT, 50 ug/ml BSA. To each reaction containing 0.5-1.0 ug of DNA, 100-200 picomoles (8-16 ul) of α-^{32}P-dATP and α-^{32}P dCTP (0.013 umol/ml), 1 nmole of cold dTTP and dGTP were added. Stock solutions of dTTP and dGTP were 10 mM in 10 mM Tris-Hcl pH 7.0, which were diluted 1/10 before use). Three to five units of E. coli DNA polymerase I and 25 ng of DNAase I (1 ul of the 1:40,000 dilution) were added to each reaction' mixture, and incubation was at 14°C for 2 hours. The reaction was then stopped by adding 1 ul of 0.5M EDTA pH 8.0. The volume was brought to 100 ul by adding TE buffer and recovery of nick translated DNA was done by using 1 ml syringe containing Sephadex G-50 (Spun column-Maniatis et al., 1982). The labeled DNA was denatured by adding 0.1X volumes of 1 M NaOH and incubating for 10 minutes at room temperature. The probe was then neutralized by adding 0.1X volumes of 2.0 M Tris-HCl pH 7.5. Determination of radioactivity was done on a

Beckman LS-800 scintillation counter with automatic quench compensating program. Counts per minutes (cpm) obtained are corrected for sample quenching by external excitation from a Cesium 137 control source. An aliquot of sample (1-2 ul) was counted in 5 ml of Aquasol (Beckman). The specific activity was 1-3 X 10^8 cpm/ug. The probes were stored at 4°C and could be used for 7-10 days.

RIBOSOMAL RNA ISOLATION

A colony of Bacillus subtilis strain 168T was incubated overnight at 37°C in Spizizen minimal medium supplemented with 0.5% glucose, 1% glutamate, 0.04% vitamine-free casamino acids (Difco) and 50ug/ml of L-tryptophan. The culture was then used as innoculum for an overnight 20 ml culture in the same medium. Eighteen millliliters of the culture was used to start a 200 ml culture, which was grown for 4-5 hours (150 Klett units). The cells were harvested by centrifugation, the pellet washed with buffer A (0.01 M Tris-HCl, pH 7.4-0.005M MgCl2), and the wet weight determined (0.3-0.5 g). Extraction of rRNA was performed according to Margulies et al., 1971. The cell pellet was frozen in dry-ice ethanol and immediately thawed in a 45°C water bath. The freezing-thawing procedure was repeated two more times, and the cells cooled in ice water. Extraction was at 4°C. Buffer A (0.6 ml) was added to resuspend the cells; 50 ul

of DNAase I (500 ug/ml in 0.1 M MgCl2), and 0.25 ml of lysozyme (8 mg/ml) were added and the suspension stirred gently for ten minutes in the cold room. The suspension was then frozen in dry ice-ethanol, transferred in a cold mortar containing cold alumina (Type 305-Sigma-2 g for each gram of cells) and ground until a liquid paste slurry was obtained (5-10 minutes). The paste was resuspended in 3 ml of buffer A and centrifuged 20 minutes at 10,000 rpm. The supernatant was then incubated with 50 ul of DNAase I at 4°C for 5 minutes. Debris was removed by centrifugation for 20 minutes at 10,000 rpm. The ribosomes were pelleted by centrifugation at 50,000 rpm for 1 hour at 4°C. The pellet was resuspended in 3 ml of buffer B (0.01M Tris-HCl, pH 7.4 - 0.05 M MgCl2 - 0.005% SDS, extracted twice with 90% phenol, 10% 0.01 M Tris, 0.005 M MgCl2, and once with ether saturated with water. Potassium acetate was then added to a final concentration of 2% and the ribosomal RNA precipitated with 2 X volumes of 100% ethanol at -20°C overnight. The rRNA was pelleted, washed with 80% ethanol and resuspended in 1 ml of buffer B. The RNA concentration was determined using a Gilford model 250 spectrophotometer (1 mg/ml = 24 O.D. 260nm).

AGAROSE GEL SEPARATION OF RIBOSOMAL RNA SPECIES

Electrophoretic separation of RNA was done according to the procedure of McMaster and Carmichael, 1977 (Maniatis et al., 1982). A 3.7 ul sample of RNA (10-20 ug) was mixed with 2.7 ul of 6M glyoxal, 8.0 ul of dimethylsulfoxide and 1.6 ul of 0.1M NaH2PO4 pH 7.0. The mixture was incubated at 50°C for 1 hour. The sample was then cooled at room temperature and 4 ul of loading buffer (50% glycerol, 0.01 M NaH2PO4 pH 7.0, 0.4% bromophenol blue) was added. Electrophoresis was in 1% agarose made in 100 mM NaH2PO4 pH 7.0 running buffer. The running buffer was recirculated to maintain the pH below 8.0 (Maniatis et al., 1982). After staining in a solution of Ethidium bromide (0.5 ug/ml) the bands corresponding to the rRNA species were visualized in a UV transilluminate. The rRNA species were then recovered from gel slices using the electroelution method.

END LABELING OF RNA

Labeling with α-^{32}P ATP was done according to Meizeles, 1977. One microgram of isolated rRNA species was hydrolyzed in 5 ul of 50 mM Tris pH 9.5, at 90°C for 20 minutes. The fragment were labeled with T4 polynucleotide kinase (1 unit) in presence of 100 pmol of α-^{32}P ATP. The reaction condition were 50 mM Tris pH

9.5, 10 mM MgCl2, 5 mM DTT, 5% glycerol. The final volume was 10-15 ul and the reaction was run for 30 minutes at 37°C. The labeled RNA fragments were then separated from α-^{32}P-ATP by using a 1 ml spun column containing Sephadex G-50 equilibrated with TE buffer. The specific activity was 1-3 X 10^7 cpm/ug.

RESULTS AND DISCUSSION

CONSTRUCTION OF INTEGRABLE PLASMIDS - INTRODUCTION

The bifunctional plasmid pJH101 constructed by Ferrari et al., 1983, was used as vector in all cloning experiments. This plasmid (Fig. 2) contains the pBR322 genes that confer resistance to Ampicillin (Ap) and Tetracycline (Tc) in *E. coli*. Plasmid pJH101 was constructed by cloning in the unique PvuII restriction site of pBR322 a 1-Kb fragment (MboI-HpaII) containing the Chloramphenicol acetyl transferase gene (CAT) of the *Staphylococcus aureus* plasmid pC194. The CAT gene is capable of expression in both *E. coli* and *B. subtilis* (Cm^r - Chloranphenicol resistance). Plasmid pJH101 can replicate in *E. coli*, but it cannot replicate in *B. subtilis*, because it lacks a replication origin for this organism (Horinouchi and Weisblum, 1982). However, if a region of homology with the *Bacillus subtilis* chromosome is cloned in pJH101, the resultant hybrid plasmid gains the ability to transform *recE*$^+$ strains of this bacterium by inserting into the chromosome via a Campbell-like integration.

CONSTRUCTION OF INTEGRABLE PLASMIDS CONTAINING EITHER 16S AND 23S OR 23S AND 5S rDNA SEQUENCES

The 5.7Kb BamHI fragment (B7) from rrnO (cloned in plasmid pMS102 by Seiki et al., 1981; Fig. 1) was double restricted with PstI and EcoRI to yield a suitable 1.2Kb fragment which includes 16S DNA sequences, the large abutment region with two tRNA genes (ile and ala), and 155 base pairs of the 5' end of the 23S cistron. A similar PstI-EcoRI 1.0Kb fragment was obtained from plasmid pBC194 (Stewart et al., 1982; Fig. 1). This fragment has a small abutment without tRNA genes. I reasoned that if the two tRNA genes play a role in the integration of the rDNA containing plasmids in the B. subtilis chromosome either by aiding the process or by limiting the integration to two rRNA gene sets, a similar DNA fragment without tRNA genes should be examined. The large and small rDNA fragments were ligated into the PstI and EcoRI sites of plasmid pJH101 to produce plasmids pGR102 and pWR103 respectively (Fig. 1, Fig. 2). Plasmid pWR103 was constructed by Russell Widom in our laboratory. These plasmids were transfected into E. coli strain HB101 to which they conferred a Tc^r, Cm^r, and Ap^s phenotype. The presence of the cloned inserts in plasmids pGR102 and pWR103 was verified by Southern blot hybridization of the plasmid DNA purified on cesium chloride-ethidium bromide gradient, cut with PstI, EcoRI, and PstI+EcoRI, probed

with end labeled 16S rRNA (Fig. 3 and Fig. 4). The presence of 16S rDNA sequences in both pGR102 and pWR103 was also confirmed by Southern blot hybridization of B. subtilis chromosomal DNA double restricted with PstI and EcoRI and hybridized to nick translated pGR102 and pWR103 (Fig. 5 and Fig. 6). The analysis of the blot of Fig. 5A reveals that plasmid pWR103 lost the PstI site during the cloning procedures.

The 2.3Kb EcoRI-HindIII fragment (cloned in plasmid p12E2 - Moran and Bott, 1979), containing more than half of the 23S cistron, the 23S/5S abutment, and the entire 5S cistron of rrnA, was inserted in the corresponding restriction sites of pJH101 to yield plasmid pYR104 (Figs. 1 and 2). This plasmid was constructed by Dr. Y. Setoguchi in our laboratory. Plasmid pYR104, upon transformation, conferred a Ap^r, Cm^r, Tc^s phenotype to E. coli. To prove the presence of the rDNA insert, plasmids p12E2, pJH101, and pYR104 were double restricted with EcoRI and HindIII, the restriction fragments were separated by agarose gel electrophoresis, and blotted on nitrocellulose paper. Hybridization of the blot with the nick translated 2.3Kb EcoRI-HindIII fragment of p12E2 showed the presence of the 2.3Kb rDNA fragment in pYR104 (Fig. 5).

CONSTRUCTION OF INTEGRABLE PLASMIDS CONTAINING EITHER PURE 16S OR PURE 23S rDNA SEQUENCES

Plasmid pGR108 carries a 1.2Kb PstI-EcoRI 23S rDNA insert (Fig. 2). The plasmid was obtained by shot gun cloning the restriction fragments, produced by double restriction of pYR104 with PstI and EcoRI, into the corresponding sites of plasmid pJH101. The ligated mixture was used to transform E. coli strain HB101, and the insertion inactivation of the plasmid Ap^r gene was used to discriminate between pJH101 (Tc^r Cm^r Ap^r) and pGR108 (Tc^r Cm^r Ap^s). To confirm the presence of the 1.2Kb PstI-EcoRI insert, DNA samples of plasmids pGR108, pYR104 and pJH101 were double restricted with PstI and EcoRI, run in a 0.75% agarose gel, blotted, and hybridized to nick translated pGR108 (Fig. 6). A 1.2Kb insert is present in both pYR104 and pGR108.

Plasmid pGR111 was obtained from plasmid pYR104 by deleting the portion of the insert containing the distal part of the 23S cistron and the entire 5S cistron (Fig. 1, 2). Plasmid pYR104 was double restricted with SmaI and HindIII. The HindIII sticky end of the linearized plasmid was filled by using the Klenow fragment of E. coli DNA polymerase I in presence of the four dNTP. The resulting blunt ended molecule was ligated and used to transform E. coli strain HB101 to Ap^r, Cm^r, Tc^s. Plasmid pGR111 contains only 23S rDNA sequences and has neither a SmaI

site nor a HindIII site. To prove that plasmid pGR111 contains a cloned insert smaller than the one present in pYR104, samples of the two plasmids were double restricted with EcoRI plus BamHI, run in a 0.75% agarose gel, blotted, and hybridized to the nick translated PstI-EcoRI fragment cloned in pYR104. As shown in Fig. 7, plasmids pYR104 and pGR111 produced respectively a 2.5Kb and a 1.8Kb band. The size difference between the two bands (0.7Kb) is in agreement with the size of the SmaI-HindIII rDNA fragment (0.65Kb) deleted from pYR104 to obtain pGR111. To confirm that the insert contains only 23S rDNA sequences, samples of chromosomal DNA from B. subtilis strains 168T and SB25 were restricted with SmaI, run in 0.75% agarose gel, and blotted on nitrocellulose filters. The autoradiogram produced probing with nick translated pGR111 (Fig. 8B) revealed the presence of only a 2.3Kb SmaI band which correspond to the rDNA sequences between the two SmaI sites present in the 23S cistron of all rRNA gene sets (Fig. 1). On the other hand the autoradiogram obtained by probing with nick translated pYR104 (Fig. 8A) showed, besides the 2.3Kb fragment, other bands (18, 14, 7.3, 5.4, 3.0 and 2.6Kb) each corresponding to the distal part of a rRNA gene set (Fig. 1; Gottlieb et al., 1985).

The same strategy was used to engineer plasmid pGR110, which contains only 16S rDNA sequences (Figs. 1 and 2). Plasmid pGR102 was double restricted with SmaI and EcoRI. The EcoRI sticky ends were filled by using the

Klenow fragment of DNA polymerase I, and the resulting molecules were recircularized by blunt end ligation. The mixture was then used to transform E. coli HB101 to Tc^r, Cm^r, Ap^s. Plasmid pGR110 has neither a SmaI nor a EcoRI site. Plasmids pGR110 and pGR102 have a HindIII site 29 bp downstream their respective inserts (Fig. 2). To prove that pGR110 contains only 16S rDNA sequences, samples of plasmid pGR102 and pGR110 were double restricted with PstI and HindIII, run in 0.75% agarose gels, and blotted. The ethidium bromide stained gel (Fig. 9A) showed that both restricted plasmids produced two fragments. One fragment is 4.6Kb long in both plasmids, while the other is 0.6Kb in pGR110 and 1.2Kb long in pGR102. The autoradiogram obtained by probing the blot with the nick translated PstI-EcoRI insert of pGR102 (Fig. 9B:b,c,d) showed a 0.6Kb band for pGR110 and a 1.2Kb band for pGR102. The autoradiogram obtained using the nick translated SmaI-EcoRI rDNA fragment of pGR102 (the fragment contains the sequences deleted from pGR102 to construct pGR110) showed only a 1.2Kb band for pGR102 (Fig. 9B:f,g,h). Since the second probe did not hybridize to the 0.6Kb fragment of pGR110, this plasmid must contain only 16S rDNA sequences. To confirm this result, samples of chromosomal DNA of B. subtilis strains 168T and SB25 were restricted with SmaI run in agarose gel, blotted, and hybridized either to nick translated pGR110 or pGR102 (Fig. 10). Plasmid pGR110 did not hybridize to the 0.7 and 0.5Kb 16S, 23S SmaI fragments

containing respectively the large and small abutment (Fig. 1).

TRANSFORMATION OF *BACILLUS SUBTILIS* STRAINS WITH INTEGRABLE PLASMIDS pGR102, pWR103, pYR104, pGR108, pGR110, AND pGR111.

The integrable plasmids were introduced into *RecE*$^+$ strains of *Bacillus subtilis* by transformation of either fresh competent cells or frozen competent cells (Rudner et al., 1967). Colonies resistant to 5 ug/ml of chloranphenicol were isolated at higher frequency when fresh competent cells were used. The strains used, and their genotypes and sources are listed in Table 1. Plasmids pGR102 and pWR103 generated more Cm^r transformants than plasmids pGR108, pGR110, pGR111 and pYR104. A comparison of the transforming efficiencies of these plasmids with plasmids pJH101 and pER102 is presented in Table 2. Plasmid pER102 (a pJH101 derivative constructed in our laboratory) has a 3.2Kb EcoRI-BamHI insert containing the 3' end of the *leuA* cistron and the entire *leuC* and *leuB* cistrons of *B. subtilis* (Fig.2). Plasmid pER102 transformed the recipient strain BD79 (*leuB1*, *pheA*) to Cm^r and Leu$^+$. The transforming efficiency was higher when selection was for *Leu*$^+$ (Table 2). Similar results were reported by Young, 1983 using plasmid p1949 (Haldenwang et al., 1980) containing a

cloned 5.7Kb EcoRI-BamHI insert (*pheA nic*) of *B. subtilis* to transform strain JH648 (*trpC2*, *pheA2 spoOB136*) either to *Phe*$^+$ (2.7×10^4 transformants/ug plasmid DNA) or Cm^r (3.8×10^2 transformants/ug plasmid DNA). The majority of the Leu$^+$transformants resulted from a double crossover event since 98% were Cm^s (LaFauci et al., 1986). The transforming efficiencies of the plasmids containing rDNA inserts were one to three orders of magnitude lower than the transforming efficiency of plasmid pER102. The highest transforming efficiencies were obtained with plasmids pGR102 and pWR103 (2.4×10^3 and 1.4×10^3 transformants/ug of plasmid, respectively). The data indicate that certain rDNA sequences mediate plasmid integration better than others.

Once the plasmids were integrated into the chromosome, the Cm^r determinant could be transferred by transformation to recipient strains of *B. subtilis* at higher frequencies (10^3-10^4 transformants/ug chromosomal DNA; Table 2).

The transformants were designed with (/) denoting Cm^r due to plasmid insertion, for example BD170/pGR102-143 is strain BD170 (*trpC2*, *thr-5*) in which the integration of plasmid pGR102 occurred and 143 is the number of the clone isolated initially.

CHROMOSOMAL INTEGRATION OF PLASMIDS pGR102, pWR103, AND pYR104 -- THEORETICAL CONSIDERATIONS

In theory, plasmid insertion into chromosomes mediated by homologous recombination (Campbell-like integration) between the rDNA insert and one of the rRNA gene sets, should result in the duplication of the region of homology. A model of Campbell-like integration of plasmid pGR102 into a rRNA gene set having a small 16S/23S abutment is presented in Fig. 11. A crossover event between the cloned rDNA fragment and a rRNA gene set results in the insertion of the entire plasmid into the gene set. Each rRNA gene set has a single BclI site at the 3' end of the 23S cistron, and no BclI sites are present in the vector plasmid pJH101. Upon restriction with BclI, the chromosomal DNA of Cm^r transformants should release a large hybrid fragment consisting of both rDNA and plasmid sequences. The length of the hybrid fragment should be equal to the sum of the length of a parental BclI rDNA fragment and the length of the plasmid. Southern hybridization analysis can be used to test this prediction. In each Cm^r transformant one BclI fragment homologous to rRNA should disappear, and a band larger than that in the parental-type strain should appear (Fig. 11:a). The expected new band should be composed of the plasmid plus the missing BclI rRNA homolog, and in case of integration of a multimeric form of the plasmid band(s)

corresponding to the insertion of these multimers should be observed. Since one SalI site is present in the Tetracycline gene of pGR102 and pWR103, and no SalI sites are present in the rRNA gene set, double restriction with BclI and SalI of the DNA of strains containing plasmids, should release two fragments homologous to the vector sequences: one having a defined size and the other having a variable size (Fig. 11:b). According to the model a fragment should be either 7.6Kb or 7.8Kb long, depending on the type of abutment (large or small) present in the gene set involved in the insertion. The variable fragment should contain unique DNA sequences present upstream to the different rRNA gene sets, and should have a minimal size of 2.5Kb since the sequence between the SalI site of the inserted plasmid and the 5' end of the 16S cistron is 2.5Kb long. In case of insertion of a multimeric form of pGR102 into a rRNA gene set, a 5.8Kb fragment, containing vector sequences and spanning between the SalI site of a pGR102 moiety and the SalI site of the next tandem repeated pGR102 moiety, should be also released. To confirm this model, DNAs of parental and transformant strains were prepared and their hybridization patterns compared. The DNAs were restricted to completion with BclI or with BclI followed by digestion with SalI, electrophoresed on agarose gels (0.75% or 0.85%), and transferred to nitrocellulose filters as described in Materials and Methods.

Integration of plasmid pYR104, containing the 2.3Kb EcoRI-HindIII 23S, 5S rDNA insert, cannot be analyzed by Southern hybridization of chromosomal DNA restricted with BclI, because a BclI site is present in the insert (Fig. 2). In fact, upon restriction with BclI of chromosomal DNA of strains with integrated pYR104, the plasmid would be released from the rRNA gene set involved in the insertion, and the resulting BclI rDNA pattern would not show any missing BclI bands.

A. SOUTHERN ANALYSIS OF BclI BLOTS OF TRANSFORMANT DNAs

The BclI blots were probed with nick translated pGR102 or pWR103 or the purified PstI-EcoRI rDNA insert of pGR102. Nick translation and Southern hybridization were performed as described in Materials and Methods

Blots of gels run at 30 Volts overnight (14-16 hours - Tris-Borate running buffer), revealed a pattern of rRNA homologs having a high concentration of fragments between the third (6.2Kb) and fourth (4.3Kb) bands of λHindIII (Fig. 12). In this region, the bands were not sufficiently resolved to allow the identification of missing fragments in some strains containing inserted plasmids (BD170/pGR102-143; Fig. 12:d). The blots, however, showed the presence of higher molecular weight band(s) corresponding to the integration of plasmids in

rDNA homologs. Strains BD170/pGR102-153 and BD170/pGR102-151 (lanes f and c) are missing, respectively, the 6.6Kb and the 4.8Kb BclI rRNA homologs. To increase the resolution, 0.75% and 0.85% agarose gels were run at low voltage (5-10 volts) for 4-5 days. Blots obtained from these gels showed a better resolution of the BclI rDNA pattern.

As seen in Fig.13 ten rDNA bands are present in the prototrophic strain of B. subtilis NCTC3610. The largest BclI homolog is 8.3Kb long and the smallest homologs appear as doublets of 4.8 and 4.9Kb. The parental strains BD170 and BD79 showed only a single 4.9Kb band instead of the doublets (Fig.13). The absence of a BclI rRNA homolog was expected in strain BD79 since Gottlieb et al., 1985 reported that this strain and others (BD29, BD80, BD73, BD47, and GSY1269) are missing the same rRNA gene set. The missing 4.8Kb band in strain BD170 might indicate that this strain has a deleted rRNA gene set. The hybridization pattern obtained with DNAs of strains having inserted plasmids, revealed as shown in Figs.13 and 14, the disappearance of parental-type bands and the appearance of new larger band(s) indicating that one, two, or three copies of pGR102 or pWR103 integrated in any given rRNA gene set. In strain BD170/pGR102-143, the 5.7Kb band was replaced by three new bands (11.5, 17.3, and 23.1Kb), the size of which corresponds to the presence of a plasmid monomer, dimer and trimer, respectively,

inserted into the 5.7Kb homolog. In strain BD170/pGR102-144, the 5.8Kb homolog was replaced by a single new band (11.6Kb) corresponding only to a monomer. The BclI rrn-homolog involved in plasmid insertion in each Cm^r transformant strain examined is listed in Table 3. The results indicate that both plasmids pGR102 and pWR103 can integrate into nine of the ten rRNA gene sets present in the B. subtilis. Among the transformants' DNA analyzed some represented repeated occurrences. For example, strains BD170/pGR102-135 and BD170/pGR102-153 revealed the displacement of the same 6.6Kb fragment, or strains BD170/pGR102-143 and BD170/pWR103-181 showed the loss of the same 5.7Kb homolog. Strains BD79/pWR103-182 (Fig. 13), 168T/pGR102-281 and 168T/pGR102-313 (Fig. 14:k and g) showed the displacement of the same 5.4Kb BclI rrn-homolog. While either the 4.9Kb or the 5.8Kb BclI rrn-homologs were lost respectively in strains 168T/pGR102-317 (Fig.14) and BD170/pGR102-144 (Fig.13), the two homologs were lost simultaneously in strains BD170/pGR102-151 (Fig.13) and GSY1269/pGR102-177 (data not shown). Strains 168T/pWR103-315 and 168T/pGR102-311 (Fig.14:f and h) showed the simultaneous loss of two other rRNA BclI homologs, namely the 4.8Kb and 5.5Kb. Strains missing only the 4.8Kb band (168T/pWR103-314) or the 5.5Kb band (168T/pWR103-316) were also found (Fig.14). The unexpected class of transformants in which two rRNA homologs (4.9 and 5.8Kb or 4.8 and 5.5Kb) are missing

showed (except strain 168T/pGR102-311) a single high molecular weight rRNA homolog, which is 10.7Kb long in strain BD170/pGR102-151. The size of this homolog corresponds to the insertion of a monomeric form of pGR102 (5.8Kb) into the 4.9Kb BclI band (5.8Kb + 4.9Kb = 10.7Kb). The 11.1Kb long BclI rRNA homolog found in strain 168T/pWR103-315, corresponds to the insertion of pWR103 (5.6Kb) into the 5.5Kb homolog (5.6Kb + 5.5Kb = 11.1Kb). In all these transformants the remaining 8(7) rRNA homologs correspond to those found in their respective parental types, and no extra bands having different mobility are observed. The inactivation or deletion of a BclI site cannot be responsible for the absence of the second BclI band. In this case, in fact, the rRNA homolog lacking the BclI site would be longer and should migrate in a different position. The deletion of rRNA gene set could explain the pattern found in these strains. This hypothesis can be tested by analyzing the rRNA pattern obtained by restricting the chromosomal DNA of these strains with other enzymes.

Among the transformants obtained with plasmid pGR102, four strains (BD170/pGR102-135, BD170/pGR102-153, BD170/pGR102-143, and 168T/pGR102-311) showed the presence of three high molecular weight rDNA fragments (Figs. 13 and 14). The sizes of these bands correspond to the presence of a plasmid monomer, dimer, and trimer, respectively inserted into the 6.6Kb BclI rRNA homolog

(BD170/pGR102-135 and BD170/pGR102-153), 5.7Kb BclI rRNA homolog (BD170/pGR102-143), and 5.5Kb rRNA homolog (168T/pGR102-311). This indicates that in each strain there are three populations of cells, each having either a monomer, dimer, or trimer inserted into a given rRNA gene set. Judging from the intensity of the bands in the autoradiograms (Fig. 13 and 14), the population having the dimeric insertion seems the most prevalent in the four strains.

B. SOUTHERN HYBRIDIZATION OF BclI-SalI BLOTS

Southern hybridization of BclI-SalI double restricted transformants' DNAs probed with nick translated plasmid pJH101 revealed, as predicted from the model (Fig. 11), the appearance of a fragment with a defined size of 7.6 or 7.8Kb (depending on the size of the 16S/23S abutment of the gene set involved) which was not present in the parental type DNA (Figs. 15 and 16). For example, digests of strains BD170/pGR102-143 and BD170/pGR102-135 released the 7.8 and the 7.6Kb fragments respectively (Fig.15:m and n). The double restriction produced a second band that had a different size in each transformant DNA. The size of these variable bands were in the range of 2.8 to 6.1Kb. The appearance of the variable bands is consistent with the proposed model of insertion of pGR102 or pWR103 into

the chromosome (Fig.11) and allows for the estimation of size and the eventual isolation of the neighboring unique 5' DNA sequences which terminate with a BclI site or with a SalI site. For example, in strain BD170/pGR102-151 (Fig.15:1), the spacer sequence upstream from the 5' end of the 16S cistron is 0.5Kb (3.0Kb minus 2.5Kb), while strains BD170/pGR102-135 and BD170/pGR102-153 have longer unique regions of 1.3Kb (3.8Kb minus 2.5Kb). Another example is strain BD170/pWR103-179 in which the unique sequence upstream from the 5' end of the 16S cistron is 3.6Kb (6.1Kb minus 2.5Kb). Since the probe used to hybridize the blots showed in Fig.15 was the original cloning vector pJH101, the control lanes of DNAs BD170, BD79, and GSY1269 yielded no hybrid bands. It was possible to conclude from the hybridization patterns of the double digestions whether the transformants contained monomeric or multimeric forms of plasmid pGR102 or pWR103. A 5.8Kb band, which is the unit size of pGR102, could only be released on digestion with SalI and BclI if the DNA harbored a multimer (Fig.15:c,e,m and n; Fig.16:h). Transformants containing a monomer did not release additional vector sequences, as was seen for strains BD170/pGR102-151, GSY1269/pGR102-177, BD170/pGR102-144 and other strains. According to the Campbell-like integration model (Fig.11), the BclI-SalI pattern of DNA BD170/pGR102-143 with a 7.8, 5.8 and 3.7Kb band clearly indicates that a multimer form of pGR102 inserted into a rRNA gene set

having a large 16S/23S abutment (7.8Kb).

The BclI-SalI blots allowed to establish that in strains BD170/pGR102-151 and GSY1269/pGR102-177 (FIg.15:1 and j) plasmid integration occurred into the 4.9Kb BclI rRNA homolog (Table 3). In fact these strains, like strain 168T/pGR102-317 (Fig. 16:i), showed a 3.0Kb BclI-SalI band. Similarly the presence of a 3.5Kb BclI-SalI band in strains 168T/pGR102-311, 168T/pWR103-315 and 168T/pWR103-316 proves that in the first two strains plasmid integration occurred into the 5.5Kb BclI rRNA homolog (Fig.16:d,e, and h; Table 3). In conclusion, double digestions with BclI-SalI provide the ultimate verification of the rRNA gene sets involved in the integration events.

SOUTHERN ANALYSIS OF EcoRI BLOTS

The Campbell-like integration model presented in Fig.11 predicts that upon restriction of transformants' DNAs with EcoRI, a fragment corresponding to the monomer size of the plasmid should be produced. The size of this fragment should be either 5.6 or 5.8Kb, depending on the type of 16S/23S abutment present in the gene set involved in the insertion. Integration mediated by homologous recombination at level of the 16S rDNA sequences would produce upon EcoRI restriction a 5.6Kb fragment if the

gene set had a small abutment, and a 5.8Kb fragment if the set had a large abutment. In case of integration of a multimeric form of pGR102 in a gene set having the small abutment, both the 5.6 and the 5.8Kb fragments should be released.

To investigate the type of abutment present in the gene sets involved in the integration, blots of transformants' DNAs restricted to completion with EcoRI were hybridized with nick translated pJH101. As shown in Fig.17 some transformants' DNAs displayed either a single band of 5.8Kb (e.g. BD170/pGR102-143 and BD170/pWR102-179) or a single band of 5.6Kb (e.g. BD170/pGR102-144 and BD79/pWR103-182), while others showed both bands (e.g. BD170/pGR102-135 and BD170/pGR102-153). In strain BD170/pGR102-144, the presence of the 5.6Kb EcoRI band proves that the gene set involved in the plasmid integration has a small abutment without tRNA genes. A small abutment must be present also in the rRNA gene set into which a multimeric form of pGR102 integrated to produce strains BD170/pGR102-135 and BD170/pGR102-153. The DNAs of these two transformants, in fact, show both the 5.6 and the 5.8Kb bands. The gene set involved in the integration of plasmid pWR103 (containing the small abutment) to produce strain BD170/pWR103-179 must have a large abutment, since this strain releases the 5.8Kb EcoRI fragment. Strain BD170/pGR102-143 which was produced by the integration of a multimer of pGR102, released only the

EcoRI 5.8Kb band (Fig.17:b). This result, together with the BclI-SalI result (Fig.15:m) proves that this gene set has a large abutment. The 5.6Kb EcoRI fragment released by the DNA of strain BD79/pWR103-182 suggests that the rRNA gene set involved has a small abutment.

Southern analysis indicates that both plasmids pGR102 and pWR103, containing respectively the large and the small abutment, are able to insert via a Campbell-like integration in rRNA gene sets having either the small or the large abutment.

CHROMOSOMAL INTEGRATION OF PLASMIDS pGR108, pGR110 and pGR111 -- THEORETICAL CONSIDERATIONS

Models of Campbell-like integration into a rRNA gene set of plasmids pGR108, pGR110, and pGR111 are presented respectively in Figures 18, 19, and 20. As in the case of integration of plasmids pGR102, and pWR103, the BclI rDNA patterns of Cm^r transformant strains should show the displacement of a parental BclI rDNA homolog. Upon double restriction with BclI + SalI, the DNAs of transformants with integrated pGR108 should release two fragments containing vector sequences (Fig. 18). One of these fragments should have a constant size (2.6Kb) independently of the site of integration, and the other should have a variable size (>7.8Kb). The model of

integration of plasmid pGR110 (Fig.19), predicts the production of a BclI + SalI fragment having a variable size (>2.0Kb) and a fragment either 7.8Kb or 7.6Kb long depending on the type of abutment (with or without tRNA genes) found in the rRNA gene set involved in the integration. Finally, the model of integration of plasmid pGR111 predicts the production of a variable BclI-SalI fragment (>4.8Kb) and a constant fragment 6.6Kb long.

SOUTHERN HYBRIDIZATION ANALYSIS OF Cm^r TRANSFORMANT STRAINS WITH INTEGRATED pGR108, pGR110, or pGR111

To investigate the validity of the models of integration presented in figures 18, 19 and 20, samples of DNAs of Cm^r transformant strains with integrated pGR108, pGR110, or pGR111 were restricted with BclI, run in 0.75% agarose gels, blotted and hybridized to rDNA probes. As expected, the rDNA BclI patterns of each transformant revealed a missing BclI band and the presence of a band having a larger size (Fig.21 and Table 4). Strains SB25/pGR108-236 and SB25/pGR108-257 (Fig.21:k and f) lost respectively the 4.9Kb and the 4.9Kb + 5.8Kb rRNA homologs. Both strains gained a 10.7Kb BclI rrn-homolog which corresponds to the insertion of a monomeric form of pGR108 (5.8Kb) into the 4.9kB BclI rRNA homolog (5.8Kb + 4.9Kb = 10.7Kb). The 4.9Kb and 5.8Kb bands were also

missing in strain BD170/pGR111-301 (Fig.21:d), where the gained BclI rDNA homolog is 11.7Kb long (insertion of pGR111 into the 4.9Kb rRNA homolog). The 5.4Kb band was replaced by 12.2Kb long BclI rrn-homolog in strain BD170/pGR111-302 (Fig.21:e). In strain SB25/pGR110-305 both the 5.4Kb and 4.8Kb rRNA homologs were replaced by a 10.6Kb rRNA homolog which corresponds to the insertion of pGR110 (5.2Kb) into the 5.4Kb homolog (5.2Kb + 5.4Kb = 10.6Kb). In strains SB25/pGR102-257 and SB25/pGR110-305 the absence of a second BclI rRNA homolog could indicate that a deletion occurred.

Upon double restriction with BclI and SalI the DNAs of these strains produced fragments containing vector sequences having the size predicted by the Campbell-like models of integration presented in Figs. 18, 19, and 20. Strains SB25/pGR108-236, (missing the 4.9Kb BclI rRNA homolog) and SB25/pGR108-257 (missing the 4.9Kb and 5.8Kb BclI rRNA homologs) both produced two BclI-SalI fragments (Fig.22) having the size of 2.6Kb and 8.1Kb. This result proves that in both strains plasmid pGR108 is inserted in the same rRNA gene set (4.9Kb BclI). As predicted, transformants having the insertion of pGR110 produced a 7.6Kb long fragment and a fragment having a "variable" size (>2.0Kb). In strain SB25/pGR110-304 the presence of the 7.8Kb fragment indicates that plasmid integration occurred into rRNA gene set having the large abutment with tRNA genes. Finally, strains BD170/pGR111-302 and

BD170/pGR111-301 produced a band having the constant size of 6.6Kb and a fragment 5.6Kb long in the former and 5.1Kb in the latter strain.

These results prove that plasmids containing either pure 16S or 23S rDNA sequences can also integrate into rRNA gene sets. While integration of plasmids pGR102 and pWR103 (both containing the 16S-23S abutment) occurred in nine of the ten rRNA gene sets present in the genome of B. subtilis (Table 3), integration of plasmids, pGR108, pGR110, and pGR111 occurred only in five different sites (Table 4). Since only eight out of 27 Cm^r strains analyzed by Southern hybridization were obtained by transformation with the latter group of plasmids, the reduced number of sites of integration is probably a statistical bias due to the small size of the sample.

GENETIC MAPPING OF INTEGRATED PLASMIDS

To map the Cm^r marker of integrated plasmids in the chromosome of Bacillus subtilis, transductional crosses using phage PBS1 were performed mostly by Erich Jarvis and the earlier crosses by Robin Eisner. The phages were prepared using as hosts the Cm^r transformants strains containing inserted plasmids. The transducing particles were crossed with the nine mapping kit strains of Dedonder et al., 1977. All crosses were performed as described in Materials and Methods, and chloramphenicol resistance was

used initially as the selective marker to establish the gross chromosomal location followed by three factors crosses. Linkage was established by replica plating for cotransfer of the integrated plasmid with at least two unselected markers. The results (Table 5) indicate that the plasmids can integrate in five different regions of the chromosome: 1) purA-cysA (linkage to Kit1 markers); 2) cysA-aroI (linkage to Kit1 and Kit2 markers); 3) dall-purB33 (linkage to Kit2 markers); 4) trel2-glyB133 (linkage to Kit3 markers); 5) aroG-thr-5 (linkage to Kit7 and Kit8 markers).

As shown in Table 5, the majority (19/28) of the transformant strains had the plasmid Cm^r determinant linked to the markers of Kit1. A close linkage to purA16 was found in strains BD170/pWR103-179 (78% cotransduction), BD170/pWR103-181 (76% cotransduction), and BD170/pGR102-143 (54% cotransduction). Strains 168T/pGR102-317, BD170/pGR102-151, BD170/pGR102-144, and others showed a closer linkage to cysA14 (77-96% cotransduction) than to purA16 (17-49% cotransduction). Strains 168T/pWR103-314 and SB25/pGR110-306, both missing the 4.8Kb BclI homolog (Figs.14 and 21) showed a weak linkage to purA16 (3-6% cotransduction) and a close linkage to cysA14 (57-84% cotransduction). Crosses with the recipient strain Kit2, revealed that Cm^r determinant of strains 168T/pWR103-314 and SB25/pGR110-306 is also linked to aroI906 (12-18% cotransduction; Jarvis, et al.,

submitted). These results represent the first genetic evidence for the existence of a previously unassigned rRNA gene set, rrnK (4.8Kb BclI band), in this region of the chromosome. Other strains (BD170/pGR110-291, 168T/pWR103-316, 168T/pWR103-315, and 168T/pWR103-311) which are missing either the 5.5Kb BclI homolog or both the 5.5Kb and the 4.8Kb homologs showed the same linkage to aroI906, cysA and purA. In strains 168T/pGR102-272 and 166/pGR102-273 the Cmr marker was linked to aroG912 (Kit7) and to thr-5 (Kit8). In this region of the chromosome rrnB was mapped by Bott et al., 1984. Strains BD170/pGR102-135, BD170/pGR102-153 and BD29/pYR104-256, showed linkage to two markers in strain Kit2, namely to dal-1 and purB33. This is the first genetic evidence for the existence of an unassigned rRNA gene set (rrnE) located in a region considerably removed from the major group of rrn genes (La Fauci et al., 1986). Finally, strain BD79/pWR103-182 revealed weak linkages to glyB133 (29% cotransduction) and to tre-12 (35% cotransduction) and none to metC3, the third marker of strain Kit3. This strain and others (BD170/pGR111-302, 168T/pGR102-281, and SB25/pGR110-305) showed also linkages to glyB133 (26-34% cotransduction) and metD1 (1.5-4% cotransduction) of strain 1A84. These results constitute proof for the existence of another unassigned rRNA gene set,rrnD (La Fauci, et al., 1986).

TRANSDUCTION CROSSES FOR MAPPING INTEGRATED PLASMIDS IN THE purA cysA REGION

To quantitate the linkage of the marker in the Kitl strain and to establish the effect of the inserted vector on the recombination frequencies, three factor crosses between the Cm^r determinant in phage PBS1 and the markers purA16 and cysA14 were performed (La Fauci et al., 1986; Jarvis et al., submitted). Table 6 presents a sample of crosses performed using donor strains with plasmid integrated into different BclI rrn-homologs. In general the number of transductants per 10^9 PFU were consistently similar and relatively high (5×10^3 to 5×10^4 per ml). All three markers were transduced at comparable frequencies in most cases, and were similar to those obtained with the parental strain BD170. The cotransfer values were comparable but not always equal in both direction of selection. For example, the cotransfer values Cm^r/Pur^+ and Pur^+/Cm^r were similar (36% and 30%) for strain BD170/pGR102-144 and quite different (28% and 78%) for strain BD170/pWR103-179. For strains BD170/pWR103-179 and BD170/pGR102-143, missing respectively the 8.3Kb and the 5.7Kb BclI rrn-homologs, the rare recombinant class was $Pur^+Cys^+Cm^s$ product of a quadruple crossover event. These results indicate that in both cases the gene order must be purA Cm^r cysA. The gene order deduced from the crosses of strains BD170/pGR102-

144, 168T/pGR102-317, BD170/pGR102-151, 168T/pWR103-316, and 168T/pWR103-314 is purA cysA Cm^r. These strains represent integration events that occurred into four different rRNA gene sets (4.8Kb, 4.9Kb, 5.5Kb and 5.8Kb BclI rRNA homologs; Tables 3 and 4). This implies that four rRNA gene sets must be present in the region of the chromosome situated immediately clockwise to cysA. The 4.9Kb, 5.8Kb, and 5.5Kb BclI rRNA homologs were previously assigned (LaFauci et al., 1986) respectively to rrnH, rrnI, and rrnG (the cluster of rrn close to attSPO2). In this report the 4.8Kb rRNA homolog is assigned to a previously unmapped rRNA gene set (rrnK). The crosses of strains BD170/pGR102-144, 168T/pGR102-317, and BD170/pGR102-151 gave similar results. The Cm^r/Cys^+ cotransfer values (79-94%) and the paucity of $Cm^rPur^-Cys^-$ and $Cm^sPur^-Cys^+$ recombinants indicate that in these strains plasmid integration occurred into rRNA gene sets situated very close to the cysA locus. Weaker linkages to cysA (49-83% cotransduction), and purA (4-6% cotransduction) were found in the crosses of strains 168T/pWR103-316, and 168T/pWR103-314 which are missing respectively the 5.5Kb and 4.8Kb BclI rRNA homologs. As shown in Table 5 these two strains showed also a linkage of the Cm^r determinant to aroI906 (12-19% cotransduction) of strain Kit 2. These data indicate that rrnG (5.5Kb BclI rRNA homolog; strain 168T/pWR103-316) is not part of a cluster with rrnH (4.9Kb BclI rRNA homolog; strain

168T/pGR102-317)) and rrnI (5.8Kb BclI rRNA homolog; strain BD170/pGR102-144), and that it is situated very close (in cluster) to rrnK (4.8Kb BclI rRNA homolog; strain 168T/pWR103-314).

GENETIC MAPPING OF rrnH, rrnI, rrnG, and rrnK

Additional transductional crosses were performed to establish the linkage relationship of the rRNA gene sets situated in the cysA-aroI region of the chromosome. Bacillus subtilis strain 1A474 (trpC2, amyE, aroI906) was transduced with PBS1 lysates made using as hosts the Cm^r transformant strains having plasmid insertions either into rrnH, rrnI, rrnG, or rrnK. The cotransductional frequencies obtained in the crosses are listed in Table 7. The values reported show that rrnK and rrnG are situated in cluster and that both are considerably apart from the two clustered rrnH and rrnI. The transductional data obtained in the crosses with Kit 1, Kit 2 and strain 1A474 were combined to construct a map for rrnH and rrnI (Fig. 25A) and rrnG and rrnK (Fig. 25B). According to Bott et al., 1984, rrnH, rrnI, and rrnG map clockwise to cysA with a cotransfer value of 45%. In this study, rrnG and rrnK show cotransfer values (54-74%) comparable to the value reported by Bott et al., 1984. The map position reported here for rrnH and rrnI is closer to cysA (78-91% cotransduction).

GENETIC MAPPING OF *rrnE*

Three transformant strains BD170/pGR102-135, BD170/pGR102-153 and BD29/pYR104-256 showed linkage of the Cm^r marker to *dal-1* and *purB33*, revealing the existence of an unassigned rRNA gene set in this region. Previous mapping data have shown that *pha-1* (resistance to SP01 phage) is localized between the *purB* and *dal-1* loci (Lepesant-Kejzlarova et al., 1975) and the *furB* marker (resistance to 5-fluorouracil in the presence of uracil) is 10% cotransduced with *purB6* (Zahler, 1978). These findings enabled us to perform a series of two and three-factor crosses which would establish the linkage relationship between *rrnE* and the nearby genes.

Resistance to SP01 phage (*pha-1*) or to 5-fluorouracil in the presence of uracil are not selective markers in PBS1 transduction crosses. Two donor strains were constructed by initially crossing the transformant strain BD170/pGR102-135 with the recipient strain 1A150 (*pha-1 hisA1 catA*) and 1A154 (*met purB6 trpC2 gutB*) and selecting recombinants that were Cm^r *pha-1* or Cm^r *furB* $PurB^+$, respectively. Transductants were colony purified twice and used to prepare PBS1-transducing lysates which were designated as IA150/pGR102-135 and IA154/pGR102-135, respectively (Table 1). The lysates were crossed with strain Kit2, and the *pha-1* and *furB* markers were either selected (the less desirable route) or used as unselected

markers among Pur$^+$, Dal$^+$, and Cm^r primary transductants. In the linkage map shown in Fig. 24, three donors BD170/pGR102-135(b), IA150/pGR102-135 (c), and IA154/pGR102-135 (d) are compared with the parental strain BD170 (trpC2 thr-5 (a). The Cm^r determinant is closely linked to pha-1 (87 to 95% cotransduction) on one side and to furB (45-49% cotransduction) on the other side. The linkage relationship between Cm^r and either purB33 or dal-1 remained essentially unchanged for all three donor lysates as did the linkage of dal-1 to purB33. The latter represents an example in which the insertion of a 5.8Kb plasmid (pGR102) does not effect the linkage relationship of the distant flanking markers. The linkage data show that the gene order is aroI dal furB (Cm^r = rrnE) pha-1 purB.

GENETIC MAPPING OF rrnD

The Cm^r determinant of strains BD79/pWR103-182, BD170/pGR111-302, 168T/pGR102-281 and SB25/pGR110-305 showed linkage to tre-12 and glyB133 of Kit3, indicating the presence of an unassigned rRNA gene set (rrnD) in this region of the chromosome. To better localize the position of rrnD on the map of the Bacillus subtilis chromosome, additional transductional crosses were performed. Two recipient strains, 1A84 (glyB133 metD1) and 1A122 (thiA78 glpK21 tre-12) were transduced with PBS1 lysate made from

strain BD79/pWR103-182, and the Cm^r determinant was found to be tightly linked to thiA78 (78% cotransduction) and weakly linked to metD1 (2% cotransduction). Fig. 25 summarizes the linkage data and shows that the gene order is tre (Cm^r = rrnD) thiA glyB metD. As mentioned above, the cotransfer of the integrated plasmid as followed by the Cm^r determinant varies with the direction of selection. Here, the percent cotransfer of Cm^r with the nearby marker thiA was lower (78 and 95% cotransduction).

ASSIGNMENT OF BclI HOMOLOGS TO MAPPED rRNA GENE SETS

As in Escherichia coli and Salmonella typhymurium (Boros et al., 1979; Lehner et al., 1984), nine rRNA gene sets of Bacillus subtilis can be assigned to distinct restriction fragments, each of which has a specific genetic locus. Table 8 summarizes the identification of nine individual rRNA gene sets with their corresponding BclI fragments. The assignment of rrnE,rrnD, and rrnB to the 6.6-, 5.4- and 8.0Kb BclI homologs, respectively, is clear cut since they are individual genes not located in tandem with others.

The rRNA gene sets rrnO and rrnA map in Kitl between purA and cysA with a close linkage to purA (Henkes et al., 1982; Wilson et al., 1981; Bott et al., 1984). The transduction crosses indicated that the rRNA gene sets that are associated with the 8.3- and 5.7Kb BclI homologs

have a closer linkage to purA than to cysA, and that the markers order for transformants BD79/pWR103-179, BD170/pGR102-143, and BD170/pWR103-181 is purA Cm^r cysA (Table 6). The analysis of the BclI-SalI and the EcoRI blots revealed, as described above, that transformants BD79/pWR103-179, BD170/pGR102-143, and BD170/pWR103-181 represent integration events that occurred into rRNA gene sets having large abutments (Figs. 15 and 17). Since Loughney et al., 1982, reported that in B. subtilis only two rRNA gene sets have abutment with tRNA genes, rrnO and rrnA must produce upon BclI restriction the 8.3Kb and 5.7Kb rRNA homologs. The region of the B. subtilis chromosome containing the origin of replication (oriC) and rrnO was sequenced by Moriya et al., 1985. The nucleotide sequence predicts that rrnO should produce upon BclI restriction a fragment having a length of 8.3Kb. This finding allowed the assignment of the 8.3Kb rRNA homolog to rrnO. Since only two gene sets have a large abutment, the second homolog (5.7Kb BclI) must therefore correspond to rrnA.

To maintain the same names for rrn gene sets that have been physically mapped previously, the 4.9Kb and 5.8Kb BclI rRNA homologs were assigned to rrnH and rrnI respectively (La Fauci et al., 1986). However, the map position of these two clustered rRNA gene sets is closer to cysA (Fig.25A) than the one reported previously (Bott et al., 1984). The 4.8Kb and 5.5Kb BclI rrn-homologs are

assigned respectively to the very closely situated rRNA gene sets which map between cysA and aroI (strains 168T/pWR103-314 and 168T/pWR103-316). No integration events were observed into the rRNA gene set responsible for the 6.0Kb BclI band. This rrn-homolog could correspond either to rrnC mapped by Bott et al., 1984 in the aroG-thr region of thechromosome or to rrnR believed to be located in the ilvBC-leu region (Gottlieb et al., 1985; La Fauci et al., 1986; Smith et al., 1968; Chilton and McCarthy, 1969). Finally the 6.0Kb BclI rrn-homolog could correspond to a rRNA gene set situated very close (in cluster) to either rrnH and rrnI or rrnK and rrnG (Chow and Davidson, 1973; Bott et al., 1984).

LABORATORY STRAINS OF Bacillus subtilis HAVING A DELETED rRNA GENE SET

Loughney et al., 1983, reported a B. subtilis strain having a deletion of a rRNA gene set. We, also, reported (Gottilieb et al., 1985) that B. subtilis strains BD29 (argA3 leuB1), BD79 (leuB1 pheA1), BD73 (argA3 pheA1), BD47 (ilvC1 pheA), and BD80 (leuB1 pheA1 argA2) are missing the same rRNA gene set. Southern hybridization analysis of chromosomal DNAs of these strains restricted either with HindIII or EcoRI or SmaI, and hybridized either with 16S or 23S + 5S probes, showed the loss of 16S rRNA homologs (5.5Kb HindIII; 2.1Kb SmaI), and the loss of

23S-5S rRNA homologs (2.9Kb EcoRI; 5.1Kb HindIII; 2.0Kb SmaI). In the present study, I found that strain BD170 (trpC2 thr-5), used as recipient for plasmid integration, is missing the 4.8Kb rrn-homolog (Fig. 13). The same BclI rrn homolog is missing in strains BD79 and GSY1269 (Fig.26A). The BclI blots revealed, also, that in another laboratory strain (strain CU420, trpC2 leuB6 ilvC4) the 5.8Kb rrn-homolog is missing (Fig.26A). Blots containing EcoRI restricted DNAs from both strains BD170 and CU420, hybridized with a 23S-5S probe (EcoRI-HindIII insert of pYR104), revealed that these strains are also missing the 2.9Kb and the 3.8Kb EcoRI rrn-homologs, respectively (Fig.26B). The results prove that in B. subtilis strains BD170 and CU420 an rRNA gene set is deleted. The rRNA gene sets involved in the deletions are rrnK in BD170 and rrnI in CU420.

Cm^r TRANSFORMANT STRAINS WITH DELETED rRNA GENE SETS

The Southern hybridization data presented above indicates that strains SB25/pGR108-257 and BD170/pGR102-151 (both missing the 4.9Kb and the 5.8Kb BclI rrn-homologs) have the plasmid inserted into the 4.9Kb BclI homolog (rrnH). Plasmid integration occurred into the 5.5Kb rrn-homolog (rrnG) in strains 168T/pGR102-311 and 168T/pWR103-315 which are missing both the 4.8Kb and the 5.5Kb BclI homologs. Finally, in strain SB25/pGR110-305,

missing the 5.4Kb and the 4.8Kb BclI bands, plasmid insertion occurred into the 5.4Kb BclI rrn-homolog (rrnD). The elimination of BclI site in a rRNA gene set cannot explain the absence of a second BclI rRNA homolog. In fact, in this case, the rRNA gene set lacking the BclI site would produce a larger fragment which would be visible in the blot as a slow migrating band. In all these strains a deletion of a rRNA gene set could be the cause of the absence of the second BclI rrn-homolog. If the hypothesis of rRNA deletion is correct, upon restriction with EcoRI the DNAs of these strains should also show a missing rrn-homolog. Accordingly, DNA samples of strains missing two BclI rrn-homologs were restricted with EcoRI, run in agarose gels, blotted and hybridized to nick translated 23S, 5S rDNA probes. Samples of EcoRI restricted DNAs from parental strains and from strains missing only one BclI rrn-homolog were run as controls. The autoradiograms shown in Fig. 27 revealed that in strains BD170/pGR102-151, GSY1269/pGR102-177 and SB25/pGR108-257 the 3.8Kb EcoRI band is missing. The 2.9Kb EcoRI rrn-homolog is missing in strains 168T/pWR103-315, 168T/pGR102-311, and SB25/pGR110-305. With the exception of the missing bands, these strains produced EcoRI patterns that correspond to the one produced by their respective parental types. In case of strains BD170/pGR102-151 and GSY1269/pGR102-177, both the 3.8Kb and 2.9Kb EcoRI rrn-homologs are missing. The parental

types of these strains (BD170 and GSY1269, respectively) are in fact strains having only 9 rRNA gene sets (lacking the 4.8Kb BclI and the 2.9 EcoRI rrn-homologs; Fig.26). The results prove that in strains BD170/pGR102-151, GSY1269/pGR102-177, SB25/pGR108-257, 168T/pWR103-315, 168T/pGR102-311, and SB25/pGR110-305 a rRNA gene set (rrnK or rrnI) is deleted.

The deleted 2.9Kb and 3.9Kb EcoRI fragments contain both 16S and 23S rDNA sequences (Wawrousek and Hansen, 1983; Widom, unpublished results). This fact implies that the 3.8Kb EcoRI fragment contains the spacer between rrnH and rrnI and that the 2.9Kb EcoRI fragment contains the spacer between rrnG and rrnK. The complete sequence of rrnB (Green et al., 1985) indicates that the distance between the 5' end of the 16S cistron and the proximal EcoRI site in the same cistron is 0.7Kb; the distance between the most distal EcoRI site in the 23S cistron and the 3' end of the 5S cistron is 2.1Kb. These data allow the determination of the length of the spacer between rrnH and rrnI (3.8Kb - (0.7Kb + 2.1Kb) = 1.0Kb). Similar calculations indicate that the spacer between rrnK and rrnG is 0.1Kb long. Spacers having similar sizes to the one calculated above are found in plasmids p14B1 (0.8Kb) and p14B8 (0.2Kb) both containing 16S and 23S sequences from adjacent rRNA gene sets (Zuber Ph.D. dissertation, 1982, University of Virginia, and Wawrousek and Hansen, 1983).

Southern hybridization shows that in 7 of 27 transformants analyzed (27%) a rRNA gene set is deleted. In six of these deletions strains the deleted operon and the one involved in plasmid integration are situated in cluster (rrnK and rrnG or rrnI and rrnH). These facts allow to formulate the hypothesis that the deletion of a rRNA gene set in a cluster is mediated by the integration of a plasmid into the adjacent gene set. Thus, the deletion in strain BD170/pGR102-151 could be the result of two simultaneous crossover events between a multimer of pGR102 and the two closely spaced rrnH and rrnI (Fig.28). One crossover would occur between rrnH and the 1.2Kb rDNA insert of pGR102, and a second between rrnI and a repetition of the insert in the multimer plasmid. The slow migrating BclI band (10.7Kb; Fig.13) gained by this strain would consist of the 5' end of rrnH, a monomeric form of pGR102, and the 3' end of rrnI up to the internal BclI site in the 23S cistron. A complete gene set equivalent becomes deleted, which explains the disappearance of the second BclI band (5.8Kb) and of the 3.8Kb EcoRI band. The hypothetical insertion-deletion of pGR110 into the rrnG-rrnK cluster is presented in Fig.29. A multimer interacts with the two closely situated gene sets. One crossover occurs between a rDNA insert of the plasmid and rrnG (5.5Kb BclI rrn-homolog) and the other between a repetition of the insert in the multimer and rrnK (4.8Kb BclI rrn-homolog). In the resulting Cm^r

transformant (Fig.29A) the 3' side of rrnG, the spacer, and the 5' side of rrnK are deleted and replaced by the plasmid insertion. Upon EcoRI restriction of the chromosomal region having the plasmid insertion, only one fragment containing 23S and 5S rDNA sequences would be produced. This fragment would correspond to the 3' side of rrnK (Fig.29B). Restriction with BclI would produce a hybrid rDNA fragment containing the 5' part of rrnG, plasmid sequences, and the 3' side of rrnK up to the internal BclI site (Fig.29C). In case of the rrnH-rrnI cluster, the model of Fig.28 predicts that in strains having plasmid insertion into the 5.8Kb BclI rrn-homolog (rrnI; BD170/pGR102-144) and the 4.9Kb BclI rrn-homolog with deletion of the 5.8Kb BclI rrn-homolog (rrnH; BD170/pGR102-151 and other strains), the inserted plasmid should be adjacent to the same downstream sequences. This prediction will eventually allow to test the validity of the model by rescue of the chromosomal sequences adjacent to pGR102 from both strains.

CONCLUDING REMARKS

Plasmids containing cloned rDNA sequences are capable of Campbell-like integration into rRNA gene sets of Bacillus subtilis. The efficiency of transformation of the bacterium with these constructs is very low (Table 2). This suggests that rDNA sequences represent "cold"

recombination spots in the chromosome. However, the 1.2 and 1.0Kb PstI-EcoRI 16S,23S rDNA inserts were able to mediate plasmid integration better than the other rDNA fragments (Table 2). The low level of recombination of certain rDNA fragments, deduced by the low efficiency of transformation of the bacterium with plasmids pGR108, pGR110, pGR111 and pYR104, can justify the erroneous assumption that rDNA sequences do not recombine (Bott et al., 1984). If upon transformation, a single strand form of a plasmid interacts with chromosomal homologous sequences, one can speculate that the capacity of forming intrastrand stem and loop structures can make it difficult for rDNA inserts to synapse and recombine with rRNA gene sets. The higher efficiency of transformation of the bacterium obtained with plasmids pGR102 and pWR103 according to this interpretation, could be due to the presence of an insert having a more relaxed secondary structure.

Plasmid integration occurred into 9 of the 10 rRNA gene sets present in the haploid genome of Bacillus subtilis. The inserted Cm^r marker allowed the transductional mapping of these genes. The position on the chromosome of rrnE, rrnD, and rrnK was not previously known. The transductional data revealed the existence of two clusters of rRNA gene sets (rrnK-rrnG and rrnH-rrnI) in the cysA-aroI region of the chromosome. The existence of a cluster of two tandemly repeated rRNA gene sets

situated 6.2Kb from attSPO2, was first reported by Chow and Davidson, 1973. Bott et al., 1984, mapped a sequence adjacent to 3' side of one of such gene sets at a site close to cysA (45% cotransduction). The same authors used the EM linkage groups formulated by Chow and Davidson, 1973 (Appendix), and the determined sequence of plasmids p14B1 and p14B8 (Zuber, Ph.D. dissertation, 1982, University of Virginia, and Wawrousek and Hansen, 1983) each containing cloned 23S,5S sequences of one gene set and 16S sequences of an adjacent gene set, to assign three gene sets, rrnI, rrnH and rrnG to this cluster. The assignment of a third gene set to the cluster was solely based on the EM linkage groups of Chow and Davidson, 1973. In the present study, no evidences for the existence of a third rRNA gene set clustered either with rrnI and rrnH or with rrnG and rrnK have been produced. However, since plasmid integration did not occur into the gene set responsible for the production of the 6.0Kb BclI rrn-homolog, it is possible that this gene set is part of a cluster. Alternatively, the 6.0Kb BclI rrn-homolog could correspond to rrnC mapped by Bott et al., 1984, in the aroG-thr region, or rrnR thought to be located in the ilvBC-leu region (Smith et al., 1968; Vold 1985; Wawrousek et al., 1984). Since no transformants having a plasmid insertion' into the 6.0Kb BclI rrn-homolog have been found, one can speculate that this gene set is somehow unique, perhaps in the way it is regulated and expressed,

and that integration into it would produce inviable bacteria.

It has been reported that amplification of integrated plasmids containing antibiotic-resistance markers, occurs in E. coli (Gutterson and Koshland, 1983), Streptococcus pneumoniae (Vasseghi and Claverys, 1983) and B. subtilis (Young, 1983). Young, 1984 reported that B. subtilis strain CL104 harbors multiple copies of the Cm^r integrable plasmid pl949 (Haldenwang et al., 1980) containing a cloned 5.7Kb EcoRI-BamHI fragment of B. subtilis DNA (pheA mic). The plasmid sequences are inserted into the pheA locus and are organized as a tandemly repeated array. Specific amplification of these sequences (up to 15 copies) occurs when the strain is grown in presence of high concentration of Cm. According to the author, amplification of integrated plasmids, containing antibiotic markers, is not of universal occurrence, and depends on the cloned insert and on the efficiency with which the antibiotic marker is transcribed. The author speculates: "if the gene (Cm^r marker) is placed in an environment where expression occurs through the agency of a relatively weak promoter, then a high level of gene expression will not be possible unless amplification takes place." This implies that transcription of the CAT gene occurs from the promoter of the B. subtilis gene into which the plasmid is inserted. Gene amplification could explain the presence of multimeric forms of plasmid pGR102

in strains BD170/pGR102-135, BD170/pGR102-153, BD170/pGR102-143, and 168T/pGR102-311. Since multimers are found only when the plasmid is inserted into either rrnE, rrnA or rrnG (with deletion of rrnK), one can speculate that the promoters of these three gene sets are weaker than the promoters of the other rrn gene sets, and that amplification is necessary in these strains to increase the expression of the CAT gene. Alternatively amplification could be possible only in strains in which a dimeric form of the plasmid integrated (most intense band). Unequal crossing over events mediated by vector sequences would occur and result in monomeric and trimeric insertions.

In a relatively high proportion of transformants (27%), plasmid insertion into a gene set was associated with the deletion of another rRNA gene set. With the exception of strain SB25/pGR110-305, in all plasmid induced deletion strains the two rRNA gene sets involved were either rrnG and rrnK or rrnI and rrnH. In these strains, according to the models presented in Figs.28 and 29, the 3' side of a gene set, the spacer, and the 5' side of the adjacent rRNA gene set, are deleted and replaced by plasmid sequences. Accordingly, in strains having a deletion at the rrnG-rrnK cluster (Fig.29) the missing 2.9Kb EcoRI and 4.8Kb BclI rRNA homologs represent respectively the 3' side of rrnG (2.9Kb EcoRI) and the 5' side of rrnK (4.8Kb BclI). Since the spontaneous deletion

strains described in this study share with the transformant deletion strains the same missing BclI and EcoRI rRNA homologs, they must have arisen via intrachromosomal recombination between clustered rRNA gene sets. Intrachromosomal recombination probably occurs between any two rRNA gene sets and results in the deletion of the sequences between the genes. The majority of these events would produce extensive deletions which are lethal, unless they are mediated by recombination between sequences of clustered rRNA gene sets. This would explain why, in both induced and spontaneous deletion strains, the deleted rRNA gene sets are always part of a cluster.

Rescue of the chromosomal sequences adjacent to the inserted plasmid of the Cm^r transformant strains produced in this study will allow the determination of the DNA sequence of nine rRNA promoter regions. The comparison of such sequences will produce important information about the regulation of rRNA synthesis in Bacillus subtilis.

SUMMARY

1) Integrable plasmids containing different cloned rDNA fragments from within the transcriptional unit of the rRNA gene set of Bacillus subtilis were constructed using the bifunctional vector pJH101.

2) All constructs were able to transform B. subtilis strains to chloramphenicol resistance at low efficiencies (10^1-10^3 Cm^r transformants per ug of plasmid). Plasmids pGR102 and pWR103, both containing cloned 16S, 16S/23S abutment, and 23S sequences, had the highest transforming efficiencies.

3) The BclI rRNA hybridization patterns obtained for the Cm^r transformants revealed the disappearance of parent-type rDNA bands and the appearance of new larger bands. This indicates that one or more copies of a plasmid integrated in a given rRNA gene set.

4) Southern hybridization showed that each Cm^r transformant has plasmid sequences inserted into the chromosome. Plasmid integration is mediated by homologous recombination and the entire plasmid is inserted into a given rRNA gene set (Campbell-like integration). Multimeric forms of plasmid pGR102 were

found inserted into rrnA (5.7Kb BclI band), rrnE (6.6Kb BclI band) and rrnG (5.5Kb BclI band).

5) PBS1 transduction crosses were done to map the inserted Cm^r determinant, using the nine mapping kit strains of Dedonder et al., 1977. The outcome of the crosses showed that the plasmids integrated in five different regions of the chromosome: I) purA-cysA; II) cysA-aroI; III) dal-1-purB33; IV) tre-12-glyB133; V) aroG-thr5.

6) Four rRNA gene sets were located in the cysA-aroI region. The map position of one of these (rrnK) was previously unknown; rrnK is linked by cotransduction to cysA (66%), amyE (62%), and aroI (12%).

7) The four rRNA gene sets that map between cysA and aroI are organized in two clusters each containing two rRNA gene sets: rrnH-rrnI and rrnG-rrnK. While rrnH and rrnI are linked by cotransduction to cysA (78%) and weakly linked to amyE (3%), rrnG and rrnK are linked to cysA (54%) amyE (62%), and aroI (12%).

8) The presence of a rRNA gene set in the dal-1-purB33 region of the chromosome was previously unknown. This gene set has been named rrnE. It is linked by cotransduction to pha-1 (87%), and furB (45%). Also unknown was the map position of rrnD in the tre-12-glyB133 region of the chromosome. This gene set is linked to thiA78 (78%), tre-12 (33%), and glyB133 (26%).

9) Plasmid integration occurred into nine of the ten rRNA gene sets present in the haploid genome of Bacillus subtilis. Nine mapped rRNA gene sets have been assigned to nine BclI rRNA homologs. The 6.0Kb BclI homolog was not involved in plasmid integration. This rrn-homolog could correspond either to rrnC, mapped by Bott et al., 1984 in the aroG-thr region, to rrnR, believed to be located in the ilvBC-leu region, or to the third rRNA gene set at the rrnH and rrnI cluster (Chow and Davidson, 1973).

10) In six out of twenty-seven Cm^r transformants analyzed by Southern hybridization, plasmid integration into a clustered rRNA gene set (either rrnG or rrnH) was associated with the deletion of the immediately adjacent rRNA gene set (rrnK or rrnI).

11) Laboratory strains of *B. subtilis* like BD170 and CU420 have only nine rRNA gene sets. The deleted sets are either *rrnK* or *rrnI*.

12) Models explaining the occurrence of plasmid induced deletions are presented.

TABLE 1

Strains and plasmids used in this study

Strain or Plasmid	Genotype	Source
Parental strains		
NCTC 3610	Prototroph	A. Sonenshein
168T	trpC2	K. Bott
ZB8	trpE46 hisH2	J. Kane
SB25	trpC2 hisH2	D. Dubnau
166	trpE26	BGSCa
GSY1269	trpE26 ilvC1	K. Bott
BD170	trpC2 thr-5	D. Dubnau
BD79	leuB1 pheA1	D. Dubnau
BD29	argA2 leuB1	D. Dubnau
CU420	trpC2 leuB6 ilvC4	A. Garro
Kit 1 to Kit 9	Mapping recipients	D. Dubnau
A122	thiA78 glpK21 glyB133 tre-12	BGSC
1A150	pha-1 catA hisA2	BGSC
1A154	met purB6 trpC2 furB gutB	BGSC
1A84	glyB133 metD1	BGSC
1A474	amyE aroI906	BGSC
1A241	cysA14 rpsE2	BGSC
Recombinant plasmids		
pJH101	Tc^r Ap^r Cm^r	K. Bott
pBC194	16S-23S rRNA Tc^r Ap^r	K. Bott
pMS102-B7	16S-23S rRNA Ap^r Km^r	K. Bott
p12E2	23S-5S rRNA Ap^r	K. Bott
pGR102	16S-23S rRNA Tc^r Cm^r	This study
pWR103	16S-23S rRNA Tc^r Cm^r	This study
pYR104	23S-5S rRNA Ap^r Cm^r	This study
pGR108	23S rRNA Tc^r Cm^r	This study
pGR110	16S rRNA Tc^r Cm^r	This study
pGR111	23S rRNA Ap^r Cm^r	This study
pER102	leu Ap^r Cm^r	R. Rudner

TABLE 1

Cont'd

Strain or Plasmids	Genotype	Source
Transformant strains[b]		
BD170/pGR102-135	trpC2 thr-5 Cm^r	This study
BD170/pGR102-143	trpC2 thr-5 Cm^r	This study
BD170/pGR102-144	trpC2 thr-5 Cm^r	This study
BD170/pGR102-151	trpC2 thr-5 Cm^r	This study
BD170/pGR102-153	trpC2 thr-5 Cm^r	This study
168T/pGR102-272	trpC2 Cm^r	This study
168T/pGR102-281	trpC2 Cm^r	This study
168T/pGR102-313	trpC2 Cm^r	This study
168T/pGR102-317	trpC2 Cm^r	This study
BD79/pGR102-174	leuB1 pheA1 Cm^r	This study
GSY1269/pGR102-177	trpE26 ilvC1 Cm^r	This study
1A150/pGR102-135	pha-1 catA hisA1 Cm^r	This study
1A154/pGR102-135	met PurB$^+$ trpC2 furB gutB Cm^r	This study
166/pGR102-273	trpE26 Cm^r	This study
168T/pGR102-311	trpC2 Cm^r	This study
BD170/pWR103-179	trpC2 thr-5 Cm^r	This study
BD170/pWR103-181	trpC2 thr-5 Cm^r	This study
BD79/pWR103-182	leuB1 pheA1 Cm^r	This study
168T/pWR103-314	trpC2 Cm^r	This study
168T/pWR103-315	trpC2 Cm^r	This study
168T/pWR103-316	trpC2 Cm^r	This study
BD170/pGR110-291	trpC2 thr-5 Cm^r	This study
SB25/pGR110-304	trpC2 hisH2 Cm^r	This study
SB25/pGR110-305	trpC2 hisH2 Cm^r	This study
SB25/pGR110-306	trpC2 hisH2 Cm^r	This study
SB25/pGR108-236	trpC2 hisH2 Cm^r	This study
SB25/pGR108-257	trpC2 hisH2 Cm^r	This study
BD170/pGR111-301	trpC2 thr-5 Cm^r	This study
BD170/pGR111-302	trpC2 thr-5 Cm^r	This study
BD29/pYR104-256	argA2 leuB1 Cm^r	This study
168T/pYR104-285	trpC2 Cm^r	This study
168T/pYR104-286	trpC2 Cm^r	This study

[a] Bacillus Genetic Stock Center, Columbus, Ohio

[b] Isolated as Cm^r transformants after plasmid transformation

TABLE 2

Transforming efficiencies of integrable plasmids and chromosomal DNA with integrated plasmids in *Bacillus subtilis*

Plasmid or chromosomal DNA	Insert (Kb) and/or relevant genotype	No. transformants per ug of DNA[2] Leu$^+$,His$^+$, or Th^r	Cm^r
Plasmids			
pJH101		0	0
pER102	(3.2) leu	9.2×10^6	7.5×10^4
pGR102	(1.2) 16S-23S rRNA	0	2.4×10^3
pWR103	(1.0) 16S-23S rRNA	0	1.4×10^3
pYR104	(2.3) 23S-5S rRNA	0	1.8×10^2
pGR108	(1.2) 23S rRNA	0	$0.5\text{-}1.0 \times 10^1$
pGR110	(0.6) 23S rRNA	0	1.4×10^2
pGR111	(1.5) 23S rRNA	0	2.5×10^2
Transformant strains			
BD170/pER102-115	trpC2 thr-5 Cm^r	9.2×10^5	4.9×10^4
BD170/pGR102-143	trpC2 thr-5 Cm^r	6.0×10^5	8.1×10^4
BD170/pWR103-179	trpC2 thr-5 Cm^r	2.3×10^5	2.5×10^4
SB25/pGR108-257	trpC2 hisH2 Cm^r	1.1×10^6	1.3×10^4
BD170/pGR110-302	trpC2 thr-5 Cm^r	9.5×10^5	4.5×10^3
BD170/pGR111-301	trpC2 thr-5 Cm^r	1.0×10^6	1.4×10^4
168T/pYR104-286	trpC2 Cm^r	2.1×10^4	5.5×10^3

[a] Recipient strains were BD79 (leuB1 pheA1), SB25 (trpC2 hisH2), and BD170 (trpC2 thr-5)

TABLE 3

BclI rrn homologs involved in plasmid insertion in strains of Bacillus subtilis transformed to Cm^r either with pGR102, or pWR103.

BclI rrn homolog involved in plasmid insertion (kb)	Transformant strains
8.3	BD170/pWR103-179
8.0	168T/pGR102-272 166/pGR102-273
6.6	BD170/pGR102-135 BD170/pGR102-153
6.0	None
5.8	BD170/pGR102-144
5.7	BD170/pGR102-143 BD170/pWR103-181
5.5	BD79/pGR102-174 168T/pWR103-316 168T/pGR1X2-311[a] 168T/pWR103-315[a]
5.4	168T/pGR102-281 168T/pGR102-313 BD79/pWR103-182
4.9	168T/pGR102-317 BD170/pGR102-151[b] GSY1269/pGR102-177[b]
4.8	168T/pWR103-314

[a] The 4.8Kb BclI rrn-homolog found in the parental type is also missing.

[b] The 5.8 kb BclI rrn-homolog found in the parental type is also missing.

TABLE 4

BclI *rrn* homologs involved in plasmid insertion in strains of *Bacillus subtilis* transformed to cm^r either with pGR108, pGR110 or pGR111.

BclI rrn homolog involved in plasmid insertion (kb)	Transformant strains
8.3	SB25/pGR110-304
8.0	None
6.6	None
6.0	None
5.8	None
5.7	None
5.5	BD170/pGR110-291
5.4	BD170/pGR111-302 SB25/pGR110-305[a]
4.9	SB25/pGR108-236 BD170/pGR111-301[b] SB25/pGR108-257[b]
4.8	SB25/pGR110-306

[a] The 4.8kb BclI *rrn*-homolog found in the parental type is also missing.

[b] The 5.8kb BclI *rrn*-homolog found in the parental type is also missing.

TABLE 5

Cotransduction Frequencies of Integrated Cm^r Element of Plasmids to Chromosomal Markers

Donor Strain	Missing BclI Homolog(s) (Kb)	COTRANSDUCTION % Recipients[a] Pur^+/Cm^r	Cm^r/Pur^+	Kit 1 Cys^+/Cm^r	Cm^r/Cys^+	Kit 2 Cm^r/Aro^+
BD170/pWR103-179	8.3	78	28	72	9	ND[b]
SB25/pGR110-304	8.3	ND	17	ND	67	ND
BD170/pGR102-143	5.7	54	57	61	57	ND
BD170/pWR103-181	5.7	76	10	83	14	ND
SB25/pGR108-236	4.9	ND	0	ND	56	0
168T/pGR102-317	4.9	17	16	77	78	0
BD170/pGR102-151	4.9+5.8	18	40	96	94	0
GSY1269/pGR102-177	4.9+5.8	30	40	84	80	0
BD170/pGR111-301	4.9+5.8	ND	20	ND	81	0
SB25/pGR108-257	4.9+5.8	13	23	44	84	0
BD170/pGR102-144	5.8	49	35	94	96	0
BD170/pGR110-291	5.5	4	11	88	70	12
168T/pWR103-316	5.5	4	4	60	49	12
168T/pWR103-315	5.5+4.8	8	4	72	73	12
168T/pWR103-311	5.5+4.8	3	4	72	73	19
168T/pWR103-314	4.8	6	5	73	84	12
168T/pWR103-306	4.8	4	3	63	57	18
SB25/pGR110-305	5.4+4.8	0	0	0	0	0
168T/pYR104-285	d	ND	9	ND	69	ND
168T/pYR104-286	-	ND	38	ND	100	ND

TABLE 5
(Cont.)

Donor Strain	Missing BclI Homolog(s) (Kb)	Recipients[a] Kit 2			
		Dal+/ Cmr	Cmr/ Dal+	Pur+/ Cmr	Cmr/ Pur+
BD170/pGR102-135	6.6	27	36	30	38
BD170/pGR102-153	6.6	32	39	28	36
BD29/pYR104-256	-	23	88	34	40

		Recipients[a] Kits 3 and 1A84					
		Tre+/ Cmr	Cmr/ Tre+	Gly+/ Cmr	Cmr/ Gly+	Cmr/ MetD+	MetD+/ Cmr
BD79/pWR103-182	5.4	ND	35	26	29	2	4
BD170/pGR111-302	5.4	ND	ND	ND	33	1.5	ND
168T/pGR102-281	5.4	ND	ND	ND	34	1.5	ND
SB25/pGR110-305	5.4+4.8	ND	ND	ND	26	0	ND

		Recipients[a]			
		Kit 7		Kit 8	
		Aro+/ Cmr	Cmr/ Aro+	Thr+/ Cmr	Cmr/ Thr+
168T/pGR102-272	8.0	54	48	43	36
166/pGR102-273	8.0	56	34	83	37

[a] Recipient strains are as follows: Kit 1, purA16, cysA14, trpC2; Kit 2, aroI906, dal-1, purB33; trpC2; Kit 3, glyB133, metC3, tre-12, trpC2; Kit 7, ald-1, aroG932, leuA8, trpC2; Kit 8, hisA1, thr-5, trpC2; 1A84, glyB133, metD1

[b] ND, not determined

[c] Selected/unselected

[d] The rDNA BclI pattern is not modified.

TABLE 6

Transduction crosses for mapping of integrated plasmids in the *purA cysA* region[a]

DONOR	Recombinant Class[b]				Cotransfer	
	Cm^r	purA	cysA	No	Type	%[c]
BD170/pWR103-179 (8.3Kb BclI[d])	1	0	0	200	Cm^r/Pur$^+$	28
	1	1	0	64		
	1	0	1	16	Cm^r/Cys^r	9
	1	1	1	20		
	0	1	0	67	Pur$^+$/Cys$^+$	21
	1	1	0	169		
	0	1	1	0	Pur$^+$/Cm^r	78
	1	1	1	64		
	0	0	1	82	Cys$^+$/Pur$^+$	17
	0	1	1	0		
	1	0	1	168	Cys$^+$/Cm^r	72
	1	1	1	51		
					gene order: *purA* Cm^r *cysA*	
BD170/pGR102-143 (5.7Kb BclI)	1	0	0	0	Cm^r/Pur$^+$	57
	1	1	0	68		
	1	0	1	80	Cm^r/Cys$^+$	57
	1	1	1	20		
	0	1	0	160	Pur$^+$/Cys$^+$	18
	1	1	0	72		
	0	1	1	0	Pur$^+$/Cm^r	44
	1	1	1	52		
	0	0	1	176	Cys$^+$/Pur$^+$	14
	0	1	1	0		
	1	0	1	60	Cys$^+$/Cm^r	36
	1	1	1	40		
					gene order: *purA* Cm^r *cysA*	

TABLE 6

(Cont.d)

DONOR	Recombinant Class				Cotransfer	
	Cm^r	purA	cysA	No	Type	%
BD170/pGR102-144 (5.8Kb BclI)	1	0	0	3	Cm^r/Pur$^+$	36
	1	1	0	6		
	1	0	1	181	Cm^r/Cys$^+$	97
	1	1	1	96		
	0	1	0	232	Pur$^+$/Cys$^+$	30
	1	1	0	113		
	0	1	1	20	Pur$^+$/Cm^r	49
	1	1	1	130		
	0	0	1	22	Cys$^+$/Pur$^+$	30
	0	1	1	18		
	1	0	1	416	Cys^r/Cm^r	94
	1	1	1	167		
					gene order: purA cysA Cm^r	
BD170/pGR102-151 (4.9-5.8Kb BclI)	1	0	0	4	Cm^r/Pur$^+$	40
	1	1	0	2		
	1	0	1	56	Cm^r/Cys$^+$	94
	1	1	1	38		
	0	1	0	38	Pur$^+$/Cys$^+$	50
	1	1	0	12		
	0	1	1	0	Pur$^+$/Cm^r	62
	1	1	1	50		
	0	0	1	8	Cys^r/Pur$^+$	32
	0	1	1	0		
	1	0	1	60	Cys^r/Cm^r	92
	1	1	1	32		
					gene order: purA cysA Cm^r	

TABLE 6

(Cont.d)

DONOR	Recombinant Class				Cotransfer	
	Cm^r	purA	cysA	No	Type	%
168T/pGR102-317 (4.9Kb BclI)	1	0	0	26	Cm^r/Cys$^+$	79
	1	1	0	0		
	1	0	1	78	Cm^r/Pur$^+$	16
	1	1	1	20		
	0	1	0	49	Pur$^+$/Cys$^+$	13
	1	1	0	2		
	0	1	1	0	Pur$^+$/Cm^r	17
	1	1	1	8		
	0	0	1	12	Cys$^+$/Pur^r	10
	0	1	1	0		
	1	0	1	34	Cys$^+$/Cm^r	76
	1	1	1	5		
					gene order: purA cysA Cm^r	
168T/pWR103-316 (5.5Kb BclI)	1	0	0	140	Cm^r/Pur$^+$	4
	1	1	0	0		
	1	0	1	124	Cm^r/Cys$^+$	49
	1	1	1	12		
	0	1	0	276	Pur$^+$/Cys$^+$	9
	1	1	0	4		
	0	1	1	20	Pur$^+$/Cm^r	4
	1	1	1	8		
	0	0	1	52	Cys$^+$/Pur$^+$	17
	0	1	1	40		
	1	0	1	140	Cys$^+$/Cm^r	60
	1	1	1	0		
					gene order: purA cysA Cm^r	

TABLE 6

(Cont.d)

DONOR	Recombinant Class				Cotransfer	
	Cm^r	purA	cysA	No	Type	%
168T/pWR103-314 (4.8Kb BclI)	1	0	0	40	Cm^r/Pur$^+$	4
	1	1	0	0		
	1	0	1	192	Cm^r/Cys$^+$	83
	1	1	1	12		
	0	1	0	164	Pur$^+$/Cys$^+$	19
	1	1	0	2		
	0	1	1	28	Pur$^+$/Cm^r	6
	1	1	1	10		
	0	0	1	16	Cys^r/Pur$^+$	22
	0	1	1	88		
	1	0	1	288	Cys$^+$/Cm^r	73
	1	1	1	0		
					gene order: purA cysA Cm^r	
BD170		1	0	356	Pur$^+$/Cys$^+$	24
		1	1	113		
		0	1	375	Cys$^+$/Pur$^+$	25
		1	1	126		

[a] The recipient was Kit 1 (purA16 cysA14 trpC2)

[b] Donor and recipient phenotypes are indicated by 1 and 0, respectively.

[c] Number cotransferred per number tested.

[d] Missing BclI rrn homolog(s).

TABLE 7

Cotransduction frequencies of integrated Cm^r element of plasmids to the chromosomal markers of *B. subtilis* strain 1A474 (*amyE*, *aroI906*)

Donor Strain	Cm^r/Amy^+	Cm^r/Aro^+	Aro^+/Cm^r	Aro^+/Amy^+
168T/pWRI03-314	78	19	18	87
SB25/pGR110-306	49	14	13	90
BD170/pGR110-291	50	32	18	84
168T/pWR103-316	74	21	16	85
168T/pWR103-315	76	14	28	88
168T/pGR102-311	78	20	15	86
SB25/pGR108-236	3	0	0	88
BD170/pGR102-144	3	0	0	87
BD170/pGR102-151	3	0	0	87

TABLE 8

Ribosomal BclI homologs and corresponding rRNA gene sets

BclI rRNA homolog (Kb)	rRNA gene set assignment	Genomic Region
8.3	*rrn0*	*purA-cysA*
5.7	*rrnA*	*purA-cysA*
4.9	*rrnH*	*cysA-aroI*
5.8	*rrnI*	*cysA-aroI*
5.5	*rrnG*	*cysA-aroI*
4.8	*rrnK*	*cysA-aroI*
6.6	*rrnE*	*dal-purB*
5.4	*rrnD*	*tre-12-glyB*
8.0	*rrnB*	*aroG-thrA*
6.0	?	?

Figure 1: The generalized restriction map of Bacillus subtilis rRNA gene sets and a collection of rDNA fragments that have been subcloned. The broken lines in plasmids p12E2 and pMS102 represent chromosomal sequences beyond the rDNA boundary. B-BamHI, H-HindIII, S-SmaI, R-EcoRI, SmaI*-site disrupted during cloning procedures. Numbers represent Kb.

pBC279, Stewart et al., 1982
p21C4, Hutchison et al., 1980
pMS102-B7, Seiki et al., 1981
pBC194, Stewart et al., 1982
p12E2, Moran et al., 1979

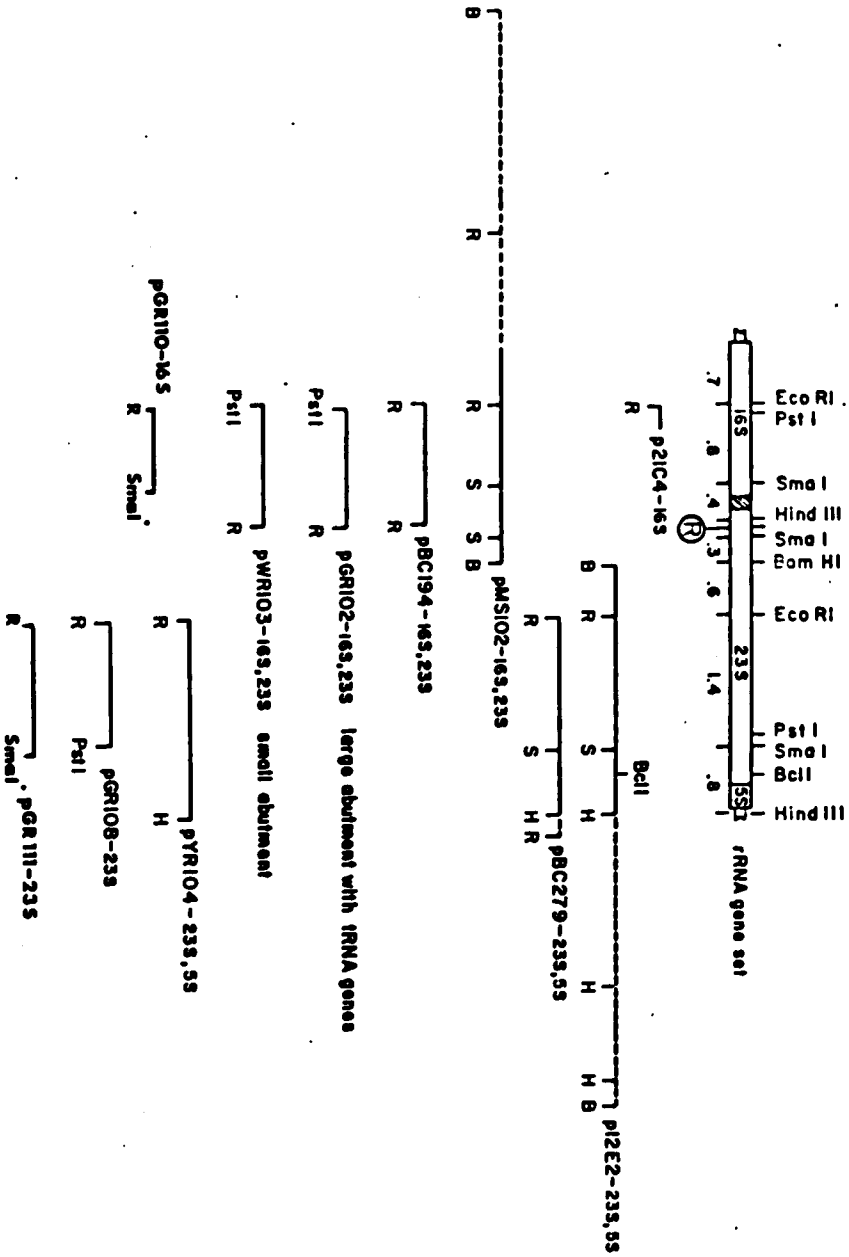

Eco RI
Pst I
Sma I
Hind III
Sma I
Bam HI
Eco RI
Pst I
Sma I
Bcl I
Hind III
16S
23S
5S
rRNA gene set
p2IC4-16S
pMSI02-16S,23S
pBC279-23S,5S
pI2E2-23S,5S
Bcl I
pBC194-16S,23S
pGRI02-16S,23S large abutment with tRNA genes
pWRI03-16S,23S small abutment
pYRI04-23S,5S
pGRI08-23S
pGR III-23S
pGRI10-16S
Sma I

Figure 2: Restriction endonuclease map of plasmid pJH101 and various integrable derivatives containing either rDNA or leu DNA sequences of B. subtilis. Abbreviations: TET, tetracycline resistance; CAT, chloramphenicol acetyl transferase; AMP, ampicillin resistance; Kb, Kilobases. B, H, P, R, and Bc indicate respectively, BamHI, HindIII, PstI, EcoRI, and BclI restrictions sites. S*, SmaI site disrupted during cloning procedures.

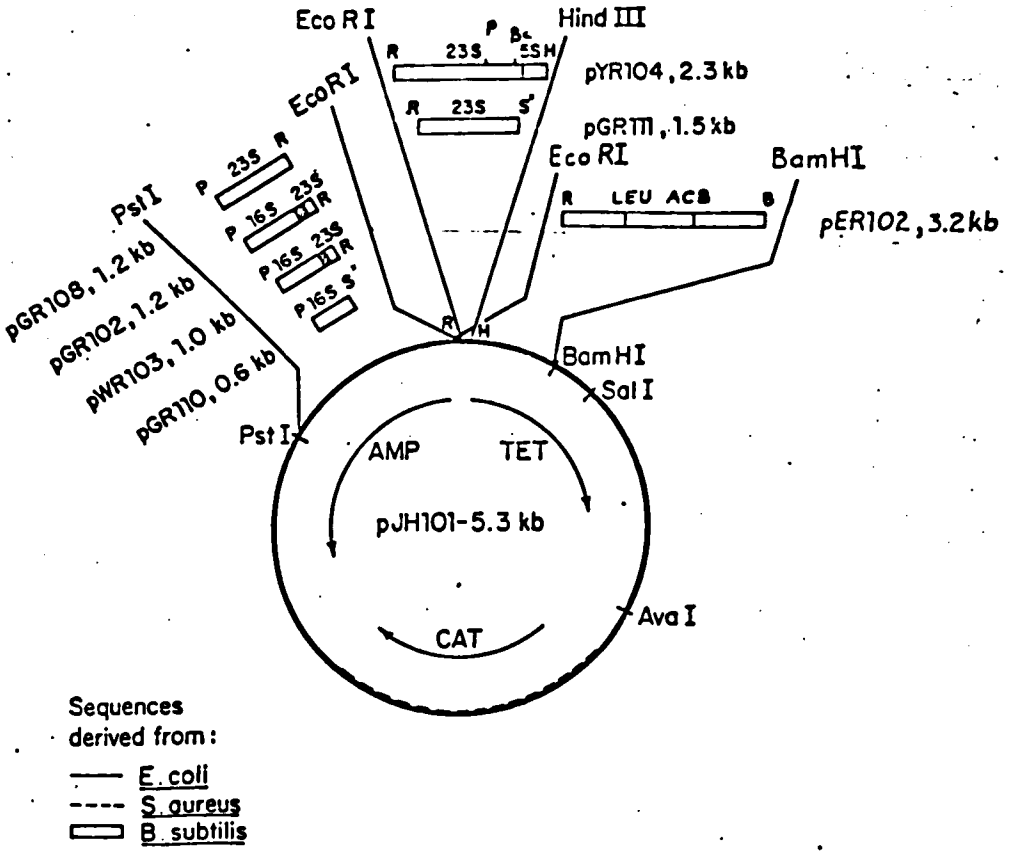

Eco R I
Hind III
pYR104, 2.3 kb
pGR111, 1.5 kb
Eco RI
BamHI
pER102, 3.2kb
EcoRI
PstI
pGR108, 1.2 kb
pGR102, 1.2 kb
pWR103, 1.0 kb
pGR110, 0.6 kb
Pst I
BamHI
Sal I
AMP
TET
pJH101-5.3 kb
CAT
Ava I
Sequences
derived from:
E. coli
S. aureus
B. subtilis

Figure 3: Southern hybridization experiments demonstrating that plasmid pGR102 contains 16S rDNA sequences.

Panels A and B: Samples of pGR102 DNA restricted with EcoRI (A:b and B:b), PstI (A:d and B:d), and PstI+EcoRI (A:c and B:c), were run in a 0.75% agarose gel, blotted to a nitrocellulose filter and hybridized to ^{32}P end labeled 16S rRNA. Panel A: Restricted DNA samples before transfer to the nitrocellulose filter. Panel B: Autoradiogram of the hybridized filter. A 1.2Kb fragment homologous to 16S rRNA is produced upon double digestion of pGR102 with PstI+EcoRI (A:c, B:c). Lanes A:a and B:a contain bacteriophage λ DNA restricted with HindIII (Molecular weight markers: 21.9Kb, 9.3Kb, 6.2Kb, 4.3Kb, 2.3Kb, 2.0Kb, and 0.5Kb).

Panel C: Samples of DNA from Bacillus subtilis strains 168T(b), SB25(c) and BD170(d) were double restricted with PstI+EcoRI run in a 0.75% agarose gel, blotted, and hybridized to nick translated pGR102. Lane C:a contains HindIII restricted bacteriophage λ DNA. The probe hybridizes to two chromosomal fragments (1.2Kb and 1.0Kb), which correspond to the rDNA fragments containing the large (1.2Kb) and small (1.0Kb) abutment, respectively (Fig. 1).

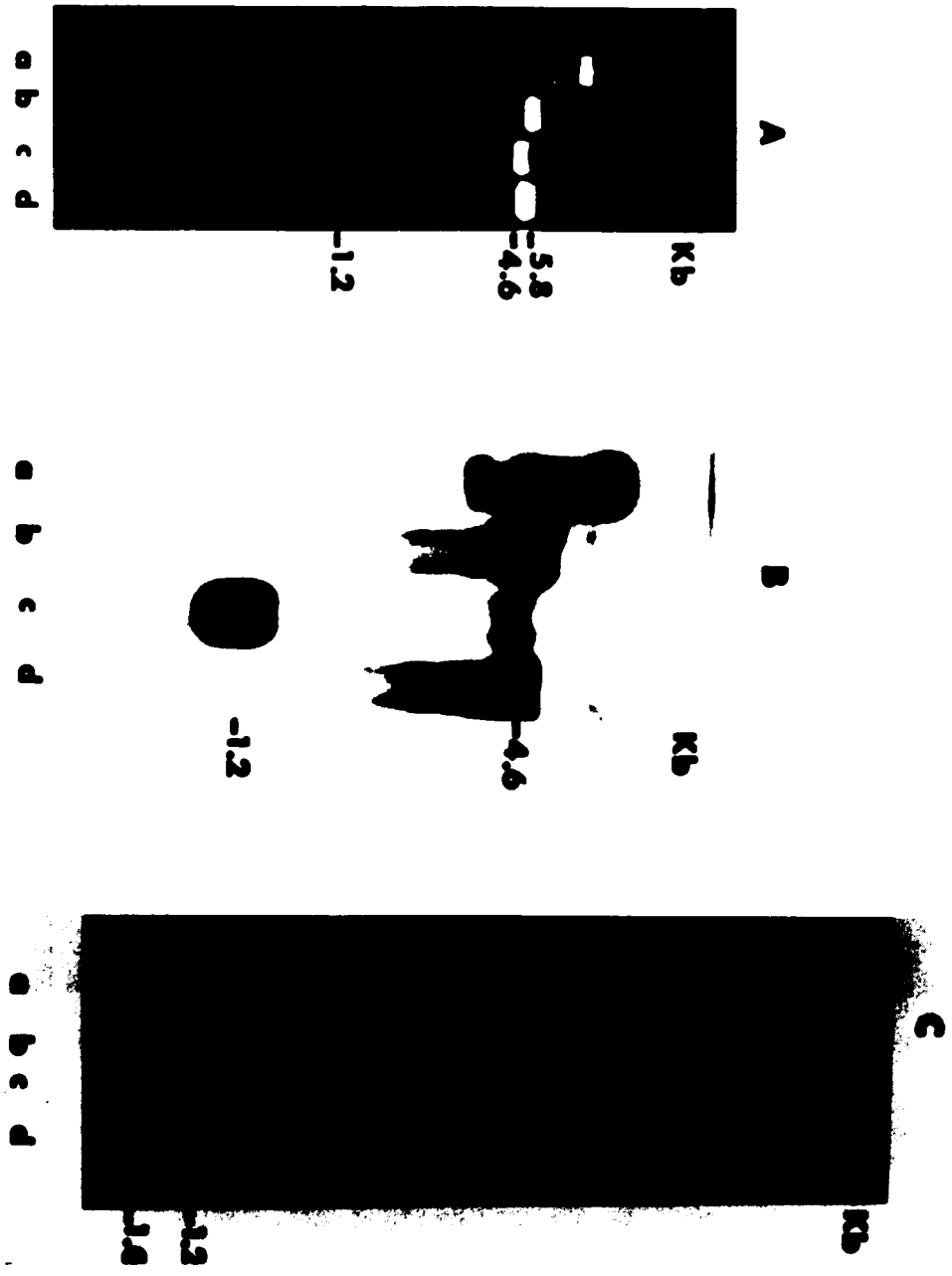

A
Kb
12
5.8
4.6
a b c d
B
Kb
12
4.6
a b c d
C
Kb
12
4.6
a b c d

Figure 4: Southern hybridization experiments demonstrating that plasmid pWR103 contains 16S rDNA sequences.

Panel A: Samples of plasmid pWR103 restricted with PstI (A:a), EcoRI (A:c), and PstI+EcoRI (A:b), were run in a 0.75% agarose gel, blotted, and hybridized to ^{32}P end labeled 16S rRNA. The expected 1.0Kb band (cloned PstI-EcoRI 16S-23S rDNA fragment) is not produced upon double restriction (A:b). Since lane A:a shows unrestricted plasmid DNA, the pJH101 PstI site is not present in plasmid pWR103.

Panel B: Samples of chromosomal DNA from B. subtilis strains 168T (B:b), SB25 (B:c), BD170 (B:d), and NCTC3610 (B:e) were double restricted with PstI+EcoRI, run in a 0.75% agarose gel, and hybridized to nick translated plasmid pWR103. Lane B:a contains bacteriophage λ DNA restricted with HindIII. The probe hybridizes to two chromosomal fragments (1.2Kb, and 1.0Kb) which correspond to the rDNA fragments containing the large (1.2Kb) and small (1.0Kb) abutment, respectively (Fig. 1).


1.2
1.0
a b c
a b c d e

Figure 5: Southern hybridization showing that plasmid pYR104 contains the 2.3Kb EcoRI-HindIII rDNA fragment cloned in plasmid p12E2. HindIII and EcoRI double restricted samples of plasmids pJH101 (b), p12E2 (c), and pYR104 (d), were run in a 0.75% agarose gel, blotted, and hybridized to the nick translated 2.3Kb EcoRI-HindIII fragment cloned in p12E2. Lane a contains bacteriophage λ DNA restricted with HindIII. The slow migrating bands (*) in lanes c and d represent partially restricted fragments. The faint band (**) in lane c is probably due to EcoRI* activity.

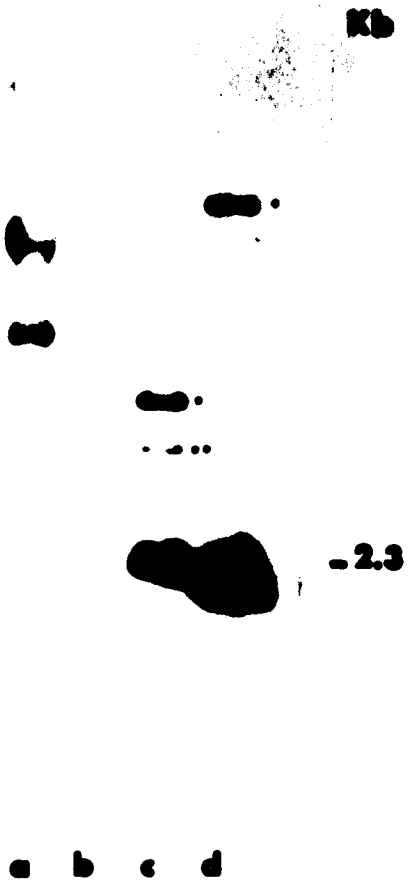

Kb
2.3
a
b
c
d

Figure 6: Southern hybridization showing that the 1.2Kb PstI-EcoRI insert of plasmid pGR108 corresponds to the 5' part of the insert of plasmid pYR104. Samples of plasmid pYR104 (b), pGR108 (c), and pJH101 (d) were double restricted with PstI+EcoRI, run in a 0.75% agarose gel, blotted, hybridized to nick translated plasmid pGR108. Lanes A:a and B:a - λ HindIII.

Panel A: Restricted DNA samples before Southern transfer.

Panel B: Autoradiogram of the blot.

A 1.2 Kb band is present in both pGR108 (A:c, B:c) and pYR104 (A:b, B:b).

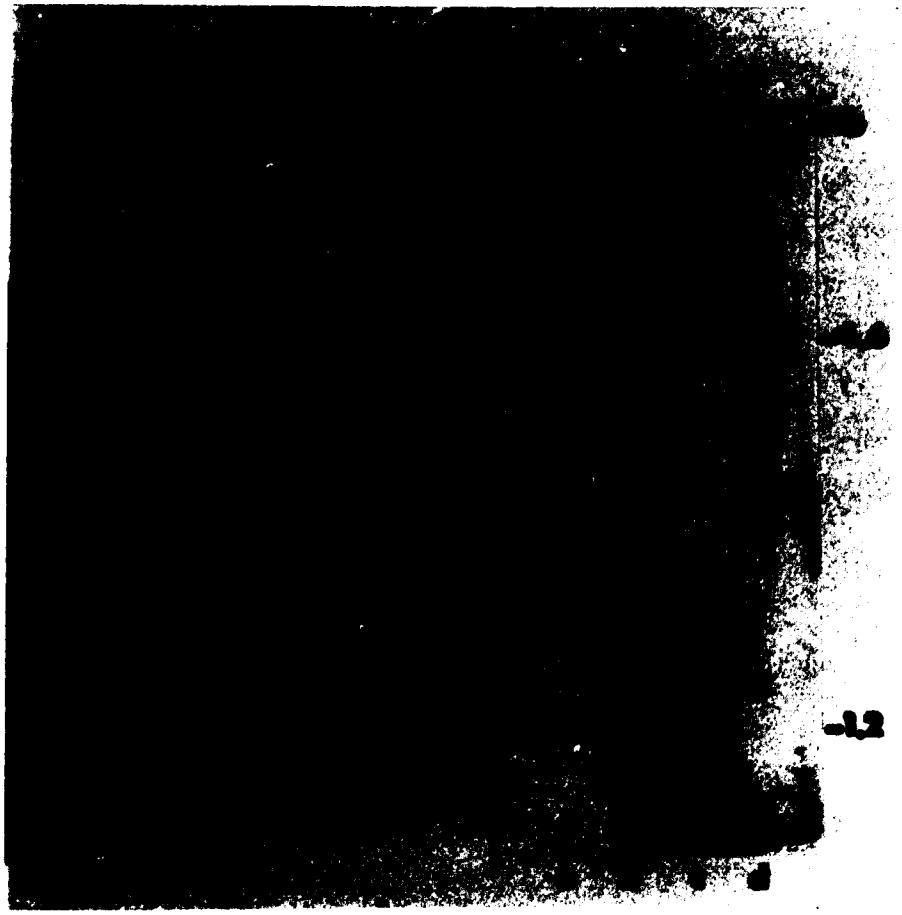


Figure 7: Southern hybridization showing that plasmid pGR111 contains an insert smaller than the one present in plasmid pYR104. Samples of plasmids pGR111 (b) and pYR104 (c) were double restricted with BamHI and EcoRI, run in a 0.75% agarose gel, blotted, and hybridized to the nick translated PstI-EcoRI rDNA fragment of pYR104. Plasmids pGR111 and pYR104 produced, respectively, 1.8Kb and 2.5Kb fragment having homology with the probe. Lane a - λ HindIII. The slow migrating bands in lanes b and c are due to partial restriction.

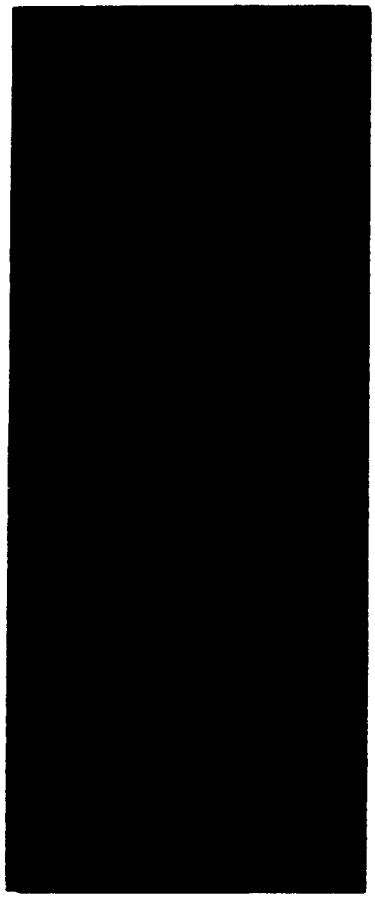


Figure 8: Autoradiograms of Southern blots containing samples of electrophoretically separated SmaI restricted chromosomal DNA of B. subtilis strains 168T and SB25.

Panel A: Blot hybridized to nick translated plasmid pYR104.

Panel B: Blot hybridized to nick translated plasmid pGR111.

lane a - λ HindIII
lane b - 168T
lane c - SB25

While pGR111 hybridizes only to the internal SmaI 23S rDNA fragmet (2.3Kb), pYR104 hybridizes also with fragments containing 23S, 5S, rDNA sequences from different rDNA gene sets (Fig.1).

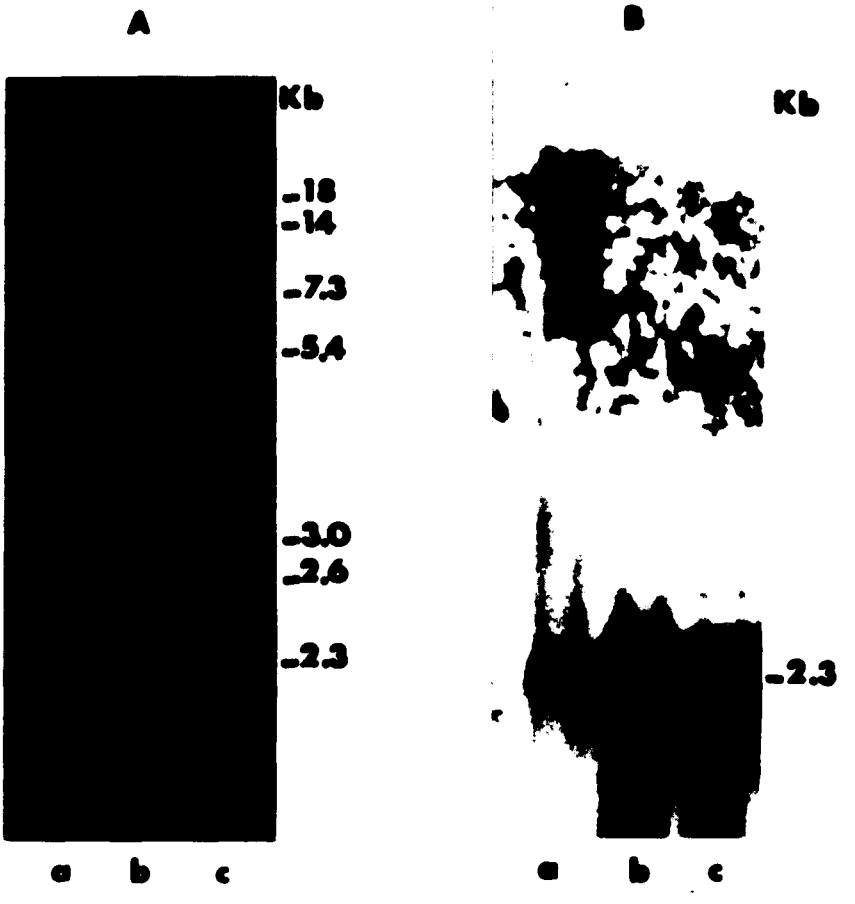

A
Kb
18
14
7.3
5.4
3.0
2.6
2.3
a
b
c
B
Kb
2.3
a
b
c

Figure 9: Southern hybridization experiments showing that plasmid pGR110 has a 0.6Kb insert homologous to the PstI-SmaI 16S rDNA fragment of the insert of plasmid pGR102 (Figs. 1 and 2). Samples of plasmids pGR102 and pGR110 double restricted with PstI+HindIII were run in a 0.75% agarose gel, blotted and hybridized either to the nick translated PstI-EcoRI insert of pGR102 (B:b, c, d) or to the nick translated SmaI-EcoRI rDNA fragment of pGR102 (B:f, g, and h).

Panel A: Ethidium bromide stained gel before Southern transfer.

Panel B: Autoradiograms obtained with the two probes.

lanes a, e - λ HindIII
lanes b, f - pGR102
lanes c, d, g, h - pGR110

The 0.6Kb PstI-HindIII insert of pGR110 is visible in the autoradiogram probed with the entire insert of pGR102 (B:c, d), but not in the one probed with the portion of the pGR102 insert (SmaI-EcoRI) deleted during the construction of pGR110.

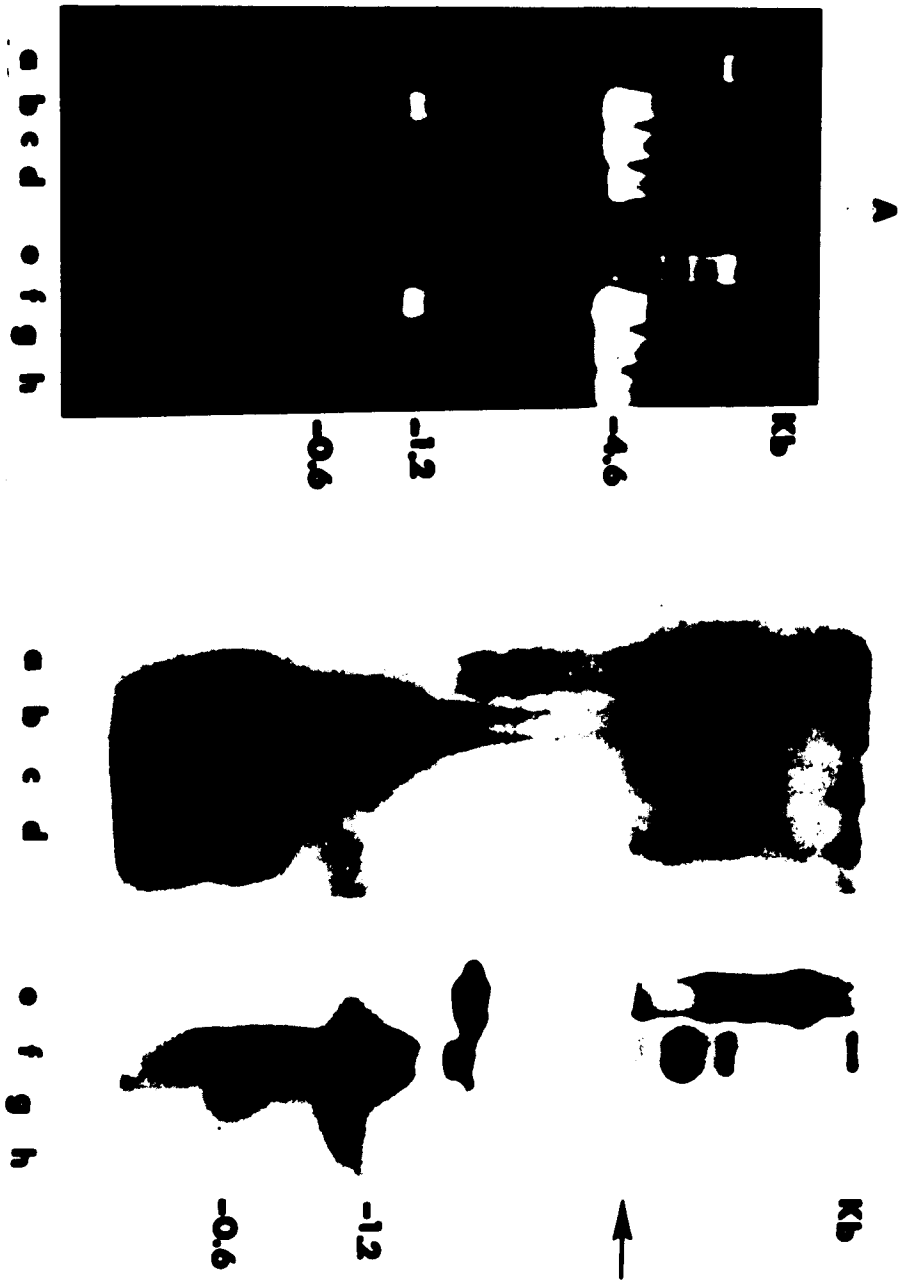

A
B
-0.6
-1.2
-4.6
Kb
-0.6
-1.2
Kb

Figure 10: Southern hybridization experiments confirming that the insert of plasmid pGR110 contains only 16S rDNA sequences. Samples of chromosomal DNA of B. subtilis strains 168T (b,d) and SB25 (c,e) were restricted with SmaI, run in a 0.75% agarose gel, blotted, and hybridized either to nick translated pGR110 (b,c) or pGR102 (d,e). Lane a - λ HindIII. The pGR110 probe hybridized to SmaI fragments (18Kb, 14Kb, 3.3Kb, 3.0Kb, 2.6Kb, and 2.1Kb) each corresponding to the 5' end of a rRNA gene set. The probe, however, does not hybridize to the 0.7 and 0.5Kb 16S, 23S SmaI fragments containing respectively the large and small abutment.

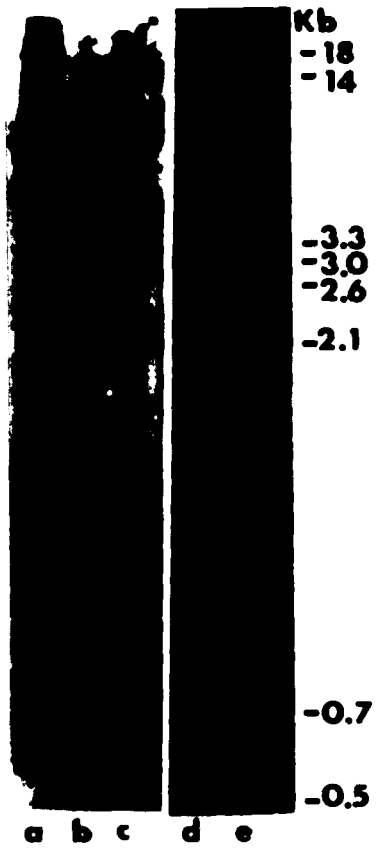

Kb
-18
-14
-3.3
-3.0
-2.6
-2.1
-0.7
-0.5
a b c d e

Figure 11: Campbell-like model of integration of plasmid pGR102 into a rRNA gene set of Bacillus subtilis containing the small abutment region. In the figure the cross-over between the plasmid and the rRNA gene set is mediated by 16S rDNA sequences. Only one integrated copy of the plasmid is shown, for simplicity, although the data indicate that two or three copies can be inserted.

A - BclI rDNA fragment containing the inserted plasmid. Only a BclI site is present in each rRNA gene set and no BclI sites are found in the plasmid.

B - Fragments containing vector sequences obtained by double restriction with BclI+SalI of the rRNA gene set having the inserted plasmid. No SalI sites are found in the gene sets, and only a SalI site is present in pGR102 (Tc^r gene).

C - EcoRI fragment containing vector sequences, produced from the gene set involved in plasmid insertion.

The expected sizes of the fragments are indicated in the figure. Integration of pGR102 into an rRNA gene set having the large abutment with the two tRNA genes would release a 7.8Kb fragment in a BclI-SalI digestion and a 5.8Kb EcoRI fragment. Restriction sites are as follows: AvaI (A), BamHI (B), HindIII (H), PstI (P), EcoRI (R), and SmaI (S).

The model is valid also in case of integration of plasmid pWR103. In this case, however, the size of the abutment is smaller.

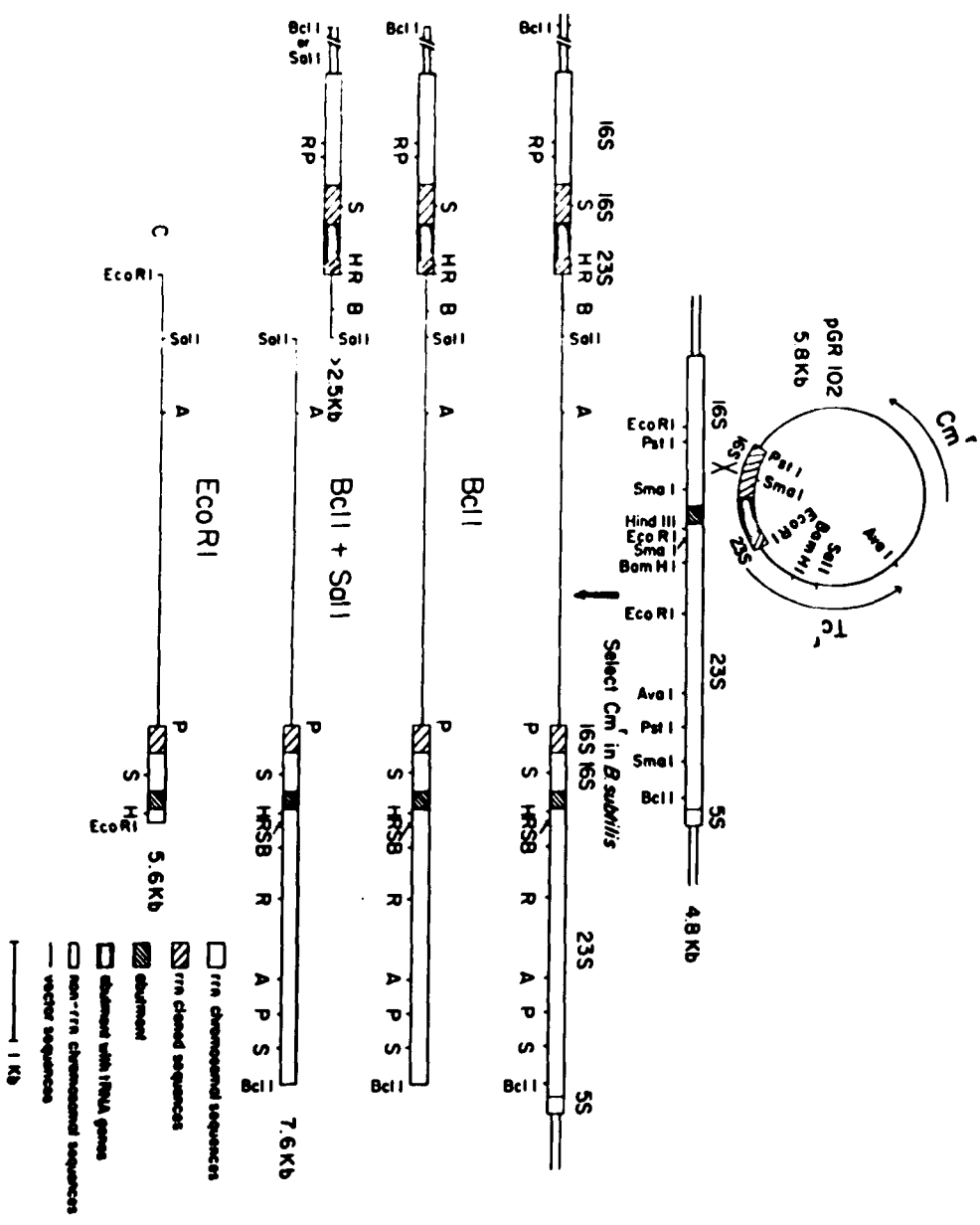

pGR 102
5.8Kb
Cm^r
Tc^r
16S
23S
5S
EcoRI
PstI
SmaI
Hind III
Eco RI
Sma I
Bam HI
Sal I
Ava I
Select Cm^r in B. subtilis
4.8 Kb
BclI
SalI
RP
S
HR
B
A
P
R
>2.5Kb
7.6Kb
BclI + SalI
5.6Kb
EcoRI
rrn chromosomal sequences
rrn cloned sequences
plasmid
plasmid with tRNA genes
non-rrn chromosomal sequences
vector sequences
1 Kb

Figure 12: Southern hybridization of chromosomal DNAs of parental and Cm^r transformant strains of B. subtilis. Samples of DNA were restricted with BclI, run in 0.75% agarose gels (30 volts, 16-18 hours), blotted and hybridized to nick translated rDNA probes (lanes b,c, and d - pGR102; lanes e and f - p12E2). Nick translated bacteriophage DNA was used to hybridize the HindIII fragments used as molecular weight markers. Only the first three bands of HindIII (21.9Kb, 9.3Kb and 6.2Kb) are visible in lane a.

lane a - λ HindIII
lane b - NCTC3610
lane c - BD170/pGR102-151
lane d - BD170/pGR102-143
lane e - 168
lane f - BD170/pGR102-153

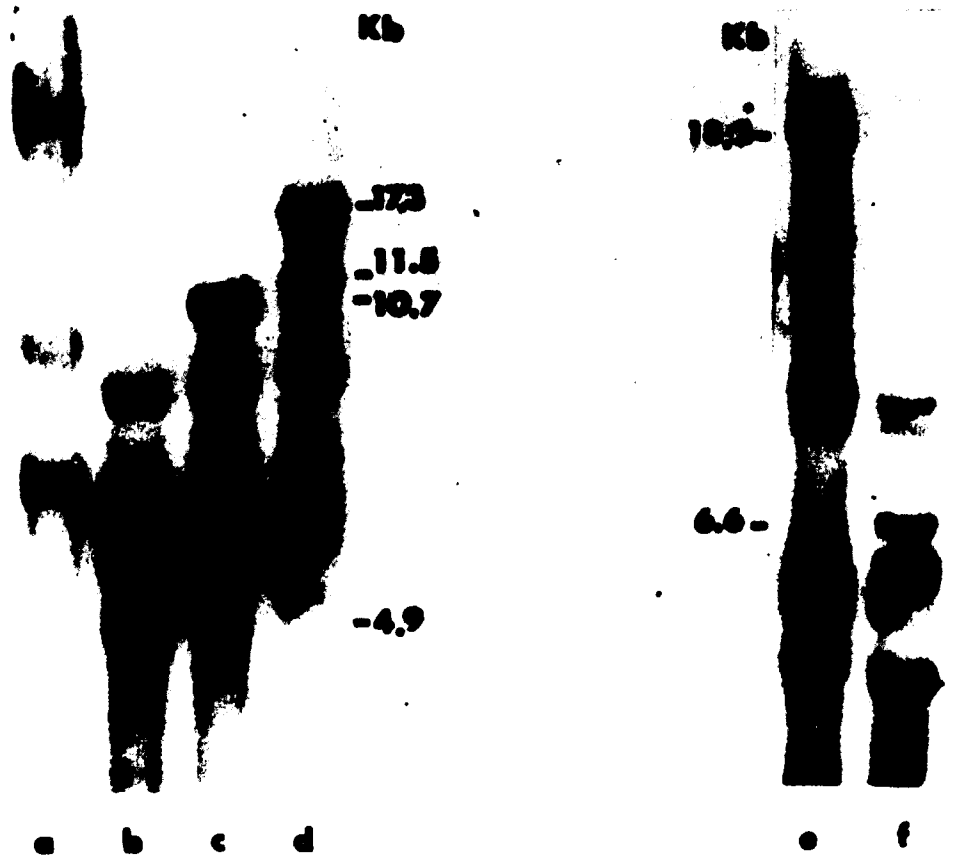

Kb
-11.8
-10.7
-4.9
Kb
10.5-
6.6-
a
b
c
d
e
f

Figure 13: Southern hybridization of chromosomal DNAs of parental and Cm^r strains of B. subtilis obtained by transformation with either pGR102 or pWR103. Samples of DNAs were restricted with BclI, run in agarose gels for four-five days at 5-10 volts, blotted and hybridized to nick translated rDNA probes. Numbers represent Kb.

Panel A - Autoradiograms of BclI digests electrophoresed and probed as follows: lanes a and b - 0.85% agarose, pWR103; lanes c, d, and e - 0.85% agarose, pGR102; lanes f and g - 0.75% agarose, pWR103.

lane a - BD170
lane b - BD170/pGR102-144
lane c - BD170/pGR102-174
lane d - BD170/pGR102-151
lane e - BD170/pWR103-179
lane f - BD170/pWR103-182
lane g - BD79

Panel B - Autoradiogram of restricted DNA samples electrophoresed on 0.75% agarose gel and probed with the purified PstI-EcoRI 16S, 23S insert of pGR102.

lane a - NCTC3610
lane b - BD170
lane c - BD170/pGR102-151
lane d - BD170/pGR102-143
lane e - BD170/pGR102-153
lane f - BD170/pWR103-181

Panel C - Autoradiogram of restricted DNA samples electrophoresed on a 0.75% agarose gel and hybridized to the PstI-EcoRI insert of pGR102. The gel was run longer than the gel shown in panel B, to increase the resolution of bands between 5.8Kb-5.4Kb. DNA fragments smaller than 5.4Kb were run off the gel.

lane a - NCTC3610
lane b - BD170
lane c - BD170/pGR102-151
lane d - BD170/pGR102-135
lane e - BD170/pGR102-143
lane f - BD170/pWR103-181


A
a b
c d e
f g

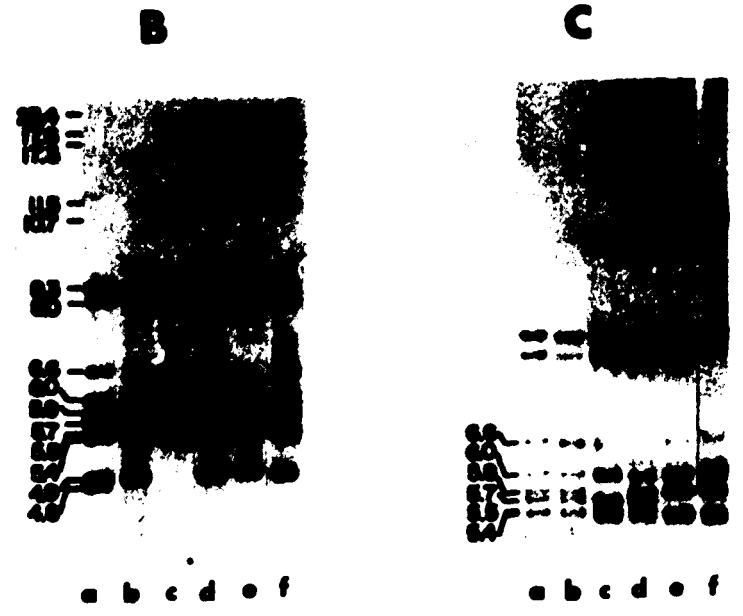

B
C
a b c d e f
a b c d e f

Figure 14: Southern hybridization of BclI restricted chromosomal DNAs of parental and Cm^r transformant strains with integration of either pGR102 or pWR103. The gels (0.75% agarose) were run for 4-5 days at 5-10 volts. The probe was the nick translated PstI-EcoRI insert of pGR102 (lanes a, b, and c) and pWR103 (lanes d, e, f, g, h, i, j, k, and l).

lane a - 166
lane b - 168T
lane c - 166/pGR102-273
lane d - 168T/pWR103-314
lane e - 168T/pWR103-316
lane f - 168T/pWR103-315
lane g - 168T/pGR102-313
lane h - 168T/pGR102-311
lane i - 168T/pGR102-272
lane j - 168T/pGR102-317
lane k - 168T/pGR102-281
lane l - NCTC 3610

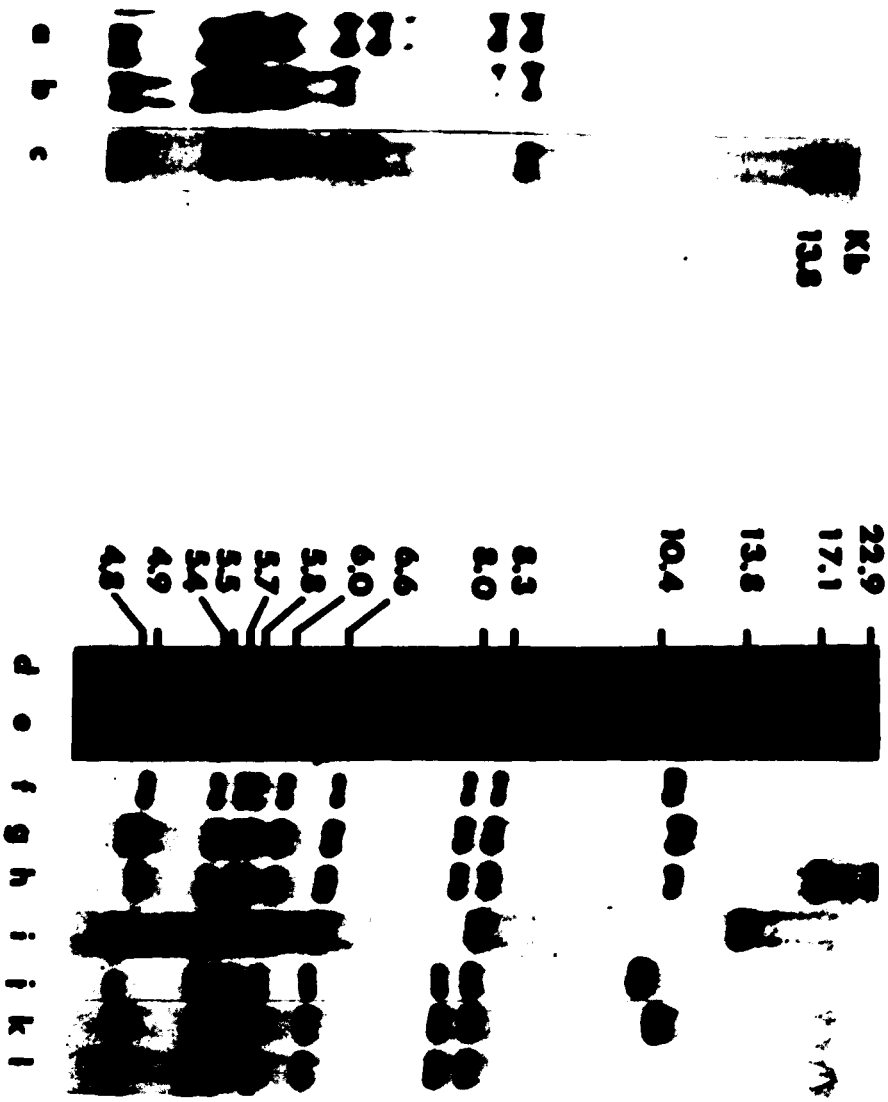
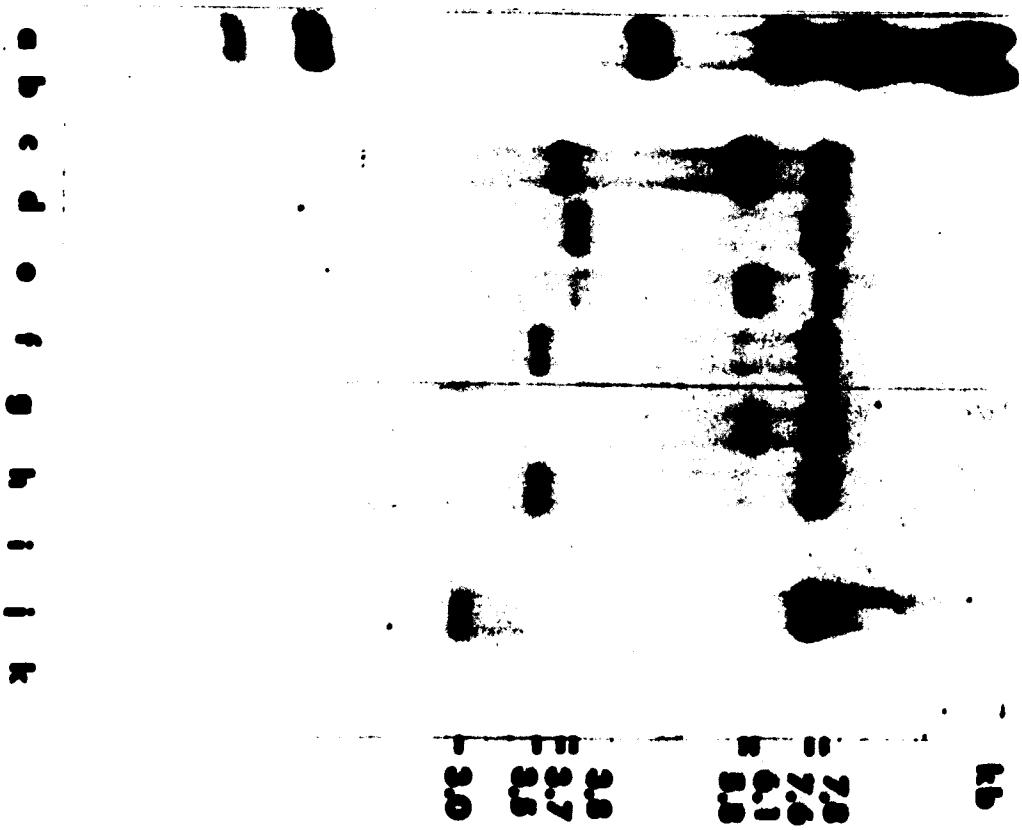


Figure 15: Southern hybridization of BclI-SalI double restricted chromosomal DNAs from parental and Cm^r transformants with integrated pGR102 or pWR103. Samples of restricted DNAs were run either in a 0.85% agarose gel (lanes a-k) or in a 0.75% gel (lanes l-n), for 14-16 hours at 30 volts, blotted, and hybridized to nick translated pJH101. As predicted (Fig. 11) a fragment with a uniform size of 7.6-7.8Kb, and a fragment with "variable size" (longer than 2.5Kb) were produced by each transformant DNA. A third fragment (5.8Kb long) was found in strains having a multimeric form of pGR102 inserted in a rRNA gene set (lanes c, e, m, and n). The lanes containing parental type DNA (b, i and k) yielded no hybrid bands.

lane a - λ HindIII
lane b - BD170
lane c - BD170/pGR102-143
lane d - BD170/pGR102-144
lane e - BD170/pGR102-153
lane f - BD170/pGR102-174
lane g - BD170/pWR103-179
lane h - BD79/pWR103-182
lane i - BD79
lane j - GSY1269/pGR102-177
lane k - GSY1269
lane l - BD170/pGR102-151
lane m - BD170/pGR102-143
lane n - BD170/pGR102-135



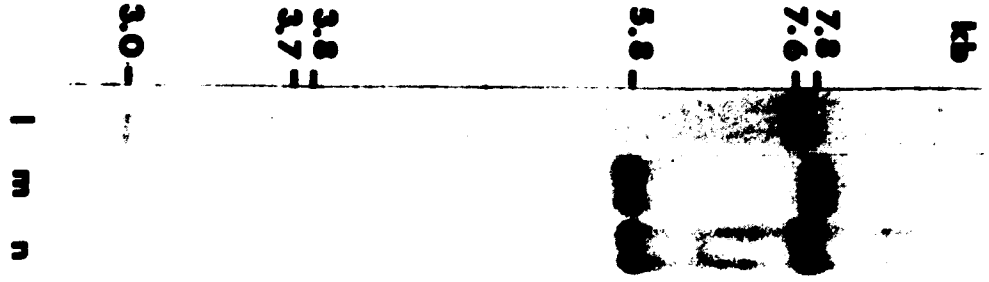


Figure 16: Southern hybridization of BclI-SalI double restricted DNAs from Cm^r transformant strains of B. subtilis having the insertion of either pGR102 or pWR103. Samples were run and hybridized as described in Fig. 15.

lane a - 168T/pGR102-272
lane b - 168T/pGR102-281
lane c - 166/pGR102-273
lane d - 168T/pWR103-316
lane e - 168T/pWR103-315
lane f - 168T/pWR103-314
lane g - 168T/pGR102-313
lane h - 168T/pGR102-311
lane i - 168T/pGR102-317

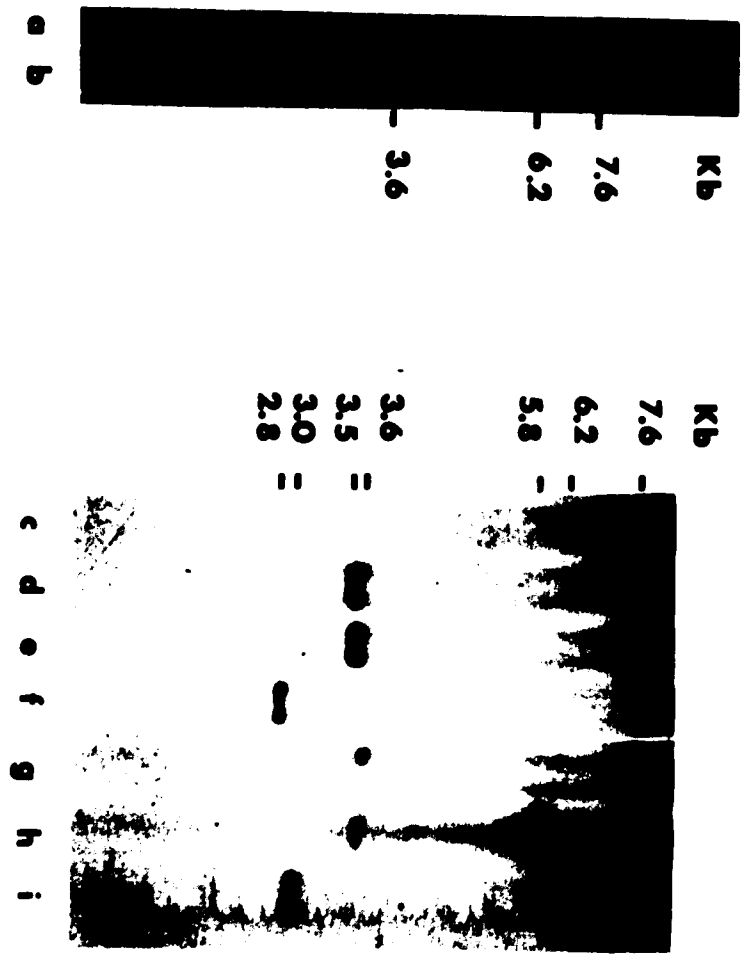

a b
Kb
7.6
6.2
3.6
c d e f g h i
Kb
7.6
6.2
5.8
3.6
3.5
3.0
2.8

Figure 17: Southern hybridization of EcoRI restricted DNAs from parental and Cm^r transformant strains, showing that plasmids pGR102 and pWR103 can integrate into rRNA gene sets having either the large or small abutment. The restricted DNA samples were run in a 0.75% agarose gel for 14-16 hours at 30 volts, blotted and hybridized to nick translated pJH101. As predicted from the model (Fig. 11c) each transformant DNA produced either a 5.6Kb or 5.8Kb band. Strains having a multimeric form of pGR102 integrated in a gene set with small abutment produced both the 5.6Kb and 5.8Kb fragments.

lane a - BD170
lane b - BD170/pGR102-143
lane c - BD170/pGR102-144
lane d - BD170/pGR102-135
lane e - BD170/pGR102-153
lane f - BD79
lane g - BD170/pWR103-179
lane h - BD79/pWR103-182
lane i - GSY1269
lane j - GSY1269/ pGR102-177

Kb
5.8
5.6

Figure 18: Model of Campbell-like integration of plasmid pGR108 into a rRNA gene set.

a - BclI rDNA fragment containing the inserted plasmid

b - BclI-SalI rDNA fragments containing vector sequences. The model predicts the production of a 2.6Kb fragment in all transformants obtained with pGR108 and of a fragment larger than 7.8Kb whose actual size depends on the site of integration.

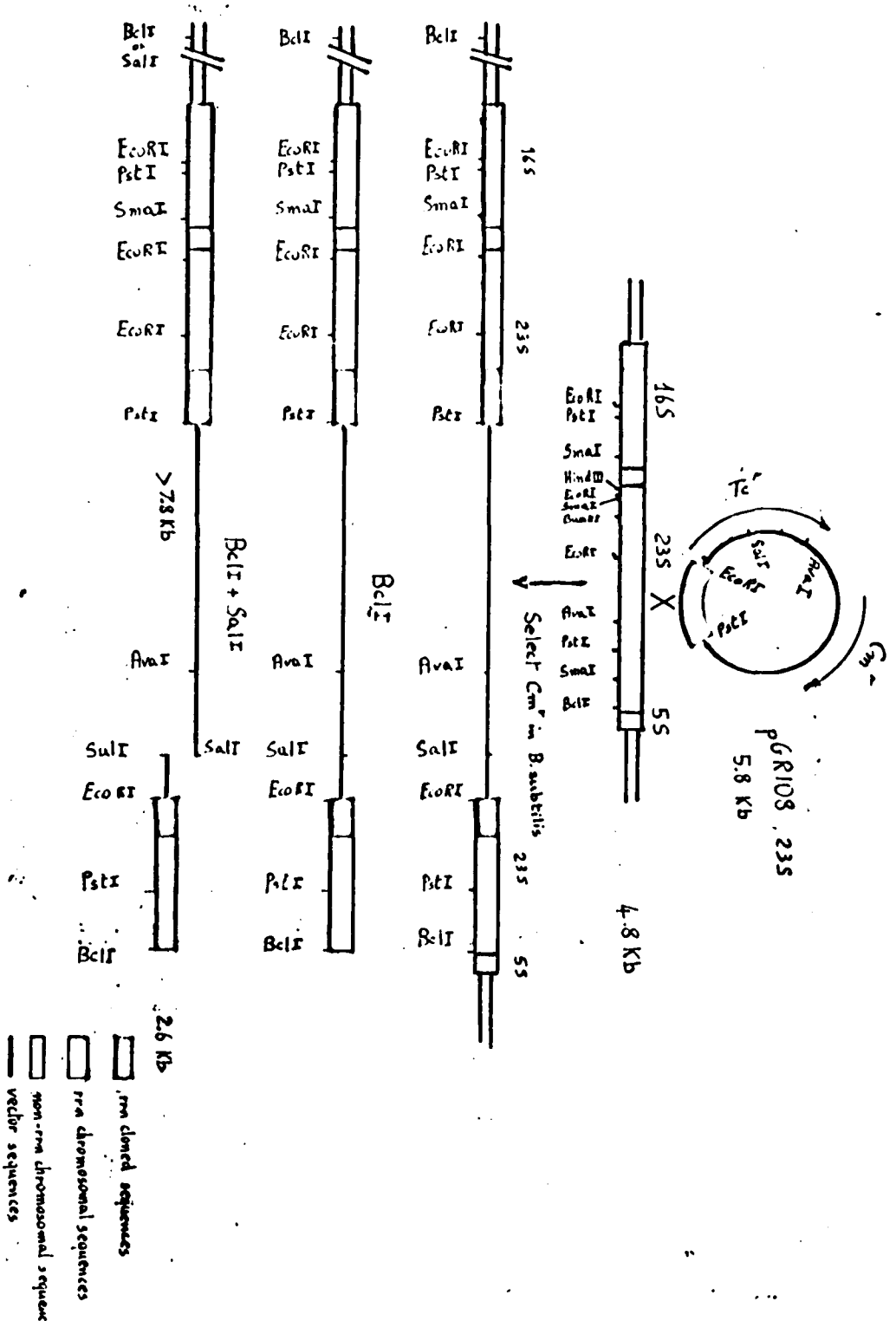

BclI or SalI
EcoRI
PstI
SmaI
EcoRI
EcoRI
PstI
>7.3 Kb
BclI + SalI
AvaI
SalI
SalI
EcoRI
PstI
BclI
2.6 Kb
BclI
EcoRI
PstI
SmaI
EcoRI
EcoRI
PstI
BclI
AvaI
SalI
EcoRI
PstI
BclI
BclI
EcoRI
PstI
SmaI
EcoRI
EcoRI
PstI
BclI
AvaI
SalI
EcoRI
PstI
BclI
165
235
55
Select Cm^r in B. subtilis
4.8 Kb
EcoRI
PstI
SmaI
HindIII
EcoRI
SmaI
SmaI
EcoRI
AvaI
PstI
SmaI
BclI
165
235
55
X
Tc^r
Cm^r
SalI
EcoRI
AvaI
PstI
pGR108.235
5.8 Kb
rrn cloned sequences
rrn chromosomal sequences
non-rrn chromosomal sequences
vector sequences

Figure 19: Model of Campbell-like integration of plasmid pGR110 into a rRNA gene set.

a - BclI rDNA fragment containing the inserted plasmid

b - BclI-SalI fragments containing vector sequences. All transformants obtained with pGR110 are expected to produce a 7.6Kb band and a band larger than 2.0Kb, whose actual size depends on the site of integration.

Restriction sites are as follows: BclI(B), PstI(P), SmaI(S). S* indicates that the SmaI site is not present.

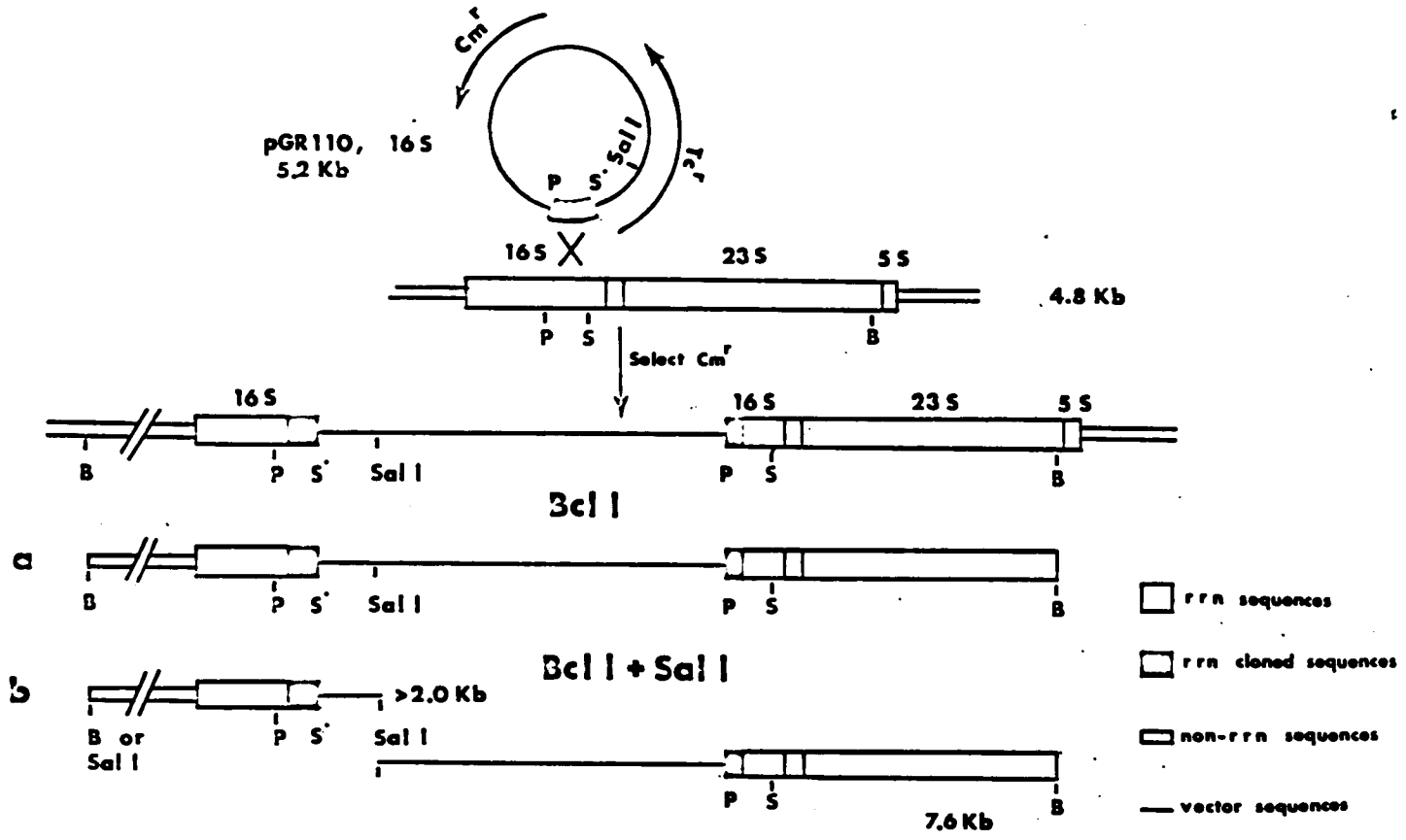

pGR110, 5.2 Kb
16S
Cm^r
Tc^r
Sal I
P S
16S
23S
5S
4.8 Kb
B
Select Cm^r
Sal I
Bcl I
a
b
>2.0 Kb
B or Sal I
Bcl I + Sal I
7.6 Kb
rrn sequences
rrn cloned sequences
non-rrn sequences
vector sequences

Figure 20: Model of Campbell-like integration of plasmid pGR111 into a rRNA gene set.

a - BclI rDNA fragment containing the inserted plasmid.

b - BclI-SalI rDNA fragments containing vector sequences. The 6.6Kb fragment is expected to be present in all pGR111 Cm^r transformants; the other fragment should have a variable size, depending on the site of integration. The minimal size of the latter is 4.8Kb.

Restriction sites are as follows: BclI (B), PstI (P), SmaI (S), EcoRI (R). S* indicates that the SmaI site is no longer present.

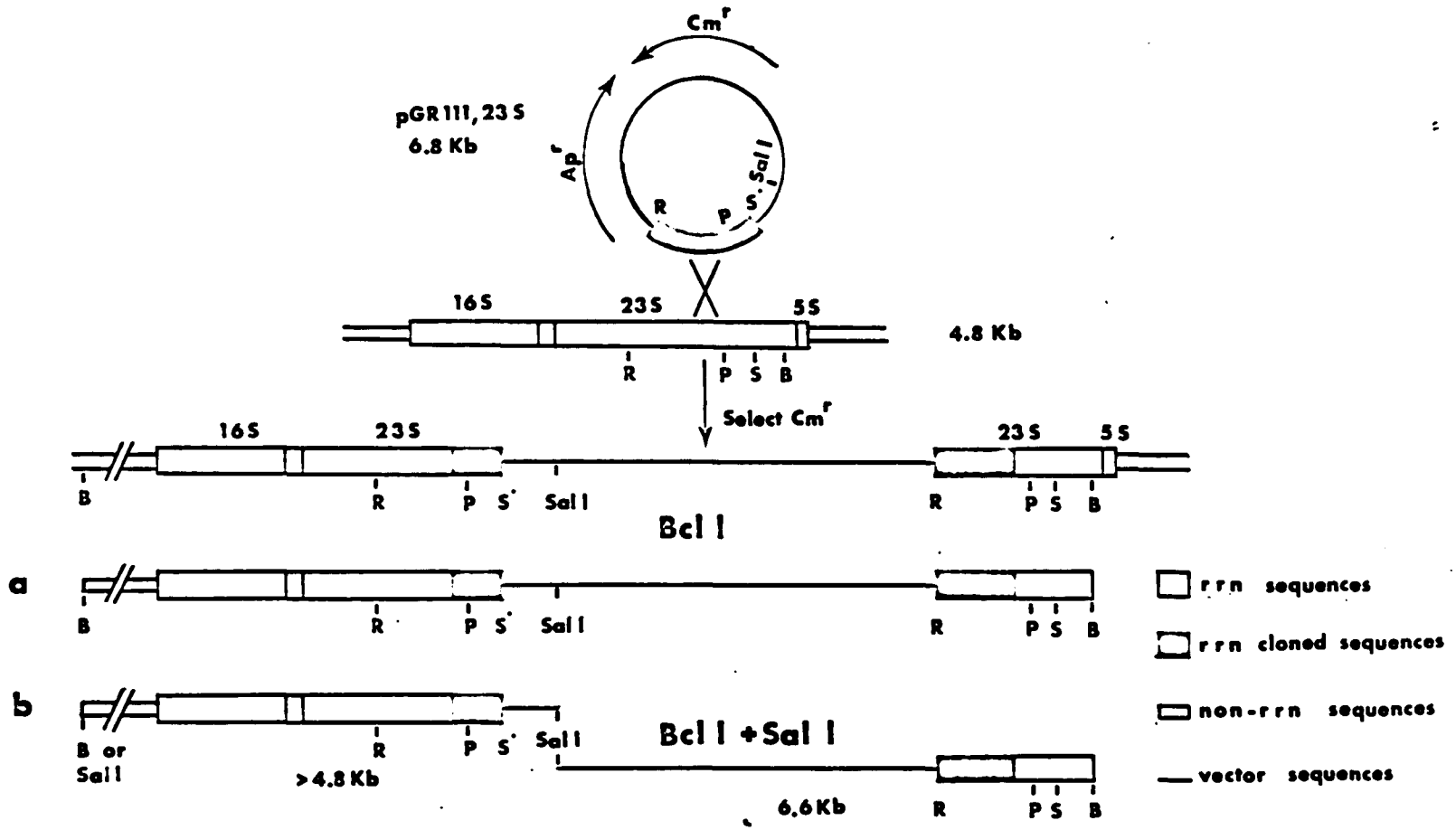

Cm^r
pGR111, 23 S
6.8 Kb
Ap^r
R
P
S
Sal I
16S
23S
5S
4.8 Kb
R
P
S
B
Select Cm^r
16S
23S
23S
5S
B
R
P
S
Sal I
R
P
S
B
Bcl I
a
B
R
P
S
Sal I
R
P
S
B
b
B or
Sal I
R
P
S
Sal I
> 4.8 Kb
Bcl I + Sal I
6.6 Kb
R
P
S
B
r r n sequences
r r n cloned sequences
non-r r n sequences
vector sequences

Figure 21: Southern hybridization of BclI restricted chromosomal DNAs of parental and Cm^r transformant strains of B. subtilis with integration of either pGR108, pGR110, or pGR111. Samples were run in 0.75% agarose gels for 4-5 days at 5-10 volts, blotted, and hybridized either to nick translated pGR102 (lanes j and k), pWR103 (lanes f-i), or the PstI-SmaI rDNA fragment of pGR102 (lanes a-e).

lane a - BD170
lane b - SB25/pGR110-304
lane c - BD170/pGR110-291
lane d - BD170/pGR111-301
lane e - BD170/pGR111-302
lane f - SB25/pGR108-257
lane g - SB25
lane h - SB25/pGR110-306
lane i - SB25/pGR110-305
lane j - SB25
lane k - SB25/pGR108-236

a b c d e
kb
12.5
11.3
10.7
10.6
10.0
8.3
8.0
6.6
6.0
5.8
5.7
5.5
5.4
4.9
4.8
f g h i
kb
10.7
i k

Figure 22: Southern hybridization of samples of BclI-SalI restricted DNAs from Cm^r transformant strains of B. subtilis with integrated pGR108, pGR110, and pGR111. Samples were run in 0.75% agarose gels for 14-16 hours at 30 volts, blotted, and hybridized to nick translated pJH101.

lane a - BD170/pGR111-302
lane b - BD170/pGR111-301
lane c - SB25/pGR110-306
lane d - SB25/pGR110-305
lane e - SB25/pGR110-304
lane f - BD170/pGR110-291
lane g - HindIII
lane h - SB25/pGR108-257
lane i - SB25/pGR108-236
lane j - λ HindIII

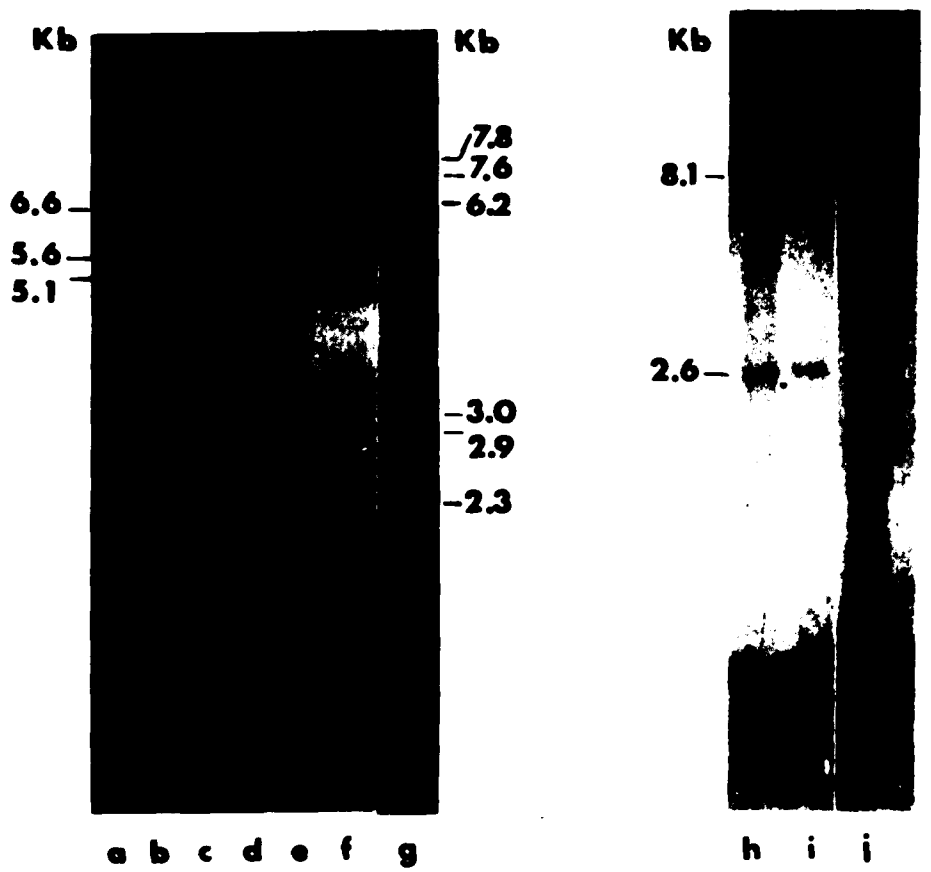
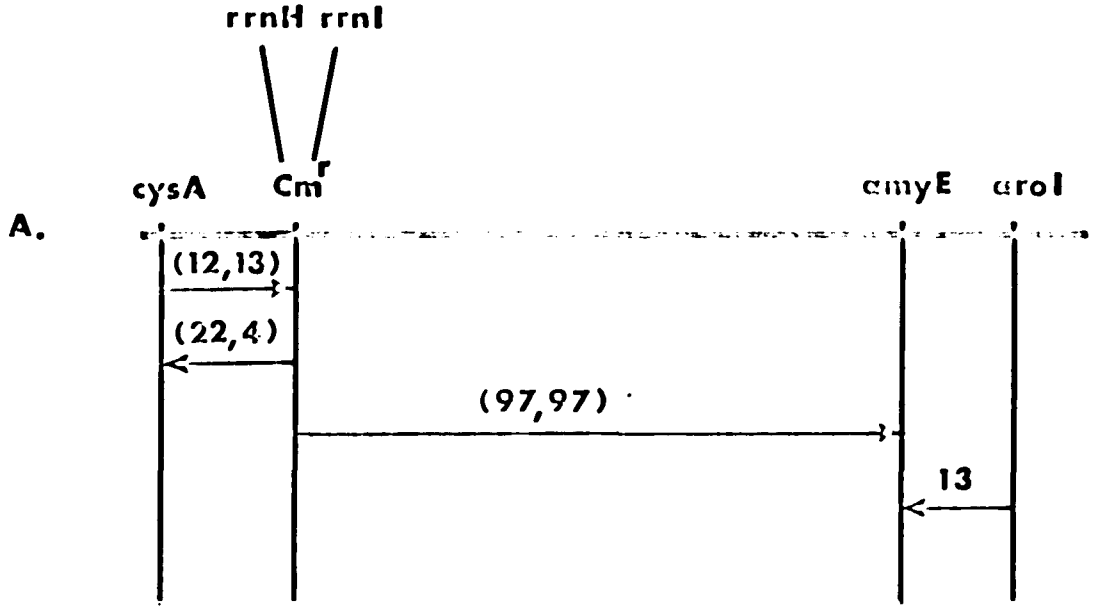

Kb
Kb
7.8
7.6
6.6
6.2
5.6
5.1
3.0
2.9
2.3
Kb
8.1
2.6
a b c d e f g
h i j

Figure 23: Genetic map of the cysA to aroI region containing four rRNA gene sets. Distance is expressed as the percentage of recombination in PBS1 transduction. The arrows point from selected to unselected markers. The linkage values in parenthesis for the paired rrnH and rrnI (A) and rrnG and rrnK (B) are given individually according to the indicated position. The linkage values are average calculated from the crosses presented in this report and other crosses (Jarvis et al., submitted).


rrnH rrnI
cysA
Cm^r
amyE
aroI
A.
(12,13)
(22,4)
(97,97)
13

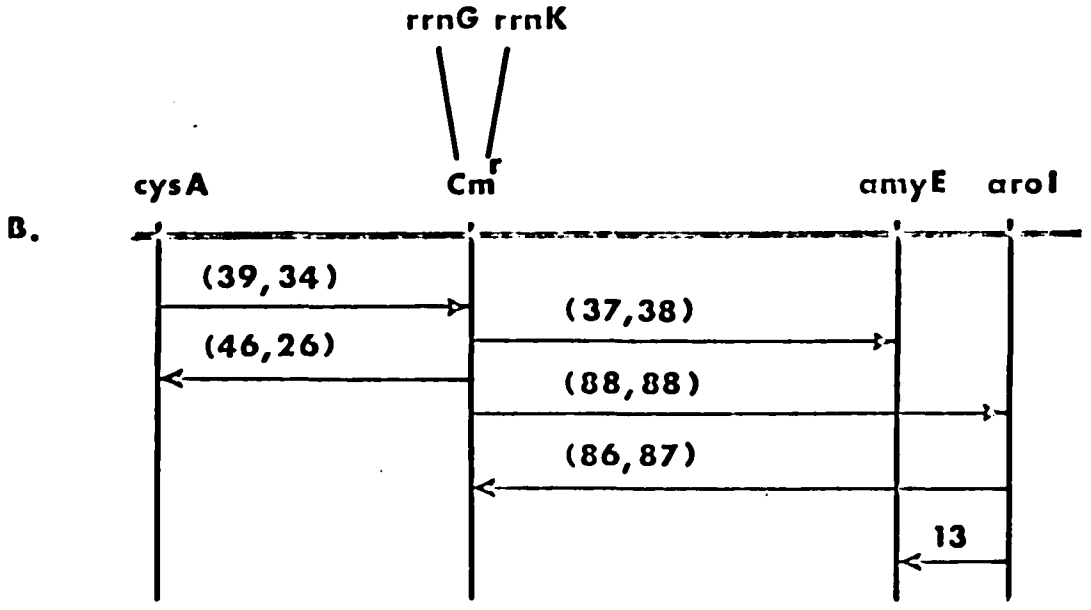

rrnG rrnK
cysA
Cm^r
amyE
aroI
B.
(39,34)
(37,38)
(46,26)
(88,88)
(86,87)
13

Figure 24: Genetic map of the aroI to purB region containing rrnE. The following donors were used: BD170 (trpC2, thr-5) (a); BD170/pGR102-135 (Cm^r, trpC2, thr-5) (b); 1A150/pGR102-135 (Cm^r, pha-1, catA, hisA2) (c); 1A154/pGR102-135 (Cm^r, met, trpC2, fur3, gutB) (d). Distance is expressed as the percentage of recombination in PBS1 transduction. The arrows point from selected to unselected markers.

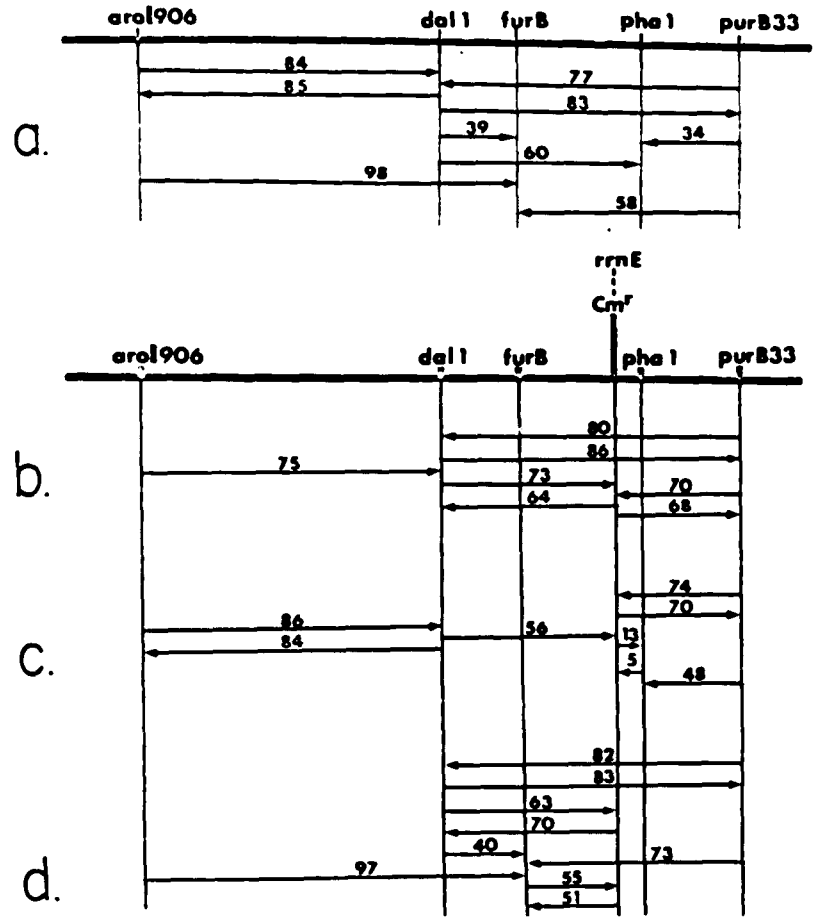

a.
aroI906
dal 1
furB
pha 1
purB33
84
85
77
83
39
34
60
98
58
b.
c.
d.
rrnE
Cm^r
aroI906
dal 1
furB
pha 1
purB33
80
86
75
73
70
64
68
74
70
86
84
56
13
5
48
82
83
63
70
40
73
97
55
51

Figure 25: Genetic map of the tre to metD region containing rrnD. Distance is expressed as the percentage of recombination in PBS1 transduction. The arrows point from selected to unselected markers.

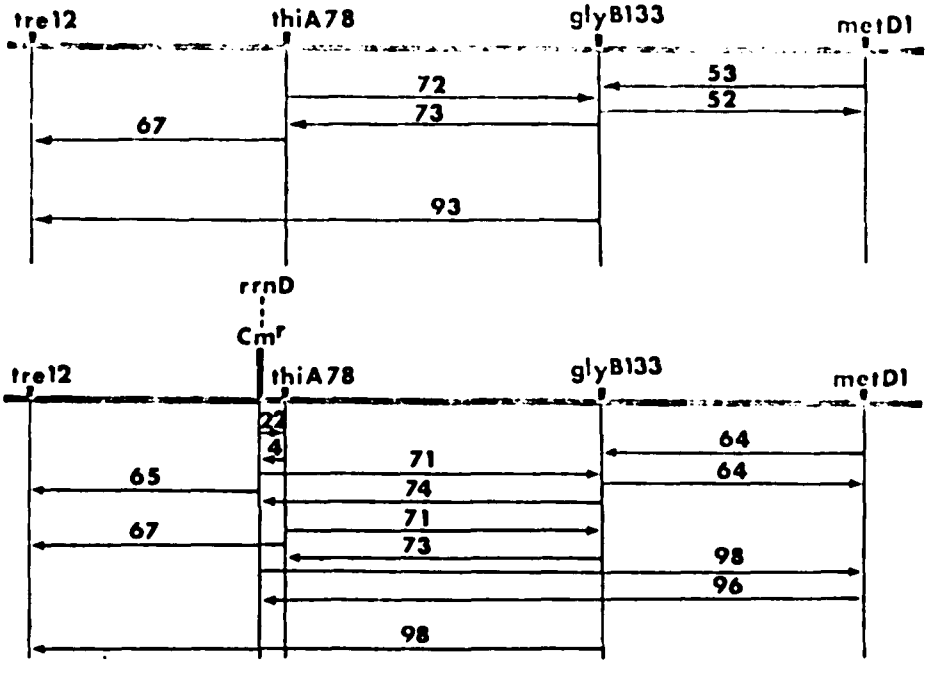

tre12
thiA78
glyB133
metD1
72
53
52
73
67
93
rrnD
Cm^r
tre12
thiA78
glyB133
metD1
22
4
64
71
64
65
74
71
67
73
98
96
98

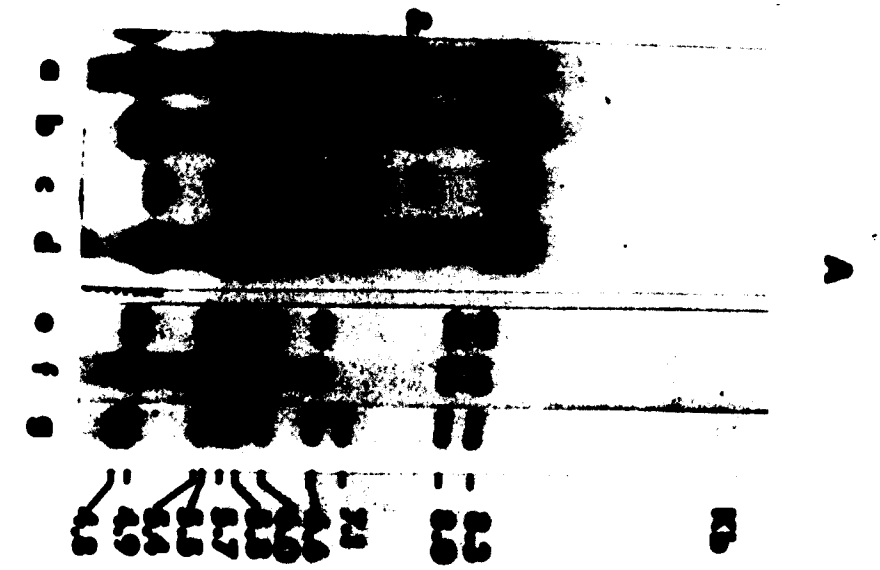
Figure 26: Southern hybridization of chromosomal DNAs of a collection of strains of B. subtilis.

Panel A - Samples of chromosomal DNAs were restricted with BclI, run in 0.75% agarose gels for 4-5 days at 5-10 volts, blotted, and hybridized to nick translated pGR102 (lanes a-d), or pWR103 (lanes e-g). Each band corresponds to the 5' part of a rRNA gene set (Fig.1) and band visible only with probes containing tRNA sequences.

lane a - GSY1269
lane b - SB25
lane c - BD170
lane d - BD79
lane e - 168T
lane f - CU420
lane g - 166

Panel B - Samples of EcoRI restricted chromosomal DNAs were run in a 0.75% agarose gel for 3 days at 15 volts, blotted and hybridized to the nick translated EcoRI-PstI 23S rDNA insert of pGR108. Each band corresponds to the 3' side of a rRNA gene set (Fig.1).

lane a - SB25
lane b - BD170
lane c - 168T
lane d - BD79
lane e - CU420
lane f - E88
lane g - 166
lane h - GSY1269



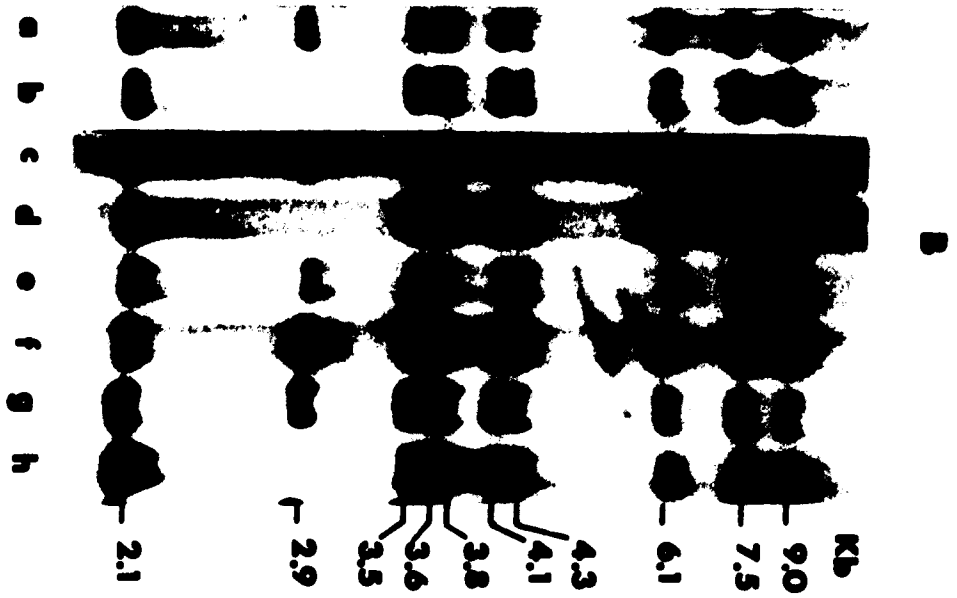

2.1
2.9
3.5
3.6
3.8
4.1
4.3
6.1
7.5
9.0
Kb

Figure 27: Southern hybridization of EcoRI restricted chromosomal DNA of parental and Cm^r strains of *B. subtilis* to 23S-5S rDNA probes. The restricted samples were run in 0.75% agarose gels for 3 days at 15 volts, blotted, and hybridized to nick translated probes as follows: lanes a-f, EcoRI-HindIII insert of pYR104; lanes g-o, pYR104. Each band represents the 3' side of a rRNA gene set (Fig. 1). Arrows indicate missing bands; & indicates plasmid sequences.

lane a - BD170/pGR102-151
lane b - GSY1269/pGR102-177
lane c - BD170
lane d - GSY1269
lane e - SB25
lane f - SB25/pGR103-257
lane g - 168T
lane h - 168T/pWR103-316
lane i - 168T/pWR103-315
lane j - 168T/pWR103-314
lane k - 168T/pGR102-313
lane l - 168T/pWR103-315
lane m - 168T/pGR102-311
lane n - SB25/pGR110-306
lane o - SB25/pGR110-305

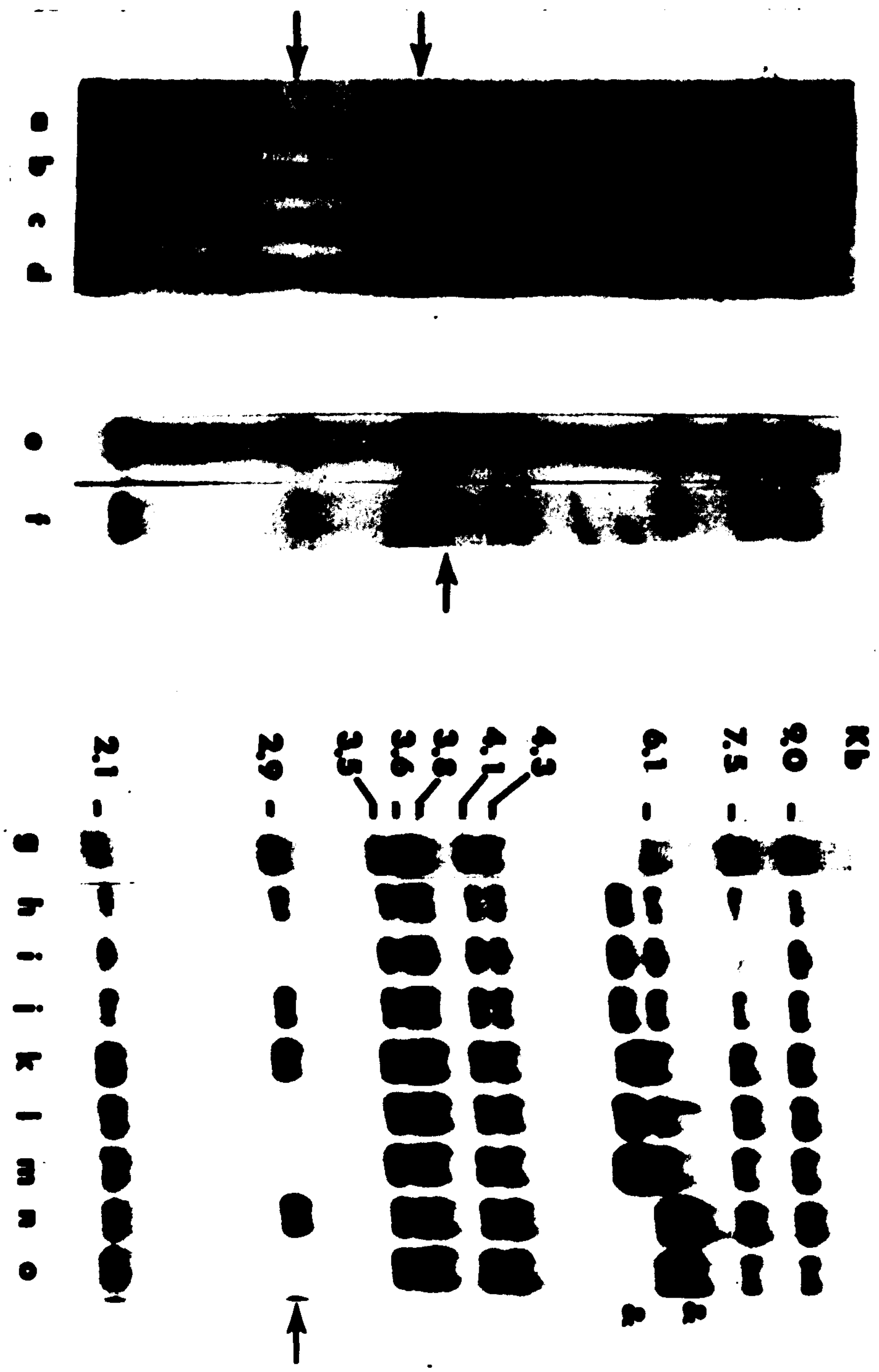

a
b
c
d
e
f
Kb
9.0
7.5
6.1
4.3
4.1
3.8
3.6
3.5
2.9
2.1
g
h
i
j
k
l
m
n
o

Figure 28: Model of integration of pGR102 into the rrnH-rrnI cluster with concomitant deletion of a rRNA gene set equivalent. A multimeric form of pGR102 (either circular or linear) interacts with the two closely situated rRNA gene sets rrnH and rrnI. Two crossovers occur; one between rrnI and the 1.2Kb DNA insert of the plasmid, and the other between rrnH and a repetition of the insert in the multimeric plasmid. The 3' portion of rrnH, the spacer, and the 5' portion of rrnI are deleted and replaced by a monomeric form of the plasmid. A multimeric insertion would result if the two interacting inserts are not immediately adjacent. Restriction sites are as follows: PstI (P); EcoRI (R); BamHI (B); AvaI (A). Symbols are as in Fig. 11.

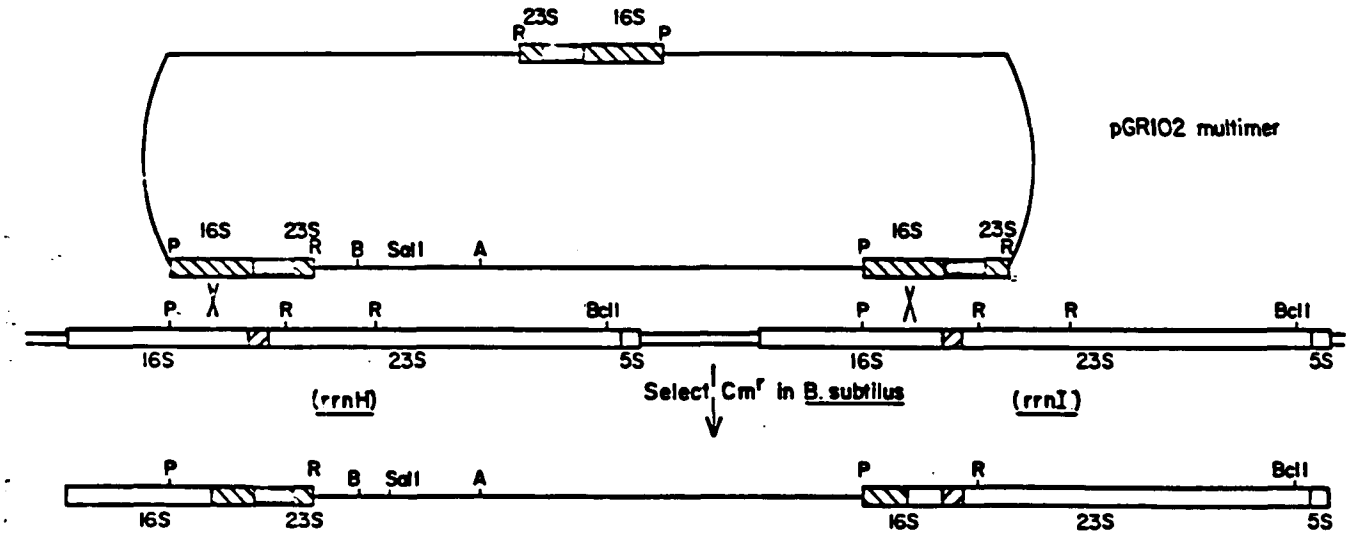
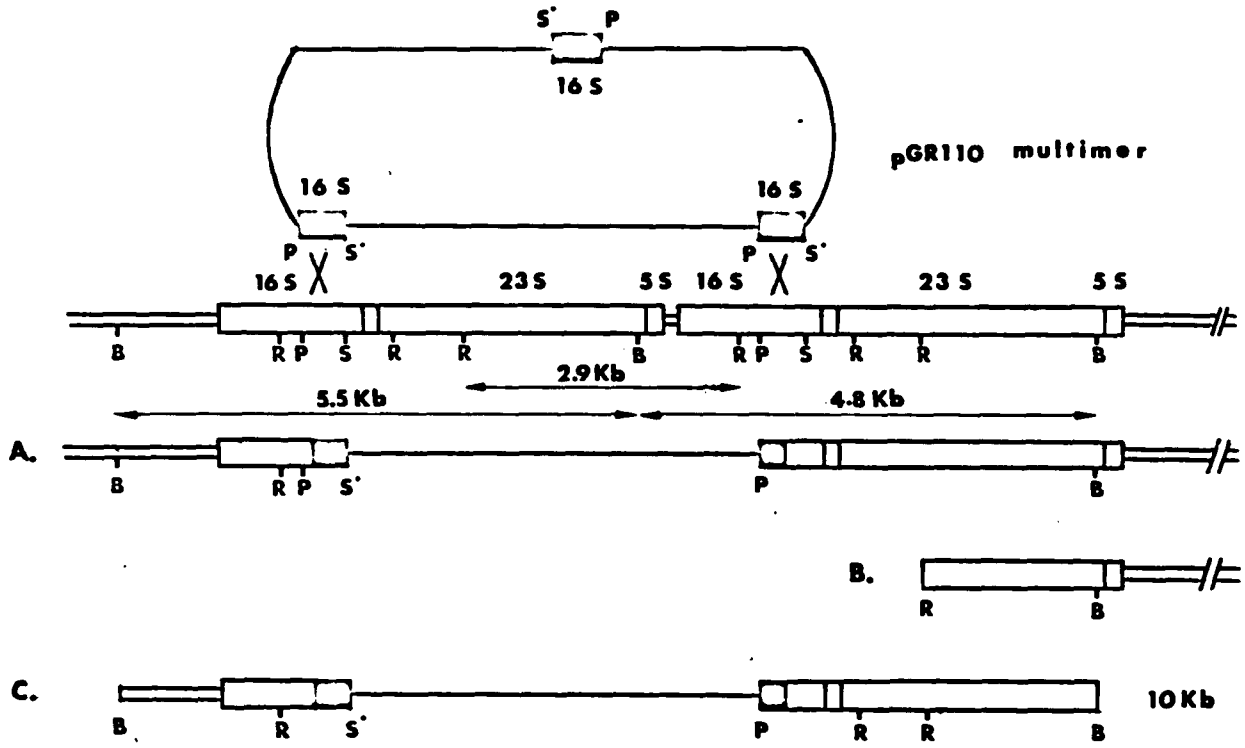

23S
16S
R
P
pGR102 multimer
16S
23S
P
R
B
SalI
A
BclI
5S
(rrnH)
(rrnI)
Select Cm^r in B. subtilus

Figure 29: Model of integration of pGR110 into the rrnG-rrnK cluster with concomitant deletion of a rRNA gene set equivalent. A multimeric form of the plasmid interacts with the two closely situated rrnG and rrnK. One crossover occurs between a rDNA insert and rrnG (5.5Kb BclI homolog); the other between a repetition of the insert in the multimer and rrnK (4.8Kb BclI homolog). Restriction sites are as follows: BclI (B); EcoRI (R); PstI (P); SmaI (S). S*, SmaI site disrupted during cloning procedures. Symbols are as in Fig. 19.

A - In the hypothetical Cm^r transformant a fragment of chromosome, containing the 3' end of rrnG, the spacer, and the 5' end of rrnK, is replaced by a plasmid insertion.

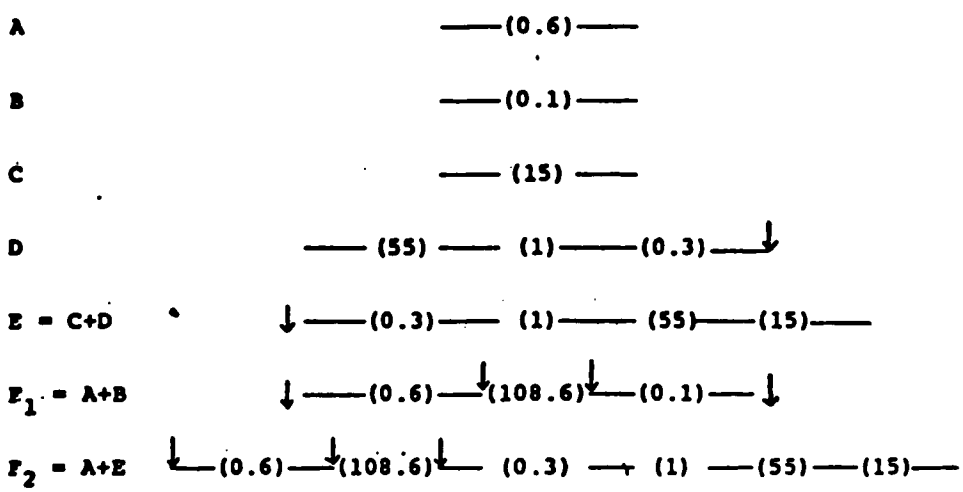
B - EcoRI restriction of "A" would produce only one fragment containing 23S and 5S rDNA sequences. This fragment corresponds to the 3' end of rrnK. The 3' end of rrnG is deleted.

C - BclI restriction of "A" would produce a hybrid rDNA homolog containing the 5' end of rrnG, plasmid sequences, and the 3' end of rrnK up to the internal BclI site in the 23S cistron. The 5' end of rrnK is deleted.


S· P
16 S
pGR110 multimer
16 S
16 S
P S·
P S·
16 S
23 S
5 S
16 S
23 S
5 S
B
R P S R R B R P S R R B
2.9Kb
5.5Kb
4.8Kb
A.
B
R P S·
P
B
B.
R
B
C.
B
R
S·
P
R
R
B
10Kb

APPENDIX

Simple (A-D) and larger (E_1, F_1 and F_2) rDNA linkage groups proposed by Chow and Davidson, 1973. Lines represent rRNA gene sets, numbers in parenthesis are in Kb and represent the spacer between rRNA gene sets. Arrows indicate the possible positions for the attachment site of phage SP02. Modified from Chow and Davidson, 1973, and Bott et al., 1984.



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