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City University of New York PH.D. 1984

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CONSERVED GENETIC REGIONS

WITHIN THE *BACILLUS* CHROMOSOME

by

Paul Gottlieb

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

1983

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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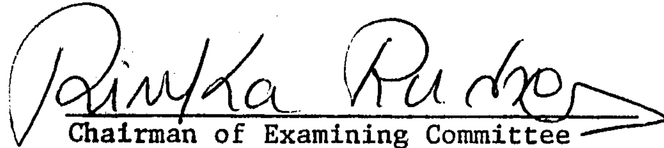

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ABSTRACT

CONSERVED GENETIC REGIONS WITHIN THE *BACILLUS* CHROMOSOME

This project analyzed conserved genomic sequences in eight members of the genus *Bacillus*. As in earlier studies (Dubnau *et al*., 1965a; Chilton and McCarthy, 1969), conserved genetic regions resistant to evolutionary change are noted, consisting of rRNA gene sets and nearby antibiotic resistance markers. Two families of these genes exist in the *Bacillus* species and strains analyzed. They differ in the presence or absence of an EcoRI sensitive site in the 5' end of the 23S gene sequence. This site is present in *B. subtilis* and *B. licheniformis* and absent in *B. globigii*, *B. pumilus*, and *B. amyloliquefaciens*. Each species has only one type 23S sequence in all its multiple rRNA cistrons, indicating a gene divergence occurred in addition to an amplification event. Otherwise, physical organization of the repeating rRNA gene sets in the genus *Bacillus* is conserved. Two abutment regions were found between all 16S and 23S determinants differing by 0.2 Kbp, the larger presumably containing tRNA sequences.

More extensive sequence divergences occur beyond the boundaries of the ribosomal RNA cistrons. Differences between strains of the same species were examined in *B. subtilis* and *B. licheniformis*.

We observed the conservation of the leucine gene first reported by Dubnau et al. (1965a) and Chilton and McCarthy (1969). Species capable of interspecific transformation of B. subtilis leu$^-$ recipients displayed greater Southern blot hybridization intensity compared to nontransforming species, when examined with a cloned leucine sequence. In contrast threonine and tryptophan markers were incapable of any interspecific transformation. Hybridization intensity of a cloned threonine sequence was less than that of leucine. A cloned B. pumilus tryptophan sequence hybridized with heterologous DNA only from B. subtilis and B. licheniformis.

Strains of B. subtilis were observed that have deletions of rRNA cistrons. Alterations were associated with a variety of mutations within the arg to leu region of the chromosome, and have led us to postulate the presence of a second rRNA gene cluster. The conservation of leu could be due to its association with conserved ribosomal genes.

Transformation of B. subtilis leu$^-$ recipients for leucine prototrophy resulted in merodiploid strains. A transformant for arginine prototrophy gained an additional rRNA gene that integrated into the chromosome between the arg and phe markers. This study suggests the arg, ilv, leu, phe region of the B. subtilis chromosome is closely associated with rRNA genes, accounting for the above effects.

This thesis is dedicated to the memory of my mother
Ruth Gottlieb (1920-1976).

The accomplishment of any difficult task requires not only perseverence but the guidance and support of many friends. I thank all the people who assisted me in this thesis project, most of all my sponsor Professor Rivka Rudner. Her dedication to the training of young scientists is exemplary and this quality has touched not only her own students but all those in the Department of Biological Sciences.

I especially thank Dr. Marvin Friedman, with whom I had many helpful discussions. In addition, it was with Dr. Shirley Raps and Dr. Marcia Brody that my own teaching abilities were developed, and the years in which we worked together in the Microbiology course were invaluable.

My father, Harvey Gottlieb, who supported my desire to become a scientist, deserves special mention. I wish to acknowledge my good friend Rhea Lesly who provided me with companionship in between the lonely hours spent writing this thesis. Not to be forgotten is Mary Ginsburg, who typed the manuscript.

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INTRODUCTION

The Genus Bacillus

Methodology in bacterial taxonomy has changed from visible description to molecular analysis. The genus *Bacillus* provides an excellent example of these trends and illustrates how classification resolution has increased by application of improved technology. Genus *Bacillus* is composed of aerobic spore-forming soil saprophytes. These gram-positive organisms, in addition to being intensively studied biochemically and genetically, perform valuable industrial tasks such as enzyme production. Varieties of some species are pathogenic to insects and are the source of industrial insecticides (Debabov, 1982).

Distinct classification of these organisms is therefore a necessary task that began in 1872, when W. Cohn first noted and designated a rod-shaped, sporulating soil bacterium as *Bacillus subtilis*. Cohn's original strain ultimately revealed itself to be composed of different bacteria, and classification and taxonomic relationships among genus members became a continuing quest. Classic work by Smith, Gordon and Clark, published in 1952, organized many of the species. *Bacillus* strains obtained by these workers numbered 1,114 and were divided into 158 species names. These were ultimately divided into 19 species which became known as the NRS collection (Smith,

et al., 1952).

Published in 1973, agricultural handbook #427, *The Genus Bacillus*, is an updating of the 1952 classifications. As in the previous efforts, the genus *Bacillus* is defined as encompassing rod-shaped bacteria capable of aerobically forming refractile endospores significantly more resistant than vegetative cells to heat, desiccation and "other destructive agencies." Authors Gordon, Haynes and Hor-Nay Pang confess inability to adhere entirely to this working definition as some of their analyzed Bacilli were non-sporulating and yet retained significant similarities to sporulating strains.

These workers arranged the strains into three groups of species based upon spore shape, swelling of the sporangium by a spore, size and shape of the cells, and "foamy or vacuolate" appearance of the protoplasm after application of a basic stain. Different Bacilli were subsequently assigned appropriate species positions on the basis of standard metabolic tests.

The quest for objective taxonomic criteria led to the exploitation of the spore as a source of distinct antigenic determinants (Norris, 1962). Spore antigens are heat-resistant and antibody response was elicited by injection of autoclaved spores into rabbits. Early work with this technology indicated the existence of serological groups. *B. cereus*, *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. coagulans*, *B. polymyxo*, *B. circulans*, *B.*

alvei, B. brevis, B. laterosporus, and B. sphaericus each possessed a spore precipitinogen characteristic of each and every strain of the respective species. B. pumilus spores contained at least two agglutinins, one of which was shared with B. subtilis in confirmation of the close physiological relationship of these two organisms.

Comparative studies of nutritional needs of the mesophilic species of Bacillus were performed (Knight and Proom, 1950). The many criteria of comparison used lend credence to the statement that the range of different bacteria may be compared to a "spectrum," a concept which well accounts for the multitude of minor differences among many species (Cowan and Steel, 1965). The technique of numerical taxonomy illustrates this clearly. Instead of classifying a strain as a genus or species, numerous morphological and physiological characteristics of one cell type are compared to those of another organism. This "numerical taxonomy" produces a coefficient of similarity, S, which may range from zero to one. The fractional expression is:

$$S = \frac{NS}{NS + ND}$$

where: NS = number of similar characteristics between two bacteria

ND = number of dissimilar characteristics between two bacteria

Ainsworth and Sneath (1962) applied this analysis to twenty-six species of Bacillus and it was noted that B.

subtilis, *B. licheniformis*, and *B. megaterium* share 70 to 79% similarity.

With the establishment of methods for the determination of DNA base composition, a new taxonomic tool was developed. Marmur and Doty in 1962 determined the correlation between mole percent guanine plus cytosine and the midpoint of thermal denaturation of DNA polymers. Schildkraut, Marmur and Doty, in that same year, determined the relationship between mole percent G + C and buoyant density in CsCl gradients. Hill in 1966 compiled a base compositon reference list of bacterial species, as determined by many different research groups. The percent guanine plus cytosine range for the entire *Bacillus* genus was 34 to 64%. DeLey, in 1970, modified the Schildkraut, Marmur and Doty relationships using both published and unpublished current percent G + C values from many bacterial species. Relevant samplings of these values appear in Table 1. Below is the relationship between mole % G + C and midpoint thermal denaturation which he derived (DeLey, 1970).

$$\text{mole \% G + C} = 2.44\ Tm - 169.25$$
$$= 1038.47(p - 1.6616)$$

where: Tm = midpoint of thermal denaturation

p = DNA buoyant density

Included in this table of base composition are values for select species of *Bacillus*. *B. amyloliquefaciens* H dis-

displays a mole percent G + C of 44.0%, *B. licheniformis* 46.9%, and *B. pumilus* 45.1%. Two strains of *B. subtilis*, W23 and 168M, possess 42.2 and 41.95% GC, respectively. *B. subtilis* strain NCTC3610 and an indole negative derivative 168i are reported to have 42.6% GC. These values greatly contrast to thermophilic strains of *B. stearothermophilus* in which the reported base composition values go as high as 56.0%. Nucleic acid analysis combined with genetic technique provides for greater species resolution. Lovett and Young (1969) reported the reclassification of *B. subtilis* strain NRRC B-3275 as a strain of *B. pumilus*. This was determined by observing its greater DNA to DNA hybridization to known *B. pumilus* chromosomal DNA than to known *B. subtilis* DNA. In addition, transformation frequencies for two amino acid auxotrophic markers, *trp* and *met*, of *B. subtilis* strain 168 with B-3275 DNA was not possible. Table 1 also lists % GC derived from their paper in contrast to the DeLey values. In some cases where the same organism is noted the values differ slightly.

B. subtilis, the genus member most often used in genetic studies and best characterized, has its own unique history. Conn (1930) inspected the strains of *B. subtilis* then available and sought to establish the true identity of the species. One strain obtained from the University of Marburg was officially established in 1936 as the neotype *B. subtilis* by the International Congress for Microbiology. Presently, the strain is designated by the American Type

Culture Collection as ATCC6051 and by the National Collection of Type Cultures of London as NCTC3610. The Bacillus Genetic Stock Center of Ohio State University catalogs it as number 3A1 (1982).

In 1947, Burholder and Giles isolated biochemical mutants from what they called the Marburg strain; however, uncertainty exists as to whether the parent was in reality ATCC6051. In 1958, J. Spizizen, using a tryptophan mutant obtained from the Burkholder and Giles study, _B. subtilis_ 168, demonstrated that the organism developed competence for transformation. This bacterium is the origin of most present transformable strains of _B. subtilis_ (Hemphill and Whitely, 1975). This organism is the best genetically characterized, and is susceptible to transfection as well as transformation. However, strain 168 possesses designations in the literature which lead to confusion of its origins. _B. subtilis_ 168M is a highly transformable derivative of 168 which originates from the laboratory of J. Marmur. It must be noted that many 168-derived strains carry prefixes denoting the laboratory in which they originated.

In contrast, prototrophic _B. subtilis_ strain W23 frequently was used as a source of transforming DNA in 168 recipients; however it is not the prototrophic progenitor of 168. The DNA of W23 is 89% homologous to that of 168 and can transform the 168 strains, but it is not itself transformable (Lovett and Young, 1969). Strains ATCC6051

(NCTC3610) and 168 have the identical host range to various phages which differs from that of W23. This suggests that 168 is similar to that of the classic Marburg strain of *B. subtilis* (Hemphill and Whitely, 1975).

Audit and Anagnostopoulos (1975) have constructed a linkage map of Burkholder and Giles strain 166, which contains the *trpE26* marker (an anthranilate synthetase requirement). Extensive rearrangements of the chromosome were noted and reversion to tryptophan prototrophy was not observed. Transformants to TrpE26$^+$ result in merodiploid cells encompassing the *thr* to *iluA* region of the chromosome.

Molecular Sequence Analysis

Woese (1980) compared ribonuclease T1 digest fragments of 16S rRNA from many bacterial and eukaryotic species and established the presence of another phylogenetic kingdom, the archaebacteria. The power of this approach lies in the evolutionary constraints placed upon components of the translational apparatus. 16S rRNA changes in sequence slowly compared to sequences coding for most proteins and serves as a common structure for comparison in all organisms.

In this thesis project, examination of rRNA gene sequences of representative *Bacilli* established the relationships of the *B. subtilis* type organisms (*B.*

subtilis, *B*. *pumilus*, *B*. *globigii*, *B*. *licheniformis* and *B*. *amyloliquefaciens*). The extensive sequence conservation between *B*. *subtilis* NCTC3610 and strain 168 presents further evidence for their common lineage.

Conserved Regions of the Bacillus Chromosome

Interspecies transformation and nucleic acid hybridization may be used to study the genetic relatedness among bacterial species. Indeed, in the genus *Bacillus* these methodologies revealed what was termed a "conserved core of genetic material" (Dubnau *et al*. 1965a). Dubnau *et al*. used as recipients *B*. *subtilis* 168-derived strains auxotrophic for the replication origin marker adenine, termination marker methionine, and tryptophan and leucine markers. With heterologous donor DNA derived from other *Bacillus* species and strains, the nutritional markers showed no transformation. DNA prepared from cells resistant to the antibiotics erythromycin, micrococcin and streptomycin, however, did allow transformation of these markers. The authors proposed that this is a reflection of only limited base sequence homology between the chromosomes of these strains.

Further demonstration by these workers of the apparent limited homology between genomes was observed by heterologous DNA hybridization. Prototrophic, antibiotic resistant DNA was hybridized to auxotrophic *B*. *subtilis* 168 DNA

(erythromycin-sensitive), density labeled in D_2O medium. CsCl gradient isolated heteroduplexes transformed only for erythromycin resistance. Interspecies transformation with B. pumilus (micrococcin resistant, streptomycin resistant) or B. licheniformis (micrococcin resistant, streptomycin resistant) DNA produced antibiotic transformants only. B. niger (Ery^r, Str^r, Mic^r) transformed (in addition to antibiotic resistance) the adenine and leucine markers. This phenomenon was termed "selective transformation" (Dubnau, 1965a) and suggests discrete regions of homology among the chromosomal DNAs, which evolved and diverged slowly if at all. Conservation of streptomycin and erythromycin genes was observed in other systems. Ravin and DeSa (1964) examined interspecies transformation of streptomycin and erythromycin resistance between Pneumococcus and Streptococcus. Chen and Ravin (1966) examined streptomycin resistant marker transformation between Pneumococcus and Streptococcus. The frequency of homospecific transformation was observed to be higher than that of heterospecific transformation. Using [^{32}P]-labeled donor DNA, these workers showed that the barrier to efficient heterologous transformation is not DNA uptake. While Pneumococcal DNA inefficiently transformed Streptococcus, the marker once integrated into Streptococcus reversed its biological identity. Integration efficiency was increased for the marker in Streptococcus, but was now greatly depressed when transformed back into Pneumococcus. It was suggested

that homology of nucleotide sequence promotes synapsis of the genetic regions. In heterospecific transformation, the probability of integration of a donor marker depends upon the degree of heterology with the recipient's chromosome regions. This heterologous barrier may be overcome by the intergenetic marker's being resident in homospecific DNA (Chen and Ravin, 1966).

Catlin (1964) reported reciprocal genetic transformation between Neisseria catarrhalis and Moraxella nonliquefaciens, with heterologous efficiency being 0.0005% that of homologous. Subsequently, a str^r region of transforming DNA from a transformant strain of Moraxella was recognized and genetically integrated by populations of Neisseria at frequencies 10,000 times higher than Moraxella DNA carrying a spontaneous mutant str^r marker. This, too, suggests that integration is highly dependent on a region of synapsis formed by homologous pieces of DNA. Nickel and Goodgal (1964) presented evidence that the streptomycin, novobiocin, and erythromycin markers of Haemophilus influenzae and Haemophilus parainfluenzae are homologous by interspecies transformation. In addition, marker linkages (which differ in the two species) were maintained in the transformations. Markers resident in H. parainfluenzae via transformation from H. influenzae display native type H. parainfluenzae linkage. These same markers, when transformed into H. influenzae, re-established native H. influenzae linkage arrangements. This supports the concept of discrete re-

gions of conservation among genomes in spite of extensive variation of other chromosomal areas.

Dubnau *et al*. (1965b) attempted to determine what types of genes are found in the conserved portions of the genome. Streptomycin resistance affects the 30S ribosome component, and erythromycin affects the 50S component. This suggested that components of the translational apparatus are likely candidates for species stability. 16S and 23S rRNA derived from *B. subtilis* 168 hybridized to other *Bacillus* species DNA at about homologous levels. Pulse-labeled RNA (representing short-lived message) were highly variable in interspecies hybridizations (Dubnau, 1965a).

Dubnau et al. (1965b) reasoned that if conserved streptomycin and erythromycin genes are involved in ribosome synthesis, then their chromosome location should be the same as ribosomal RNA cistrons. *B. subtilis* strain W23 was density labeled in D_2O medium and the time at which 16S, 23S, and tRNA could hybridize to chromosomal DNA was compared to the time of appearance of nutritional markers in the CsCl gradient hybrid position. The rRNA sequences appeared shortly after the erythromycin marker and before the adenine marker. In addition, the streptomycin and erythromycin markers co-transformed at a frequency of 50%. Conserved sequences were therefore found in the early replicating region of the chromosome.

In 1969 Chilton and McCarthy analyzed the efficiency with which *B. globigii* and *B. subtilis* strain W23 donor

DNA transformed B. subtilis 168 DNA at a variety of genetic loci. They discovered two regions that appear most similar within these three genomes, one of which surrounds the streptomycin marker and the other the leucine marker. These regions form what the authors termed a "gradient of conservation." Markers surrounding these two are also conserved but to a lesser degree, i.e., a series of adenine markers in the streptomycin vicinity and two isoleucine-valine markers in the leucine vicinity showed a high transformation frequency.

Chilton and McCarthy attempted to assay whether the transformation variations reflected actual points of DNA base sequence similarity. Single-stranded DNA from the heterologous strains was annealed to B. subtilis 168M DNA. The melting temperatures observed produced a similar gradient where streptomycin and leucine had the highest melting temperature. The temperature at which 50% transforming activity was lost was termed Tmi (mean thermal dissociation temperature). This value displayed the least variation for these two markers between homo- and hetero-duplexes. These determinations were made by noting loss of transforming activity for each marker at a particular temperature.

The conservation of the adenine and leucine markers could be accounted for by placing them in the vicinity of rRNA gene clusters. Density transfer confirmed this with the adenine marker. Smith et al. (1968), using density

transferred DNA from germinating spores, placed 60 to 80% of the 16S, 23S rRNA and tRNA sequences to the "left" of the *purB6* marker. The remaining 20 to 40% of these sequences appeared in the vicinity of the leucine marker. Harford and Mergeay (1973) examined the rifampicin resistance marker, which is the genetic determinant for RNA polymerase. Seven of eight *Bacillus* species could effect heterologous transformation for this marker, as well as the erythromycin marker. Linkage between the rifampicin and erythromycin loci was also maintained to some degree in interspecies crosses, thus extending the concept of conservation of a region of *Bacillus* gene to another essential locus.

Goldberg *et al*. (1966) performed interspecies transformation between *B. subtilis* 168 strains and *B. licheniformis*. These transformations were performed with either species as donor or recipient. The streptomycin marker transformation frequency when *B. licheniformis* was the donor was 10^{-1} to 10^{-2} times that of a homologous cross. When *B. subtilis* was the donor, the Str^r frequency was 10^{-4} times the homologous transformation. Arginine, histidine and adenine markers were found not to be conserved at all between these two species. Copeland and Marmur (1968) noted conservation of two temperature-sensitive markers, one which displayed a phenotypic alteration related to the sporulation process and another which blocked cell division. The sporulation marker was

located in the early replicating conserved region. The cell division marker was positioned in the later replicating region in the vicinity of arg, leu, and phe where rRNA and tRNA cistrons have been postulated to be.

Harford and Sueoka (1970) mapped antibiotic resistance loci which inhibit protein synthesis by both density transfer analysis and PBS1 transduction. Most of these markers are clustered near the replication origin adjacent to genes for rRNA and tRNA. However, eleven spectinomycin resistant (ribosomal protein S5 determinant) mutants were located in the latter part of the chromosome at about 72% of the replication order map. This coincided with the region of the second peak of rRNA and tRNA, i.e., near the arg, phe, and leu region. Pai and Dabbs (1981) noted a kasugamycin resistant loci mapping near leucine by PBS1 transduction. This antibiotic interferes with binding of fMet-tRNA to the ribosome. The exact position is not yet determined; its approximate position is noted in Fig. 1.

The conservation of ribosomal rRNA cistrons was specifically compared in B. subtilis species (Doi and Igarashi, 1965a). Chromosomal DNA from B. subtilis, B. cereus, B. megaterium, B. stearothermophilus and B. macerans were annealed with pulse-labeled RNA of sporulating and log-phase cells of B. subtilis and B. cereus. The heterologous hybridizations ranged from 1 to 6% of the homologous. B. subtilis-derived rRNA produced in heterologous hybridizations 47.5 to 62.9% of the

homologous results. Base composition analysis indicated that among B. subtilis, B. cereus, and B. stearothermophilus, the %GC ranged from 48.6 to 51.7 in the rRNA sequences. Hybrid competition studies indicated that B. stearothermophilus rRNA could compete completely against B. subtilis rRNA for hybridization to B. stearothermophilus DNA. However, it competed only partially against B. subtilis rRNA hybridizing with B. cereus DNA (Doi and Igarashi, 1965b). This study indicated some heterogeneity existed within the ribosomal DNA sequences among the Bacillus. The lack of DNA restriction and sequencing techniques at this time prevented a more definitive answer to this question.

The extent of evolutionary stability of the rRNA sequences was indicated by Takahashi et al. (1967). B. subtilis Marburg strain rRNA hybridized to chromosomal DNA of Staphylococcus epidermidis, Alcaligenes faecalis and Escherichia coli at 55%, 33% and 20% the homologous level, respectively.

A comparative study of ribosomal RNA cistrons by similar techniques within species of the Enterobacteria and Myxobacteria indicated again the evolutionary stability of the translational apparatus genes as compared to other sequences (Moore and McCarthy, 1967).

Sequence Conservatism in Gram-Negative Species

Anilionis and Riley (1980) and Harshman and Riley (1980) looked at conservation and variation in portions of the genome of select gram-negative bacteria. The Southern transfer technique was used with DNA-to-DNA hybridizations. This allowed observation of the distribution of restriction enzyme target sites near and within limited portions of the genome. Sizes of chromosome restriction fragments were used as an estimate of the extent of nucleotide variation within a region. The rationale was that size differences result from changes causing loss or gain of a restriction site sequence or changes in the numbers of base pairs that lie between them. The hybridization probes were lambda transducing phage carrying markers from the *E. coli* genome. In comparison of different strains of *E. coli*, portions of the genome that were homologous to phage DNA and that presumably are inactive, displayed more variability than regions with functional genes. Endonuclease cleavage sites in the regions of the tryptophan operon, *lacZ*, thymidylate synthetase and tryptophanase genes were conserved.

These same workers assessed the degree of relatedness of these portions of the *E. coli* genome to those of other enteric bacteria. The lambda homologs and *lacZ* gene were noted to be variant. As in *Bacillus*, strict conservation was observed in ribosomal RNA genes. Labeled *E. coli* rRNA hybridized across species boundaries and produced identical DNA fragment size with EcoRI restrictions

(Ostapchuck, et al., 1980). Nakamura et al. (1979) found the mRNA to an outer membrane lipoprotein of E. coli hybridized to EcoRI and HindIII restriction endonuclease fragments from chromosomes of nine bacteria in the family Enterobacteriaceae. Overall, functional constraints appear to often retard the rate of divergence in particular genes. However, not all gene alterations involve gradual base substitutions; in enteric genomes major chromosome rearrangements have effected evolutionary events. Case et al. (1973) noted inversion of a large segment of the genome, including the trp operon, in E. coli compared to S. typhimurium. Duplications of particular regions are observed frequently in E. coli and Salmonella, often involved with increased enzyme expression. Such conditions are reported to occur in the lactose operon of E. coli and histidine operon of Salmonella. The gene for glycyl-tRNA synthetase in E. coli shows this effect also. This phenomenon is usually manifest in the form of tandem duplications and might provide a form of gene amplification (Anderson and Roth, 1977).

Organization of rRNA Gene Sets in B. subtilis 168

The fine structure of the ribosomal gene sets of B. subtilis has been analyzed in terms of its genomic position and internal structure. There are at least two clusters of ribosomal RNA gene sets in B. subtilis. The major

cluster was mapped by Chow and Davidson (1973) very near the attachment site for the lysogenic bacteriophage SP02. This is the same general region as the ribosomal protein genes. The localization was performed by denaturation and renaturation of chromosomal DNA with visualization by electron microscopy. Multiple rRNA gene sets form duplex regions separated by denatured loops. The chromosome segments are renatured out of register and clearly display the homologous regions representing the multiple rRNA gene sets. The cell line used was a lysogen of SP02, and the series of tandem rRNA gene sets began 6.2 Kbp from one phage end. These researchers were able to draw general conclusions on the nature of these gene sets in addition to their position. It was estimated that there were 7 to 9 sets per chromosome. The average length of each was 4.83 Kbp, and the order of 16S and 23S sequence is the same in all sets. The spaces between the gene sets are not homologous, as they vary in length from 0.2 to at least 55 Kbp (Chow and Davidson, 1973).

Oishi and Colli (1969) produced evidence for a physical linkage between 16S and 23S rRNA sequences. Using DNA strands separated by MAK (methylated albumin Kieselguhr) chromatography, the same sheared *B. subtilis* chromosomal DNA fragments hybridized to 16S rRNA could also be rehybridized after alkaline removal of RNA to 23S rRNA. The DNA sequences complementary to both RNA's were observed to be on the same DNA fragment. DNA fragments able to accept hybrid-

ization with rRNA showed no hybridization with pulse-labeled mRNA. The physical linkage among 16S, 23S and 5S sequences was confirmed by Colli, Smith and Oishi (1971) in a similar manner. DNA was sheared and hybridized to rRNA species. On the average, 3 kilobase fragments could not co-hybridize 16S and 23S, but could contain both 23S and 5S rRNA. 16S and 5S sequences rarely co-hybridized. Ten kilobase DNA fragments contained all three rRNA species. Zingales and Colli (1977) demonstrated the transcriptional order of 16S, 23S and 5S for the *B. subtilis* rRNA.

Mature rRNA species were estimated to be of the size 1.67 Kbp (16S), 3.33 Kbp (23S) and 0.12 Kbp (5S) (Attardi and Amaldi, 1970). In *B. subtilis*, Hecht *et al*. (1968) noted the immediate precursor of mature 23S rRNA is about 5% greater in molecular length. Precursor 16S rRNA is about 10% larger than the mature molecule. Pace *et al*. (1973) found the presence of two distinct 5S precursors, one 50% longer than the mature molecule, and the other 25% longer. RNase II has been implicated in the maturation process of the precursors. The expression of ribosomal RNA genes during sporulation in *B. subtilis* has been examined. Testa and Rudner (1975) reported that during periods of increased RNA synthesis in sporulating cells 65 to 80% of the transcripts are copies of stable RNA genes. Edgell, Hutchison and Bott (1975) observed that rRNA's derived from vegetative or sporulating cells hybridized to the same Hae III restricted chromosome fragments. They concluded that

rRNA's transcribed during both phases of the Bacillus life cycle are from the same cistrons or from cistrons with identical HaeIII sequence distribution.

The Restriction Map of the Bacillus subtilis rRNA Gene Sets

With the advent of restriction site enzyme analysis and the Southern hybridization techniques, the organization of these gene sets was better resolved. Moran and Bott (1979) observed that BamHI restriction fragments co-hybridized both 23S and 5S rRNA sequences. The 16S rRNA hybridizing fragments were not equivalent to the 23S, 5S ones; therefore, 5S is more closely linked to 23S than to 16S, as early studies suggested. This linkage was also visualized upon a cloned *B. subtilis* BamHI fragment which was able to hybridize to both 23S rRNA and 5S rRNA.

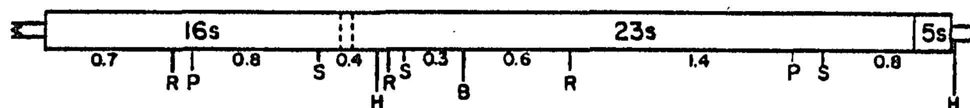
Biological selection for ribosomal rRNA gene sets is not possible as with antibiotic markers. Indeed strains of *B. subtilis* deleted for an rRNA gene set have been isolated displaying no viability loss (Loughney *et al.*, 1983; Gottlieb and Rudner, unpublished). In order to directly map these gene sets by PBS1 transduction, selectable markers were transformed into the chromosome at the rRNA sites. This technique is based upon the observation of unique DNA sequences beyond the boundary of the rRNA gene (spacer DNA). A plasmid carrying an rRNA gene along with its associated unique spacer DNA may integrate at its native position

within the chromosome during recombination with proficient recipients. An rRNA operon carried by chimeric plasmid p12E2 (chloramphenicol resistance) was found by this technique to be resident in the early replicating chromosome region. It maps near the guanine marker located between purA and cysA on the B. subtilis chromosome, and was designated rrnA (Bott et al, 1980; Wilson, et al., 1982). This is not within the gene set group observed by Chow and Davidson (1973) adjacent to the SPO2 attachment site.

Henckes et al. (1982) located a ribosomal gene set (rrnO) which overlaps the replication origin region of the chromosome. Its presence was noted upon a cloned 5.7 Kbp BamHI chromosome fragment of the initially replicating region. Two tandem promoters are found at the 5' end of this 16S gene sequence. The two promoters are contained within a 500-base-pair sequence, which when cloned into E. coli plasmids provided a cis inhibitory effect on their replication. Elimination of the second promoter prevented the replication inhibition and altered transcriptional activity. Nucleotide sequences place the promoters 98 base pairs apart. Downstream from the second promoter are two identical sequences of about 80 nucleotides, the second of which codes for the 5' end of the mature 16S ribosomal RNA. In E. coli transcription for ribosomal RNA is initiated from tandem promoters. A first is located about 300 nucleotides upstream from the 5' end of mature 16S RNA. The second promoter is located 190 nucleotides from the 16S RNA

(Brosius et al., 1981). This dual promoter arrangement appears a common feature of the rRNA gene sets.

Detailed restriction mapping of the ribosomal RNA genes of B. subtilis was performed by Stewart, Wilson and Bott (1982). These workers made extensive use of gene banks of B. subtilis inserted in E. coli plasmid vectors and lambda charon 4A vectors. Ribosomal RNA-containing clones were identified with [^{32}P]-labeled rRNA or previously identified rDNA clones. Of all the examples analyzed representing a variety of gene sets throughout the chromosome, restriction sites in the coding sequences for 16S, 23S and 5S are homologous. Overall restriction maps of these regions display the following construction, which is diagrammed below (map is based upon Stewart et al, 1982).



The rDNA lacks cleavage sites for the enzymes SalI, BglI and BglII. There is a single BamHI site (in the 23S sequence) and a single HindIII site (in the 23S sequence also). The only restriction site which has been found to be variable is the HindIII site distal to the 5S rRNA determinant. Apparently, there are two distinct precursor 5S rRNAs (Pace et al., 1975); one of these seems to possess the distal HindIII site (Stewart et al., 1982). The 16S sequence contains an EcoRI site located 0.7 Kbp from its 5' end. The

23S coding sequence contains two EcoRI sites, one 2.2 Kbp from the 3' end of the gene set and the other 0.9 Kbp proximal to the first. Three SmaI sites are found, one at the 3' end of 16S, the second at the 5' end of 23S, and the third at the 3' end of 23S. Distal to the 16S determinant EcoRI site is found a PstI recognition sequence. One additional PstI site exists in the 23S sequence at the 3' end proximal to the third SmaI site. It must be noted that if a complete chromosomal restriction is performed with either EcoRI or SmaI, internal sequences will be cleaved out of all the gene sets. These can be identified with appropriate radio-labeled probes on Southern blots and allow conclusions to be made on internal organization within the gene sets.

Wilson _et al_. (1982) report that there are at most ten rRNA gene sequences in _B. subtilis_ strain 168M. Total chromosome restriction with EcoRI reveals 13, 23S homolog bands of which three represent internal operon sequences. HindIII restriction produces eight bands with 23S or 16S rRNA probes, two of which are wide and dark, suggesting they represent co-migrating but different DNA fragments (i.e., at least two cryptic operons to produce the total number, ten). Using the same methodology, Kiss, Sain and Venetianer (1977) estimated that _E. coli_ contains seven rRNA gene sets.

Moran and Bott (1979) observed that the SmaI restriction fragments of _B. subtilis_ that hybridized to 5S rRNA

rRNA or 16S rRNA also hybridized tRNA sequences. Their plasmid p12E2 containing a 23S, 5S rDNA BamHI insert carried a 400 base pair fragment that hybridized both 5S and tRNA sequences. These workers concluded that tRNA sequences are closely linked to 5S rRNA sequences and are only found between individual rRNA gene sets. In *E. coli* tRNA sequences are found in spaces between the 16S and 23S genes in addition to the 3' end of the whole operon (Morgan, 1978; Young *et al.*, 1980). The initial report claiming tRNA intraoperon sequences were not present in *B. subtilis* was in error.

There are two types of rRNA gene sets in *B. subtilis*, differing in respect to the size of the piece bridging the 16S and 23S sequences (termed the abutment fragment). The estimated ratio of the small to large type was 7:3 (Stewart *et al*, 1982). Loughney *et al.* (1982) cloned the 16S, 23S abutment fragments and determined the larger contains an extra 180 base pairs. These extra nucleotides contain sequences for transfer RNA, tRNA ile and tRNA ala. Using a tRNA ile-specific sequence as a molecular probe, this group calculated that at least two of the ten total rDNA abutment spaces carry tRNA. Ogasawara *et al.* (1982) noted the presence of two ribosomal RNA operons in the replication origin region of the *B. subtilis* chromosome. One corresponds to *rrnA* (previously mapped by Bott *et al.*, 1981) with its linkage to the *guaA* marker. The other overlapping the earliest replicating region is *rrnO*. Both operons show

the presence of tRNA sequences in the abutment space.

In fact, such insertions within the central region of the gene set prove to be very common. Transfer RNA sequences are also located between the 16S and 23S regions in chloroplast rDNAs of *Euglena gracilis* (Graf *et al.*, 1980) and *Zea mays* (Koch *et al.*, 1981). Recently, the 16S and 23S sequences of the archaebacteria, *Methanococcus vannielii*, were seen to be separated by a spacer of two size classes, differing by about 100 base pairs; tRNA sequences are contained in both types (Jarsch *et al.*, 1983). The strong sequence homology in this part of the rRNA cistron of gram-positive and gram-negative bacteria, eucaryotic chloroplasts, and archaebacteria, indicates functional significance resulting in structural constraints.

Chromosomal Rearrangements Mediated by Ribosomal RNA Cistrons

The presence of multiple ribosomal RNA sequences at numerous positions in the chromosome suggests a mechanism of genetic alteration. In the case of *E. coli* there are seven copies of rRNA distributed about the chromosome (Ellwood and Normura, 1982) and unequal recombination between these homologous regions can lead to gene rearrangement. Such alterations can be in the form of deletions, tandem duplications, inversions or transloca-

tions. Hill and Harnish (1981) constructed mutants that have an inversion of the segments between rrnD and rrnB (and rrnE). In *E. coli* rrnB and rrnE are less than 1 minute apart on the chromosome map (Ellwood and Normura, 1982). Mutants were also constructed which had the chromosomal segment directly between these two sequences deleted from their normal position and transposed into another one of the seven rRNA genes. The mechanism proposed suggests that the transposition of a chromosomal segment involves three redundant ribosomal genes. A chromosome segment which is flanked by rRNA sequences is carried to a recipient cell (by a transducing particle). Within this cell, the extensive homology allows the segment to form a circle with the joint being the rRNA sequences. This circle, with the markers it carries, could integrate at any available rRNA gene (Hill and Harnish, 1982). Lehner and Hill (1980) studied a family of tandem duplication mutants in *Salmonella typhimurium*. The duplications were generated by unequal recombination between pairs of similarly oriented rRNA operons. Physical isolation of duplicated regions as circular DNA fragments was achieved. A given duplication mutant gave forth a circle with a size equal to one copy of the duplication, and each circle contained a joint formed by a hybrid rRNA operon. Anderson and Roth (1981) noted that the frequency of spontaneous duplication in *Salmonella typhimurium* is high throughout the region of the chromosome bounded by directly repeated

rRNA cistrons. All tandem duplications noted had end points within rRNA cistrons.

Cohen *et al.* (1978) observed the occurrence of a deletion in a recombinant plasmid that contained yeast ribosomal DNA. A pMB9 *E. coli* vector with a yeast DNA insert containing a tandem rDNA repeat lost one of these repeats and retained only a single copy. The deletion event occurred with greater frequency in Rec^+ hosts than in *recA*$^-$.

Overall, the mechanism of duplication and rearrangement presented above could be of adaptive significance to an organism by permitting amplification of rRNA gene copies. Such an event may be utilized during periods of extremely rapid growth or at other less favorable times to promote the discard of "excess baggage" from the genome.

Specific Aims

This project examines conserved genetic regions of the chromosomes of eight species and strains of *Bacillus*. Analysis by nucleic acid hybridization of the genes of the translation apparatus further illuminates previous observations of the depressed rate of divergence within these sequences. We also seek to explain why some auxotrophic markers (*arg*, *ilv*, and *leu*) also show some resistance to evolutionary change in this genus. Does their proximity to stable genes (rRNA cistrons) influence their rate of

change?

The rRNA gene sets of *B. subtilis* are found primarily in the early replicating region of the chromosome but others have been postulated to exist within the late replicating region. With our nucleic acid hybridization assay coupled with transformation and transduction techniques we seek to locate rRNA genes within the *arg*, *ilv*, *leu*, *phe* region of the chromosome. The presence of reiterated nucleotide sequences throughout the genome may allow intrachromosome recombination events. The presence of rRNA genes might effect the organization of the chromosome in this manner. *B. subtilis* strains possessing mutational markers and chromosome rearrangements are analyzed for any change within their ribosomal gene sets. Correlations between genetic changes and loss or gain of rrn genes is of interest regarding possible mechanisms of evolutionary change. Conventional concepts of gradual base sequence changes as driving evolution may not be entirely valid. Major genetic changes may also result through translocation, transposition, and recombination. Mediating elements of such events need to be identified and the *Bacillus* system provides a useful tool to do so.

MATERIALS AND METHODS

Bacterial Strains

Transformable mutant derivatives of *B. subtilis* 168M were strains obtained from numerous researchers. Table 2 presents relevant genotypic information on them. Prototrophic strains W23 and NCTC3610 were obtained from D. Dubnau and A. Sonenshein, respectively. Transformant strains constructed for this study are listed in Table 3. *B. subtilis* strains with chromosomal rearrangements, GSY1269 and 166, (American Stock) were gifts from K. Bott. *B. subtilis* strain BD224, a *recE4* strain, was used for maintaining cloned fragments of the *B. subtilis* chromosome without recombination. *B. pumilus* RUB502 (*rif*^{r}, *str*^{r}, *ery*^{r}, *bio*$^-$) and *B. licheniformis* FD01 (*str*^{r}) were from D. Dubnau. *B. amyloliquefaciens* H (prototroph) and *B. licheniformis* 8480 (*str*^{r}) were obtained from A. Garro. *B. globigii* RUB562 (prototroph) was from C. Tackney. *E. coli* strain HB101 (*hsdm*$^-$, *hsdr*$^-$, *recA*$^-$, *gal*$^-$, *pro*$^-$, *leuB*$^-$, *rpsL*) was used to maintain and amplify select plasmids used in this study. *E. coli* C600 (*hsdm*$^-$, *hsdr*$^-$, *thi*-1, *thr*-1, *leuB6*, *lac41*, *tonA21*, *SupE44*) was supplied to us as the host for plasmid RSF2124·B-leu (Nagahari and Sakaguchi, 1978).

Plasmid Hybridization Probes

Plasmids p21C4-16S, pBC279-23S, p12E2-23S,5S and p14B1-16S,23S,5S carry cloned ribosomal DNA sequences of *B. subtilis* 168M and were a generous gift of K. Bott. All show "relaxed control" of replication and could be amplified to high copy number in *E. coli* hosts by stoppage of protein synthesis with chloramphenicol (Clewell and Helinski, 1972).

RSF2124'B-leu carries an EcoRI piece of the *B. subtilis* 168M chromosome and was a gift of K. Nagahari and K. Sakaguchi (1978), who constructed it. Plasmid pBS02a-threonine carries a fragment of the *B. subtilis* 168M threonine operon. It was a gift of A. Inglesias, who obtained it from G. Rapoport's library of sheared chromosomal DNA (Rapoport *et al.*, 1979). These two chimeric plasmids were isolated by the chloramphenicol amplification method. Plasmid pRR106 was constructed in this laboratory by Tackney and Rudner (1981). It carries an EcoRI fragment of the *B. pumilus* tryptophan operon in vector pUB110 and replicated in *B. subtilis*.

Culture Media

Bacillus species were routinely grown in Penassay broth (Difco) from small volume starter cultures. Large-scale growth was in veal infusion broth (Difco) supple-

mented with 0.5% yeast extract (Difco). Tryptose blood agar base (Difco) contained an additional 0.5% agar (Difco) and was supplemented with 5.9 mM $MgCl_2$, 0.02 mM $MnCl_2$, which enhanced sporulation for storage purposes. Tryptose blood agar base plus 0.5% agar was also used for streaking.

Minimal medium consisted of Spizizen salts buffer (1958) supplemented with 0.5% glucose, 1% sodium glutamate and 100 ug/ml each of the required amino acids. Plating was performed on this same medium with 1.5% agar added. Spizizen salts 1X are: 0.44 M KH_2PO_4, 0.08 M K_2PO_4, 0.0034 M Na citrate, 0.001 M $MgSO_4$. VY medium is Veal Infusion broth (Difco) supplemented with 0.5% yeast extract (Difco).

E. coli was grown on 1X YT medium, which consisted of 0.8% Bactotryptone (Difco), Bacto yeast extract 0.5% and 0.5% NaCl. This same formulation was used on spreading plates with 1.5% agar added. The minimal medium was M9, consisting of 0.042 M Na_2HPO_4 (anhydrous), 0.022 M KH_2PO_4, 0.009 M NaCl, 0.019 M NH_4Cl, 0.0001 M $CaCl_2$, 0.001 M $MgSO_4 \cdot 7H_2O$ and 0.2% glucose. 1.5% agar was added for preparing plates.

Enzymes

Restriction enzymes HindIII and BamHI were purchased from Miles Laboratories. EcoRI and SmaI were from New England Biolabs. Polynucleotide kinase-T4 came from P.L.

Biochemicals. DNA polymerase I (Kornberg polymerase) was from Boehringer Mannheim. Lambda DNA 1 ug/50 ul was shipped with each restriction enzyme and was used routinely to check the quality of each new purchase. The digest was compared to the standard restriction pattern for each enzyme as supplied by the manufacturer. In addition, purified phage lambda cI857 S7 DNA (500 ug/ml) was purchased from New England Biolabs.

DNAase I was Worthington type DP dissolved in 0.2 M $MgSO_4$ to 100 ug/ml. RNAase A and RNAase T1 were also from Worthington and were made up to 2 mg/ml stock and 10,000 units/ml solution, respectively. Pronase, from Calbiochem, was brought to 5 mg/ml stock. This solution was brought to pH 5.0 with HCl and heated to 80°C for 10 minutes. The pH was then adjusted to pH 7.0 and NaCl was added to 1 M. The RNAase A was brought to 80°C for 10 minutes to inactivate contaminating DNAase.

Miscellaneous Compounds Used

Tris base, bovine serum albumin, ethidium bromide were from Sigma. Unlabeled ribo- and deoxy-ribonucleoside triphosphates were from P.L. Biochemicals. Phenol was purified crystals of ANALA-R. Amino acids (L) were from Calbiochem. Biofluor was from New England Nuclear.

Isotopes

Deoxyadenine-5'-triphosphate (α-^{32}P) and deoxycytidine-5'-triphosphate (α-^{32}P) (600 to 800 Ci/mM) were from New England Nuclear. It was shipped in 0.01 M Tricine (N-tris[hydroxymethyl]methylglycine) at a concentration of 0.013 umol/ml, and could be used in the reactions without prior desiccation.

Adenosine-5'-triphosphate (γ-^{32}P) (3000 to 4500 Ci/mM) was from ICN Chemical and Radioisotope Division. The stock solution was in a 1:1 ethanol:water combination at a concentration of 0.0025 umol/ml. It was desiccated to dryness prior to use.

Thymidine-5'-triphosphate (methyl-^{3}H) (78.1 Ci/mM) was from New England Nuclear. The stock solution was a 1:1 ethanol:water combination at a concentration of 0.012 umol/ml, and was desiccated to dryness prior to use. Drying, when necessary, was under vacuum at room temperature.

Transformation of Competent B. subtilis Cells

The method used is a modification of Anagnostopoulos and Spizizen (1961). A 5.0 ml Penassay culture was started from a single colony isolate. The colony was incubated at 37°C for 6 to 8 hours and was then diluted 10^{-2}. One-tenth ml was inoculated into a 20 ml Penassay starter culture and incubated overnight at 37°C in a shaking water bath (New Brunswick Scientific). Ten ml of this

culture was centrifuged in 15 ml sterile glass tubes in a clinical centrifuge. The cell pellet was resuspended in 2.5 ml Spizizen minimal medium I. This consisted of Spizizen salts supplemented with 0.5% glucose, 0.02% vitamin-free casamino acids, 0.1% yeast extract, 0.8% L-arginine, 100 ug/ml of each required amino acid, depending on cell strain being transformed. Twenty ml of medium I in a side arm flask was brought to Klett units of 25-35 (red filter, 660 nM) with the concentrated starter culture and shaken at 37°C in the water bath. Growth was followed with the Klett-Summerson colorimeter (red filter). After 4 to 4.5 hours stationary phase was reached. The stationary culture was diluted 1:10 in Medium II which consisted of 1X Spizizen salt, 0.5% glucose, 0.01% vitamin- free casamino acids, 0.05% yeast extract, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM spermine tetrahydrochloride and 50 ug/ml of each required amino acid. The second culture was incubated for 90 minutes after which maximum competence is obtained. Often this procedure was performed at 10-fold greater volume, 200 ml medium II, being incubated for 90 minutes followed by rapid centrifugation. Concentration of the competent cells was 10 times in their own medium II supernatant, and glycerol was added to 5%. Rapid freezing was in a dry ice-acetone slurry followed by storage at -70°C. The cells could be thawed and reconstituted with medium II for the transformation assay.

From 0.1 to 10 ug of transforming DNA was added in a

volume of 0.1 ml (1X SSC) to a 12 x 100 mm Wasserman tube. To this was added 0.9 ml of the competent cell suspension. The tube was incubated on a roller for 30 minutes at $37^{o}C$, followed by hydrolysis of remaining DNA by addition of 0.1 ml of DNAase I to a final concentration of 10 ug/ml. The sample tubes were diluted into dilution saline (0.15 M NaCl and 0.02 M K_2HPO_4, pH 7.0) and vortexed; 0.1 or 0.2 ml of appropriate dilutions were spread with an alcohol-flamed glass rod on selective agar medium. Incubation was for 48 hours at $37^{o}C$. A transformation control tube was included containing 0.1 ml 1X SSC alone as a revertant control.

Transformation of E. coli with Plasmid DNA

The transformation procedure used is a modification of that of Mandel and Higa (1970). An overnight 1X YT broth culture of *E. coli* HB101 was used as an inoculum. Three-tenths ml of this was added to 25 ml YT broth at $37^{o}C$ and grown to Klett = 100. This took about 2.5 to 3 hours. Ten ml of the culture were chilled on ice for 10 minutes. It was then centrifuged 5 minutes in sterile capped glass tubes in a clinical centrifuge. The supernatant was poured off and the cells were gently resuspended in 10 ml 0.01 M $CaCl_2$. This was recentrifuged, the supernatant was poured off and resuspension was with 5 ml of cold 0.03 M $CaCl_2$. The suspension was placed on ice for 20 minutes,

then pelleted again and resuspended in 1 ml of cold 0.03 M $CaCl_2$. To 200 ul of Ca^{2+} prepared cells in sterile glass "Kimax" capped tubes, DNA was added (0.1 to 1.0 ug) and mixed gently. The samples were incubated 30 minutes at 0°C. The tubes were then transferred to 42°C for a two-minute "heat shock." They were then brought to room temperature. To each sample was added 2.7 ml of YT broth, and shaking was for 90 minutes at 37°C. This allows phenotypic expression of the antibiotic resistance marker. The cells were suspended in dilution saline and spread on YT broth plates with antibiotic supplement, or if nutritional markers were being examined they were plated on M-9 minimal medium containing casamino acid. Additions or deletions of growth factors were appropriate for the cell strain being used. Plate incubation was for 24 hours at 37°C.

AR9 Phage Generalized Transduction

Generalized transduction was performed with the temperate phage AR9. AR9 is a temperate virus which can lysogenize, and the cells so produced are UV inducible. The virus also possesses transducing activity and can transfer a chromosome segment 1.5 times larger than PBS 1 (Belyaeva and Azizbekyan, 1968). To prepare a transducing lysate, overnight cultures of motile *B. subtilis* were prepared first. These were diluted 3 ml culture into 7 ml

veal yeast extract medium and grown at 37°C in a shaking water bath. When the culture reached a Klett value of about 130, they were infected with 0.1 ml phage suspension per 10 ml culture. The culture was followed until the Klett readings dropped indicating lysis had occurred. One to two ml of chloroform was then added to the lysed cultures and they were left overnight at 37°C. Chloroform and debris were then removed by centrifugation. The supernatant was sterile filtered through a Millipore apparatus and stored over chloroform. For transduction, overnight cultures of motile B. subtilis recipients were grown in VY extract medium. The culture was diluted (3 ml culture into 7 ml veal-yeast extract medium) and grown to about 300 Klett units. One ml bacteria was added to 0.1 ml transducing lysate and incubated for 30 minutes at 37°C with aeration. After incubation, the mixture was pelleted in a clinical centrifuge and the cells were resuspended in 1 ml dilution salt. Samples of 0.05 to 0.1 x 10^{-1} dilution were spread on appropriate Spizizen minimal selective medium. To isolate motile bacteria (swarming cultures), a swarmer plate was used which consisted of 5 ml tryptose blood agar base medium plus 5 ml veal-yeast extract medium autoclaved and poured into a plastic petri dish.

Preparation of Chromosomal DNA of Bacillus Species

Aliquots of overnight Penassay cultures were diluted

10^{-2} times and 0.2 ml was added to 250 ml volume of veal infusion broth supplemented with VY extract. Up to 2 to 4 liters were cultured at a time. Incubation was overnight at 37°C with shaking. Cells were harvested by 5-minute centrifugation at 7000 RPM, and washed once and resuspended in 0.15 M NaCl-0.1 M EDTA (pH 8.0). DNA isolation was by the procedure of Marmur (1958) as modified by Rudner *et al.* (1967). Phenol (ANALA-R) was dissolved in 1 M tris-HCl (pH 7.5) and used immediately. High molecular weight DNA had OD 280/260 ratios of about 0.5. Dialysis was with 10 mM Tris, 0.1 mM EDTA and the stock was stored over a drop of chloroform. With some preparations the cells were grown in Spizizen salt supplemented minimal medium. The DNA isolation procedure was the same but the yield of product was typically less.

Plasmid Isolation from B. subtilis

Plasmids pUB110 and pRR106-*trp* replicate in *B. subtilis* and have a kanamycin resistance gene (Gryczan *et al.*, 1978; Rudner and Tackney, 1981). Bifunctional plasmids pHV33 and pBS02a when replicating in *B. subtilis* express their chloramphenicol gene (Rapoport *et al.*, 1979; Erlich, 1978). Large-scale isolation of these plasmid DNA from *B. subtilis* was carried out according to Gryczan *et al.* (1979). A 5-10 ml starter culture contained Penassay broth supplemented with 5 ug/ml of kanamycin or chloram-

phenicol. This was diluted by a factor of 10^{-2} and 0.1 ml of the dilution was inoculated into 250 ml veal-yeast extract broth also supplemented with either antibiotic. This was incubated with shaking for 16 hours at 37°C. Cells were harvested by centrifugation and washed once with 0.1X volume 25% sucrose, 0.1 M NaCl, and 0.05 M tris-HCl (pH 7.5) (Buffer A). Resuspension was at 0.1X volume Buffer A. Lysozyme was added to 0.5 mg/ml from a 5 mg/ml stock; incubation was at 37°C for 15 minutes. To the 25 ml of protoplasted cells were added 6 ml 5 M NaCl, 1.5 ml 0.5 M EDTA, and 32.5 ml 2% SLS-0.7 M NaCl. The flask was inverted and left on ice at least 18 hours. The lysate was centrifuged at 18,600 RPM for 30 minutes at 4°C in a Spinco 30S rotor. The resulting cleared lysate was brought to 0.3 M sodium acetate and two volumes of 95% ethanol were added. This was left overnight at -20°C. It was subsequently centrifuged at 5000 RPM for 30 minutes and residual ethanol was desiccated by vacuum. The pellet was gently resuspended in 6.25 ml 30 mM tris-HCl (pH 7.5), 50 mM NaCl and 5 mM EDTA (TES buffer). Pancreatic ribonuclease A (50 ug/ml) and 1 unit T1 ribonuclease were added and the solution was incubated 30 minutes at 37°C. Pronase was used to visibly clear the solution. Plasmid DNA was separated from chromosomal within a CsCl-ethidium bromide density gradient (Clewell and Helinski, 1972).

Where minimally grown cells were required, the medium was 250 ml of Spizizen's minimal medium supplemented with 100

ug/ml of required amino acids and 5 ug/ml antibiotic. The starter cultures went through one additional minimal medium stage after Penassay growth.

Plasmid Isolation from E. coli

A 50 ml overnight culture of 2X YT broth was added to 450 ml 2X YT broth, supplemented with 100 ug/ml ampicillan and 25 ug/ml tetracycline, in a 2-liter flask and the culture grown at 37°C until a Klett reading (red filter) of 180-200 was reached; then chloramphenicol was added to the culture to 180 ug/ml. Shaking at 37°C was continued overnight to amplify the ColE1 type plasmids (Clewell and Helinski, 1972). Cells were harvested by centrifugation, washed, and resuspended in 5 ml cold 50 mM tris-HCl (pH 8.0) and 25% sucrose. The suspension was cooled in an ice slurry within a polypropylene tube. Seven mg lysozyme, usually solid, was added, mixed, and left on ice 15 minutes, followed by addition of 1.5 ml of 250 mM EDTA; this very viscous suspension remained in the ice bath an additional 15 minutes; 20 ml lytic mix was added, and it was kept at room temperature 5 minutes. (Lytic mix consisted of 0.05% Triton-X, 50 mM tris-HCl (pH 8.0), 62.5 mM EDTA and 1% SLS.) Ten ml of 5 mM NaCl was added and the SLS plus NaCl precipitated a substantial amount of protein and RNA. The suspension was left at 0°C at least 20 minutes. Large molecular weight species were pelleted by

centrifugation at 17,500 RPM for 30 minutes. The very viscous chromosomal DNA was discarded. Resulting volume was in the range of 5-20 ml. Precipitation was with 0.54 volumes isopropanol left at $0^{o}C$ for 45-60 minutes. This was spun at 17,500 RPM for 20 minutes, the supernatant was discarded and the pellet vacuum dried. Resuspension was in 3 ml TE buffer (10 mM tris-HCl (pH 8.0), and 0.1 mM EDTA) by aspiration with a pipet. Debris was cleared if necessary by further centrifugation in a 15 ml siliconized Corex tube at 10.000 RPM for 5-10 minutes. Final plasmid isolation was by CsCl-ethidium bromide density centrifugation.

CsCl-Ethidium Bromide Density Gradients

For plasmid isolation of lysates of either *B. subtilis* or *E. coli*, CsCl-ethidium bromide density gradients were prepared as follows: Six ml of lysate was gently poured onto 7.35 g cesium chloride (biological grade, Schwartz/Mann) in a 40 ml beaker. Gentle swirling dissolved the CsCl crystals. To this was added 2 ml (1 mg/ml stock) ethidium bromide in subdued light. The resulting solution was poured into 8 ml polyallomer tubes (Beckman), pretreated by boiling 10 minutes in 1 mM EDTA. Centrifugation was at 38,000 RPM for 48 hours in a T150 fixed-angle rotor at $15^{o}C$.

Visualization of the intercalated fluorescent dye was by exposure to short-wavelength ultraviolet illumination

(UVL-21, Blak-Ray). The lower gradient band, representing ccc DNA, was removed by puncturing the tube with a 2.0 ml syringe with attached 18-gauge needle after opening the tube cap vent closure. The ethidium bromide in the sample was removed by extracting three times with isopropanol saturated with water-CsCl. The aqueous solution containing plasmid was dialyzed against 1 liter 10 mM tris-HCl (pH 7.5), 4 mM NaCl and 0.1 mM EDTA overnight with two changes of dialysate solution. Dialysis membrane tubing was previously boiled in $NaHCO_3$ buffer and re-boiled in 10^{-3} M Na_3EDTA solution. Plasmid concentration was determined by UV spectroscopy at 260 nm (1 OD = 50 ug/ml).

Restriction Enzyme Reactions

Chromosome DNA samples from all *Bacillus* species were adjusted optically to 2 units OD_{260} (100 ug/ml) in 10 mM tris, 0.1 mM EDTA. Aliquots were withdrawn as needed and brought to 1X restriction buffer conditions from 10X stock solutions. Restriction buffers were made using supplier's specifications. The enzymes used along with their recognition sequences are described in Table 4.

Five to ten micrograms of chromosomal DNA were restricted at 3.0 units enzyme per microgram. Time of digestion was from 12 to 16 hours at 37°C. Restriction reaction vessels were 1.5 ml polypropylene (Eppendorf) snap cap tubes. The reactions were stopped by heating to 65°C

for 10 minutes.

Plasmid restrictions were performed in a similar manner; however stock concentrations varied. Only 1 to 2 ug were digested per reaction.

Gel Electrophoresis

Agarose gel electrophoresis was conducted in a horizontal configuration when the DNA was to be transferred to nitrocellulose. The gel apparatus was 22 x 13 cm and could accomodate two 10 cm long slab gels simultaneously (Aquebogue, Long Island). The comb had twelve teeth and formed wells capable of containing a 50 ul sample. The loading buffer was 5% glycerol, containing 0.025% of both bromphenol blue and xylene cyanol. Running buffer was 89 mM tris (pH 8.3), 89 mM boric acid and 2.5 mM Na_2EDTA, with 0.2 to 0.5 ug/ml ethidium bromide. Agarose was type II low endo-osmotic (Sigma) and was added to 150 ml of running buffer to form an 0.75% solution after boiling and agitating on a magnetic stirrer with heating element. The resulting solution was de-gassed with a vacuumm pump. When the solution cooled to 50°C it was poured onto the electrophoresis apparatus gel base and allowed to solidify at room temperature. Running buffer was poured over the gel slab, which was then allowed to age overnight at 4°C to form a proper matrix. Prior to sample loading, pre-electrophoresis of the gel was at 10 mA for 30 minutes.

Sample migration was from cathode to anode, generally at 35 mA and 100 volts; occasional overnight runs were at 9 mA. The gel was stopped when the bromphenol blue marker was within 1 cm of the gel end. Because of the presence of the fluorescent dye, ethidium bromide, within the running buffer, the gel could be examined immediately over U.V. transilluminator (C-63 [Ultraviolet Products]). The restricted DNA pattern was visualized by light emission at 590 nm in the red-orange region of the visible spectrum. The gel was routinely photographed by a Polaroid MP-4 system onto Polaroid Type 57 film (ASA 3000) through Wrattan 25A and 8 gelatin filters (Kodak). If a negative were required, Polaroid Type 55 P/N film (ASA 300) was used with much longer exposure time.

For rapid visualization of plasmid DNA or RNA, samples were run on a vertical gel apparatus (12 cm x 15 cm) (Aquebogue, Long Island). In this case, at 100 volts the bromphenol blue marker migrated to one cm of the bottom in about two hours. The gel running buffer contact was at two reservoirs at the top and bottom of two 12 x 15 plates of glass that held the gel. Current was therefore carried entirely by the gel matrix in contrast to the previous submarine mode. Combs formed loading wells at the gel top, the finest of which provided 20 lanes. Running buffer was 40 mM tris (pH 8.1), 20 mM acetic acid, and 2 mM Na_2EDTA with ethidium bromide included. Loading buffer and U.V. visualization were as described. On a 0.75% gel of this

type, as little as 100 to 200 ng of nucleic acid sample could be visualized in a lane.

Polyacrylamide gel electrophoresis was used to visualize the extent of rRNA hydrolysis prior to T4 kinase end labeling. Ten percent gels were poured between two (12 x 15 cm) pieces of glass held apart by plastic spacers and were run in the vertical mode. Stock solutions of 30% acrylamide consisted of 29% acrylamide and 1% N,N'-methylene bis acrylamide dissolved in deionized water. Stocks of 3% ammonium persulfate in deionized water were kept no more than one week at $4^{o}C$. Phosphate running buffer, 50X stock, was made of 500 mM mono- and di-basic sodium phosphate mixed in proportions to give pH 7.0. To fill the gel plates, 50 ml of polyacrylamide solution were made from stock solution, employing the following proportions: 16.7 ml 30% acrylamide, 1.1 ml 3% ammonium persulfate, 1 ml 50X phosphate stock, 31.2 ml deionized water. To start polymerization, 15 ul TEMED (N,N,N',N'-tetramethylethylene diamine) was added to the 50 ml. This was poured into the space between the two glass plates, in which the small gaps around the plastic spacers had been sealed with agar. Polymerization was at room temperature for 60 minutes. The gel was run at 80 to 100 volts and was stained after electrophoresis by submerging in 1X tris-borate buffer with 0.5 ug/ml ethidium bromide for 60 minutes.

Low melting point agarose (BRL) was used to extract electrophoretically separated restriction fragments of

plasmids. LMP agarose does not have as great a gel strength as standard agarose at identical concentrations; therefore, 1% gels were employed, and were carefully cooled to 50°C before pouring to prevent shrinking and subsequent cracking. Otherwise, running and loading buffers were the same as with conventional submarine gels. Occasionally, minigels were run in the submarine mode, with a gel tray of 12 x 8 x 1 cm filled to 40 ml with LMP agarose, and submerged in the horizontal gel apparatus.

DNA fragments were visualized by U.V. transillumination and desired gel regions were excised and heated (65°C) in a polypropylene screw-top scintillation vial until the agarose melted. Two volumes of 0.5 mM Na_2EDTA and 0.05 M tris-HCl (pH 8.0) were added to the melted gel and the solution was transferred to a 37°C water bath. An equal volume of phenol saturated with 0.1 M tris HCl (pH 8.0) was mixed with the melted gel. Centrifugation in an Eppendorf microfuge for 5 minutes caused the agarose to form an insoluble layer at the interface of the aqueous and organic phases. The ethidium bromide partitions into the phenol phase. Residual phenol is removed from the aqueous phase by extraction with an equal volume of ether. Sodium acetate (pH 7.0) is added to the aqueous phase to a final concentration of 0.3 M DNA was precipitated by addition of 3 volumes of ice-cold ethanol and left in a slurry of dry ice and acetone for at least 15 minutes. The ethanol was poured off and residual amounts were removed in a desic-

cator jar with vacuum applied. Resuspension of the sample was in 10 mM tris-HCl (pH 7.5), 0.1 mM Na_2EDTA, 4 mM NaCl.

Extraction of Radiolabeled DNA from Agarose Gels

Radiolabeled DNA was electrophoresed through 0.75% agarose gels. The gel lane was excised and sliced into 1 mm pieces with an automatic slicing apparatus (Mickel). Each gel fragment was dissolved in 0.2 ml NH_4OH at $80^{\circ}C$ followed by scintillation counting in Bray's solution.

Southern Blot Transfer

DNA fragments separated according to size by electrophoresis through an agarose gel were transferred to a nitrocellulose filter and immobilized, preserving their relative position (Southern, 1975). Each gel lane was loaded with 0.5-2.0 ug of restricted chromosome. Ethidium bromide (0.2 to 0.8 ug/ml) was included in the buffer and after electrophoresis each gel was photographed under short-wave U.V. light, as described. Lambda fragments of HindIII cleavage were run on each gel as molecular weight standards and their migration was measured in millimeters from the well base. They were left on the gel for nitrocellulose transfer.

The gel slab was soaked in 500 ml 1.5 M NaCl and 0.5 N

NaOH with agitation for sixty minutes. It was neutralized in 1 M tris-HCl (pH 7.5) and 1.5 M NaCl for one hour with constant shaking. The gel was placed on top of a wick support of a blot transfer system (BRL model H_2/H_3). A strip of Whatman 3M paper under the gel made contact with 20X SSC in the blot system reservoir. A nitrocellulose sheet (B85, Schleicher and Schuell) was cut to the size of the gel and soaked in deionized water. This was placed on top of the gel and air spaces between the gel and nitrocellulose surfaces were squeezed out by rolling over with a 2 ml glass pipet. One sheet of 3 mm paper (the same size as the filter) was placed on top of the nitrocellulose filter. A stack of paper towels about 6 to 7 cm in height was placed on top of the nitrocellulose. The stack was compressed with a piece of plastic on top of which was placed a 200 g glass sheet. Transfer occurred for 12 to 24 hours.

After transfer the nitrocellulose was peeled away from the compressed gel matrix and soaked for 10 minutes in 2X SSC. It was soaked another 10 minutes in 0.5X SSC and dried with a hand-held hair dryer. The filter was then baked for two hours at $80^{\circ}C$.

Hybridization

Filter hybridization was performed using the conditions of Ostapchuk and Riley (1980). The baked filter

was floated on the surface of 4X SSC and wetted from beneath. It was then placed in a heat-sealable plastic bag (Daisy "Seal-a-Meal") and 2 to 5 ml of prehybridization solution was added. As much air as possible was removed from the bag. Prehybridization solution was 40% formamide, 4X SSC, 50 mM sodium phosphate buffer (pH 6.5), 250 ug/ml sonicated calf thymus DNA, 1% glycine and 1/20 volume 100X Denhardt's solution (0.02% each Ficoll, polyvinylpyrolidone, and BSA, prepared as 100X stock and stored at -20°C until use).

The nitrocellulose was prehybridized from one to twelve hours at 42°C in a water bath. After this time, the prehybridization fluid was squeezed out of the bag after cutting off one corner. For each 100 cm^2 of filter, 4 ml of the following hybrid solution was prepared: 40% formamide, 4X SSC, 20 mM sodium phosphate (pH 7.5), 100 ug/ml sonicated calf thymus DNA, 1/100 volume 100X Denhardt's solution and 10% Dextran sulfate. When the labeled probe used was RNA, 0.1% SLS was included. Inserted into each hybridization bag was 5-200 ng ^{32}P-labeled probe either nick-translated (if DNA) or end labeled (if RNA). In general, each 100 cm^2 filter was hybridized with about 1 to 2 x 10^6 cpm of probe. The bag corner was resealed and hybridization was at 42°C for 12 to 24 hours.

After hybridization the bag was sliced open and the filter was removed. It was washed successively for 15 minutes in the following: a) 4X SSC-0.1% SLS b) 2X

SSC-0.1% SLS c) 3 mM tris-HCl (pH 7.5)-0.1% SLS. Drying was with the hand-held hair dryer and the filter was wrapped in Saran wrap to prevent it from adhering to the X-ray film during exposure.

The wrapped filter was placed in an X-ray cassette box (Picker) with top and bottom Cronex Lightening plus intensifying screens. The temperature at which filming took place was determined empirically. If a hand-held radiological survey meter recorded 1000 or less cpm when the probe was about 2 cm from the filter surface, filming was at -70°C for 12 hours. With more than 1000 cpm, filming was at room temperature for no more than 12 hours. Kodak X-omat AR film was used. With intensifying screens, the majority of events recorded by the film are long-wave photons resulting from fluorescence when ^{32}P decay beta particles strike the screen. Exposure at -70°C prolongs the period of fluorescence (Laskey and Mills, 1977).

Northern Blots Transfer

Transfer of ribosomal RNA to nitrocellulose filters was performed according to Thomas (1980). RNA (1-2 ug in a 15.3 ul volume) was added to dimethylsulfoxide (25 ul). This was followed by the addition of 7.1 ul of 7 M glyoxal. The mixture was incubated at 50°C in a polypropylene tube, after which the sample was loaded and run on an agarose gel (0.75%, phosphate running buffer).

Molecular Weight Determinations

Molecular weights of restriction fragments were calculated by comparison with known size fragments of the same ionicity. Plots of mobility versus molecular weight raised to the -0.666 exponent use the relationship of Bearden (1979). Graphic display is linear over a greater size range than with the conventional inverse of logarithm (base 10) (See Figure 2). Lambda DNA restriction with Hind III produces seven fragments which serve as size standards. These could be transferred to Southern blot filters along with the sample DNA, and nick-translated lambda DNA was included in hybridization buffers. This produced an accurate size measure on each blot and effects of slight gel shrinkage during transfer could be ignored. Ultimately in this study, the best molecular weight standards were provided by EcoRI restricted chromosomal DNA (strain NCTC 3610/168) hybridized to 23S rRNA sequences. Once these fragment size values were accurately determined, comparison to DNA of other species was employed. It produced 13 hybridization bands from 9.0 to 0.9 kilobases in size (See Figure 12A). Figure 2 illustrates employment of the Bearden relationship to six lambda HindIII fragments electrophoresed on a 0.75% agarose gel. Linearity is evident except at largest fragment size. The seventh lambda HindIII band (about 480 base pairs) usually ran off the gel. Within the

linear range, measurements could easily be made to within 100 base pairs DNA on the 10 cm length gels used.

Nick Translation

Labeling by nucleotide replacement synthesis was based on the procedure of Rigby *et al*. (1977). Reactions were in a buffer of 50 mM tris-HCl (pH 7.5), 10 mM $MgSO_4$, 1 mM DTT, and 50 ug/ml BSA at a volume of 25 to 100 ul. The stock solution of pancreatic DNAase I (Worthington) was at 1 mg/ml in 50 mM tris (pH 7.5), 10 mM $MgSO_4$, 1 mM DTT and 50% glycerol stored at -20°C. At the time of use, this was diluted 1:200 in dilution buffer [50 mM tris (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DTT and 50 ug/ml BSA]. A further dilution was dependent upon the duplex length of the DNA fragment to be labeled. A final concentration of 0.025 ug/ml was used for duplex length less than 5 kilobases, 0.0025 ug/ml if the DNA duplex was 5 to 20 kilobases.

DNA, 0.5 to 2 ug, was added to a 1.5 ml polypropylene reaction tube which contained 100 to 200 picomoles each of α-^{32}P-dATP and α-^{32}P-dCTP, aqueous stock solutions. Tritiated (methyl-^{3}H) deoxythymidine triphosphate for analysis of nick-translation kinetics was desiccated from a 1:1 ethanol:water stock prior to reagent additions. Cold deoxyribonucleotides were kept at 10 mM stocks in 10 mM tris (pH 7.0) and 500 to 1000 pmol of these (other than the labeled species) was added to the reaction mixture.

Sterile water was added to bring the reaction to the appropriate volume, and 0.012 or 0.0012 ng of previously diluted DNAase I was added. One to ten units of E. coli polymerase I was added and the reaction was incubated at 14°C for 2 hours. For kinetic studies, 1 to 3 ul of the ^{3}H-methyl TTP labeled reaction was removed at timed intervals and added to 0.2 ml of sonicated calf thymus DNA at 200 ug/ml, followed by precipitation with 1 ml of 6% trichloroacetic acid.

The reaction was stopped by adding an equal volume of stop solution consisting of 0.02 M Na_3EDTA, 2 mg/ml sonicated calf thymus DNA, and 0.2% SLS.

Recovery of Labeled Nick Translated Product

The reaction was loaded on a 0.7X 20 cm Sephadex G-50 (medium) column pre-equilibrated with TE buffer (10 mM tris and 1 mM Na_3EDTA at pH 7.5). Column washing was with TE buffer by gravity flow from a reservoir above the column. One half ml effluent samples were collected with DNA eluted after about 2 ml of wash. The location of the ^{32}P-labeled DNA was noted with a hand monitor (radiological survey meter). Alternatively, 0.5 ul samples from the fractions were withdrawn and counted in glass scintillation vials containing 5 ml biolfluor. Two radiolabeled peaks could be noted: the first peak containing labeled DNA was saved and pooled from the fractions (See Figure 9); the

second peak of unincorporated nucleotides was discarded.

An alternative method to Sephadex chromatography involved ethanol precipitation of labeled product. The nick translation reaction was brought with sterile water to 100 ul (if not already at this volume). Carrier yeast tRNA was added to 25 ug/ml concentration. One hundred ul of PIC solution (50% phenol, 2% isoamyl alcohol, 48% chloroform) was added and the reaction mixture vortexed. The two phases were separated in an Eppendorf microfuge and the aqueous phase was withdrawn into another polypropylene tube. To the PIC phase 100 ul of 4 M NH_4 acetate was added to remove residual labeled product, and vortexed. The aqueous phase was again separated by centrifugation and added to the first aqueous pool. Extraction with NH_4 acetate was performed on the PIC once more to produce an aqueous phase of 300 ul. The labeled nucleic acids were precipitated with 0.7 ml absolute ethanol in a dry ice-acetone slurry for 30 minutes. The nucleic acid was pelleted by 15-minute centrifugation. The pellet was washed once with 70% ethanol, and collected with a final 5-minute centrifugation and resuspended in 10 mM tris (pH 7.5), 4 mM NaCl and 1 mM EDTA.

The labeled DNA was denatured for hybridization studies by adding 0.1X volume 1 M NaOH for 10 minutes at 25°C. This was neutralized with 2.0 M tris-HCl (pH 7.5). The specific activity of the product obtained was from 0.5 to 2 x 10^8 cpm/ug.

Ribosomal RNA Isolation

B. subtilis 168 cells were grown in VY medium to a Klett of 150 to 200 units. The culture volume was 100 to 250 ml. Pellet mass was about 0.2 g after washing in 0.01 M tris, 0.01 M acetate at pH 7.4. RNA was isolated according to Margulies *et al*. (1971). The pellet was ground with mortar and pestle with 2X pellet weight of alumina (Type 305, Sigma). The alumina cell paste slurry was resuspended in 3.0 ml 0.1 M tris, 0.005 M magnesium acetate pH 7.4. This was centrifuged 20 minutes at 10,000 RPM in a refrigerated Sorvall model 2C centrifuge. To the supernatant was added 0.1 ml of 100 ug/ml DNAase. Incubation was for 5 minutes at 4°C. Debris was removed by centrifugation for 20 min at 10,000 RPM. The ribosomes were pelleted by centrifugation of the supernatant at 50,000 RPM for 60 minutes at 4°C. The ribosome pellet was resuspended in 0.9 ml 0.01 M tris, 0.005 M Mg acetate, 0.005% SLS (sterile filtered). Two extractions were performed with 90% phenol, 10% 0.01M tris, 0.005 M Mg acetate. Each phenol extraction was followed by an ether extraction. Ribosomal RNA was precipitated overnight at -20°C in the presence of 2% potassium acetate with 2X volume 100% ethanol. The pellet was stored under ethanol until needed.

Agarose Gel Separation of Ribosomal RNA Species 16S, 23S

and 5S

Low melting point agarose (low EEO) was made to 1% in 10 mM phosphate buffer, pH 7.0. Ethidium bromide (10 ug/ml stock solution) was added to 0.2 ug/ml. This was boiled on an electric heater, with gentle stirring, until dissolved. Forty milliliters of this solution were poured into a U.V. transparent tray (12 x 8 x 0.75 cm) of a horizontal gel apparatus and allowed to gel completely overnight. A ten-tooth comb was used to make a well capable of accommodating a 25 ul volume. Ethanol precipitated rRNA was re-pelleted in an Eppendorf centrifuge and resuspended in the 0.01 M tris, 0.005 M Mg acetate, 0.005% SLS buffer to a concentration of 20 ug/ml (0.5 /25 ul) (1 mg/ml = 24 OD_{260nm}.) Twenty-five ul samples in a solution of 5% glycerol and marker dyes (0.001% bromphenol blue, 0.001% xylene cyanol) were loaded into wells. The running buffer was the same as that used to dissolve the agarose. Each gel was used to separate at most 5 ug rRNA. Gels were run at 100 volts and 35 maA. When the bromphenol blue dye had migrated 6 to 7 cm the current was stopped and the rRNA visualized by U.V. transillumination. 23S and 16S species ran between the two dyes and the 5S species ran ahead of the bromphenol blue marker (See Figure 7).

RNA Extraction from the Low Melting Gel

The procedure for nucleic acid isolation from low melting agarose was modified as follows for RNA extraction. Appropriate strips of gel containing each separated species were excised and placed within a 1.5 ml polypropylene tube. The agarose was melted at 65°C and 0.5 ml of 0.01 M tris, 0.005 M Mg acetate, 0.005% SLS (sterile filtered) was added. Extraction was with 90% phenol, 10% tris-Mg acetate, SLS. High-speed centrifugation in the Eppendorf microfuge partitions the ethidium into the phenol phase and the agarose as an interface layer. Two ether extractions of the upper aqueous phase removed residual phenol. RNA was precipitated in the presence of 2% potassium acetate and stored under ethanol at -20°C until needed.

The recovery of rRNA was checked after resuspending the ethanol precipitated pellet in the tris, Mg acetate, SLS buffer and running an aliquot estimated to contain 0.5 to 1.0 ug through a vertical 1% agarose gel (12 x 15 cm) under the same phosphate buffer conditions with 0.2 ug/ml ethidium bromide. Electrophoresis continued until the bromphenol blue marker migrated about one-half of the gel distance, and visualization was by U.V. transillumination. RNA concentration was estimated by comparison with an unfractionated sample of known concentration run in another gel lane. In general, the recovery process resulted in the species breaking down to the 16S size position. This was not of consequence as the partial sequences represented 23S rRNA and ultimate rRNA labeling procedure involved exten-

sive hydrolysis of the nucleic acid, as will be described.

End Labeling of RNA

The procedure for ^{32}P labeling of rRNA is that of Maizels (1977). One microgram of a single rRNA species was hydrolyzed in 5 ul of 50 mM tris, pH 9.5, at 90°C in an 0.5 ml polypropylene tube. Hydrolysis was for 10 to 20 minutes and the size of degradation product was calibrated on a 10% acrylamide gel (Peacock and Dingman, 1968). Molecules about 100 bases in length migrated between bromphenol blue and the xylene cyanol dye markers (See Figure 3). Hydrolysis of 1 ug of RNA to this length produces about 30 pmol of 5'-hydroxyl ends. RNA fragments were then added to a 1.5 ml polypropylene tube containing 30 to 100 pmol of -^{32}-ATP (previously desiccated to dryness). The reaction conditions were brought to 50 mM tris, pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 5% glycerol at a final volume of 10 to 15 ul. One unit T4 polynucleotide kinase was added and the reaction ran for 30 minutes at 37°C.

The labeled RNA was separated from unincorporated -^{32}P-ATP by chromatography on a 1 ml Sephadex G-50 column. The elution buffer was 10 mM tris, pH 8.0, 0.1 mM EDTA. The labeled product was then precipitated with 0.5 ug yeast tRNA carrier in the presence of 2% potassium acetate with 100% ethanol. It was stored in a lead shielded container at -20°C until use. Specific activity

obtained ranged from 1 to 3 x 10^7 cpm/ug.

Scintillation Counting

Determinations of radioactivity were on a Beckman LS-800 counter with an automatic quench compensating program. Counts per minute obtained are corrected for sample quenching by external excitation from a cesium 137 control source. Samples precipitated with cold 6% trichloroacetic acid (TCA) were applied to glass fiber discs (2.4 cm diameter, GF/C) with suction through a Millipore apparatus. The filters were washed three times with 15 ml cold 6% TCA followed by two 3 ml washes with 95% ethanol. Filters were air dried and placed in borosilicate scintillation vials for counting. Filter bound samples were counted in BBOT/toluene, prepared at a concentration of 4 mg/ml. An external tritium standard had a specific activity of 1.738 x 10^4 cpm/10 ul. This allowed a machine efficiency of 48.75% to be calculated. Liquid samples were counted in 5 ml Aquasol (Beckman).

Spectrophotometry

A Gilford model 250 spectrophotometer equipped with an automatic cuvette positioner model 2451-A was used for optical measurements. Cuvettes were quartz with a 1 cm path length. One mg/ml RNA is equal to 24 OD_{260} units and

1 mg/ml DNA is equal to 20 OD_{260} units. DNA free of protein contamination gives an $OD_{280/260}$ ratio of 0.5.

Stock solutions ribo- and deoxyribo-nucleotides were prepared optically in 10 mM tris-HCl (pH 7.0). For a cell with a 1 cm path length, absorbance = /M, where is the molar extinction coefficient. Values for were given by the nucleotide supplier (P.L. Biochemicals).

Densitometry Measurements

Autoradiogram films were scanned for hybridization intensity. A linear transport drive with a holder for X-ray film strips was inserted between the spectrometer monochromatic light source and the spectrophotometer. The scan rate could be varied from 1 to 4 cm per minute. Densimetric measurements were made at 580 nM. Scan profiles were made by a recorder model 6051 (Gilford) equipped with a "pyr-o-scribe" (heated stylus) device, onto heat-sensitive chart paper (Honeywell). The chart was set to run at 20 cm/minute. The paper calibration grid was from 0 to 100 with major horizontal time lines spaced 7 cm apart. The covered range in OD units represented by the vertical 0 to 100 pattern was set on the spectrometer with a range control (See Figures 11 and 19).

Peak area was determined with a planometer (Noris Instruments, Germany) equipped with a mobile cursor to scan the densimetric tracings. Relative areas of the peaks are

reported in planometer units measured, since absolute optical density units per radiogram were not needed.

RESULTS AND DISCUSSION

I. Selective Transformation of the Bacillus Chromosome

This study begins with an analysis of the conserved "core" of genetic markers with eight strains and species of the genus *Bacillus*. Table 5 presents the results of interspecific transformations. Most clearly heterologous DNA transformation of *B. subtilis* 168 was virtually non-existent for the tryptophan or threonine markers. For genes A, C, B of the leucine operon, transformation with *B. globigii* DNA ranged from 2.5 to 5.4 x 10^3 transformants per ml. Frequency for the *arg15* marker was 4.1 x 10^2 transformants, significantly higher than the results of Chilton and McCarthy (1969), who report insignificant interspecific transformation for this marker. The *ilvC* marker displayed 1.3 x 10^3 transformants per ml and phenylalanine at about the same frequency. These markers are found on the *Bacillus subtilis* chromosome in the 260 to 245 degree region (Henner and Hoch, 1980) in the order *arg*-*ilv*-*leu*-*phe*. Rifampicin and streptomycin markers were donated by *B. pumilus* RUB502 and *B. licheniformis* 8480, respectively, in interspecies crosses. In comparison to transformation with *B. subtilis* W23 for streptomycin resistance (*str*^{r}) the *B. licheniformis* donor displayed 0.04 the homologous frequency. The *strA* marker is an alteration in ribosomal protein S12 and is at 10 degrees on

the B. subtilis chromosome map (Dubnau et al., 1969). The strB and C markers are at 130 and 220 degrees respectively (Henner and Hoch, 1980) and display the resistant phenotype only during vegetative growth phase. Sensitivity appears during sporulation and germination (Staal and Hoch, 1972). The mode of action of these two markers is not clear and they could be a permeability mutant as well as a ribosomal one (Staal and Hoch, 1980). We did not identify which of the three markers was transformed in this experiment.

B. subtilis strain BD29 contains the leu-1 and argA2 markers. The leu-1 marker is a lesion in leucine gene B (S. Zahler, personal communication). It was isolated by Barat et al. (1965) and we heretofore refer to it as leuB1. It could be complemented in transformation crosses with DNA containing the leuA marker. The argA2 marker was complemented by the arg15 marker donated by strain RM125 (leuA8, arg15). B. pumilus RUB562 and B. licheniformis 8480 display a very low ability to transform the leu-1 marker, yet it is above the reversion level which is essentially zero (Table 5).

The second gradient of conservation was postulated (Chilton and McCarthy, 1969; Smith et al., 1968) to be mediated by sequences designating components of the essential translational apparatus, ribosomal RNA in particular. The presence of such conserved sequences similarly affected other markers in the vicinity. Harris-Warrick and Lederberg (1978) noted heterologous DNA could be made to behave

in transformation as homologous DNA if it were "flanked" by recipient homologous sequences. Conceivably, such a process could operate in a natural fashion using ribosomal DNA as a "universal" source of homology which flanks particular markers (i.e., *arg*, *ilv*, *leu*, and *phe*).

II. Analysis of Conserved Regions of the Bacillus Chromosome

The degree of nucleotide sequence conservation in the vicinity of the rRNA gene sets was assessed. The number and physical map of rRNA gene sets in the genome of *Bacillus subtilis* strain 168M has been reported (Stewart *et al*., 1982), and this served as a basis of comparison for the other seven species and strains of *Bacillus* which we examined (See Fig. 6).

As shown biologically, the markers with the greatest degree of conservation were those associated with the translational apparatus. Similarly, the genomic sequences coding for the ribosomal RNA are strongly conserved among species (Introduction). *In vitro* labeled rRNA of *Bacillus subtilis* and several probe rDNAs were used to explore the relatedness of select segments of the genomes. We used the Southern hybridization technique of restricted chromosomal DNA as our assay and it proved to complement the transformation results presented. The hybridizations were performed using *in vitro* labeled nucleic acid probes.

A. Labeling of the 5' ends of hydrolyzed rRNA

T4 polynucleotide kinase was used to transfer the γ-phosphate of γ-^{32}P-ATP to a 5'-OH terminus in rRNA previously hydrolyzed. Degradation of the rRNA species to about 100 base pairs in length produces about 30 pmol of 5-OH ends (Maizels, 1977; Materials and Methods). Figure 3 is a 10% acrylamide gel on which were run aliquots from the progressive hydrolysis of a fractionated sample of 23S rRNA. The hydrolysis was at 90°C, pH 9.5, from 0 to 30 minutes. As the labeled polynucleotides were to be used as Southern hybridization probes, disruption to this small size was permissible. Figure 4 represents the time course for the labeling of 1 ug of hydrolyzed 23S rRNA, with γ-^{32}P-ATP at 10 uM concentration. The reaction reached plateau after about six to ten minutes. Routinely, 5 units of T4-polynucleotide were used. One unit is defined as that catalyzing the transfer of one nanomole of phosphate from ATP to the molar equivalent polynucleotide in 30 minutes at 37°C (Richardson, 1971). Therefore our reactions contain an enzyme excess. Purification of the labeled RNA was by exclusion chromatography through a 1 ml Sephadex G-50 column. A typical column profile is presented in Figure 5. The percent nucleotide incorporation is seen to be 27%. The first column peak represents labeled polynucleotide. To insure no unincorporated nucleotides in the hybridization product, the first peak was precipitated in 100% ethanol with 2.5 ug yeast tRNA carrier prior to use.

B. The structure of the cloned ribosomal DNA sequences

The initial Southern blot hybridizations all employed rRNA end labeled probes. This procedure resulted in at most 1 to 2 x 10^7 cpm per microgram specific activity of nucleotide. The single-stranded RNA is exceptionally susceptible to degradation and often the 16S and 5S rRNA isolates were contaminated by fragments of the larger species during isolation. This problem was controlled in hybridization solutions by adding unlabeled competing RNA of the contaminating species, usually at 10X concentration of the probe. Needless to say, such a procedure consumed much rRNA and was cumbersome. The best way to alleviate this problem was through the use of cloned ribosomal DNA sequences. These could be labeled to high specific activity by nick-translation (up to 1 to 2 x 10^9 cpm per microgram) and the plasmid isolation procedure was rapid and easy, yielding a homogenous product. Finally, the clones we chose to use share homology with select portions of the *B. subtilis* rRNA gene sets, thus enabling regions internal to the cistrons to be visualized. The following plasmids were used as a source of cloned rDNA in this study. Figure 6 displays only the *B. subtilis* chromosomal inserts of each lined up next to the restriction map of a rRNA gene set determined by Stewart *et al*. (1981).

Plasmid p21C4-16S: The rDNA insert is derived from a *B. subtilis* gene bank made by Hutchinson and Halverson

(1980). Random cloning was performed from sheared fragments of chromosome employing the pMB9 plasmid vector (tetracycline resistance). DNA was inserted by the oligo(dA)-oligo (dT) tailing method at the HpaI site of the vector. The plasmid selected for this study contains the region around the EcoRI site in the 16S rRNA determinant. It served as a good hybridization probe for any enzyme chromosomal digest except EcoRI. Because of the nature of construction, the exact size of the oligo(dT) tailed fragment is undetermined. A kpnI site reported to be about 200 base pairs "leftward" of the 16S EcoRI site is not on it (G. Stewart, personal communication). Therefore, it is a poor probe in EcoRI restrictions. This plasmid was maintained in *E. coli* strain HB101.

Plasmid p12E2-23S,5S: This clone was constructed by ligating BamHI-digested *B. subtilis* DNA into the BamHI site of the *E. coli* plasmid pBR313. The resulting plasmid was identified as a clone carrying *B. subtilis* rDNA sequences by *in situ* hybridization with ^{32}P-labeled *B. subtilis* rRNA (Moran and Bott, 1979). Restriction fragments homologous to either 23S or 5S rRNA were identified upon Southern blot sheets. As noted in Figure 6, the rightward part of the insert carries regions homologous to 3.5 Kbp of interoperon (spacer) DNA, distal to the 5S determinant. Bott, Wilson and Stewart (1981) identified this rRNA sequence as one derived from between the *purA* and *cysA* markers on the chromosome. They designated it

rrnA. This clone served as a good probe for 23S sequences up to the 23S BamHI site, and it included all the 5S sequence.

Plasmid pBC279-23S,5S: The chromosomal fragment within this chimera was originally subcloned by Stewart, Wilson and Bott (1982) from a lambda charon 4A gene bank of B. subtilis (Ferrari et al., 1981). The EcoRI fragment contains the 3' last half of the 23S sequence plus all of the 5S gene.

Plasmid pMS102-B7'-16S,23S: Plasmid pMS102-B7 carries a BamHI fragment from the origin of replication region of the B. subtilis 168M chromosome. This region is homologous with 16S and 23S rDNA sequences. It is a bifunctional plasmid with ability to replicate in either B. subtilis or E. coli. This unusual chimera was constructed by Seiki, Ogasawara and Yoshikawa (1981), from whom it was obtained. Replication of this plasmid in B. subtilis is supported by the origin of replication determinant of the pUB110 part of the plasmid, which also imparts a kanamycin resistance determinant. Replication in E. coli is due to the pBR322 portion of the vector which also carries the determinant for ampicillin resistance. The insert carries 3.5 Kbp of "spacer" DNA sequence proximal to the 5' end of the 16S sequence. This plasmid was recently determined by Ogasawara, Seiki and Yoshikawa to contain tRNA sequences in the region between the 16S and 23S determinants. The ribosomal gene set it carries is designated rrnO.

Plasmid p14B1-23S,5S,16S: This clone contains fragments from two gene sets separated by a spacer sequence (0.5 Kbp). The insertion is derived from BamHI cleaved *B. subtilis* chromosomal DNA. The rDNA sequence it carries includes the total rRNA determinants but not in their native order (Stewart *et al.*, 1982).

The presence of rDNA insertion sequences in all these plasmids was proven by Northern blotting. Glyoxal denatured rRNA was transferred after electrophoresis to nitrocellulose sheets. Hybridization conditions were as described in Materials and Methods. The results presented in Figure 7 indicate the cloned fragments have the correct rDNA sequences.

C. Nick translation of DNA

High specific activity probes were made from the ribosomal DNA cloned on *E. coli* plasmids. We exploited the ability of DNA polymerase I to add nucleotide residues to the 3'-hydroxyl terminus created when one strand of a double-stranded DNA molecule is nicked. Replacement of pre-existing nucleotides with radioactive $^{32}P(\alpha)$-labeled dNTPs as described in the Materials and Methods section is by the procedure of Rigby *et al.* (1977).

Preliminary assays with lambda DNA and plasmid pUB110 were performed prior to actual hybridization probe preparation to determine optimum conditions with our materials for the nick translation. Measurement of tritium incorporation

(TTP-methyl-^3H) into plasmid pUB110 and commercial stocks of lambda phage DNA produced the following parameters subsequently followed. Plasmid pUB110 (1 ug) served as a control template in these initial reactions. Nicks to introduce internal 3'-OH termini were created with 0.025 ng DNAase I in this reaction. As seen in Figure 8, the reaction proceeded at the greatest rate and appeared to plateau after about two hours at 10°C, with 5 to 10 units of polymerase. The radioactive nick translation product was purified by Sephadex G-50 exclusion chromatography from unincorporated dNTPs and the distribution of the 10-unit reaction appears in Figure 9. The excluded high molecular weight material calculated by summation of cpm within each peak (or planometer area integration) amounted to 45% incorporation. The second peak represents unincorporated nucleotide. Rigby _et al_. (1977) report the method produces 25 to 50% nucleotide replacement in DNA molecules, indicating our conditions are optimal.

These initial studies and previous work in our laboratory (Tackney, PhD dissertation, 1982) employed TTP, either ^3H- or ^{32}P-labeled, and the question arose as to uniformity of labeling with only this one nucleotide. One microgram of lambda phage DNA was nick-translated in a similar manner with ^3H-TTP. After purification by exclusion chromatography, the labeled product was completely digested with endonuclease HindIII and electorphoresed through 0.75% agarose. The gel lane was excised and sliced

into 90 1-mm pieces in which the radioactivity was estimated. Figure 10 is a gel profile in cpm of the seven lambda peaks so obtained. The cpm constituting each peak were summed. Table 6 presents the molecular weight of six of the lambda pieces. Designating the 2.0 Kbp fragment as a unit, the multiplicity of the five larger peaks was determined. Table 6 presents the idealized molecular weight ratio of each fragment in relation to the 2.0 Kbp piece. The tritium-incorporated fragments do not show the appropriate ratios, indicating non-uniformity of labeling (Table 6). Assuming non-biased incorporation would result by nick translating two non-complementary precursors, deoxy-ATP and deoxy-CTP (α-^{32}P) were used for all hybridization probe nick translations. One microgram of lambda HindIII restricted DNA was transferred to nitrocellulose by the Southern procedure. The α-^{32}P-dATP, dCTP-labeled lambda served as the hybridization probe. The resulting autoradiogram was scanned by our densitometer and peak areas measured wtih the planometer (Figure 11A). Fragment ratios now better approached the idealized standard (Table 6). As a control for the planometer measurements, 1 ug of lambda (also HindIII digested) was electrophoresed and the gel stained with 0.2 ug/ml ethidium bromide. A photograph of the stained bands was made on Polaroid type 55 negative film and densitometry peaks were measured (Figure 11B). The areas so obtained also better conform to the idealized ratios than do the tritiated results (Table 6). The

intercalated fluorescent stain shows no base pair bias, and intensity is seen to be related to the molecular weight of the fragment. Labeling with both a purine and pyrimidine deoxynucleotide compensates for non-uniform distribution of the bases within our hybridization probes.

Skalka (1966) reports the left and right halves of the linear molecule contain 55% and 45% GC base pairs respectively. Maniatis and Ptashne (1973) observed concentrations of AT base pairs within the left and right operator sites bound by CI repressor molecules. Reference to the lambda DNA restriction map (Maniatis, 1982), reveals this region to be carried by the 6.19 Kbp, HindIII fragment. Figure 11A shows "fusion" of the lambda HindIII peaks, while they seem completely separated in the fluorescent photograph scan (11B). Band width is somewhat larger on the radiograms but the "missing" slopes were extrapolated for the area determinations.

D. Conservation of ribosomal RNA genes among members of the genus Bacillus

To characterize conserved sequences, Bacillus species chromosomes were probed with 16S, 23S and 5S rRNA or with the above cloned rDNA segments. After a Southern transfer of electrophoretically separated restriction endonuclease fragments, hybridization patterns were obtained with the α-^{32}P-

dATP and α-^{32}P-dCTP or γ-^{32}P-ATP labeled hybridization probes. Unique and reproducible multiple-band patterns were noted with each restriction endonuclease. The results of B. subtilis strain 168T were used as a basis of comparisons with the other species and strains. 168T is an indole-negative mutant of the classic 168 Marburg strain.

As noted in Tables 7, 8, 9, 10 and Figures 12, 13, 14, 15, regardless of the restriction endonuclease used the NCTC 3610 pattern was identical to the 168T pattern. All 168M type strains, with three exceptions (discussed in a later chapter), behaved as did NCTC3610/168T. As with the phage host range results reported by Hemphill and Whiteley (1975), this provides equally strong evidence favoring the classic Marburg strain as the direct ancestor of current 168M strains. On the other hand, B. subtilis strain W23, although a suitable DNA donor in transformation experiments, displays hybridization patterns different from the Marburg group.

Among the other species, the probes (all derived from B. subtilis) hybridize with equal intensity. A mere examination of the autoradiogram patterns for EcoRI, HindIII, BamHI and SmaI reveals little dissimilarity in terms of hybridization intensity (Figures 12, 13, 14, 15). The salient point to be made is that among the species and strains, the multiple band hybridization patterns indicate variation not in the rDNA sequences but in the spacer regions. Every band appearing on the radiogram has only

two possible derivations. The first results from multiple restriction sites within the gene set cutting out internal cistron fragments that hybridize with the probe. This occurs, but not for all the endonucleases used, as reference to the standard gene set shows (Figure 6). The second type of band has one restriction site within the gene set and a second beyond it in the intergenic spacer region. Complete restriction with EcoRI produces twelve 23S homologs in *B. subtilis* NCTC3610/168M (Table 7 and Figure 12). The two lowest are rRNA cistron segments extending from the central part of the 16S determinant to the 5' end of the 23S determinant. The observation that both 16S and 23S rRNA probes display bands of 1.2 and 1.4 Kbp is due to the fact that every cell carries two families of "abutment regions" between 16S and 23S genes (Stewart *et al.*, 1982). The larger region contains tRNA sequence inserts (Dahlberg and Loughney, 1983). The 23S internal homolog of 0.9 Kbp is also visible on radiograms probed with 23S rRNA, extending from the 5' EcoRI site to the one central to the 23S determinant. The other nine bands on the 23S autoradiogram are derived by the second method. One EcoRI site is within the gene set and the second one is within spacer DNA. Variation in spacer DNA is evident as different positions of the distal EcoRI sites produce individual hybridization bands with any one of the probes.

The endonuclease SmaI produces internal fragments also. There are three such sites in each gene set, one at the 3'

end of the 16S determinant (Figure 6) and another at the 5' end of the 23S sequence. These designate an 0.5 to 0.7 internal fragment encompassing the intragenic abutment space. The third SmaI site at the 3' end of the 23S determinant will produce internal 23S sequences of 2.3 Kbp. All other higher SmaI bands are bordered (as with the EcoRI situation) by spacer DNA SmaI-containing sites (Table 10 and Figure 15). Enzymes HindIII and BamHI have only one site in each gene set, thus all their homolog bands are produced by variation in the spacer sequence.

The only restriction site on the standard gene set of Figure 6 which is found to be variable is the HindIII site immediately distal to the 5S determinant (Stewart et al., 1982). At least two precursors of 5S rRNA have been reported for B. subtilis (Pace et al., 1973); one of these possesses this distal HindIII site. This recognition sequence is located one base outside the coding sequence for mature 5S rRNA. According to the gene set restriction map, a 23S homolog ranging from the 5' HindIII site to the variable one should be about 3.0 to 3.1 Kbp in length. Table 8 and Figure 13 indicate the presence of a 2.9 Kbp 23S or 5S homolog within B. subtilis strains NCTC3610/168T. B. subtilis W23 possesses a 3.1 Kbp homolog with these two probes. Both are the smallest sizes observed with probes pBC279-23S,5S and p12E2-23S,5S, and must be derived from the right half of the operon. Using a 23S rRNA probe, a 2.4 Kbp homolog which is extremely faint and below the 2.9

band is noted in _B. subtilis_ 168. It presumably is derived from the 23S 5' HindIII site extending leftward to the 23S 5' border, a span of about 400 base pairs. (The p21C4-16S probe produces a 2.4 Kbp homolog also of greater intensity, so this must be a fragment derived from the left half of the cistron.) It was noted only in the Marburg strains. These results suggest the maintenance of a 5S distal HindIII site. The slight variation in size (about 200 bp) could reflect a greater leftward position of the W23 23S HindIII site simultaneously obliterating the faint 23S rRNA 2.4 Kbp fragment present in the Marburg strains. Alternatively, the 5S distal HindIII may be slightly rightward in W23. According to the gene set map determined by Stewart _et al_. (1982), there should be produced a 3.1 EcoRI Kbp band. Our 2.9 Kbp band may be an underestimate of this band. _B. globigii_ RUB562 produces a 2.9 Kbp band also, with one of the two bands beneath it possibly derived from the 23S sequence extending leftward on the gene set. Plasmid p21C4-16S also produces a 2.7 Kbp band probably equivalent to the 23S homolog. In contrast, _B. amyloliquefaciens_ H produces no band within the 2.9 to 3.1 Kbp range with 23S sequences; therefore the 5S distal HindIII site has not been conserved in this species (Table 8 and Figure 13).

The p21C4-16S probe indicates that the first HindIII site proximal to the 16S sequence is variant in position in the species. The sizes range from 1.7 Kbp to 2.7 Kbp with

the minimal sized homologs. Both *B. licheniformis* FD01 and 8480 display a 2.4 Kbp minimal size. Indeed the hybridization pattern for these two strains is closer than that between *B. subtilis* strains NCTC3610/168 and W23 with either 23S or 16S specific probes. They differ with respect to their EcoRI and HindIII homologs. Note strain FD01 has a 6.5 Kbp 23S EcoRI homolog not present in 8480. In addition, the second FD01 EcoRI 23S band of 9.0 is 800 base pairs greater in strain 8480 (Table 7 and Figure 12). Strain FD01 contains a 4.0 Kbp 23S,5S specific HindIII homolog not present in strain 8480. On the other hand a 2.8 Kbp band not present in the FD01 pattern appears in the 8480 pattern (Table 8 and Figure 13).

Digestion of the chromosomal DNA with endonuclease BamHI produced many high molecular weight fragments ranging from 14 to 30 Kbp (Table 9 and Figure 14). The very large fragments are poorly resolved on the agarose gels and do not transfer as efficiently to the nitrocellulose sheets (Maniatis, 1982). Of the species examined with this enzyme, the well resolved minimal size homolog fragments suggest the strict retention of only one BamHI site within each of the rRNA gene sets (Table 9). With each probe, no sizes smaller than 2.6 Kbp were noted. Among the species, none were observed to display the identical small homologs, suggesting extensive variation in "spacer" DNA. The pattern produced by *B. subtilis* NCTC3610/168T with 23S or 5S probes revealed as its minimal size 2.9 Kbp. Calculation

of the size of a fragment ranging from the BamHI site to the 5S 3' end of the gene set is 2.8 Kbp (Figure 6). This indicates a BamHI site in one or more cistrons immediately distal to the 5S sequence. B. subtilis strain W23 displays a minimal size of 3.6 Kbp placing the distal BamHI site further out into the spacer region. B. licheniformis 8480, with a 3.4 Kbp minimal 23S,5S homolog, also displaces the distal BamHI site at least 0.5 Kbp into spacer DNA. On the other hand, B. pumilus and B. globigii RUB562 with a 3.0 Kbp sized homolog, allows postulation of a BamHI fragment in the immediate 5S 3' distal point. The species have not conserved the position of their BamHI sites proximal to the cistron. Probe p21C4-16S displays a lack of equivalence of these minimal homologs among the species (Table 9 and Figure 14). The p21C4-16S HindIII pattern shows the same result. The overall conclusion must be that the rRNA cistron's internal arrangement is equivalent in all the cell lines and evolutionary deviation took place beyond their boundary.

The enzymes with multiple sites of sensitivity within the operon allowed a more direct visualization of intraoperon conservation. EcoRI and SmaI were used to specifically focus upon the rRNA cistrons themselves (See Section F).

E. Gene order in the rRNA gene sets of the genus Bacillus

The physical map of a B. subtilis 168M rRNA gene

set and linkage studies indicate the gene order of this cistron is 16S, 23S and 5S (Stewart, _et al_., 1982; Colli _et al_., 1971). This arrangement is preserved in other strains and species examined. Chromosomal DNA restricted with endonuclease EcoRI, HindIII, BamHI or SmaI produce fragments homologous to 16S, 23S and 5S sequences (Tables 7, 8, 9, 10). In every case, those fragments homologous to 5S sequences were also homologous to 23S sequences. With _B. subtilis_ strains NCTC3610/168M and W23, chromosomes restricted with EcoRI, HindIII or BamHI 5S rRNA end-labeled with ^{32}P served as a hybridization probe (Materials and Methods). The equivalence of the 23S and 5S fragments is directly observed (Tables 7, 8, 9, 10, and Figures 12, 13, 14, 15). The 23S rRNA EcoRI sequences display three additional multiple minimal bands derived from intragenic scission. Probe pBC279-23S,5S behaves in the hybridization pattern just as 5S, displaying no minimal bands. The reason for this is evident, as its rRNA sequence extends only to the 23S determinant central EcoRI site (Figure 6). While it is homologous to distal 23S sequence and the entire 5S sequence included, it behaves as a good 5S substitute in EcoRI, HindIII and BamHI hybridizations. In all species examined with these endonucleases, comparison of actual 23S rRNA homologous fragments to pBC279-23S,5S fragments can also be used to infer 23S,5S linkage. There is a SmaI site at the 3' "end" of the rRNA gene set (Figure 6); therefore, pBC279-23S,5S and 5S rRNA hybridization

probes do not "behave" identically in SmaI digestions (Table 10, Figure 15). Hence, with Southern blots produced by this enzyme, we inferred closer 23S,5S linkage because the 23S rRNA pattern appeared identical to pBC279-23S,5S patterns. The p21C4-16S pattern differed from both (Table 10 and Figure 15).

In the case of HindIII, BamHI and SmaI chromosomal restriction, the 16S (p21C4-16S) hybridization pattern was never equivalent to the 23S,5S (pBC279-23S,5S) one (Tables 8, 9, 10, and Figures 13, 14, 15). Probe p21C4-16S provides a poor hybridization probe for EcoRI restricted chromosome for reasons previously described. 16S rRNA, when used as a hybridization probe, had to be used in the presence of unlabeled 23S inhibitor. Trace 23S contaminant labeled along with 16S probe and hybridized. Plasmid pMS102'B7-16S,23S was used as a substitute 16S probe. It contains this sequence and shares homology only with the 0.9 Kbp minimal 23S EcoRI piece (Figure 6). Comparison of a 16S rRNA EcoRI hybridization pattern in strain NCTC3610/168M indicates that pMS102'B7'-16S,23S produces the 0.9 Kbp minimal and an additional 6.1 Kbp fragment (Table 7 and Figure 12). The latter homolog clearly is derived from the leftward spacer DNA on the probe and represents a part of the origin region of the chromosome. Subtracting these extra homologs reproduces an acceptable 16S EcoRI pattern. Futhermore, the EcoRI piece of 1.2 Kbp or 1.4 Kbp (dependent on the intragenic spacer size) con-

necting the 16S to 23S sequence is visible. It is also visible in B. pumilus, B. globigii and B. amyloliquefaciens as a 1.9 or 2.1 Kbp piece for reasons discussed in the next section (Table 7 and Figure 12).

The lack of absolute equivalence of the pMS102'B7-16S, 23S homologs with the 23S rRNA of pBC179-23S,5S homologs and the two common EcoRI 16S and 23S homologs observed in all species/strains, confirmed the 16S, 23S and 5S order of the gene sets (Figures 6 and 12 and Table 7).

F. Dichotomy of rRNA cistron sequences

Comparison to the standard rRNA gene set restriction map constructed by Stewart et al. (1982) of B. subtilis 168 to the restriction sites we observed reveals one intragenic variation (Figure 6). In the eight Bacillus species and strains analogous restriction sites, i.e. EcoRI and SmaI, were observed in strains NCTC3610, 168T, W23 and B. licheniformis (FD01 and 8480). However, in the case of B. globigii RUB562, B. pumilus RUB502 and B. amyloliquefaciens H the EcoRI site within the 5' end of the 23S sequence is not present. In all of the cell types studied, mixtures of these two categories of rRNA gene sets were not observed. Amplification was only of one type or the other. In addition, in spite of the presence or absence of this EcoRI site, two types of rRNA gene sets are preserved in each strain. Variation in size results by differences in the 16S,23S abutment space. In the B. subtilis and B.

licheniformis strains, this EcoRI fragment was 1.2 or 1.4 in molecular size. In B. globigii, B. pumilus and B. amyloliquefaciens, the EcoRI site elimination resulted in larger abutment fragments of 1.9 or 2.1 Kbp. This is precisely the sum obtained from the standard gene set when the variable EcoRI is eliminated.

Of all the hybridization bands observed, the multiple dark minimal ones were focused on, as they represent fragments resulting solely from internal cleavage sites in each gene set. Table 11 presents the sizes of only these internal fragments and the hybridization probes that produced them. Figures 16 and 17 are autoradiograms of EcoRI and SmaI restrictions with the relevant bands indicated. The site loss was deduced by combining the results observed with all the hybridization probes which share homology with select regions of the standard gene set (Figure 6). Comparisons to the restriction pattern of B. subtilis 168 were applied to the other strains and species. Probe p21C4-16S has only a partial 16S sequence, mostly rightward of the 16S EcoRI site. The fragment introduced into the plasmid from a sheared chromosome preparation is of undetermined size as the overall insert contains poly(dA,dT) "tails." Therefore, the plasmid is homologous to only the minimal fragment from its EcoRI site to the next distal one in the 23S 5' region. It produces in the B. subtilis strains the predicted 1.2 and 1.4 Kbp fragments, while in B. amyloliquefaciens, B. globigii, and B. pumilus 1.9 and 2.1 Kbp are observed

(Figure 16A). The probe p12E2-23S,5S contains 23S sequence extending to the 23S BamHI site, as noted in Figure 6. As a result, an 0.9 minimal size homolog is produced in EcoRI restriction in B. subtilis and B. licheniformis. The 0.9 Kbp fragment spans the region from the central 23S EcoRI site 900 base pairs to the variable 5' one. However, B. globigii, B. pumilus and B. amyloliquefaciens display the 1.9, 2.1 abutment fragment (Figure 16C), the sizes resulting from the 23S 5' EcoRI site elimination from each gene set independent of the 16S,23S spacer size.

Plasmid pBC279-23S,5S contains in its cloned sequence 23S rDNA extending only to the central EcoRI site (Figure 6). The EcoRI and BamHI extension present in p12E2-23S,5S is missing in this probe. As a result, in B. subtilis 168 strains no multiple minimal sizes are predicted or observed in EcoRI restrictions. In Figure 16D, neither the B. subtilis nor B. licheniformis strains display the 0.9 minimal size, and B. globigii, B. pumilus and B. amyloliquefaciens do not display their enlarged abutment region as this probe shares no homology with it.

Plasmid pMS102'B7-16S,23S affords elegant proof of the results obtained with the prior probes. Its BamHI insert derived from the origin of replication region contains the entire 16S sequence and extends to the BamHI site in the 23S sequence (Figure 6). The minimal sizes in EcoRI restrictions are the 1.2 and 1.4 abutment fragments as well as the 0.9 Kbp 23S fragment (Figure 16B).

In the case of B. globigii, B. pumilus and B. amyloliquefaciens, only the enlarged 1.9 and 2.1 abutment regions appear. The plasmid "mimics" the hybridization pattern observed with end-labeled 23S rRNA probe, which also shows these three internal operon fragments in B. subtilis and B. licheniformis (Figure 16B and Table 11). Here B. globigii and B. pumilus also display the two large EcoRI pieces. B. amyloliquefaciens H, which was not probed with 23S rRNA, presumably would display the same pattern as the other two species.

Overall, these minimal sizes observed can be accounted for on the presence or absence of the 23S 5' EcoRI site (labeled R, Figure 6). In addition, it is concluded that the 16S EcoRI site and the central 23S EcoRI site are conserved in both their presence and position in the cistron.

Restriction of chromosomal DNA with the restriction enzyme SmaI confirmed the conservation of the SmaI sites (Table 11 and Figure 17). The 23S sequences from RNA or cloned probes indicated conservation of the two SmaI sites in the 23S rDNA. The distinct 2.3 Kbp fragment was present in B. subtilis, B. globigii and B. pumilus. The gene set restriction map indicates the abutment region between 16S and 23S is flanked by two SmaI sites, the one in 23S being the 5' border of the 2.3 Kbp fragment. On our earlier 23S RNA blots, these small pieces apparently ran off the edge of the gel. Probing with pMS102'B7-16S,23S revealed, in

all species examined, 0.5 and 0.7 Kbp fragments (Table 11 and Figure 17). The size difference suggests that abutment size variation is preserved in the small spacer region between 16S and 23S, presumably the result of tRNA sequence inclusion in the larger piece (Loughney and Dahlberg, 1982). This effect is preserved in B. globigii and B. pumilus. Thus, this observation allows the conclusion to be drawn that within the rRNA cistrons of B. subtilis, B. globigii and B. pumilus the three SmaI sites are conserved as to both presence and position. The only variation observed was the elimination (or gain) of the 23S sequence 5' EcoRI site in these species.

The limited dichotomy of the rRNA gene sequences among these species suggests an evolutionary scheme for the genus members. Figure 18 is a dendrogram which illustrates a proposed derivation and relationship among these Bacillus. From a prototype gene type, a bifurcation of cell lines must have led to two strain types either with or without the EcoRI site in the 23S sequence. The model indicates amplification of the rRNA cistrons, resulting in a family of nine to eleven genes per cell. Each family is composed entirely of a 23S sequence with or without the EcoRI site. "Horizontal evolution" via amplification of one variant cistron could produce this result. Unequal crossing-over can fix a given sequence type within a population of heterogeneous sequences (Smith, 1974). The observation that only one or the other rDNA sequence is found allows

the groupings of organisms as presented in Figure 18.

Two families of the eight Bacillus are postulated. The first includes B. globigii, B. pumilus and B. amyloliquefaciens; the second contains the B. subtilis and B. licheniformis strains. The evolutionary positions are presented here without a time parameter, as only one small sequence alteration is being looked at (EcoRI recognition site). Divergence between the species and strains within each family is by changes in sequences beyond the rDNA boundary ("spacer regions").

From published accounts of the derivation of the present strains of B. subtilis (Hemphill and Whiteley, 1975), other positions in the dendrogram can be assigned. B. subtilis strain NCTC3610 gave rise after ultraviolet and ionizing radiation to strains 166 and 168, which differ in chromosome segment arrangement and auxotrophic requirements (Figure 1). B. subtilis 168M comprises the Marmur transformable strains derived from 168.

We noted the two B. licheniformis strains differ in the spacer regions between rRNA cistrons. This variation is limited to one 4.0 Kbp 23S,5S HindIII fragment present in strain FD01 but not in 8480 (Table 8). EcoRI restrictions reveal that strain 8480 possesses a 9.8 Kbp 23S,5S homolog where FD01 has a comparable 9.0 and 6.5 Kbp homolog. Plasmid pMS102'B7-16S,23S in EcoRI restrictions displays FD01 as having an additional 1.0 Kbp 16S sequence containing fragment not observed in strain 8480. Hori and

Osawa (1979), in sequence comparison of 5S rRNA from many species of eukaryotes and prokaryotes, constructed an extensive phylogentic tree. Evolutionary distance was determined for select *Bacillus* species. Two distinct classes of *B. licheniformis* are noted and recorded as diverging from the same common ancestral type that also deviated into *B. subtilis* at the same time. The scheme derived by these workers appears to correlate with the sequence of events we derived (Figure 18). Osawa and Hori (1979) constructed a similar phylogenic tree based upon ribosomal protein composition. By this method they observed that *B. subtilis*, *B. megaterium* and *B. cereus* share no identical ribosomal proteins. On the other hand they report *E. coli* and *Salmonella* as very similar by this criterion, differing in only seven proteins. These facts suggest that taxonomic criteria such as genus are not always equivalent in different bacterial groups. Fox *et al.* (1980) used the 16S rRNA sequence to examine prokaryotic phylogeny. The oldest groups are anaerobes such as *Clostridia*. *Bacillus*, these workers report, evolved with the occurrence of the aerobic atmosphere presenting a point of appearance of the genus. Our dendrogram focusing on strain and species derivations, records far more recent events.

G. The similarity coefficient

In order to determine the extent of conservation of

sequence in the spacer regions between the ribosomal DNA gene sets of the species and strains, the following analysis was performed. We define the "similarity coefficient" as a fractional estimate (s) of conserved fragment sizes between two chromosome digests. The relationship is expressed as:

$$s = \frac{2a}{(x + y)}$$

where a equals the pairs of size-conserved fragments in paired digests of chromosomal DNA; x and y are the total number of homolog fragments observed in paired autoradiogram lanes.

The enzyme digests used for this comparison were EcoRI and HindIII which had given the best homolog band resolution. In the case of EcoRI chromosomal restrictions, the minimal internal operon fragments were not counted as they were not representative of extra operon enzyme sites.

For the purposes of this analysis, bands within one hundred base pairs in size, as determined by Beardon analysis (Materials and Methods), were considered of identical size. In the linear region of the Beardon relationship this repesents a 4 to 5 mm difference in migration distance on our 0.75% agarose gels (See Figure 2). This is within the limits of good resolution as each autoradiogram band appears about 2 mm in width (see Figures 12A and 13A). Dark bands representing two or more co-migrating species were counted as one, since two or more

fragments from the same chromosome cannot be identical.

This analysis is used only within the confines of the following parameters: (1) The Bacillus chromosome was shown to have about ten restriction fragments carrying ribosomal DNA sequences. (2) The sizes of the operon restriction fragments ranged from about 10,000 to 900 base pairs. Thus, any one of the ten operons has (10,000 - 900) ÷ 100, or 91, intervals in which we could visualize it. The probability of any one band being in a given interval is 1/91, assuming random placement of the restriction sites bordering each DNA fragment. Ten bands resulting from a complete digest clearly have 10/91 chances of occupying a given size interval. Conceptually, we think of the restriction fragments produced by one species as setting the sizes to be matched by the second strain or species (the "test set"). Therefore, of the bands produced by the test set, we calculate the probability that one of its 10 will match in size any one of the other set's 10. The fraction is:

(10 test set homologs/91 size intervals) X 10 interval choices = 1.09.

Clearly, a match of two bands is most likely a random occurrence without the intervention of an evolutionary conservation. We are obligated to choose a point where the number of size-conserved bands designates a real evolution-

ary constraint. The probabilities calculated in this manner for size-conserved bands from 1 to 9 are entered in Table 12. At size conservation for 8 bands of the 10, the probability fraction is 0.10. In other words, this point represents, under these conditions, 90% probability of true evolutionary constraint on the placement of spacer restriction sites. Similarly, by this criterion, the chance of all 10 homologs appearing equal is 0.01. This estimate allowed the following decision to be made about the spacer variation.

Table 12 presents all possible paired comparisons of the eight species and strains examined. With restriction enzyme EcoRI, the probe is pBC279-23S,5S and with endonuclease HindIII it is p21C4-16S. We are in effect hybridizing to DNA fragments both left and right of the rDNA cistrons. For the purpose of this analysis we define s values of 0.50 or greater (representing 41% or less random homolog size matches) as conserved.

The following notable observations are made: 1) In chromosomal restrictions, B. subtilis NCTC3610 is identical to B. subtilis 168M, as indicated by an s value of 1.00. 2) The NCTC3610/168M strains are quite homologous to B. subtilis W23, which shares values of 0.53 and 0.86 for EcoRI and HindIII, respectively. 3) The relationship between the two B. licheniformis strains shows a close and and expected homology with 0.77 EcoRI and 0.90 HindIII homolog conservation (Table 12). However the relationship

between the *B. subtilis* and *B. licheniformis* strains appears random in the gene "spacer" regions. These s values range from 0.14 to no more than 0.50 (Table 12).

The preceding comparisons involved only species which contain the type I rRNA cistron with the EcoRI site within the 5' end of the 23S determinants. Similarly compared are the species with the type II rRNA cistron which do not possess this EcoRI site. Table 12 reveals no greater degree of conservation within this group of *Bacillus*. The only exception is the *B. pumilus* and *B. amyloliquefaciens* comparison, within the EcoRI restriction. The value of 0.80 is significantly high by our criteria, but the corresponding HindIII value of 0.36 is within the "random" range.

An interesting observation is found when comparisons are made between species which have different type rRNA gene sets. As shown previously, the one variation observed in this conserved sequence set apart the two groups of *Bacillus*; thus one would not expect anything other than random spacer conservation between them. Table 12 indicates the occurrence of seeming "depots" of conservation. NCTC3610/168 vs *B. globigii* displays a similarity of 0.57 with a HindIII restriction, while the corresponding EcoRI value is clearly of random nature (Table 12). The same is the case with *B. subtilis* W23 and *B. globigii* (Table 12). *B. globigii*, compared to both *B. licheniformis* strains, suggests conservation of

the EcoRI homologs (Table 12). This effect could, on the genetic level, account for the designation under the initial heading of B. subtilis organisms which were in reality different species (Introduction). This similarity analysis (along with the thr and leu hybridization patterns) suggests the true identity between B. subtilis strains NCTC3610 and 168M. The fact that with both enzymes all homolog fragments are of identical size provides strong evidence that they are in reality the same.

H. Densitometry autoradiogram calibration

Through the use of autoradiogram homolog band intensities, we estimated the proportion of hybridizable sequences within the genome. The calibration of the densitometry system was done by estimating the range of linear response of Kodak X-omat film. A dry nitrocellulose filter was "spotted" with ^{32}P-nucleotide in five positions. The counts per minute within each 2 ul spot ranged from 150 to 2,400. Each spot was 3 millimeters in diameter, spaced 1 cm apart. Two such standards were made, one of arithmetic increase of cpm per spot, the other of geometric increase. The filter was dried and applied against autoradiogram film with a Cronex screen. After a twelve-hour exposure at room temperature, the developed film was sliced into strips containing the spots and scanned at 580 nm. Figures 19A and B are the recorded

peaks of the arithmetic and geometric standards. Figure 20 is a plot of cpm versus peak areas obtained by planometer tracings. As can be seen, the film response under these conditions appears linear up to about 1,400 cpm.

An estimate was made of the approximate number of counts per minute bound to a homolog fragment on a typical Southern blot. For the calculation the 5 Kbp size of an entire rRNA gene set is used, representing 0.13% of the 3787 Kbp within the chromosome. Two micrograms of restricted chromosome are added to each gel lane of which 20 to 30 ng are rDNA sequences. For complete hybridization of this region, it must hybridize to an equal mass of labeled probe. The specific activities of the probe made by nick translation or end-labeling for this purpose were about 1×10^3 cpm/ng. To each hybridization reaction to be quantitated 20 to 30 ng of labeled probe was used per blot lane. This is equal to 2 to 3×10^4 cpm per lane. A typical Southern hybridization had from 8 to 13 ribosomal DNA homologs per lane (Figures 12A and 13A). Each homolog subjected to complete hybridization would carry 2 to 3×10^3 cpm. In addition EcoRI restriction of the gene set creates 23S rRNA homologs representative of 44% of the total rDNA sequence (Figure 6; note the position of the 3' EcoRI site). Therefore the resulting homologs are estimated to hybridize to about 800 to 1200 cpm, within the appropriate range.

I. Quantity of ribosomal RNA gene sets in the genus *Bacillus*

Wilson *et al.* (1982) determined on the basis of EcoRI restriction that *B. subtilis* 168 has about 10 rRNA gene sets per chromosome. Their estimate was determined by counting 23S rRNA homologs observed upon Southern blots. This technique is limited by the fortuitous migration of different fragments of the same molecular weight. On this basis, "darker" radiogram bands were counted as two, although it is possible that dark bands are composed of more than two gene sets. In this study, EcoRI digests probed with p12E2-23S,5S or 23S rRNA upon Southern blots were subjected to densitometry measurements.

EcoRI restriction of *Bacillus* DNAs was chosen for the following two reasons: first, the internal fragment's appearance clearly indicated that a complete restriction had taken place; second, the minimal internal bands were of two size classes based upon the tRNA sequence insertion. Each blot could be used to estimate the number of gene sets and the proportion of them containing tRNA sequences (As shown in Figure 12).

Intensity of each homolog band in this case was independent of its molecular weight because all EcoRI bands greater than minimal are bordered by the 23S central (or *only*, in *B. globigii*, *B. pumilus* and *B. amyloliquefaciens*) EcoRI site and an EcoRI site rightward of the cistron in "spacer" regions. Each band regardless of size carries

only about 2.1 Kbp of 23S and 5S sequence (See Figure 6).

Two micrograms of restricted chromosomal DNA were loaded onto each gel lane and all hybridization filters received an equal amount of radioactivity as measured in cpm. The area of the smallest peak on each scan was designated as one. All other peak areas are presented as a multiple of the small peak estimating their multiplicity. Thus, each blot lane carries its own internal control. Table 13 presents area estimates in planometer units with estimated multiplicity of each band. Absolute areas of the peaks are divided by the peak with the smallest area, where a multiple of less than 1.5 is considered equal to 1 and greater than 1.5 equal to 2. Figure 21 is a series of densitometer tracings of EcoRI restrictions of each species probed with 23S rRNA sequences. For the purposes of clarity, the minimal internal fragments are not shown. In these scans, the B. subtilis NCTC3610/168 4.3, 3.7 and 3.5, 3.4 homologs are poorly resolved and were recorded as two peaks only (Figure 21A).

All the Bacillus appear to have 9 to 11 gene sets. B. licheniformis 8480 is estimated to have 9 to 10, but B. licheniformis FD01 displays an additional band (6.5 Kbp) (See Figure 12 C and D) in an otherwise identical EcoRI pattern. Therefore, it is concluded that the latter strain may possess about 10 to 11 gene sets. In the B. licheniformis FD01 scan chart, the additional 6.5 Kbp band is interpreted in the densitometry tracings as a fusion

with the 6.0 Kbp band (Figures 21F and 21G). The area of the fusion peak is increased by about one unit. Multiplicity values with fractional additions of about 0.5 units are indicated as being within a one-unit interval, and account for the range of total rRNA gene sets per species presented.

J. The number of rRNA gene sets with internal genes

As noted in previous hybridization patterns, the conservation of two families of rRNA gene sets in each species is evident. The two classes, i.e. those containing tRNA sequences and those not, differ in size by about 180 to 200 base pairs. These fragments were visualized with most of the cloned hybridization probes. Reference to Figure 6 indicates that p21C4-16S overlaps the fragments from the 16S side of the operon.

Plasmid pMS102'B7-16S,23S overlaps these pieces on both the 16S and 23S side, with the actual spacer sequence included. Densitometric tracings were performed only on regions of the Southern blot autoradiograms corresponding to these abutment-containing pieces i.e., 1.2 and 1.4 or 1.9 and 2.1 Kbp. Figures 21 A-G present the tracings of all 23S bands and many display fusion of peaks. Figures 22A and 22B display tracings of minimal internal EcoRI fragments from the two types of rDNA gene sets. Bifurcation of the two peaks was by extrapolation of the slope between each peak and then planometer areas were taken.

The total area of both peaks was computed and the ratios presented are of the larger gene sets divided by total gene sets. For the purpose of calculation the autoradiograms were scanned at a slower speed. This resulted in an expanded peak width displayed in Figure 22.

Table 14 presents the area ratios of the two forms of the gene sets for seven of the eight species and strains. The values obtained for the four hybridization probes were about equal within a species. Each probe was tested upon a species at least three times. Both strains of _B. subtilis_ have a value of 0.31 in agreement with the Stewart _et al._ (1982) estimation. Suprisingly, _B. licheniformis_ strain FD01 provided a ratio of 0.45, significantly higher than _B. subtilis_ but also higher than _B. licheniformis_ 8480 (0.28). As noted in the last section, _B. licheniformis_ FD01 has at least one gene set more than 8480, which might contain insertion sequences accounting for this ratio variation. _B. globigii_ RUB562 shows a ratio of 0.34 and _B. pumilus_ RUB502 is higher still at 0.42. However, the data presented show no correlation between abutment size and the variation of the 23S EcoRI site. Of the ten rRNA genes present in _B. subtilis_, Stewart _et al._ (1982) estimated at least three are of the larger class while Loughney and Dahlberg (1982) calculated the number to be two. Chromosomal DNA restricted with EcoRI and hybridized upon Southern blots with either 16S or 23S rRNA sequences displays the two size classes in the form of small sized

doublet fragments. The two size classes are the 1.2 and 1.4 fragments of B. subtilis and B. licheniformis or the 1.9 and 2.1 fragments of B. globigii, B. pumilus and B. amyloliquefaciens.

III. Analysis of Conserved and Non-conserved Nutritional Markers

The transformation assays performed by us and others (Chilton and McCarthy, 1969) indicated biological conservation of the leucine, arginine and phenylalanine markers in comparison to many other "nutritional" markers. It was postulated that this resulted from the proximity of this gene to conserved regions of the chromosome (i.e., translation and transcription genes). We analyzed the physical counterpart of this phenomenon by hybridizing the leucine sequence to the restricted DNA of the Bacillus genomes. The hybridization of the leu marker sequence was compared to that of two non-conserved nutritional markers threonine and tryptophan. We asked to what extent the restriction site positions of these markers are maintained in the eight Bacillus. We also determined the degree of interspecific hybridization by analyzing band intensity for each sequence.

A. Structure of plasmid RSF2124'B-leucine

The source of cloned leucine A C B D genes used in

this study was plasmid RSF2124'B-leu. Construction was by Nagahari and Sakaguchi (1973). Vector RSF2124 was constructed as a derivative of the plasmid colE1, with transposon A (Tn A), the resistance factor for ampicillin, inserted into it. The plasmid has one EcoRI site which is within the determinant for colicin biosynthesis, so it is unable to produce colicin upon ligation to foreign DNA. It is chloramphenicol amplifiable in the *E. coli* C600 host.

Although a restriction map along with biological activity is presented by the researchers who constructed it in this study these properties were re-examined and two new qualities were noted.

The chromosomal fragment is derived from a *B. subtilis* 168 leucine prototrophic strain. It was inserted into the vector's EcoRI site, with selection for ampicillin and leucine prototrophy in *E. coli* C600 (*hsdR*$^-$, *hsdM*$^-$, *leuB*) (Nagahari and Sakaguchi, 1978). Table 15 indicates the molecular size in kilobases obtained with restriction of this plasmid. Figure 23 is a photo of an ethidium-stained 0.75% agarose gel with digestions in each lane as noted. EcoRI produces two fragments; the smaller, a 6.2 Kbp fragment, carries the leucine operon. Total size of the composite plasmid is 17.3 Kbp. Restriction with BamHI produces two fragments which are poorly resolved on the gel photo and appear as one thick band. Actual identity of each was noted on 0.5 percentage gels which allowed separation. Digestion with HindIII yields six fragments of 10.5,

2.6, 1.6, 1.5, 0.7 and 0.4 Kbp. The vector possesses no HindIII sites (Nagahari and Sakaguchi, 1978), therefore the leucine insert must have six sites susceptible to restriction by this enzyme. On the gel photograph presented, the last two fragments ran off the gel terminus and had to be visualized on gels on which molecular migration was not as far. SmaI restrictions produced two fragments, one of 13.3 Kbp and another of 4.0 Kbp. This was not noted by Nagahari and Sakaguchi. Vector RSF2124 contains one SmaI site derived from its colE1 progenitor. The insert must also possess one SmaI site in order to generate two fragments.

Double restrictions were performed to map the SmaI sensitive site, as shown in Table 15. Staggered restrictions with EcoRI and SmaI produced four fragments as expected. Two bands, 3.3 and 2.9 Kbp, appear as one on the photo presented and had to be resolved on 0.5 percent gels. Their molecular sizes summed equals the total insert size of 6.2 Kbp, placing the SmaI site roughly central in the leucine piece. Nagahari and Sakaguchi (1978) present the BamHI positions in their restriction map. Staggered BamHI, SmaI restriction allowed the SmaI site to be placed relative to the insert's BamHI site. An 8.5 Kbp fragment present in BamHI restrictions remained intact after additional SmaI treatment. However, the 8.8 Kbp BamHI piece resolved into three pieces of 4.5, 4.0 and 0.3 Kbp (Not all shown in Figure 23; the 0.3 Kbp piece ran off the gel on the photo presented). Placement of one SmaI site in the vector and

the second one 300 base pairs counterclockwise from the insert's BamHI site resulted in the restriction map presented in Figure 24.

The hybrid plasmid transforms *B. subtilis leuA*, *leuC* and *leuB* auxotrophs to Leu^+ while the linked genes *ilvB* and *ilvC* were not rescued (Nagahari and Sakaguchi, 1978). Table 16 verifies this complementing activity but indicates that *leuD* auxotrophs were also rescued during plasmid transformation. The expression of the amp^r in *B. subtilis* did not occur (as expected) (Erlich, 1978). The transformation event observed must be due to an actual chromosome integration of the leucine fragments, but whether the vector section was within the chromosome was not examined. The markers on the "borders" of the conserved chromosomal segment *argA2* or *pheA* were not represented on the plasmid either, not surprising in light of the physical size of the *B. subtilis* insert.

On the other hand, the transformation efficiencies in *E. coli* HB101 (*leuB*) to leucine prototrophy and to both amp^R and Leu^+ were equal but considerably lower than the leucine frequency in *B. subtilis*. Homologous marker integration into the host chromosome was more efficient than plasmid-directed complementation of a resident *E. coli* mutation. The *leuB* marker in *E. coli* corresponds to the *B. subtilis leuC* which codes for the enzyme β -isopropylmalate dehydrogenase.

B. Survivals of gene markers in the leucine region of the B. subtilis chromosome following restriction cleavages of prototrophic DNAs

The restriction site organization of the leucine gene cluster in the genomes of *B. subtilis* strains W23 and NCTC 3610/168M was evaluated. Survival levels of the four *leu* genes and the linked *argA*, *ilvB* and *pheA* markers after restriction of the two donor DNAs with endonucleases EcoRI, HindIII and SmaI were estimated and compared to the cleavage pattern of plasmid RSF2124'B-*leu*. Survival levels are expressed as relative transforming activity (RTA) which is the percent biological activity of restricted DNA to its native counterpart. Table 17 indicates that the genes of the *leu* region and other linked markers *ilvB2*, *pheA1* exhibited different survivals after digestion with EcoRI. Markers in the middle of the operon *leuC*, *leuB* gave higher values, not unexpected, since the plasmid construction suggests that *leuA* and *leuD*, the operon flanking genes, are physically closer to EcoRI sites.

The survival levels of the *leu* gene cluster after restriction with HindIII were uniformly low, as expected, when compared to the plasmid which displayed multiple HindIII sites (Figure 23). One of the sensitive sites seems to be located in the *leuC* gene, based on the lowest RTA values obtained. The other five HindIII cleavage sites are harder to locate and possibly might be within promoter-operator and terminator regions or intragenic spacers.

The survivals obtained after restriction with SmaI were relatively low, in agreement with the placement of this site central to the plasmid's chromosome insert. The survival pattern also suggests a gradient in RTA values from the lowest determined for leuA to the highest for leuD (Table 17). It is therefore predicted that the SmaI cleavage site is located nearest the leuA gene.

C. Molecular size and number of chromosomal restriction fragments that hybridize to the leucine probe within members of the genus Bacillus

The conservation of the leucine operon was analyzed by Southern hybridizations using as a probe the nick translated RSF2124'B-leu plasmid. The nick translation reaction conditions were adjusted such that the specific activity of the probe was at 1 to 2 x 10^8 cpm per ug. Restriction pattern autoradiograms indicate intense hybridization of the B. subtilis 168 derived probe across the species boundaries. The number and molecular sizes of leucine EcoRI homologs are presented in Figure 25A and Table 18. Size variation with EcoRI appears to correlate with the transformation conservation. The three B. subtilis strains display absolutely the same size homolog of 6.2 Kbp. The most efficient heterologous donor DNA, B. globigii, displays this size homolog, 6.2 Kbp, also. B. licheniformis strains 8480 and FD01 both produce smaller EcoRI homologs. Within these two B. licheniformis strains an EcoRI site is posi-

tioned within the area encompassed by the leucine probe. The restriction site positions are therefore not conserved between B. subtilis and B. licheniformis. These produce no leucine transformants in B. subtilis. Transforming DNA from B. pumilus RUB502 yield a few leucine transformants in B. subtilis recipients. Its leucine homolog was at an intermediate size range in comparison to the other species at 5.2 Kbp.

When the specific activity of the hybridization probe was raised one order of magnitude to about 1 to 2 x 10^9 cpm/ug, a small fragment of about 1.2 to 1.3 Kbp was detected on the Southern blots beneath the main band, of the three B. subtilis chromosomes. It was in the size range of one of the internal 23S EcoRI sequences. The cloned probe contains no EcoRI sites internal to the chromosome insert (Figure 25A), so these small pieces must represent other regions homologous to the plasmid. The B. licheniformis strains also displayed lower bands in the 1.2 to 1.4 Kbp range (in addition to the secondary band of 2.7 Kbp) beneath its main 4.2 Kbp homolog. High specific activity probes reveal in B. pumilus RUB502 and B. globigii RUB562 homologs of 2.7 Kbp and 3.0 Kbp, respectively.

Of course, the most direct explanation is that the EcoRI positions in the leucine operons of other species is different and the plasmid probe shares homology with resulting small fragments. But this cannot account for the small B. subtilis sizes observed. Another explanation would

postulate occasional chromosome degradation by shearing or nuclease action, etc.; however, this type of event would produce a continuation of fragments visualized as a smear on a Southern blot.

Smith et al. (1968) suggested the occurrence of rRNA sequences in the leucine region of the genome. Chilton and McCarthy (1969) suggested that if these sequences appear where predicted they could easily account for the interspecies transformation ease of the leucine marker.

Conceivably, our leucine plasmid probe carries such conserved sequences upon it. A simple calculation reveals that the chromosomal insert of RSF2124'B-leu carries additional sequences beyond the operon itself. Each protein produced by genes of the leucine operon has a size which can be translated into mRNA kilobase size (i.e., DNA kilobase pair size). The conversion factor is 10,000 Daltons protein, equivalent to 270 base pairs DNA (Maniatis, 1983).

Gene	Enzyme	Daltons Protein	Kbp DNA
leuA	β-Isopropylmalate synthetase	55,000	1.49
leuC	β-Isopropylmalate dehydrogenase	55,000	1.27
leuB	Isopropylmalate isomerase	39,300	1.06
leuD	Isopropylmalate isomerase[a]	21,600	0.58
		leader	0.15
		terminator	0.20
		TOTAL	4.75

a--not absolutely confirmed (Ward and Zahler, 1973)

The calculated excess DNA in the cloned sequence is 6.2-4.8, or about 1.4 Kbp.

Our results and those of Nagahari and Sakaguchi (1978) place the leuA gene in the vicinity of the insert's SmaI, BamHI sites (Figure 24). The approximately 3.0 to 3.1 kilobases needed to contain the rest of the operon could be accounted for by the 3.0 to 3.3 kilobases in the clockwise direction on the plasmid map, extending to the bordering EcoRI site. The excess DNA in the clone appears to be proximal to the leuA gene, and presumably is composed of conserved sequences, possibly rDNA. The nick translated probe was hybridized to plasmid p14B1-23S,5S,16S. This plasmid contains sequences for all three species of rRNA and is composed of two tandem rRNA operons connected by a

0.6 Kbp spacer region (See Figure 6). The operons are derived from the major ribosomal RNA cluster in the early replicating part of the chromosome. They have been designated rrnI and rrnH (Wilson et al., 1982) (See Figure 6). Thus, this cloned fragment contains a complete gene set, but not in the usual order. The RSF2124'B-leucine insert did not hybridize to these ribosomal sequences. The conserved parts of the RSF2124'B-leucine clone may be hybridizing to similarly conserved peripheral regions surrounding rRNA sequences with the later replicating part of the chromosome. As will be shown below, B. subtilis strains auxotrophic for the genes in the arginine, isoleucine-valine, leucine, phenylalanine region occasionally display losses of rRNA operons.

Restriction enzyme HindIII was shown to have multiple sensitive sites within the cloned insert of RSF2124'B-leu. Therefore, the probe was hybridized to HindIII digests of the species' chromosomes as an estimate of the operon's internal sequence conservation. Table 18 and Figure 25B display the number and sizes of these homolog bands. It is evident that presence of multiple HindIII sites is maintained, but the position of these sites is altered. B. subtilis strains NCTC3610 and W23 are different with W23 displaying different band sizes. The NCTC3610 and 168 patterns are the same, indicating again the common origin between these two strains. As in the EcoRI restrictions with high specific activity probe, higher (albeit faint) RSF2124'B-leu

homologs are observed (Figure 25B). The NCTC3610/168M pattern shows bands at 2.1, 3.8 and 5.5 Kbp, roughly corresponding to HindIII rRNA 23S and 16S homologs. Such minor bands are included on the autoradiogram photograph (Figure 25B, lane 1). The sizes of the 168 pattern correlate to those observed after RSF2124'B-leu restriction itself. The largest plasmid fragment of 10.5 Kbp contains vector sequence; the other five pieces clearly correspond to the 2.0, 1.7, 1.5, 0.85, and 0.83 Kbp chromosome fragments (Table 15). The 0.85 and 0.83 fragments are barely resolved on our gel system. They are quite clearly below the 100 base pair limit of resolution we have adhered to. At this size, we are no longer in the linear range for measurement by the Beardon (1977) method, but these size values are listed for comparative purposes. The smallest plasmid pieces of 0.7 and 0.4 Kbp are probably the 0.85 and 0.83 chromosome fragments with about 150 to 450 base pairs still fused at the EcoRI points of the vector.

The HindIII restriction pattern difference between B. licheniformis strains 8480 and FD01 is dramatic. FD01 displays three bands at 4.30, 1.64 and 0.96 Kbp, while 8480 only produced two bands of 3.2 and 2.4 Kbp after HindIII restriction (Table 18 and Figure 25B, lanes 4 and 5). B. pumilus and B. globigii also exhibited variant patterns. The largest HindIII fragment of each differed by at least 100 base pairs at 2.7 Kbp for B. pumilus and 2.8 Kbp for B. globigii. A second fragment of B. globigii was intense

(1.4 Kbp) while two smaller remaining ones displayed themselves faintly at about 1.00 and 0.96 Kbp (Table 18; Figure 25B, lane 7). In contrast, B. pumilus had a second homolog HindIII fragment which was significantly fainter (1.6 Kbp) but displayed a third one at 1.1 Kbp distinctly darker than its other two (Table 18; Figure 25B, lane 6).

The clone insert in RSF2124'B-leu was noted to have one central SmaI site. Restriction of chromosome DNA with this enzyme not surprisingly revealed this site in both B. subtilis strains NCTC3610 and 168M (Table 18; Figure 25C). Strain W23 produced two bands indicating one internal SmaI site also. The B. subtilis strains all showed a 9.2 Kbp band, but the lower band size varied. W23 had a band size of 6.2 Kbp while the second band of NCTC3610/168T was 4.3 Kbp (Table 18 ; Figure 25G, lanes 1 and 2). To produce two SmaI homologs, the intraoperon SmaI site appears conserved along with one beyond the operon boundaries. The third site in "extracistronic" space is variant between the strains, accounting for the difference in lower band size. The other two species hybridized, B. globigii and B. amyloliquefaciens, displayed one band each, 10.0 and 4.0 Kbp, respectively (Table 18; Figure 25C, lanes 3 and 4). The leucine determinant, at least in terms of restriction site position, appears to be non-conserved. On the other hand, Figure 25 indicates that autoradiogram bands remain intense, regardless of the species.

D. Structure of plasmid pBS02a-threonine

Plasmid pBS02a-threonine was used as a cloned marker from a non-conserved region of the *B. subtilis* genome. This plasmid is derived from a cloned library of *B. subtilis* chromosomal DNA constructed in the following manner. Plasmid vector pHV33 is a bifunctional vector composed of pBR322 and pC194 fused at their single HindIII site (Erlich, 1978). *E. coli* plasmid pBR322 carries the antibiotic determinants for ampicillin and tetracycline resistance and a functional replication origin for that organism. Plasmid pC194 replicates in *B. subtilis* and confers resistance to chloramphenicol. The composite plasmid continues this behavior in each organism; however, while *E. coli* expresses chloramphenicol resistance, *B. subtilis* is unable to express either ampicillin or tetracycline resistance functions (Michel *et al.*, 1980).

Construction was by the poly(dA), poly(dT) joining method with mechanically sheared *B. subtilis* DNA (Rapoport *et al.*, 1979). A 2.17 Kbp fragment was inserted into the BamHI site which resides in the tetracycline resistance factor of the vector. The BamHI site was thereby obliterated from the plasmid and initial selection was for tetracycline sensitive, ampicillin resistant transformed *E. coli*. Ultimate isolation of the threonine complementing plasmid from the library was via transformation of recombinant plasmids into a *B. subtilis* threonine auxotroph and selection for threonine complementing activity. Maintenance

of any clone from this library in *B. subtilis* requires the *recE4* mutation which prevents "loss" of the recombinant by integration into the chromosome. We received the plasmid within *B. subtilis* strain BD224 (*trpC2*, *thr-5*, *recE4*).

Since the plasmid was intended to be a hybridization probe for *B. subtilis* DNA, it had to be completely isolated from contaminating homologous chromosome which would obscure the Southern blots. The bifunctional characteristic was exploited and the plasmid isolated from strain BD224 was transformed into *E. coli* HB101. *E. coli*-derived plasmid was of hybridization quality, without additional purification by gel electrophoresis.

Table 19 summarizes results on plasmid transformation in Rec$^+$ and *recE4* recipients with two pBS02a-threonine preparations, one isolated from *B. subtilis* BD224 and the other isolated from *E. coli* strain HB101. As shown, the frequencies for the nutritional marker (Thr$^+$) are highest in the Rec$^+$ recipients (BD170, *trpC2*, *thr-5*) due to the ease with which the gene is integrated into the bacterial chromosome. Recently, Iglesias and Trautner (1983) examined *thr-5* gene conversion within pBS02a-threonine from a *thr-5* chromosome. They postulate that in Rec$^+$ proficient cells, transient synapsis of chromosome and plasmid allows this process. We did not examine to what extent such allelic conversion took place in our plasmid preparations.

In *recE4* recipients, the nutritional marker cannot

complement the existing mutation thr-5, suggesting that the insert lacks its own promoter and is located a considerable distance from the Cm^r promoter, or may be in an opposite orientation. Only the Cm^r marker is expressed in the recE4 recipient at a reduced frequency, i.e., in 10-20% of isogenic Rec^+ strain. Indeed, plasmid transformations in recE4 strains appear reduced in frequency whether or not the plasmid carries some chromosomal homology. Figure 26 is a dose response curve in strains BD224 (trpC2, thr-5, recE4) and BD170 (trpC2, thr-5, Rec^+). Plasmid pUB110 (CCC form) with a kanamycin resistance determinant was the donor DNA. Saturation for the Rec^+ strain is at 1 to 2 ug transforming DNA while it appears to be greater than 4 ug for the isogenic recE4 recipient. The recE4 mutation allows uptake and processing of DNA by competent cells, but formation of stable donor-recipient DNA association is blocked (Dubnau et al., 1973). The depression in BD224 for plasmid transformation may also be affected by decreased viability of this strain.

Selection for both Cm^r and the nutritional marker (BD170; Table 19) yielded lowest frequencies, indicating a preference of Thr^+ to integrate into the host chromosome. Finally, the Cm^r transformation by pBS02a-threonine observed is practically equivalent in plasmid derived from either E. coli HB101 or B. subtilis BD224.

Table 20 and Figure 27 list sizes of the plasmid fragments obtained after restriction with the endonucleases

HindIII, EcoRI, and BamHI. Included is the HindIII fragment size produced by parental vector pHV33 in which the pBR322 fragment is 4.0 Kbp. In pBS02a, the fragment size increased after insertion to 6.1 Kbp. The pC194 moiety remains constant at 2.8 Kbp. EcoRI restriction yielded two fragments (4.6 and 4.3), allowing us to place another such site in the threonine insert not reported previously.

Comparison of the fragment sizes with the published restriction maps of pBR322 and pC194 (Bolivar and Backman, 1979; Erlich, 1978) places the EcoRI site almost central in the insert. Figure 28 is a restriction map of pBS02a in which this EcoRI site is noted to be 1.2 Kbp from the former BamHI site ("Bat" in the diagram) reading in the clockwise direction. BamHI treatment of this plasmid yields the open circle, linear and CCC supercoiled forms indicating that the enzyme recognition site is not present and native plasmid remains (Figure 27B, lane 1).

E. Marker survival of two non-conserved genes, thrA and trpC, following EcoRI, HindIII and SmaI restriction of B. subtilis DNA

The non-conserved marker *thrA* is located at 290 map degrees of the *Bacillus* chromosome (Henner and Hoch, 1980) and the percent RTAs of this marker are presented for three strains of *B. subtilis*: W23, NCTC3610 and 168M (Table 21 I). In accordance with the cloned plasmid restriction map,

high survivals after digestions with HindIII correlate with the lack of this restriction site internal to the marker. SmaI restriction yielded an intermediate survival level, suggesting no internal SmaI sites, although this enzyme was not tested on the plasmid. The EcoRI restriction produces a dramatically low survival in comparison, as expected based on the calculated position of the EcoRI site within the pBS02a-thr insert.

Table 21 II also shows the biological survival of another non-conserved gene, the tryptophan marker. The opposite survival emerged with respect to high EcoRI and low HindIII. This SmaI pattern is of the intermediate range. The tryptophan operon of B. pumilus RUB502 was cloned into the EcoRI site of plasmid pUB110 (Tackney and Rudner, 1981). Chimeric plasmid pRR106 contains an EcoRI chromosomal segment of B. pumilus RUB502, inserted into the one EcoRI site of plasmid pUB110. The size of the tryptophan segment is 3.6 Kbp and complements B. subtilis strains with mutations in trpE, D, C and F. Restriction endonuclease cleavage site mapping by DiGiovanni and Rudner is presented in Figure 29. The biological survivals observed again correlate well with the structure of the cloned insert. An internal HindIII site accounts for the extremely low survival with this enzyme. The peripheral EcoRI sites explain the high survival observed with this restriction. No SmaI sites are found within this operon and the RTA values with this enzyme are intermediate.

Previously survival patterns of the B. subtilis aroma-

tic gene cluster were compared to this plasmid (Rudner *et al.*, 1982). As will be shown by hybridization studies, tryptophan displays a highly non-conserved sequence among *B. subtilis* species and strains. RTA values placed the first EcoRI site in proximity to the *trpE* gene in both *B. subtilis* strains W23 and 168M; the second EcoRI is more distal and variable. In W23, the HindIII site appears to be at the *trpC* gene and probably between the *trpF* and *trpB*. In *B. subtilis* NCTC3610, a rough placement of restriction sites based on biological survival is possible.

F. Restriction site organization of non-conserved genes in the Bacillus

Hybrid plasmid pBS02a-*thr*, after nick translation, served as a probe of the nonconserved gene threonine. The results obtained are in agreement with the organization of the marker upon the cloned plasmid. In each of the *B. subtilis* strains examined, HindIII restriction resulted in only one fragment, indicating the lack of intraoperon sites (Table 22; Figure 30B). Inspection of the autoradiogram indicates that in comparison to the ribosomal sequences the hybridizations are far less intense. Size conservation is not observed and between *B. subtilis* strains NCTC3610 and W23 the HindIII sizes are 5.1 and 10.6 Kbp, respectively. This correlates with the percent RTA of each organism (Table 21), in which the marker survival of W23 is about

two times greater than NCTC3610/168M. NCTC3610 and 168M both have the same hybridization size values with all enzymes used, indicating their common identity. B. *licheniformis* strains 8480 and FD01 are almost identical in terms of rRNA operon distribution but display distinct homology with threonine HindIII fragments. The FD01 value of 2.7 Kbp is significantly different from the 8480 result of 4.2 Kbp.

EcoRI restriction of the same chromosomal DNA preparation (Table 22; Figure 30A) indicates an intraoperon sensitive site in four of seven *Bacillus* species and strains. The EcoRI conservation in NCTC3610/168M and W23 appears quite strict, with both types producing two bands of 3.9 and 1.4 Kbp. *B. licheniformis* FD01 and 8480, on the other hand, are variant. FD01 displays two homologs of 3.6 and 1.4 Kbp, while 8480 shows only one 5.6 Kbp band. In the case of this probe, the non-conservation of the intraoperon EcoRI fragments was confirmed in the following manner. High specific activity probe (1 to 2 x 10^9cpm/ug) of 100 to 200 ng per hybridized filter was used. The total mass of the insert is about 2.17 Kbp, or 24% of the total 8.9 Kbp plasmid. When the chromosome and plasmid vector was "tailed" with poly(dA), poly(dT) (Rapoport *et al*., 1979), about 500 extra nucleotides were added, thus the actual chromosome insert is 2.17 - 0.5 = 1.67 Kbp. The threonine portion, therefore, is 1.67/8.9, or 19% total chimeric plasmid. Assuming homogeneous nick translation, the real specific activity of the hybridizable portion must be about 1.9 x 10^8 cpm/ug. The

calculated size of the *B. subtilis* chromosome is 2.5 x 10^9 Daltons (2,500 Md) (Henner and Hoch, 1980) or about 3,787 Kbp. The threonine insert is 1.67/3,787, or 0.04% total chromosome. Therefore, in the 2 ug of chromosome added per gel lane (assuming 100% transfer to nitrocellulose) 0.8 ng sequence of interest is present, at most. The amount of probe used is at least 100 times the saturation amount in a completely homologous system.

SmaI cleavage of six species and strains is presented in Table 22 and Figure 30C. In this case, size conservation was not observed, between *B. subtilis* W23 and NCTC3610/168M (9.4 and 13 Kbp, respectively). Overall, the values range from 9.4 to 14 Kbp. The BamHI digest sizes were compared within the three *B. subtilis* strains. Between NCTC3610/168T and W23, they differed by about 1.9 Kbp (Table 22 and Figure 30D).

The hybridization pattern is confirmatory of the transformation survival observed. As expected, the *B. subtilis* strains suffer EcoRI inactivation due to the intraoperon scission. This is evident in Table 21 which records the low threonine survival after digestion of the chromosomal DNA with EcoRI.

Fragment size difference within the *trp* region among the *Bacillus* strains was determined by hybridizations to the *B. pumilus* tryptophan chromosomal piece cloned by Rudner and Tackney (1982). Because this plasmid replicates in and is isolated from *B. subtilis*, any chromosomal contaminant would

obscure the Southern blots. Therefore, additional purification was carried out by isolating the EcoRI insert from a 1.0% low-melting agarose gel prior to nick translation (Materials and Methods). Most notably, only hybridization patterns could be obtained with five of the species and strains. Intensity was variant with the greatest degree of hybridization to the completely homologous *B. pumilus* chromosome followed by *B. subtilis* strains W23 and NCTC3610/168. In addition, all fragment sizes obtained were quite different. Sizes of the restriction fragments with *trp* homologs are summarized in Table 23 and Figure 31, which present the relevant heterologous hybridization.

In the EcoRI digests, the sizes varied from 4.5 to 19.3 Kbp. W23 DNA gave rise to a smaller fragment (8.74 Kbp) than did NCTC3610/168M (14.8 Kbp). HindIII digests produced two hybrid bands in the *B. pumilus* chromosome as expected (8.55 and 4.89 Kbp). It was shown by Tackney and Rudner (1981) that the smaller fragment shares homology with the *trpE D C* portion of the probe while the larger is homologous to the rest of this operon. The conservation of this HindIII site in *B. licheniformis* is suggested by the HindIII homologs of 2.73 and 1.98 Kbp. EcoRI-HindIII double restrictions only displayed a 1.86 Kbp band. Perhaps the EcoRI site in this species chromosome is closer to the intraoperon HindIII site. The small piece between these two sites may not be visualized simply because of the probe's inhomology to the chromosome.

Double EcoRI-HindIII digestion of W23 chromosomal DNA produced two bands, 2.95 and 5.11 Kbp. The lowest was very dark (Figure 31C) and the higher was exceptionally faint. This suggests retention of the HindIII site within the trpC region in the B. subtilis strain. The transformation data of Tackney and Rudner (1981) support this, as prototrophic strain W23 is frequently used as a source of DNA for transformation into 168M strains and is reported to have contributed a segment to the aromatic amino acid cluster of strain 168i$^-$ (Hemphill and Whitely, 1975). Relative transforming activity data support the view that the W23 sequence was conserved in many 168M strains (Tackney and Rudner, 1982). The average EcoRI fragment size for the trp homologs is 16 Kbp in NCTC3610/168M strains, suggesting regions flanking these transformed areas are still conserved from the Marburg progenitor. This relationship is schematized on Figure 18 as a broken line connecting strains W23 and 168. Analysis of the B. subtilis genetic structure must take into account this limited contribution of strain W23 into the present 168 genome.

G. Leucine and threonine heterologous hybridizations: densitometry comparisons

Comparison was made between the leucine and threonine markers by their degree of hybridization to homologous vs. heterologous chromosomal DNA. Autoradiograms were scanned as described in Methods and the band intensities were calculated

as planometer units. These intensities provided a measure of the total amount of DNA present in a particular chromosomal restriction fragment that was well-matched with the corresponding DNA sequences in the probe preparation. The sum of the measured intensities for all hybrid bands in each bacterial chromosome served as an estimate of DNA sequences in the chromosome that shared homology with either probe, RSF2124'B-leu or pBS02a-thr. The areas under the peaks of leucine hybrid bands were determined for HindIII digests and those for threonine with EcoRI digests. In each case, restriction sites are internal to the operon.

The values are presented as the ratio of heterologous hybridization to the homologous hybridization or the "H index." These have been entered on Tables 18 and 22, under their appropriate restriction digests. The densitometer tracings for probe RSF2124'B-leu and pBS02a-threonine are presented in Figures A1 and A2, respectively. The values indicate the greater degree of leucine homology over that of threonine. Significantly, chromosomal DNA derived from *B. globigii* RUB562 and *B. pumilus* RUB502, the two species which produced detectable leucine transformants, have values of 0.43 and 0.39, respectively. Contrast this to *B. licheniformis* FD01 with a value of 0.27, a strain that produced no detectable leucine transformants (Table 5). The threonine heterologous hybridizations were all within a lower range. Surprisingly, *B. globigii* DNA, with an index of 0.06, the lowest of all, was in marked contrast

to the leucine conservation. The indices for *B. subtilis* W23 DNA were 0.90 and 0.84 for leucine and threonine, respectively; in close agreement with the overall homology of 89% (Hemphill and Whiteley, 1975) with the Marburg derived *B. subtilis* strains. Riley and Anilionis (1980), using the same technique, noted similar variations in homology for the *trp*, *tna*, *thy*, and *lac* gene markers in select gram-negative species.

The mole percent G + C values for the mesophilic *Bacillus* DNAs are all within about 10% (DeLey, 1970; Lovett and Young, 1969; Table 1). This represents results from sequences distributed throughout the chromosome which include the completely conserved regions. The lower degree of relationship we observe in our discrete markers results from the isolation of the cloned regions from neighboring sequences. In the case of the ribosomal gene sets, which are conserved, the H indices would be prejudiced by variation in the absolute number of gene sets. The presented results with leucine and threonine are inherently more accurate for this type presentation because they represent only one copy per chromosome, as opposed to the nine to eleven for the rRNA gene sets.

IV. Genomic Alterations in the ArgA to PheA Conserved Region

Our study had revealed a mutant of *B. subtilis* with a

deletion of an rRNA gene set. Transformants of this *Bacillus* displayed duplications of the leucine marker and alterations of ribosomal DNA homologs. Two strains of *B. subtilis* with noted translocations and inversions indicated novel rDNA homolog patterns on Southern hybridization. These effects are examined and noted within this section with an emphasis upon the association of the nutritional markers involved with the conserved sequences.

A. Deletion of an rRNA gene set in Bacillus subtilis

As shown in Table 24 and Figure 32A, among the EcoRI chromosomal fragments which are homologous to 23S rRNA sequences a 2.9 Kbp homolog was missing. The loss was observed in strains BD29 and BD79, which share the *leuB1* mutation. The closely linked *ilvC1* mutation was resident in strain GSY1269 which also displayed the band loss. Table 24 and Figure 32A indicate the presence of the 2.9 Kbp homolog in the wild type NCTC3610 and 168M strains. The 2.9 Kbp homolog was also present in the 166 strain. A series of 168 strains with mutations in the *arg* to *leu* region were similarly hybridized. Strains MT119 and 127 both possess a mutation, *leuB6*, and yet contain the variable 2.9 Kbp band. Strain RM125, with both a *leuA8* and *arg15* marker also produces a wild type hybridization pattern. Strain Cu373 3 contains a deletion extending from the *azal* marker (aza leucine resistance; map position 250°) to the proximal part of closely linked *ilvB* (map

position 250°). This deletion spans 200 base pairs of the chromosome (C. Mackey, personal communication) and also gave wild type hybridization results. T. Henkin and G. Chambliss recently noted that a mutation causing an alteration in ribosomal protein S4 mapped between argA (map position 265°) and aroG (map position 270°) (personal communication). A strain they supplied to us, 1A92, with these two markers, produced only wild type rRNA gene fragment homologs. The mutational change of many of the mutations we used is not well known. Their origin of production is in many cases obscure, and we suspect that only mutants derived by a postulated intrachromosomal recombination should have lost an rRNA gene set.

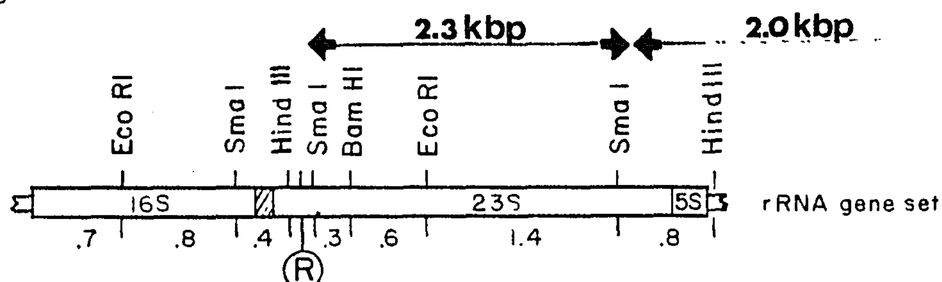
All the Eco RI fragment losses noted also occurred with the 5S sequence, not unexpected in view of its linkage with the 23S rDNA. This loss was visualized directly with 5S rRNA probes. Probes pBC279-23S,5S and p12E2-23S,5S carry this sequence and in the deletion strains the same 2.9 Kbp band was not visible.

HindIII chromosome digestions probed with plasmid p21C4-16S allowed examination of the 16S side of the cistron. In this case, the leuB1 mutant strains BD29 and BD79 lost a 5.5 Kbp homolog (Table 24 and Figure 32B). Strain BD73 argA3, pheA1 showed loss of the same band. The GSY1269 strain behaved as the other three. The wild type strain and select 168M auxotrophs clearly displayed the homolog. As with the EcoRI restriction, strain RM125

with the leuA8 marker produced the wild type pattern. Strain BR54, with the leuC7 marker, displayed the 5.5 Kbp homolog. Curiously, strain 166 had one additional HindIII 16S homolog of 3.3 Kbp, giving it one additional gene set. This band was also observed in GSY1269 in spite of the 5.5 Kbp loss (See Table 24 and Figure 32D).

The HindIII restriction patterns were also probed with 23S and 5S sequences, both as rRNA and cloned rDNA. Table 24 and Figure 32C indicate the loss of a 5.1 Kbp homolog with strains BD29, BD79 and GSY1269. Strains NCTC3610/168M display the presence of this band. A BamHI sensitive site is found in the 23S determinant of the rRNA gene set (Figure 6). Strain BD29 strain probed with p21C4-16S displayed loss of a 5.4 Kbp homolog (Figure 32G). SmaI restrictions with the mutant strains are presented in Table 24 and Figure 32D and E. In this case, with the relevant mutant strains, 2.3 Kbp and 2.0 Kbp bands are lost with 16S and 5S probes, respectively. Figure 32F is a comparison of a wild type and strain BD29 SmaI chromosomal restriction probed with a 23S rDNA sequence. The lack of any lost homologs appears to be a contradiction, especially since the 5S sequence displays the deletion. However, reference to the standard restriction map of the rRNA set (Figure 6) explains the phenomenon. The 23S determinant has a SmaI sensitive site within its 5' end and a second one a few hundred base pairs proximal to the 5S determinant. The two sites are separated by about 2.3 Kbp and in complete chromosomal

restrictions produce a multiple minimal band of this size. Its gross size on a radiogram is obliterating the 2.0 Kbp band present in the wild type cell. The 2.0 Kbp piece is bordered by the 3', 23S SmaI site and extends to a spacer SmaI site beyond the 5S sequence. This piece, which is lost in the mutants, carries not only several hundred base pairs of 23S homology, but the entire 5S sequence. Finally, the pure 5S rRNA probe shares no homology with the 2.3 Kbp intragenic piece. Thus, the two lost bands, while only evident with the 16S and 5S probe, still support the lost operon observation. The area of the chromosome with this spacing of SmaI sites is schematized below.



K. Loughney *et al.* (1983) characterized a *B. subtilis* strain which showed the deletion of an rRNA gene set. The genotype of this deletion strain is not well described (other than a tryptophan requirement) and the exact position of the deletion is not reported. The autoradiograms by these workers are identical to those observed in our series of deletion strains, leading to the conclusion that the same cistron may be involved. Furthermore, it has been suggested that the strains involved in this deletion are derived from the rearranged genome of strain 166 *trpE26* and this mutant simply has one less rRNA cistron (S. Zah-

ler, personal communication). The linkage maps of strains 168 and 166 are presented in Figure 1. The trpD to ilvA segment is translocated in trpE26 strains and occupies a position near tre or thr (Trowsdale and Anagnostopoulos, 1975). The chromosomes of strain 168 and trpE26 also differ in the inversion of the top section of the chromosome (180°). Inside the rearranged chromosomal segments the linkage arrangements are the same in both types of strains. Strain BD29 was obtained from the laboratory of D. Dubnau, who reports its original source as the laboratory of C. Anagnostopoulos. The original designation of this strain was GSY384 (Audit and Anagnostopoulos, 1973). Strains BD79 (leuB1, pheA1) and BD73 (argA3, pheA1) probably have the same origin. If these strains are of 166 trpE26 lineage, they seem to lack some unusual qualities of this strain. Haploid Trp^{+} transformants (or transductants) from 166 (with 168-type donors) have never been obtained (Audit and Anagnostopoulos, 1976). Only diploid tryptophan prototrophs occur, involving the thr to ilvA region and represent about one-third of the chromosome. Audit and Anagnostopoulos (1976) report that trpE26 mutants do not revert to Trp^{+}, so this event must be ruled out in strain BD29. In Table 25 and Figure 32B are compared rDNA hybridization patterns from the six strains. The overall homolog band sizes are reiterated for purposes of comparison. Note the distinct appearance in GSY1269 (trpE26, ilvC1) and 166 (trpE26) of the 3.3 Kbp,

HindIII 16S homolog not found in the NCTC3610/168 group. Strain 166 displays the wild type 5.5 Kbp 16S homolog as do wild types of 168 origin; while strain GSY1269 bearing *ilvC1* contains the 3.3 Kbp band and also exhibits the loss, the strains BD29, BD79 and BD73 lose the 5.5 Kbp band but contain no 3.3 Kbp one. The linkage relationships in the *arg* to *phe* region remain the same in 168 and 166 type strains (See Figure 1). The band loss events we report take place within this segment. The argument of the existence of the second ribosomal gene set group could well be made within the confines of cells of 166 *trpE26* lineage.

Finally, genomic deletions or rearrangements within the first conserved region near the chromosome origin of replication may not be tolerable. Seiki *et al.* (1983) place rRNA gene sets containing tRNA sequences in the early replicating region. Rearrangements resulting in loss of these sequences could be extremely unfavorable for cell viability.

B. Double restriction with EcoRI and HindIII in the deleted operon strain

Double restriction of NCTC3610/168T and BD29 chromosomal DNA was performed. Hybridization was with 23S or 5S end labeled rRNA. This technique identified the 23S and 5S 2.9 Kbp homolog lost in strain BD29 as an EcoRI fragment without internal HindIII sites. Reciprocally, the 5.1 Kbp piece lost in BD29 contains at least one EcoRI

site. Table 26 and Figure 33 present an EcoRI-HindIII hybridization pattern in which, in strains NCTC3610 and 168T, the 2.9 Kbp 23S,5S homolog has not changed in size after secondary restriction with HindIII. In strain BD29 it is the lost fragment in either EcoRI or EcoRI-HindIII restrictions. It is noted that double restriction cuts the 9.0 to 6.1 Kbp 23 EcoRI bands to much smaller fragments which either run off the gel or co-migrate with other bands. The 3.4 Kbp EcoRI fragment also displays an internal HindIII site and is not seen in double restrictions. The accuracy of the method is indicated by looking at the EcoRI 1.2 and 1.4 abutment sequences. Double restriction cuts off about 100 base pairs to trim them to 1.1 and 1.3 Kbp, respectively. The gene set restriction map places the one HindIII site about 0.1 Kbp proximal to the 5' EcoRI site of the 23S sequence (Figure 6).

In 23S homolog patterns of HindIII-restricted chromosomal DNA, the 5.1 Kbp fragment is lost in strain BD29. Double restriction reveals that this band has at least one EcoRI site as its loss coincides with the loss of the 3.1 Kbp EcoRI-HindIII band. The sizes of the 23S homologs indicate that the lost rRNA operon cannot be a member of the group of operons containing a HindIII site distal to the 5S sequence (Stewart et al., 1982). Reference to the gene set restriction map accounts for at most 2.9 Kbp extending from the intraoperon HindIII site to the 5S distal one. Clearly, to lose a 23S,5S 5.1 Kbp HindIII

fragment the extraoperon HindIII site must be at least 2.2 Kbp beyond the 5S end.

C. Alterations in leucine homologs of Leu$^+$ transformants of B. subtilis 168

Southern hybridizations indicated extensive alteration of the leucine region in Leu$^+$ transformants of *B. subtilis*. It was asked: to what extent do these transformation events alter restriction site positions? Does the donor DNA completely replace the mutant's sequence, or only partially? If transformation to leucine prototrophy may be mediated by neighboring conserved sequences (i.e., rDNA), is it possible that recipients are ectopically transformed (which would result in the transformant carrying donor DNA sequences at different positions on the chromosome)? Would this subsequently result in merodiploid cell lines?

Donor DNA was isolated from leucine prototrophic cell lines. The interspecific transformations were from strains and species that had given some detectable transformation frequency with this marker, i.e., *B. subtilis* W23, *B. globigii* and *B. pumilus*. Figure 34 and Table 27 record fragments and their sizes resulting from restriction of the chromosomes of *B. subtilis* transformants. The parental patterns are presented for comparison. DNA patterns from strains NCTC3610/168M form two SmaI hybridization bands at 9.2 Kbp and 4.3 Kbp as expected with RSF2124'B-leu probe (Table 27, Figure 34A lanes 1 and 3). This had been noted

earlier and the SmaI site was mapped within the plasmid insert (See Figure 24). *B. subtilis* strain W23 produced the 9.2 Kbp homolog also, but the second one was at 6.2 Kbp (Table 27, Figure 34A lane 2). Separate transformants of *B. subtilis* BD29 were isolated and the chromosomal DNA extracted (See Table 3). Two of those transformed with W23 DNA (BD29 Leu^{+W23} *argA2* (1), (2) show a composite pattern which consists of three bands derived from both strains. The hybridization suggests a multiple-site transformation, with recipients carrying both their native and W23 derived sequences (Figure 34A Lanes 4 and 5). Alternatively, a third transformation was performed with a lower DNA concentration (1.0 ug) (transformant Leu^{+W23} (3). In this case, the native *B. subtilis* 168M pattern was obtained (Table 27, Figure 34A Lane 6). BD29 (*leuB1*, *argA2*) transformed for arginine prototrophy with 5 ug W23 DNA was indistinguishable from the 168-type hybridization (Table 27, Figure 34A, lane 7). It must be noted that the *leuB1* genetic lesion is not visibly different on Southern hybridizations. The mutant is non-revertible and presumably the results of a deletion, but any size alteration appears beneath the limits of resolution of the gel electrophoresis system (Rudner, 1981).

Endonuclease HindIII with six sites of sensitivity within the *B. subtilis* 168 *leu* operon was used in the same manner. Table 27 and Figure 34B present the hybridization patterns of the same three isolates that were examined with

SmaI. Strain BD29 Leu^{+W23} argA has bands attributable to both strains. The 2.0 Kbp 168 band is replaced with one slightly smaller (1.9 Kbp) clearly derived from strain W23 (Table 27, Figure 34B, lane 4). The smallest 168 0.83 Kbp homolog is replaced by one at 0.78 Kbp, and a 3.8 Kbp piece is added at the top; both are W23 derived. The 0.85 penultimate homolog is of indeterminate origin, as it is found in both strains. The 168 type homologs, 1.7 Kbp and 1.5 Kbp, remain on the radiogram lane. A second transformant isolate, BD29 Leu^{+W23} argA2 (2), has obtained W23 type bands also. Strain BD29 Leu^{+W23} argA2 (3) has replaced both its 2.0 Kbp and 0.83 Kbp fragments with the W23 1.9 Kbp and 0.78 Kbp pieces, respectively (Table 27, Figure 34B, lane 6). These results are compared to the transformant obtained with high DNA concentration of NCTC3610 DNA (BD29 Leu^{+3610} argA2) which clearly maintains all the 168 type HindIII leucine homologs (Table 27 Figure 34B Lane 8). The leuB1 lesion presumably would be found within the 2.0 or 0.83 Kbp (or both) leucine homologs of strain 168 as the analogous W23 fragments are always integrated. At higher transforming DNA concentrations, other regions of the operon could also be replaced by a clustering of integration events.

The HindIII leucine fragments of strain 168 total to 6.9 Kbp. The single strand donor moiety in transformation integration is reported to have a weight average of 7.6 to 9.1 Kbp (Henner and Hoch, 1980). Replacement of the entire

operon is certainly not beyond the capabilities of the recipient organism.

It is possible (as the SmaI restrictions suggest) that these operon fragments are not going to their original positions but to other sequences similar enough to receive them. One transformant, BD29 Leu^{+W23} argA2 (4), demonstrates this. Transformation in this case was with 10 ug DNA. Its HindIII leu homologs display the "obligatory" 0.78 and 1.9 Kbp pieces of W23 derivation, but numerous additional pieces are obvious (Table 27 and Figure 32B Lane 7). With a high specific activity probe (1 to 2 x 10^9 cpm/ug), a multitude of high molecular weight fragments are evident. The "darkest" is 3.8Kbp, one of the W23 homologs; the other seven range from 2.3 to about 7.2 Kb. At least two of these bands are possibly the same phantom homologs observed in 168 native patterns with high specific activity probe and associated with ribosomal DNA correspondence (2.3 Kbp, 5.5 Kbp). One might conclude that leucine area is extremely receptive to multiple transformation events resulting in tandem-type integrations.

The interspecies transformants were compared in the same way. Strain BD29 Leu^{+562} argA2 (1 and 2) transformants with B. globigii DNA. The close correspondence between the donor DNA HindIII pattern and that resulting from the transformant immediately indicates that the low efficiency transformation is not a mis-identified revertant (Table 27; Figure 34C, Lane 5). The integration appears to

be of a replacement type with the *B. subtilis* intergenote retaining at most only one of its original pieces. The BD29 *leuB1* Arg^{+562} transformant (arginine donated by *B. globigii*) maintains the classic 168 fragment pattern with the leucine probe. Three of the Leu^{+} transformants of *B. pumilus* RUB502 were similarly analyzed by Southern hybridization. Figure 34C Lanes 1, 2 and 3 and Table 27 indicate that the resident 1.5 Kbp *leu* homolog is eliminated in the transformant. The native *B. pumilus* RUB502 pattern possesses a 1.6 Kbp band and Figure 34C, lane 3 indicates apparent density increase of the 1.7 Kbp homolog in the transformant. The integration event might have substituted the 1.5 Kbp 168 type homolog with the 1.6 Kbp *B. pumilus* type. These blots derived from DNA of interspecific transformants demonstrate entrance of a foreign sequence in the *B. subtilis* genome.

D. Mass increase of the leucine sequence after transformation

The transformant cell lines which on Southern blots indicated multiplication of their leucine operon sequence were analyzed by densitometry. Figure 35A presents the densitometer scans of SmaI restricted chromosomes. Transformation with W23 DNA has the effect of increasing the mass of the leucine sequence. This was observed in two transformants, BD29 Leu^{+W23} *argA2* (1) and (2). Refer-

ence to the densitometer tracing indicates the molecular weight of the SmaI leucine homologs remains unaltered after transformation with strain W23 DNA. The resulting insertion adds the W23 derived 6.2 Kbp homolog into strain BD29. The total area of each set of scanned peaks was determined and the results are presented in Table 28. The two leucine transformants (#1 and #2) possess 1.65 times the hybridizable sequence of the parental strains. This result is presented as a multiple of the average mass of parental strains BD29 and W23. The mass of the homolog bands change in relation to each other from that observed in the parental types. Most noticeably, the 168 type 4.3 Kbp homolog became less intense to 56% of its native density in transformants BD29 Leu^{+W23} arg2 (1) and (2) (Figure 35A). Estimation of the mass of the leucine operon contained within the 2 ug of DNA applied to the agarose gel was performed. For this calculation we presume this gene of 6.2 Kbp total is represented once per genome. This comes to about 3 to 4 nanograms of leucine DNA sequence for every 2 ug of chromosome (assuming a total chromosome size of 2.5×10^9 daltons). The probe added for these hybridizations was in this range, i.e., 20 to 40 ng for each 10-lane Southern blot. The added mass of the additional sequences could result in a competition between homologs for available hybridization probe. In future work, exploitation of this phenomenon could allow a titration of genomic sequence quantity, with carefully

controlled addition of labeled probes. In addition, this loss of hybridization intensity, if only due to the above effect, has artificially depressed our gross cistron mass estimates. The value of 1.65 which we derived from total densitometry peak sum has most likely erred in the low direction.

The transformants were analyzed in the same way with HindIII restrictions. The sum of peak areas in each strain is presented in Table 28, and representative densitometer scans are recorded in Figure 35B. Transformants BD29 Leu^{+W23} argA2 (1) and (2) have an estimated parental sequence multiplicity of 1.49. This number is lower than the 1.65 previously derived and represents an 11% error between estimates. The average multiplicity with both restrictions is 1.57 times average parental sequence mass. The increase in absolute mass (in planometer units) of the HindIII fragments appears greater than the SmaI restriction fragments (Table 28). The different autoradiograms utilized for each estimate had variation in overall band intensity among them. The multiplicity of sequence units is the more significant figure.

Transformants BD29 Leu^{+W23} argA2 (1) and (2), as in the SmaI analysis, display, in addition to homolog fragment insertions, a change in relative proportion of peak areas. In Figure 35B, the 1.7 Kbp homolog is significantly depressed in area. The inserted 1.9 Kbp band which replaced the endogenous 2.0 Kbp band, is of equal intensity in BD29

Leu^{+W23} argA2 (1) and (2) and the W23 parent. As in the SmaI restriction, we presume the inserted band carries some of the same (or similar) sequences as the 1.7 Kbp one.

A fourth leucine transformant, BD29 Leu^{+W23} argA2 (4), produced nine HindIII homologs to RSF2124'B-leu (Figure 34B, lane 7). The hybridizable mass sum is about 2.7 times the wild type parental strains. This value by our criteria could represent three copies. The DNA of transformant BD29 Leu^{+3610} argA2 shows no homolog band insertion or size change upon our Southern blots (Figure 34B, lane 8). This unaltered leucine HindIII homolog pattern was subjected to densitometry analysis (Figure 35B). The 2.0 Kbp band increased in area, resulting in a total mass increase about 1.27 times parental type. The largest increase is within the 2.0 Kbp homolog, which increases in peak area about 1.6 times. The 1.7 Kbp homolog does not vary and the 1.5 Kbp piece actually decreases to 81% its original value. The last two bands, not well resolved, together are at parental type unit amount. Should this strain be displaying a reiteration of leucine sequence, it appears to encompass only part of the leucine cistron.

In summary, this set of experiments demonstrates that the leucine determinant shares enough homology with areas beyond its native position. The effect is to produce multiple integrations of the marker, in some cases creating cell lines with duplications. This observation will be

further developed by genetic analysis in a later section.

E. Ribosomal DNA homolog alterations in an arginine transformant of B. subtilis

The argA marker was able to undergo transformation with heterologous DNA in B. subtilis recipients (Table 5). This biological conservation suggests, as with the Leu^+ determinant, association with gene sequences of the translational apparatus. Accordingly recipient strain BD29 (leuB1, argA2) was transformed with prototrophic W23 DNA. Of the several transformants selected for arginine prototrophy, one transformant leuB1 Arg^{+W23} (See Table 3) displayed a distinct alteration of its EcoRI 23S,5S hybridization pattern on Southern blots (Figure 36 lane 4). As noted previously, parental strain BD29 is lacking one of nine to ten rRNA operons found in B. subtilis NCTC3610/168M. Arginine selection did not restore the lost operon (designated 2.9 Kbp band in EcoRI restrictions). The native 7.7 Kbp disappeared and simultaneous appearance of a 9.9 and 17.0 Kbp band was noted (See Figure 36, lanes 3 and 4). It is concluded that this strain exhibited a gain of one rRNA operon, thereby providing it with a total of at least ten operons.

The change when noted with plasmid probes of pBC279-23S,5S or 5S rRNA indicates the following two events. The probes are only homologous to the right side of the rRNA operon. The central 23S sequence EcoRI site

borders the 5' end of each homolog fragment and the distal restriction site is obligated to be in spacer DNA beyond the operon terminus (Figure 6). In addition, probe pBC279-23S,5S contains a 2.2 kilobase insert with very little spacer DNA and will reveal no extraneous bands. The first possibility provides for the W23 DNA donation merely to repair the argA2 lesion and thereby alter the position or remove an EcoRI site. This might account for a 7.7 Kbp band becoming a 9.9 Kbp band.

The alternative possibility better accounts for the arrival of the additional rRNA operon. The model proposed by Lehner and Hill (1980) for chromosomal rearrangements mediated by tandem duplicated rRNA cistrons allows such an event. Should the arginine segment be flanked by rDNA, an unequal recombination would create duplicate regions. Yet another conceptualization has the donor DNA segment fusing into a circular conformation linked by recombination via the flanking repeated regions. Such a circle might translocate into a recipient rRNA cistron bringing arginine and also adding one rRNA sequence to the chromosome.

F. Transformants to Leu^+ prototrophy correlated with rDNA alterations

Strain BD29 Leu^{+W23} argA2 (4) (Table 3), which produced a leucine homolog pattern on the Southern blots indicating multiple sites of insertion for this marker (Table 27, Figure 34B), was examined for its rDNA pattern

as well. Probed with pBC279-23S,5S, the EcoRI homolog pattern of this transformant strain indicated a distinct increase in intensity of the 7.7 Kbp band and a faint return of a homolog in the region of 2.9 Kbp (Figure 37A). The apparent return band does not appear completely analogous to the original lost 2.9 Kbp band as it is lighter and in a slightly lower position (Figure 36, lane 6). This was compared to control strains NCTC3610, BD29, and to another transformant BD29 Leu^{+W23} argA2 (3) which had showed the alteration of one leucine HindIII homolog fragment only (Figure 36, lanes 1, 2, and 5). All three displayed no evident change in the ribosomal homolog pattern. As in the parent strain BD29 leuB1, argA2, the 2.9 Kbp fragment is still absent, and no other homologs have changed intensity (Figure 36E). Quantitation of both of these alterations was by densitometry.

The 7.7 Kbp homolog of BD29 Leu^{+W23} argA2 (4) underwent about a twofold intensity increase, indicating a doubling of its copy number (Figure 37A). The percent return of the variable rDNA homolog was determined in the following manner. The 2.9 Kbp native homolog is estimated as one copy under native conditions. In the case of BD29 Leu^{+W23} argA2 (4), we calculated its return as 0.38 times one copy. Inspection of the autoradiogram scan indicates the band is within a slightl4)lower molecular weight position than the native band and is spread over a wider area (Figure 37, A and D). The donor strain W23 does

not normally possess a 2.9 Kbp 23S rRNA homolog in EcoRI restrictions. We suggest that upon this transformation for the leucine marker, one of the multiple inserts went to the native position in the recipient chromosome, bringing back some associated conserved sequences. On the other hand, BD29 Leu^{+W23} argA2 (3), which was initially isolated at low DNA concentration (Table 3), had no multiple leucine hybridization pattern. Consequently, we see no rDNA homolog alterations (Figure 37B).

The multiple insertion event observed in our leucine W23 transformants may be explained as follows (See Figure 34 and Table 27). The favored position of integration of the leucine marker is the native position at 250° map degrees. Note that the transformant BD29 Leu^{+W23} argA2 (3) showed integration of one leucine HindIII fragment (Figure 34B). At high DNA transforming concentration it is possible that after the native positions have been filled, integration events may form in a variety of regions. Should sufficient homology exist on incoming DNA fragments by virtue of areas of conserved sequence, such synaptic regions could occur. It must be noted that in the transformant BD29 Leu^{+W23} argA2 (4) the implicated ribosomal DNA band is near the band (2.9 Kbp) previously associated with leucine, isoleucine-valine, and arginine (see Table 24). Additions and alterations noted on our Southern blots would be expected to be manifest in genetic dose and linkage changes. (See below)

G. Linkage analysis

Transformant cell strains that displayed alterations in their hybridization patterns were analyzed by transduction and transformation. We asked whether insertions within the genome (as with our Arg^{+W23} transformant, Figure 36) would be manifest by linkage alteration. Would multiple site transformations (Figure 34) be displayed by a dosage effect? Finally, we attempt to denote the positions of the transformed sequences within the chromosome. The AR9 transducing particles and transforming DNA were prepared from donors carrying alterations in the *arg*, *ilv*, *leu*, *phe* region.

Table 29 presents the linkage relationships among three of our transformant strains. Transductants were selected for one marker and replica plated to Spizizen's minimal medium for the second marker. Generally, selection was performed in two directions, i.e., one marker selected first and colonies replica plated for second marker selection. Most notable in its biological behavior is strain BD29 *leuB1* Arg^{+W23} (See Table 3). This strain was seen to have obtained a 17 Kbp rDNA homolog and increased one other rDNA fragment by 0.2 Kbp from the transformation event (Figure 36). In total it has added 19 Kbp of new sequence to its genome. The co-transduction frequency displays a decrease from the wild type value provided by the 168T donor. The *argA* to *pheA* co-transduction frequency

in the BD80 (argA2, leuB, pheA1) recipient implies that the addition occurred between the arginine and phenylalanine markers. Control co-transduction values are presented for the trpC2 (map position 205) and thr-5 (map position 290) (Henner and Hoch, 1980). These as expected are zero (Table 29). Similarly, the argA-pheA linkage decreases when the transforming activity of the strains is examined (Table 30). The transforming fragment is noted to be 10 to 20% the size of the transducing fragment (Henner and Hoch, 1980). Percent co-transformation indicates the argA to pheA distance has increased in this Arg^{+W23} transformant strain (Strains BD40 and BD80).

To better locate the actual place of addition, another recipient strain BD47 (ilvC1, pheA) was employed (Table 30). The control donor in this case is strain NCTC3610. The arginine transformant's co-transformation percent (averaging selection in either direction) was less than half the prototroph's. The average percent co-transformation with the W23 donor equals that of the transformant strain. This indicates an insertion between these two markers which is at least 10 degrees distal from arginine on the B. subtilis 168 chromosome map (Figure 1). Such an addition would account for the linkage change between arg and phe also. Ten degrees on the B. subtilis map represents slightly over 100 kilobases of genome (with a 2.5×10^9 dalton chromosome). The event that took place in this transformant led to both arginine prototrophy and the added

DNA mass of 19 Kbp. This genetic acquisition could result from one large insertion or one or more clustered integrations in the region. The acquisition of additional rDNA sequences may be favored if such sequences are normally resident with the arg, ilv, leu, phe chromosome segment.

The leucine transformants were analyzed to see if the leucine mass increase observed by hybridization had biological consequences. Donor DNA derived from strain BD29 Leu^{+W23} argA2 (4) (See Table 3) displayed almost double the Leu^+ transformants per microgram as native donor DNA in recipient BD79 (leuB1, pheA1). The frequency is enhanced 1.7 times in recipient BD80 (Table 30). With strain BD79 the pheA frequency was unaltered.

Should these altered frequencies be results of sequence duplications, the linkage frequencies should indicate it. Transduction analysis is presented in Table 29. Selection of BD80 recipients for Leu^+-Phe^+ co-transduction significantly increased over that of 168T when BD29 Leu^{+W23} (4) was the donor. Higher co-transduction indicates that the leucine marker is in closer proximity to the phenylalanine marker. If the DNA mass increase of about 2 to 3 is skewed in the phe direction, this linkage alteration might well result. Strain BD29 Leu^{+W23} argA (2) (See Table 3), which by Southern hybridization indicated a leucine diploid state gave a Leu^+-Phe^+ co-transduction frequency 5.7% higher than the native donor. This again implicates an

integration towards phenylalanine. The amount of the insertion (in agreement with the hybridization result) is less than with the transformant strain BD29 Leu^{+W23} (4). The transformant BD29 Leu^{+3610} argA2 (See Table 3) gave a Leu^{+}-Phe^{+} average co-transduction of 60.9, somewhat greater than the 168T control. Integration in the phenylalanine direction may account for this. Our Southern autoradiograms detected this event as a mass increase (See Table 28). Insertion of additional DNA in the leu to phe region appears to have decreased the ilv-phe linkage (See Table 29). When strain BD47 (ilvC1, pheA1) is the recipient the average co-transduction frequency in either direction is 55.3% with the 168 donor. This compares to an average of 42.4 with the BD24 Leu^{+W23} argA2 (4) donor. BD29 Leu^{+3610} argA2 produces a drop in the co-transduction frequency, again suggesting the integration of the NCTC3610 leucine in the region. The transformation event that produced these strains resulted in a decrease in the leuB-pheA distance with a concomitant increase in the distance between ilvC-pheA. We conclude from the above data that the added DNA is Leu sequences present in a tandem array.

In transformation assays, the leuB-pheA linkage increases in strain BD29 Leu^{+W23} argA2 (4) significantly (Table 30). The recipients were either BD79 (leuB1, pheA1) or BD80 (argA2, leuB1, pheA1) (Table 30). Our autoradiogram results of the leucine transformant produced by B. globigii

RUB562 (BD29 Leu^{+562} *argA2*; see Table 3) indicated extensive substitution of *B. globigii* leucine sequence for *B. subtilis* sequence. Its *pheA*-*leuB* co-transformation frequency is less than half that of NCTC3610 with the BD79 recipient. The transformation frequency of the intergenotic marker is 6.9% of the homologous phenylalanine marker (3×10^4 and 4.3×10^5, respectively). This linkage decrease must be a function of the partial inhomology of *B. globigii* leucine as compared to the 168 strains. The *trpC2* and *hisH* markers (map position 205, Henner and Hoch, 1980) are unaffected in the transformant strains and display no significant co-transformation alterations (Table 30).

H. Relationship between genetic and physical distance; changes in transformant cell strains

Absolute physical distance was estimated for transformant cell strains. The calculation which relates the frequency of co-transduction of markers to their physical distance from each other on a transducing piece of DNA is that of Kemper (1974). This analytical method presumes the two markers are contiguous on a single fragment of transducing DNA; and as the distance between them increases, their chance of being carried on the same transducing fragment decreases. In addition, as the markers become closer to the ends of the transducing fragment, the size of the pieces of DNA outside of the markers in which crossing-over might occur becomes limited, reducing the number of co-transduc-

tants. The transducing particles must be of uniform length; in our case, phage AR9 has a molecular size of 431 Kbp (Belyaeva and Azizbekyan, 1968). The transducing particles must also be randomly produced. The relationship employed is:

$$c = (1-t) + t \ln t$$

where: c = co-transduction frequency

t = fractional length of the transducing fragment separating the markers

The genetic distance is converted to physical distance (D) by the relationship:

$$D = t(T)$$

where T is the molecular size of AR9.

As t can only range from 0 to 1.0, the standard curve for the function is generated (Figure 38). From an expanded curve such as this, our observed c values were converted to corresponding t numbers. The resulting physical distances calculated are entered on Table 29. The theoretical physical distances for the genetic segments were estimated as a guideline. The *argA2* to *pheA1* markers span from 260 to 245 map degrees. This converts to 159.1 Kbp with a chromosome of molecular weight 2.5×10^9 daltons. The *leuB1* to *pheA1* distance is 5 map degrees, converting to 52.6 Kbp.

By the Kemper (1974) relation, with a 168T donor the

argA to pheA length is 200.4 Kbp. In comparison transformant strain BD29 leuB1 Arg^{+W23} is 218.9 (Table 29). The difference between the two, 18.5 Kbp, corresponds quite closely to the added 19 Kbp observed by Southern hybridization (Figure 33). Therefore, the insertion to this genome is made evident by two independent means which show significant agreement. Strain BD29 Leu^{+W23} argA2 (4) presents its leuB1 determinant 27.2 Kbp from the pheA1 one. The wild type control, 168, gives this span as 70.3 Kbp. The suggestion is that the tandem duplications extend 70.3 - 27.2, or 43.1, Kbp in the direction of pheA. Transformant BD29 Leu^{+3610} shows a similar, although not as pronounced, effect, with a calculated physical separation from pheA of 55.6 Kbp. Strain BD29 Leu^{+W23} argA2 (2), one of the transformant strains that indicated a leu diploid condition by hybridization, separated its leuB marker from pheA by 48.3 Kbp. Complementing these results are those indicating a widening of the span betwen the ilvC and pheA markers. These results are presented in Table 29. The control stain, 168T, indicates a 65.9 Kbp separation between the ilvC and pheA markers. Transformant BD29 Leu^{+W23} argA2 (4), with the comparable distance of 99.9 Kbp, places 34 additional kilobase pairs in this region. (This compares favorably with the 43.1 Kbp calculated for the leuB to pheA length decrease.) BD29 Leu^{+3610} indicates the ilvC and pheA markers are now 50.9 Kbp (116.8 - 65.9) further apart, in poor agreement with the previous calculation of 14.7 Kbp for

the *leuB* to *pheA* length decrease. This figure appears aberrantly high, as it represents a wide range of the co-transduction frequencies of *pheA* to *ilvC* and *ilvC* to *pheA* which were 44.5% and 29.5% , respectively (Table 29). Suffice it to say that in this case, too, the *leu* insertion showed the expected qualitative result of decreasing one linkage with the simultaneous increase of another.

The transduction technique further indicates that our Leu^{+} transformants with multiple insertions are in a tandem array. The increased transformation dosage could result from additions in numerous places on the chromosomes, with congressive events increasing linkage estimates. The transduction results negate that assumption. The t values show that the linked markers we observe are at most 50% of the AR9 molecular size in separation; that is, about 200 Kbp in the theoretical range of the *argA*, *ilvC*, *leuB*, *pheA* segment by map unit calculation. To summarize these events, Figure 39 is a detailed map of this region with the postulated points of chromosome insertion noted. The co-transduction frequencies (c) are converted by the relationship 1-C to recombination units on the figure. The altered co-transductions in the BD29 Leu^{+W23} *argA2* (4) and BD29 *leuB1* Arg^{+W23} are presented below the native in values in parentheses. The area of *leu* insertion is schematized as involving multiple crossover events of homologous regions on donor and recipient DNA distal to the native leucine site. The resulting increase in linkage

between *leuB* and *pheA* results from the position of the most rightward *leuB* insert's closer proximity to the *pheA* marker. The linkage increase is therefore resultant not of a deletion event which would have shortened the wild type distance, but of the new location of the *leu* marker.

I. Concluding remarks

The scheme we presented for the relationship among the eight Bacillus members leaves the mechanism by which it resulted undescribed (Figure 18). The heterogeneity noted within the rRNA gene sequences could have taken place either before or after the amplification event that resulted in about 9 to 11 sequence repeats in each species and strain. It is convenient, conceptually, to place the loss or gain of the 5' 23S EcoRI site prior to gene multiplication. In such a mechanism, the two alleles were present in prototype cells which subsequently amplified them to present levels.

Alternatively, a second model would have an entire gene cluster replaced by a different type of gene copy, an event that would occur after gene set multiplication. Smith (1974) has presented a model "crossover fixation" which could effectively propagate a variant sequence with the elimination of another. This event depends upon the occurrence of unequal crossing-over among tandem repeats. Genes in a tandem array of a fixed size and undergoing unequal crossing-over will eventually replace others by duplication of one of the original genes with others eventually eliminated by deletion. As applied to our system of rRNA genes, the mechanism could independently fix one or the other of two types of rDNA sequences. Figure 40 is a schematic illustration of the crossover fixation event based on that proposed by Smith (1974).

The figure is designed to show the fixation for the type I rRNA sequences (containing the EcoRI site), although the argument is also made in the same manner for fixation of the type II sequence. It is evident that a gene variant originally in the minority can ultimately become the predominant or only type.

The model allows the extreme heterogeneity of the intergenic spacers by supposing that deleterious mutations in critical rRNA genes would be rapidly eliminated while changes in intergenic spacer sequences are better tolerated. Presumably, the degree of selective force is less in these regions and changes will accumulate.

The fact that we, in this study, noted mutants of _B. subtilis_ displaying an rDNA deletion is evidence that a mechanism such as this might occur. The addition, alteration, or replacement of rDNA in two transformants, BD29 Leu^{+W23} _argA2_ and BD29 _leuB1_ Arg $^{+W23}$, lends further credence to the dynamic nature of the rDNA repeats. Reference to a eukaryotic example is in order. The bobbed mutants of _D. melanogaster_ are the result of deletions of ribosomal DNA (Ritossa _et al._, 1966). These deletions may be compensated for by increasing the number of rDNA repeating units (Tartof, 1973), and such increases could be the result of unequal crossing-over between sister chromatids. Recombinants produced magnified and reduced chromosomes, that manifested as flies with increased and reduced rDNA (Tartof, 1974).

Finally, the events we recorded occurred only in the part of the *B. subtilis* chromosome from the *argA* to *pheA* markers, the site of the postulated late replicating rDNA cluster (Smith *et al.*, 1968). The other rDNA clusters are in the early replicating region rDNA cluster almost superimposed at the origin of replication (Ogasawara, Seiki and Yoshikawa, 1983). Such events leading to chromosomal rearrangements or even deletions in this region, if they occur, could suffer from unfavorable selective pressure rendering them inviable.

We noted propensity for transforming DNA with the *leuB* marker to integrate at multiple positions within this region. This argues strongly for the presence of multiple homologous domains. Biological conservation of this region in comparison to other parts of the chromosome must result from the presence of sequences of critical function to the cell.

The existence of multiple copies of a gene in a chromosome could impart a potential for rapid change. Duplications are frequent occurrences in regions containing rRNA cistrons in gram-negative organisms. Such gene sets are involved in tandem duplications of the *glyT* locus in *E. coli* (Hill *et al.*, 1977). The position of the *rrnH* ribosomal RNA cistron was postulated to be within the *leu* to *pro* region of the *Salmonella typhimurium* and *Escherichia coli* chromosome by the propensity for duplications at this site (Anderson and Roth, 1981).

Should these duplications occur by spontaneous recombination between *rrn* loci under rapid growth conditions, cells harboring such structures may increase in frequency in the population. The selective advantage of duplication-containing cells could be due to both an increased rRNA gene dosage and amplification of genes found within the duplicated region.

Figure 41 is a schematic version of how these rearrangements may occur. In Figure 41A two *rrn* sequences of the same polarity undergo intrachromosome recombination. A circular structure is excised creating a deletion of the region between the two rRNA gene sets. Such a circular structure may subsequently undergo recombination with yet another *rrn* sequence at another point within the chromosome resulting in a translocation (Figure 41B). In Figure 41C the unequal recombination postulated occurs between two post-replication chromosome segments containing *rrn* genes. This event would result in one chromosome with a duplication and another with a deletion. Should two *rrn* genes be of opposite polarity, the effect could be to produce an inversion via the looped intermediate structure diagrammed in Figure 41D. These mechanisms form an appropriate model for the events we have observed.

Transformation for the *leuB* marker with its multiple sites of insertion was postulated by us to involve homologous pairing of conserved sequences flanking the leucine marker. The enhanced hybridization observed on Southern

blots could well be driven by flanking conserved regions on the probe. This reaction could mimic the synapsis of leucine transforming fragments *in vivo*, with stabilization of the donor-recipient complex by conserved sequences. The hybridization probe, like donor DNA (after uptake by the recipient cell), is single stranded (Dubnau and Cirigliano, 1972). Ultimately this donor piece will base pair to a homologous region on the recipient chromosome. This model requires a heterologous sequence to be "held in position" by adjacent regions of homology (See Figure 41E). Such an intermediate structure could be resolved by replication producing two homoduplexes of donor and recipient type. Mismatch repair could also be employed generating one type homoduplex only (Dubnau, 1976). Should conserved regions promoting this base pairing be suitably spaced in other positions of the chromosome, this event could take place at multiple spots. A mechanism of this type explains the tandem integrations of the *leuB* determinant we isolated from transformant *B. subtilis* strains.

Elucidation of the evolutionary relationship of all organisms requires examination of a molecule that occurs commonly and functions the same in all. Ribosomal components serve this function as they are the only mechanism for protein assembly. Construction of phylogenetic trees by the 5S rRNA sequence (Hori and Osawa, 1979), the 16S rRNA sequence (Fox *et al.*, 1980) or even ribosomal protein composition (Osawa and Hori, 1979) have led to increased

resolution among many related species. Indeed the genes that code for the translational apparatus may not be mere recipients of change but perhaps also augment it. Active participation by rDNA in evolution through genetic rearrangement by translocation or enhancement of recombination must also be considered.

Summary

1) Conservation of genes of the translation and transcription apparatus in the *Bacillus* is observed. Interspecific transformation with heterologous DNA for the *str* and *rif* genes is possible. Heterologous transformation of nutritional genes does not occur with threonine and tryptophan but does with leucine and arginine.

2) The sequence of the rRNA gene sets is highly conserved with hybridization of *B. subtilis*-derived rRNA to other strains and species very efficient. Organization of the cistron's genes is 16S, 23S, 5S in all cases analyzed.

3) There are two abutment region types between the 16S and 23S determinants in the rRNA gene sets. They differ in size by about 0.2 Kbp. In each *Bacillus* species the predominant cistron type has the smaller abutment piece.

4) Only one rRNA intracistronic EcoRI site is variable in the *Bacillus*. It is found in the 5' end of the 23S determinant and is present in *B. subtilis* and *B. licheniformis*. This site is absent in *B. globigii*, *B. pumilus* and *B. amyloliquefaciens*.

5) In all species and strains nine to eleven rRNA cistrons are found per chromosome. *B. licheniformis* FD01 has an additional gene not present in *B. licheniformis* 8480.

6) Southern blot hybridizations and autoradiogram densitometry measurements indicate the leucine sequence is conserved across strain and species boundaries to a greater extent than the threonine and tryptophan genes. In some hybridizations identical chromosome restriction fragments carried both leucine and rRNA sequences. The presence of rDNA within the arg-ilv-leu-phe gene cluster is postulated.

7) One rRNA cistron is deleted in B. subtilis strains possessing the leuB1, ilvC1 or argA2 mutation. Strains with the trpE26 mutation exhibit a gain of one gene set.

8) Transformation of the rDNA deleted strain for Leu^+ resulted in partial restoration of the lost gene set.

9) Transformation for Arg^+ resulted in the addition of one rRNA gene set and alterations of the rDNA restriction pattern of the recipient. It gained about 19 Kbp of new DNA sequence.

10) Leu^+ transformants revealed on Southren blot hybridizations (leucine probed) multiple sites of donor insertion with loss and substitution of resident DNA.

11) Densitometry analysis of these transformants indicated a mass increase per chromosome of the transformed sequence.

12) Genetic analysis by transduction and transformation revealed an increase in gene dosage and alterations in linkage characteristics.

13) The Arg^+ transformant was estimated to have gained 18.5 Kbp of additional DNA sequence between the *arg* and *phe* genes. This compares favorably with the 19 Kbp of additional rDNA homologs observed in the hybridization study.

14) The presence of rRNA genes in the *arg*-*ilv*-*leu*-*phe* cluster may promote an increase in duplications and other chromosomal aberrations which could arise by unequal crossing-over between these reiterated sequences.

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Table 1

Mole Percent Guanine Plus Cytosine of Select Species

	%GC[a]	%GC[b]
B. subtilis		
W23	42.2	45.6
168i⁻	42.6	43.9
168	41.9	ND
NCTC3610	42.6	ND
B. amyloliquefaciens H	44.0	ND
B. pumilus		
ATCC6631	45.1	ND
NRRL B-3275	ND	43.4
ATCC945	ND	43.2
ATCC14884	ND	43.2
ATCC1	ND	42.2
ATCC70	ND	41.2
ATCC7061	ND	42.9
RUB502	ND	ND
B. licheniformis		
FD0-12	ND	47.3
ATCC9789	46.9	ND
B. globigii	ND	ND
B. stearothermophilus 10	56.0	ND
E. coli		
K12	51.2	51.5
B	50.9	ND
Lambda	48.6	ND

(a) Based on U. DeLey, J. Bacteriol. 101(3):738-754, 1970.

(b) Based on P. Lovett and F. Young, J. Bacteriol. 100(2):658-661, 1969.

Table 2

B. subtilis 168 Derivatives and Other *Bacillus* Species Used In This Study

B. subtilis	Markers
W23	prototrophic (*str*^{r}, *ery*^{r}, *mic*^{r})
ATCC(6051),NCTC3610	prototrophic
168T	*trpC2*
SB25	*trpC2*, *hisB2*
BD170	*trpC2*, *thr-5*
BD29	*argA1*, *leuB1*
BR151	*trpC2*, *metB10*, *lys-3*
BD224	*trpC2*, *thr-5*, *recE4*
BD366	*trpC2*, *thr-5*, pUB110
RM125	*arg15*, *leu-8*, r$^-$, m$^-$ (Bsu)
Mu8u5u16	*purA16*, *leuA8*, *metB5*
GB7044	*leuA169*, *ilvB2*
BR54	*leuC7*, *trpC2*
BD79	*leuB1*, *pheA1*
Cu315	*leuD117*, *trpC2*
166	*trpE26*
GSY1269	*trpE26*, *ilvC1*
BD80	*argA2*, *leuB1*, *pheA1*
BD47	*ilvC1*, *pheA1*
1A92	*argA2*, *aroG932*, *bioB141*, *sacA321*
MT119	*leuB6*, *trpC2*, r$^-$, m$^-$
MT127	*leuB6*, *trpC2*
Cu373	*trpC2*, *ilvB*Δ*3*
BD73	*argA3*, *pheA1*
B. globigii	
RUB562	prototrophic
B. pumilus	
RUB502	*rif*^{r}, *str*^{r}, *ery*^{r}, *bio*$^-$
B. licheniformis	
FD01	*str*^{r}
ACTC8480	*str*^{r}, prototroph
B. amyloliquefaciens	
H	prototroph

Table 3

B. subtilis 168 Strains Constructed By Transformation For This Study

Strain Designation[a]	Transformed Genotype	DNA Donor x Recipient
BD29 Leu^{+W23} *argA* 1,2,3,4 [b]	Leu^{+}	W23 x BD29
BD29 Leu^{+3610} *argA*	Leu^{+}	3610 x BD29
BD29 *leuB1* Arg^{+W23}	Arg^{+}	W23 x BD29
BD29 Leu^{+562} *argA* 1,2	Leu^{+}	RUB562 x BD29
BD29 Leu^{+502} *argA* 1,2,3	Leu^{+}	RUB502 x BD29
BD29 *leuB1* Arg^{+562}	Arg^{+}	RUB562 x BD29

a. Numbers after strain designation indicate individual transformant isolates.

b. 1 to 2 transformed with 5ug/ml donar DNA
 3 transformed with 1ug/ml donar DNA
 4 transformed with 10ug/ml donar DNA
 All other transformations were with 5ug/ml donar DNA

Table 4

Restriction Endonucleases and Other Enzymes Used

Nuclease	Source	Recognition Site
EcoRI	*E. coli* RY13	G'AA*TTC
HindIII	*H. influenzae*	A*'AGCTT
BamHI	*B. amyloliquefaciens*	G'GAT*CC
SmaI	*S. marcescens*	CCC*'GGG
Polynucleotide kinase	T4 infected *E. coli* B	5'-OH
DNA polymerase I (Kornberg polymerase)	*E. coli* MRE 600	

Specificity data and reaction conditions for the restriction endonucleases were as cited by the manufacturer. Polynucleotide kinase conditions were those of Maizels (1977). DNA polymerase I was used for nick translation as described by Rigby et al. (1977).

Table 5 Transformation of B. subtilis Recipient Cells
By Homologous and Heterologous DNA

Recipient	Donor and Genotype	Transformants (no./ml)				
		Leu$^+$	Arg$^+$	Trp$^+$	Thr$^+$	Drug Resistance
BD29 leu-1 argA2	W23 prototroph, str^r	1.7×10^{6}	1.9×10^{6}			7.5×10^{3}
"	Mu8u5u16 purA16, leuA8, metB5	1.9×10^{4}	2.0×10^{6}			
"	RM125 leuA8, arg15	6.6×10^{4}	5.3×10^{4}			
"	B. globigii RUB562, prototroph	2.6×10^{3}	6.0×10^{2}			
"	B. pumilus RUB502, Rif^r	50	-			3.5×10^{2}
"	B. licheniformis 8480, str^r	95	-			2.9×10^{2}
BD170 trpC2 thr-5	W23 prototroph, str^r			8.2×10^{5}	3.2×10^{6}	3.7×10^{3}
"	B. globigii RUB562, prototroph			-	-	
"	B. pumilus RUB502, Rif^r			-	-	1.9×10^{2}
"	B. licheniformis 8480, str^r			-	-	1.2×10^{2}

Recipient	Donor and Genotype	Leu$^+$	Arg$^+$	Ilv$^+$	Phe$^+$
RM125 leuA8 arg15	B. globigii RUD562, prototroph	5.4×10^{3}	4.1×10^{2}	-	-
GB7044 leuA169 ilvB2	"	2.6×10^{3}	-	1.3×10^{3}	-
BR54 leuC7 trpC2	"	3.0×10^{3}	-	-	-
BD79 leuB1 pheA1	"	2.5×10^{3}	-	-	1.7×10^{3}
CU315 leuD117 trpC2	"	(n.d.)	-	-	-

Table 6: Distribution of radioactivity in lambda DNA HindIII digests after labelling by nick translation

Band	Molecular size(kbp)	Molecular size(ratio)[a]	cpm(^{3}H-TTP) nick translated[a]	Southern blot[b] band density[a]	Ethidium stained gel[a]
1	21.86	10.93	5.66	15.73	12.64
2	9.29	4.65	2.82	8.26	6.76
3	6.19	3.09	2.19	4.50	3.88
4	4.27	2.14	2.10	3.07	2.35
5.	2.27	1.14	1.04	1.23	1.29
6.	2.00	1.00	1.00	1.00	1.00

a. Ratio of each band to the 2.00 kbp band

b. lambda DNA was nick translated with α-^{32}P-dATP and α-^{32}P-dCTP Specific activity 1×10^{8} cpm/ug

Table 7: Molecular size and number of chromosomal restriction fragments which hybridize to rRNA in the Bacillus.

B. subtilis				B. subtilis			B. licheniformis	
EcoRI : NCTC3610/168M				W23			FD01	
16s rRNA	16s, 23s pHS102-B7'	23s rRNA pBC279 p12E2	5s rRNA	16s, 23s pHS102-B7'	23s rRNA pBC279 p12E2	5s rRNA	16s, 23s pHS102-B7'	23s rRNA pBC279 p12E2
								13.5
					9.9	9.9		
9.0	9.0	9.0	9.0	9.0				9.0
		7.7	7.7					
					7.2	7.2		
								6.5
	6.1	6.1	6.1	6.1	6.1	6.1		
								6.0
				5.8				
		4.3	4.3		4.3	4.3	4.3	
							4.1	
4.0	4.0			4.0				
								3.8
		3.7m	3.7m		3.7m	3.7m		
							3.6	
		3.5	3.5					
3.4	3.4	3.4	3.4					
					3.3	3.3		
2.9	2.9	2.9	2.9	2.9				
							2.7	
				2.5				2.5
								2.3
2.1	2.1	2.1	2.1				2.1	
1.8	1.8			1.8			1.8	
1.4m	1.4m	1.4m[b]		1.4m	1.4m[b]		1.4m	1.4m[b]
1.2m	1.2m	1.2m[b]		1.2m	1.2m[b]		1.2m	1.2m[b]
							1.1	
							1.0	
	0.9m	0.9m[ab]		0.9m	0.9m[ab]		0.9m	0.9m[ab]

a- band visible with p12E2- 23s, 5s probe

b- band visible with 23s rRNA probe. m- multiple band; Molecule sizes are in kilobase pairs.

Table 7: continued

B. licheniformis 8480: 16s, 23s pMS102-B7'	B. licheniformis 8480: 23s rRNA pBC279 p12E2	B. globigii RUB562: 16s, 23s pMS102-B7'	B. globigii RUB562: 23s rRNA pBC279 p12E2	B. globigii RUB562: 5s rRNA	B. pumilus RUB502: 16s, 23s pMS102-B7'	B. pumilus RUB502: 23s rRNA pBC279 p12E2	B. pumilus RUB502: 5s rRNA	B. amyloliquefaciens H: pMS102-B7'	B. amyloliquefaciens H: 23s rRNA pBC279 p12E2
	13.5		13.6	13.6					
	9.8		10.2m	10.2m	11.7	10.2	10.2		10.2
		7.3							
		7.0	7.1	7.1					
	6.0					6.5	6.5	6.5	6.1
		5.2				5.6m	5.6m		5.6
					4.9				
4.3					4.4	4.8	4.8	5.0	4.8
4.1		3.9							
	3.8		3.8m	3.8m		3.8	3.8	3.8	3.8m
3.6					3.7				
		2.9						3.3	
2.7	2.5		2.6	2.6	2.8				
					2.5				
	2.3	2.2							
2.1		2.1m	2.1m[ab]		2.1m	2.1m[ab]		2.1m	2.1m[ab]
1.8		1.9m	1.9m[ab]		1.9m	1.9m[ab]		1.9m	1.9m[ab]
1.4m	1.4m[b]				1.8				
		1.3			1.6				
1.2m	1.2m[b]							1.5	
1.1		1.1						1.3	
0.9m	0.9m[ab]								

Table 8: Molecular size and number of chromosomal restriction fragments which hybridize to rRNA in the Bacillus.

HindIII:	B. subtilis NCTC3610/168M			B. subtilis W23			B. licheniformis FD01	
	16s p21C4	23s rRNA pBC279 p12E2	5s rRNA	16s p21C4	23s rRNA pBC279 p12E2	5s rRNA	16s p21C4	23s rRNA pBC279 p12E2
					9.9	9.9		
	9.3			9.3				
	9.1							
		8.3	8.3					
					7.7	7.7		
							7.5	
	6.5m			6.5m				
					6.4m	6.4m		
								6.0
	6.0m			6.0				
		5.9	5.9				5.8	
	5.5							
		5.1	5.1					
								5.0
							4.4	4.5
					3.9	3.9		
								4.0
		3.2[a]	3.2					
					3.1	3.1		3.3
	2.7	2.9[b]	2.9	2.7				
							2.9	
	2.4	2.4		2.4				
							2.4m	2.4
	2.0			1.9				

a - minimal size band observed with pBC279- 23s, 5s probe.

b - Faint 23s rRNA minimal band.

m — multiple band

Molecular sizes are in kilobase pairs.

Table 8: continued

B. licheniformis 8480		B. globigii RUB562		B. pumilus RUB502	B. amyloliquefaciens H	
16s p21C4	23s rRNA pBC279 p12E2	16s p21C4	23s rRNA pBC279 p12E2	16s p21C4	16s p21C4	23s rRNA pBC279 p12E2
		9.3				
7.5						
		6.5	6.9	6.8		
5.8	6.0				5.6	6.0
						5.5
	5.0	4.9	5.0	4.9	4.9	4.7
4.4			4.7			
	4.3				4.1	4.2
			3.8	3.7		3.8
		3.4				
	3.3				3.2	
2.9	2.8		2.9	3.2		
2.4m	2.4	2.7	2.7	2.7	2.4	2.5
			2.4			
		2.0				
					1.7	

Table 9: Molecular size and number of chromosomal restriction fragments which hybridize to rRNA in the *Bacillus*.

	B. subtilis			*B. subtilis*			*B. licheniformis*		
BamHI:	NCTC 3610/168M			W23			8480		
	16s p21C4	23s rRNA pBC 279	5s rRNA	16s p21C4	23s rRNA pBC 279	5s rRNA	16s p21C4	23s rRNA pBC 279	5s rRNA
	30 to 22	30 to 22	30 to 22	30 to 22	20	20			
					17	17			
	16.1			16.1					
							14	14	14
		12.5	12.5		12	12		12	12
					9.5	9.5	9.9		
	8.0								
		7.3	7.3		7.3	7.3	7.5		
	6.5			6.5			6.5	7.0m	7.0m
	6.2	5.9	5.9	6.2					
					6.0m	6.0m			
	5.4m						5.8	6.0m	6.0m
					4.5	4.5	4.6		
	4.4	4.3	4.3	4.4				4.2	4.2
		3.9	3.9				3.6	3.8	3.8
		3.5	3.5		3.6	3.6		3.4	3.4
		2.9	2.9						

Table 9: continued

BamHI:	B. pumilus RUB502			B. globigii RUB562		
	16s p21C4	23s rRNA pBC279	5s rRNA	16s p21C4	23s rRNA pBC279	5s rRNA
					19	19
		17	17			
					16	16
				15.3		
	14					
					12	12
					11	11
				9.7		
	9.0					
		8.8	8.8		8.5	8.5
	8.0	8.0	8.0			
		7.0m	7.0m			
				6.1m	6.0m	6.0m
				5.9m	5.9m	5.9m
	4.6	4.4	4.4	4.4	4.3	4.3
	3.5	3.5	3.5			
		3.0	3.0	2.9	3.0	3.0
				2.6	2.6	

Table 10: Molecular size and number of chromosomal restriction fragments which hybridize to rRNA in the Bacillus.

SmaI:	B. subtilis NCTC3610, 168M			B. subtilis W23	B.globigii RUB562	B. pumilus RUB502
	p21C4- 16s	23s rRNA pBC279- 23s,5s p12E2- 23s,5s	5s rRNA	p21C4- 16s	p21C4- 16s	p21C4- 16s
	33.8					
					24.0	22.6
	16.9					
				13.6		
	12.6				12.8	12.1
				11.0		
		9.2	9.2			
				8.5		
		8.0	8.0		8.0	8.0
		6.5	6.5		5.2	5.4
		5.0	5.0		4.0	4.1
	3.5	4.2	4.2			
	3.2			3.2		
	2.9			2.8	3.0	
	2.7	2.6	2.6			
		2.4	2.4			
	2.1	2.3m	2.0			

m, multiple band

Table 11: Molecular size and number of restriction fragments internal to the rRNA gene sets in the *Bacillus*.

Species Strain	p21C4- 16s	23s	5s	pBC279- 23s, 5s	p12B2- 23s, 5s	pHS102- 16s, 23s
EcoRI:						
B. subtilis						
NCTC3610, 168T, W23	1.4 1.2	1.4 1.2 0.9	-	-	0.9	1.4 1.2 0.9
B. licheniformis						
FD01, 8480	1.4 1.2	1.4 1.2 0.9	ND	-	0.9	1.4 1.2 0.9
B. globigii						
RUB562	2.1 1.9	2.1 1.9	-	-	2.1 1.9	2.1 1.9
B. pumilus						
RUB502	2.1 1.9	2.1 1.9	-	-	2.1 1.9	2.1 1.9
B. amyloliquefaciens						
H	2.1 1.9	ND	ND	-	2.1 1.9	2.1 1.9

Table 11: continued

Species Strain	p21C4- 16s	23s	5s	pDC279- 23s, 5s	p12E2- 23s, 5s	pMS102- 16s, 23s
SmaI:						
B. subtilis						
NCTC3610, 168T, W23	-	2.3	-	2.3	2.3	2.3 0.7 0.5
B. globigii						
RUB562	-	2.3	-	2.3	2.3	2.3 0.7 0.5
B. pumilus						
RUB502	-	2.3	-	2.3	2.3	2.3 0.7 0.5

- No rRNA operon internal fragments observed.

ND, Not determined

Molecular sizes are in kilobase pairs.

Table 12: Similiarity coefficients for rDNA homologs.

Species/strain comparisons

I	II	S(EcoRI)	S(HindIII)
B. sub. NCTC3610	B. sub. 168M	1.00	1.00
" "	B. sub. W23	0.53	0.86
" "	B. lich 8480	0.27	0.15
" "	B. lich.FD01	0.38	0.14
B. sub. W23	B. lich 8480	0.50	0.18
" "	B. lich.FD01	0.30	0.17
B. sub. NCTC3610	B. glob.RUB562	0.14	0.57
" "	B. pum.RUB502	0.14	0.15
" "	B. amylo.H	0.28	0.29
B. lich.8480	B. lich.FD01	0.77	0.90
B. sub. W23	B. glob.RUB562	0.36	0.67
" "	B. pum.RUB502	0.18	0.18
" "	B. amylo.H	0.36	0.00
B. glob.RUB562	B. pum.RUB502	0.40	0.36
" "	B. amylo.H	0.40	0.17
B. pum.RUB502	B. amylo.H	0.80	0.36
B. glob.RUB562	B. lich.8480	0.55	0.00
" "	B. lich.FD01	0.50	0.00
B. pum.RUB502	B. lich.8480	0.18	0.00
" "	B. lich.FD01	0.33	0.00
B. amylo.H	B. lich.8480	0.36	0.00
" "	B. lich.FD01	0.33	0.00

Table 12: continued

Probability of homolog size conservation between two species or strains.

Pairs equivilent	Probability random	Theoretical S value
1	1.09	0.10
2	0.90	0.20
3	0.72	0.30
4	0.56	0.40
5	0.41	0.50
6	0.29	0.60
7	0.19	0.70
8	0.10	0.80
9	0.05	0.90
10	0.01	1.00

The homolog size matched pairs are presumed to be derived from chromosomal DNA restricted with EcoRI and hybridized with 23s specific probe (see figure 12) for this theoretical presentation.

Table 13: The multiplicity of 23s rRNA homologs in EcoRI digests of Bacillus DNAs.

	kbp	X unit area	band number
B. subtilis	9.0	1.09	1
	7.7	1.29	1
NCTC3610/	6.1	1.05	1
168M	4.3/3.7	2.02	2
	3.5/3.4	2.48	2 to 3
total = 9 to 10	2.9	1.37	1
	2.1	1.00	1
B. subtilis	9.9	1.00	1
	7.2	1.74	2
W23	6.1	1.08	1
	4.3	1.36	1
total = 9	3.7/3.3	3.69	4
B. globigii	13.6	1.00	1
	10.2	1.23	1
RUB562	7.1	2.78	3
	3.8	1.66	2
total = 9	2.6	1.86	2
B. pumilus	10.2	2.09	2
	6.5	2.17	2
RUB502	5.6	2.53	2 to 3
	4.8	1.61	2
total = 9 to 10	3.8	1.00	1
B. amyloliquefaciens	10.2	1.00	1
	6.1	2.48	2 to 3
H	5.6	2.13	2
	4.8	1.25	1
total = 10 to 11	3.8	3.98	4
B. licheniformis	13.5	1.59	1 to 2
	9.8	1.70	2
8480[a]	6.0	2.23	2
	3.8	2.21	2
total = 9 to 10	2.5	1.00	1
	2.3	1.00	1

a. B. licheniformis FD01 has one additional band of 6.5 kbp.

Band sizes are presented as kilobase pairs (kbp).

X unit area = band multiplicity of the 1.00 unit homolog. Band number = estimated gene set number from the multiplicity.

Table 14: Ratio of the large to small 16s, 23s abutmant fragments in the rRNA gene sets of the *Bacillus*[a]

Type I; ratio of the 1.4 to 1.2 kbp bands

B. subtilis	168M	0.31
	W23	0.31
B. licheniformis	FD01	0.45
	8480	0.28

Type II; ratio of the 2.1 to 1.9 kbp bands

B. globigii	RUB562	0.34
B. pumilus	RUB502	0.42

a. average of ratios performed with both cloned and rRNA hybridization probes

Table 15: Fragments produced by restriction endonuclease digestion of the plasmid RSF2124.B-leucine

Enzyme	A	B	C	D	E	F	Sum of fragment sizes (kbp)
EcoRI	11.1	6.2					17.3
BamHI	8.8	8.5					17.3
HindIII	10.5	2.6	1.6	1.5	0.7	0.4	17.3
SmaI	13.3	4.0					17.3
SmaI-EcoRI	10.0	3.3	2.9	1.1			17.3
BamH-SmaI	8.5	4.5	4.0	0.3			17.3

Table 16

Transformation of E. coli and B. subtilis strains with RSF2124-leu plasmid DNA

Strain used	Relevant genotype	No. transformants/ml[a] Leu$^+$	Leu$^+$, Am^r
E. coli			
HB101	leuB[b]	4.3×10^4	3.2×10^4
B. subtilis			
RM125	leuA8	1.5×10^5	0
BR54	leuC7	5.2×10^6	0
BD29	leuB1	4.5×10^6	0
CU315	leuD117	1.6×10^6	0
B. subtilis			
BD29	argA2	0	
GB7044	ilvB2	0	
BD79	phe1	0	

(a) The concentration of RSF2124-leu ccc DNA was 2.0 μg per assay.

(b) The leuB in E. coli corresponds to the B. subtilis leuC which codes for the enzyme β- isopropylmalate dehydrogenase

Table 17

Survivals of genes in the leu region of the B. subtilis chromosome following restriction cleavages of prototrophic DNAs[a]

Recipient and genotype		RTA%[b] W23DNA EcoRI	HindIII	SmaI	3610 or 168M DNA EcoRI	HindIII	SmaI
RM125	leuA8	2.9	0.27	0.25	0.72	0.17	0.07
BR54	leuC7	3.3	0.06	0.42	2.3	0.02	0.28
BD29	leuB1	3.8	0.21	0.98	7.5	0.21	0.15
CU315	leuD117	3.1	n.d.	2.2	4.0	n.d.	1.1
BD29	argA2	0.38	0.32	0.51	1.9	0.21	0.27
RM125	arg15	1.5	0.11	0.27	n.d.	0.03	0.31
GB7044	ilvB2	1.1	n.d.	0.31	n.d.	n.d.	0.06
BD79	pheA1	1.3	n.d.	2.5	n.d.	n.d.	0.15

(a) Transforming DNA (10-20 μg) digested overnight at 37°C with endonucleases in the appropriate buffer at 3 U/μg DNA. The competent cell suspension (0.9 ml) containing 2 x 10^8 cells/ml was mixed with native or restricted DNA.

(b) RTA is Relative Transforming Activity expressed as percentage.

n.d. = not determined

Table 18: Molecular size and number of chromosomal restriction fragments which hybridize to the leu probe within members of the genus *Bacillus*.

	B. subtilis			*B. licheniformis*		*B. pumilus*	*B. globigii*	*B. amyloliquefaciens*
	NCTC3610	168T	W23	FD01	8480	RUB502	RUB562	H
EcoRI:	6.2	6.2	6.2				6.2	ND
						5.2		
				4.2	4.2			
							3.0[a]	
				2.7	2.7	2.7[a]		
				1.4[a]	1.4[a]			
	1.2[a]	1.2[a]	1.2[a]	1.3[a]	1.3[a]			
HindIII:	5.5[a]	5.5[a]						ND
			4.1	4.3				
	3.8[a]	3.8[a]	3.8		3.2	2.7	2.8	
					2.4			
	2.1[a]	2.1[a]						
	2.0	2.0	1.9					
	1.7	1.7				1.6		
	1.5	1.5		1.64			1.4	
				0.96		1.1	1.0	
	0.85	0.85	0.85				0.96	
	0.83	0.83						
			0.78					
H index	1.00		0.90	0.27	ND	0.39	0.43	ND
SmaI:	9.2	9.2	9.2	ND	ND	ND	10.0	
			6.2					
	4.3	4.3						4.0

Molecular sizes are in kilobase pairs. The H index is determined from the HindIII restriction fragments (see figure A1). a. Band observed with probes of 1×10^9 cpm/ug.

Table 19

Transforming activity of plasmid pBS02a isolated from *B. subtilis* and from *E. coli*

Host where plasmid was isolated	Selection for[a]	Transformants/ml[b]	
		BD170 $RecE^+$	BD224 recE4
I. *B. subtilis* BD224	Cm^r	4.7×10^5	4.4×10^4
	Thr^+	2.4×10^6	0
	Cm^r and Thr^+	4.2×10^4	0
II. *E. coli* HB101	Cm^r	2.5×10^5	4.8×10^4
	Thr^+	4.1×10^6	0
	Cm^r and Thr^+	7.8×10^3	0

(a) The concentration of chloramphenicol was 5 μg/ml.

(b) The concentration of plasmid DNA was 2 μg per transformation assay.

Table 20: Fragments produced by restriction endonuclease digestion of the plasmids pBS02a-threonine and pHV33

	Enzyme	A	B	Sum of fragment sizes (kbp)
pBS02a-threonine:	EcoRI	4.6	4.3	8.9
	HindIII	6.1	2.8	8.9
	BamHI	native form		
pHV33	EcoRI	6.8		6.8
	HindIII	4.0	2.8	6.8
	BamHI	6.8		6.8

Table 21

Marker survival of two non-conserved genes thrA and trpC following EcoRI, HindIII and SmaI digestion of B. subtilis DNAs.

DNA	Genotype	RTA% EcoRI	HindIII	SmaI
I. ThrA+				
W23	prototroph	0.24	7.5	2.8
3610	prototroph	0.28	3.8	2.3
168M	leuB1, argA2,[a] and derivatives	0.15	3.1	2.9
II. TrpC+				
W23	prototroph	13.2	0.26	4.7
3610	prototroph	35.1	2.3	8.9
168M	leuB1, argA2, and derivatives	9.5	0.14	7.4

(a) Strain BD29 and its various Leu$^+$ transformants.

Table 22: Molecular size and number of chromosomal restriction fragments which hybridize to the thr probe within members of the genus Bacillus.

	B. subtilis			B. licheniformis		B. pumilus	B. globigii	B. amyloliquefaciens
	NCTC3610	168T	W23	FD01	8480	RUB502	RUB562	H
EcoRI:							6.5	ND
					5.6			
						4.3		
	3.9	3.9	3.9	3.6				
	1.4	1.4	1.4	1.4				
H index	1.00		0.84	0.20	ND	0.27	0.06	ND
HindIII:			10.6					
						5.9		
	5.1	5.1			4.2			
							3.8	3.1
				2.7				
SmaI:	13.0	13.0		14.0	ND	14.0		ND
							11.0	
			9.4					
BamHI:	5.2	5.2		ND	ND	ND	ND	ND
			3.3					

Molecular sizes are in kilobase pairs. The H index is determined from the EcoRI restriction fragments (see figure A2).

Table 23: Molecular size of chromosomal restriction fragments which hybridize to the *trp* probe within members of the genus *Bacillus*.

Species/strain	EcoRI	HindIII	EcoRI-HindIII
B. subtilis			
W23	8.74	4.59	a. 5.11 b. 2.95
NCTC 3610	14.83	4.45	4.30
SB25	15.19	ND	ND
BR151	15.91	4.64	4.79
BD170	19.34	4.17	4.17
B. pumilus			
RUB502	4.45	a. 8.55 b. 4.89	a. 2.58 b. 1.06
B. licheniformis			
FD01	11.36	a. 2.73 b. 1.98	1.86

Molecular sizes are in kilobase pairs

The probe is the *trp* insert isolated from plasmid pRR1C6-tryptophan.

ND, not determined

Table 24: *B. subtilis* strains displaying loss or gain of rRNA gene sets.

Strain	Genotype	probe digest band lost	23s, 5s EcoRI 2.9 kbp	p21C4-16s HindIII 5.5 kbp	23s, 5s HindIII 5.1 kbp	p21C4-16s SmaI 2.3 kbp	5s SmaI 2.0 kbp
NCTC3610	wild type		+	+	+	+	+
168T	*trpC*-		+	+	+	+	+
BR151	*trpC2*, *met310*, *lys-2*		+	ND	ND	ND	ND
BR54	*trpC2*, *leuC7*		ND	+	ND	ND	ND
RM125	*leuA8*, *arg15*, r^-, m^-		+	+	+	ND	ND
MT119	*leuB6*, *trpC2*, r^-, m^-		+	ND	ND	ND	ND
MT127	*leuB6*, *trpC2*		+	ND	ND	ND	ND
CU373	*trpC2*, *ilvBΔ3*		+	ND	ND	ND	ND
IA92	*argA*, *aroG*, *bioB*, *sacA*		+	ND	ND	ND	ND
166	*trpE26*		+	+[a]	ND	ND	ND
BD29	*leuB1*, *argA2*		-	-	-	-	-
BD79	*leuB1*, *pheA1*		-	-	-	-	-
BD73	*argA3*, *pheA1*		ND	-	ND	-	ND
GSY1269	*trpE26*, *ilvC*		-	-[a]	-	ND	ND

\+ homolog present

\- homolog absent

ND, not determined

a. appearance of a 3.3 kbp homolog band

Table 25: Comparison of the rDNA homolog pattern in B. subtilis strains with chromosomal rearrangements.

Strains	NCTC3610, 168		BD29, 79, 73		166		GSY1269	
probes	16s[a]	23s, 5s[b]	16s[a]	23s, 5s[b]	16s[a]	23s, 5s[b]	16s[a]	23s, 5s[b]
	9.3		9.3		9.3	ND	9.3	
	9.1	8.3	9.1	8.3	9.1		9.1	8.3
	6.5		6.5		6.5		6.5	
	6.0m	5.9m	6.0m	5.9m	6.0m		6.0m	5.9m
	5.5	5.1	-	-	5.5		-	-
		3.2		3.2	(3.3)		(3.3)	3.2
	2.7	2.9	2.7	2.9	2.7		2.7	2.9
	2.3	2.3	2.3	2.3	2.3		2.3	2.3
	2.0		2.0		2.0		2.0	

All genomic DNA is HindIII restricted

- homolog missing

( ) appearance of an additional homolog band

m, multiple band

a. 16s sequence carried by plasmid p2104- 16s

b. 23s and 5s sequences carried by plasmid pBC279- 23s, 5s

Table 26: Molecular size of chromosomal restriction fragments which hybridize to 23s rRNA after digestion with EcoRI and HindIII.

Strains of <u>B. subtilis</u>

NCTC3610/168			BD29		
EcoRI	EcoRI/HindIII	HindIII	EcoRI	EcoRI/HindIII	HindIII
9.0			9.0		
		8.3			8.3
7.7			7.7		
6.1			6.1		
		5.9			5.9
4.3	4.3	5.1	4.3	4.3	-
3.7m	3.7m		3.7m	3.7m	
3.5	3.5		3.5	3.5	
3.4		3.2	3.4	-	3.2
	3.1				
2.9	2.9	2.9	-	-	2.9
		2.3			2.3
2.1			2.1	2.1	
1.4			1.4		
	1.3			1.3	
1.2			1.2		
	1.1			1.1	
0.9	0.9		0.9	0.9	

Molecular sizes are in kilobase pairs.

Table 27: Distribution of HindIII restriction fragments which hybridize to the leucine gene cluster among parental *Bacillus* species and their interspecific transformants.

	I. Parental types		II. Leu$^+$ transformants of BD29 leuB1, argA2				III. Arg$^+$ transformant of BD29 leuB1, argA2
Species	B. subtilis						
Strains	NCTC3610, 168T, BD29	W23	W23(1),(2),	W23(3)	NCTC3610		W23
SmaI:	9.2	9.2	9.2	9.2	9.2		9.2
		6.2	6.2				
	4.3		4.3	4.3	4.3		4.3
Species	B. subtilis						
Strains	NCTC3610, 168T, BD29	W23	W23(1),(2)	W23(3)	W23(4)	NCTC3610	W23
HindIII:					7.2		
					6.2		
					5.5		
		4.1			4.3		
		3.8	3.8		3.8		
					3.5		
	2.0				3.0	2.0	2.0
		1.9	1.9	1.9	2.9		
	1.7		1.7	1.7	2.3	1.7	1.7
	1.5		1.5	1.5	1.9	1.5	1.5
	0.85	0.85	0.85	0.85	0.85	0.85	0.85
	0.83	0.78	0.78	0.78	0.78	0.83	0.83
Species	B. pumilus	B. globigii					
Strains	RUB502	RUB562	502(1),(2),(3)	562(1),(2)			562
		4.2					
	2.6	2.7		2.7			
			2.0				2.0
	1.6		1.7				1.7
	1.2	1.3		1.3			1.5
	1.0	0.91		1.0			
	0.88	0.85	0.85	0.91			0.85
			0.83	0.85			0.83

Table 28: Leucine sequence multiplicity in transformant strains[a] of B. subtilis.

SmaI:

Strains	NCTC3610	BD29	W23	Leu^{+W23} (1) & (2)	Leu^{+W23} (4)	Leu^{+3610}
Planometer total	0.649	0.648 (average = 0.634)	0.604	1.046	ND	ND
Sequence unit multiplicity[b]		1.00		1.65		

HindIII:

Strains	NCTC3610	BD29	W23	Leu^{+W23} (1) & (2)	Leu^{+W23} (4)	Leu^{+3610}
Planometer total	0.925	0.931 (average = 0.869)	0.807	1.299	2.333	1.100
Sequence unit multiplicity		1.00		1.49	2.68	1.27

a. Transformants are derived from strain BD29 leuB1, argA2 (see table 3).

b. One unit sequence is defined as the average of the amount in the parental strains.

Table 29: Linkage relationships of the arg-ilv-leu-phe genes in AR9 transduction with donars carrying various substituted segments.

Recipient genotype	Donar genotype	Selected phenotype	Unselected class	Total colonies tested	Percent cotransduction	Physical distance (kbp)[a]
BD80 argA2, leuB1, pheA1						
	168T trpC2	Arg$^+$	Arg$^+$Phe$^+$	1,440	18.0	200.4
		Leu$^+$	Leu$^+$Phe$^+$	948	58.0	70.3
		Phe$^+$	Phe$^+$Leu$^+$	436	49.3	
	BD29 Leu^{+W23}, argA2(4)	Leu$^+$	Leu$^+$Phe$^+$	681	73.7	27.2
		Phe$^+$	Phe$^+$Leu$^+$	330	75.8	
	BD29 Leu^{+W23}, argA2(2)	Leu$^+$	Leu$^+$Phe$^+$	350	63.7	48.3
	BD29 Leu^{+3610}, argA2	Leu$^+$	Leu$^+$Phe$^+$	1,955	59.8	55.6
		Phe$^+$	Phe$^+$Leu$^+$	384	62.0	
	BD29 leuB1, Arg^{+W23}	Arg$^+$	Arg$^+$Phe$^+$	2,001	14.7	218.9
		Phe$^+$	Phe$^+$Arg$^+$	174	0	
BD47 ilvC1, pheA1						
	168T trpC2	Phe$^+$	Phe$^+$Ilv$^+$	508	57.5	65.9
		Ilv$^+$	Ilv$^+$Phe$^+$	321	53.0	
	BD29 Leu^{+W23}, argA2(4)	Phe$^+$	Phe$^+$Ilv$^+$	248	46.4	99.9
		Ilv$^+$	Ilv$^+$Phe$^+$	190	38.4	
	BD29 Leu^{+3610}, argA2	Phe$^+$	Phe$^+$Ilv$^+$	519	44.5	116.8
		Ilv$^+$	Ilv$^+$Phe$^+$	614	29.5	

a- The physical distances were determined by the Kemper (1974) formula.

Table 29: continued; Transduction frequency of two unlinked genes.

Recipient genotype	Donar genotype	Transductants/ml Trp$^+$	Thr$^+$	Percent cotransduction
BD170 trpC2, thr-5				
	168T trpC2	0	7.1×10^2	0
	NCTC3160 prototroph	6.4×10^2	6.6×10^2	0

The percent cotransductants were scored by replica plating onto selective media.

Table 30: Transforming activity and linkage relationships of genes transformed with donars containing deletions, rearrangements and substitutions in the arg-ilv-leu-phe gene cluster.

Recipient (relevant genotype)	Donar (relevant genotype)	Transformants/ug DNA		Percent
		Selection	Number	Cotransformation[a]
BD40 argA3, pheA12				
	NCTC 3610 prototroph	Phe^+	4.6×10^5	3.3
	BD29 leuB1 Arg^{+W23}	Phe^+	5.6×10^5	1.2
	CU371 ilvBΔ2	Phe^+	5.4×10^5	5.4
	GSY1269 ilvC1	Phe^+	4.3×10^5	3.6
BD80 argA2, (leuB1), pheA1[b]				
	NCTC 3610 prototroph	Arg^+	2.5×10^5	1.5
	BD29 leuB1 Arg^{+W23}	Arg^+	2.6×10^5	0.65
	CU371 ilvBΔ2	Arg^+	3.1×10^5	3.8
	GSY1269 ilvC1	Arg^+	2.6×10^5	1.9
BD80 (argA2), leuB1, pheA1[c]				
	NCTC 3610 prototroph	Leu^+	2.5×10^5	3.7
	BD29 Leu^{+W23} argA2(4)	Leu^+	4.3×10^5	5.3
	BD29 Leu^{+3610} argA2	Leu^+	2.7×10^5	3.9
	CU371 ilvBΔ2	Leu^+	2.2×10^5	6.1

a- scored by replica plating; no. doubles/no. singles x 100

b- linkage between argA2 - pheA1

c- linkage between leuB1 - pheA1

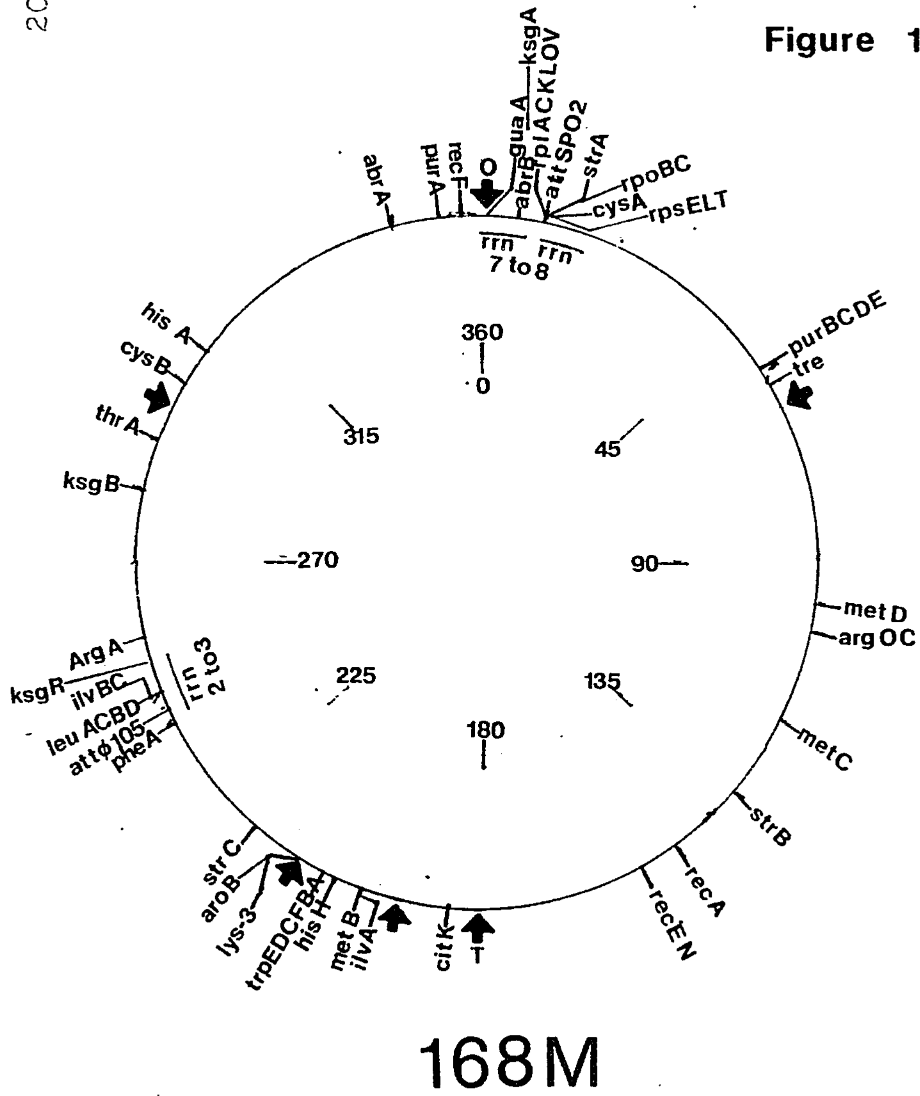
(Table continued on next page)

Table 30: continued

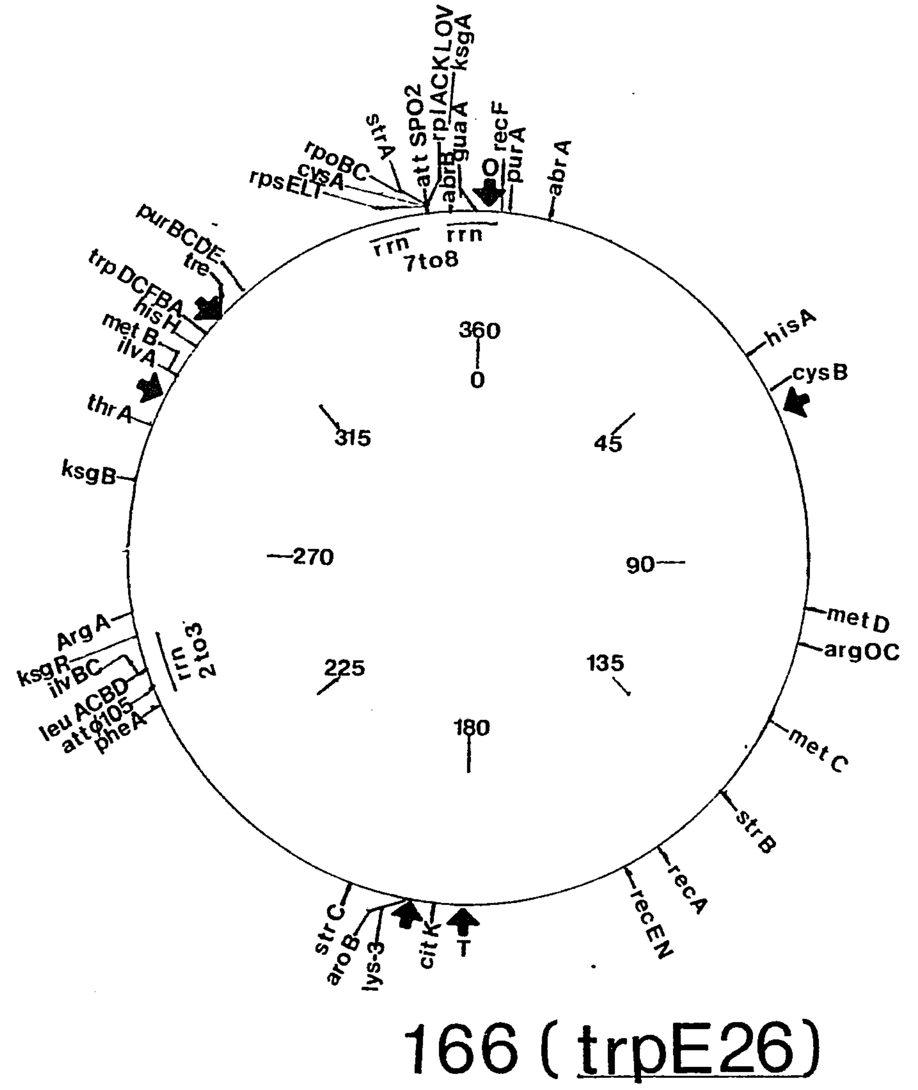
Recipient (relevant genotype)	Donar (relevant genotype)	Transformants/ug DNA Selection	Transformants/ug DNA Number	Percent Cotransformation [d]
BD47 <u>ilvC1</u>, <u>pheA1</u>				
	NCTC3610 prototroph	Ilv^+	1.4×10^5	3.4
		Phe	1.3×10^5	4.1
	W23 prototroph	Ilv^+	4.7×10^4	2.6
		Phe^+	8.6×10^4	1.2
	BD29 <u>leuB1</u>, Arg^{+W23}	Ilv^+	7.9×10^4	1.4
		Phe^+	1.1×10^5	2.3
BD79 <u>leuB1</u>, <u>pheA1</u>				
	NCTC3610 prototroph	Leu^+	4.3×10^5	6.5
		Phe^+	3.9×10^5	7.8
	W23 prototroph	Leu^+	4.0×10^5	2.5
		Phe^+	4.1×10^5	2.6
	BD29 Leu^{+W23}, <u>argA2</u>(4)	Leu^+	<u>7.8×10^5</u>	<u>11.0</u>
		Phe	3.6×10^5	<u>13.0</u>
	BD29 Leu^{+3610}, <u>argA2</u>	Leu^+	3.8×10^5	5.2
		Phe^+	4.4×10^5	7.1
	BD29 Leu^{+562}; <u>argA2</u>	Leu^+	3.0×10^4	0
		Phe^+	4.3×10^5	3.5
SB25 <u>trpC2</u>, <u>hisB2</u>				
	NCTC3610 W23 prototrophs	His^+	4.6×10^5	46.0
	BD29 Leu^{+W23}, <u>argA2</u>(4)	His^+	5.6×10^5	52.0
	BD29 Leu^{+3610}, <u>argA2</u>	His^+	3.4×10^5	54.0
	BD29 Leu^{+501}, <u>argA2</u>	His^+	4.7×10^5	46.0
	BD29 Leu^{+562}, <u>argA2</u>	His^+	3.2×10^5	55.0

Figure 1: Genetic maps of the chromosomes of B. subtilis strains 168 and 166 (trp E26). The arrows mark the positions of replication origin, termination, and points of linkage alterations between the two strains. The positions of the ribosomal RNA clusters are noted. The position of the ksg R marker is approximate(Pai and Dabbs 1981). The maps are based upon that of Henner and Hoch (1980) and Anagnostopoulos and Trowsdale (1976). Map degrees are noted on the central circle.

Figure 1



168M



166 (trpE26)

Figure 2: Standard curve of electrophoretic mobility vs. molecular weight on an 0.75% agarose gel. Bacter-lambda DNA is digested to completion with HindIII endonuclease and subjected to electrophoresis as described in Methods. Anode is on the right of the migration scale. Ethidium bromide stained band distances from the gel origin are measured and plotted vs. M.W. raised to the (-) 0.666 power by the procedure of Beardon (1977).

Lambda DNA fragment sizes after restriction

kbp.	Md.	$MW^{-0.666}$
21.86	14.40	0.169
9.29	6.13	0.299
6.19	4.09	0.391
4.27	2.82	0.501
2.27	1.50	0.763
2.00	1.32	0.831

Figure 2

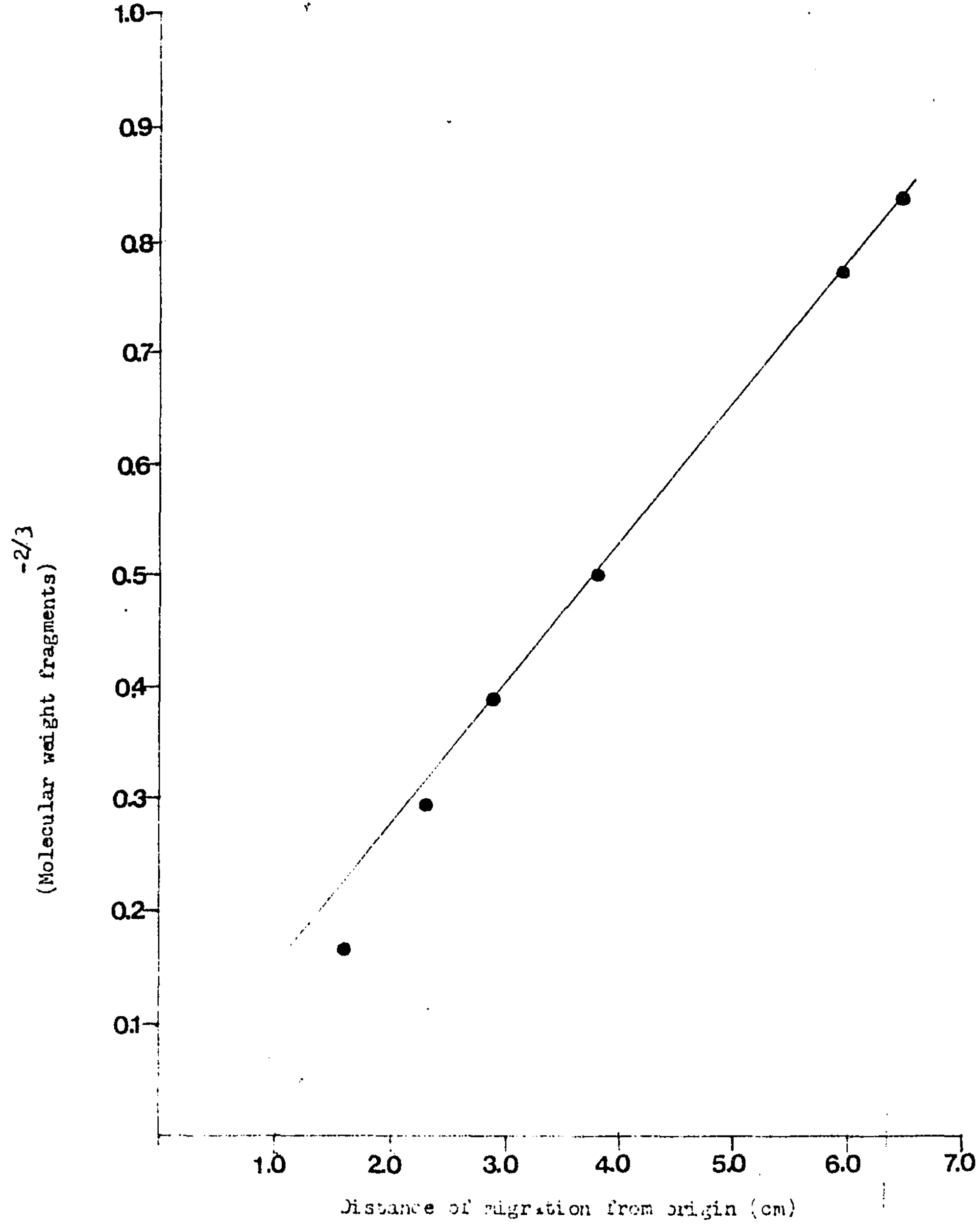
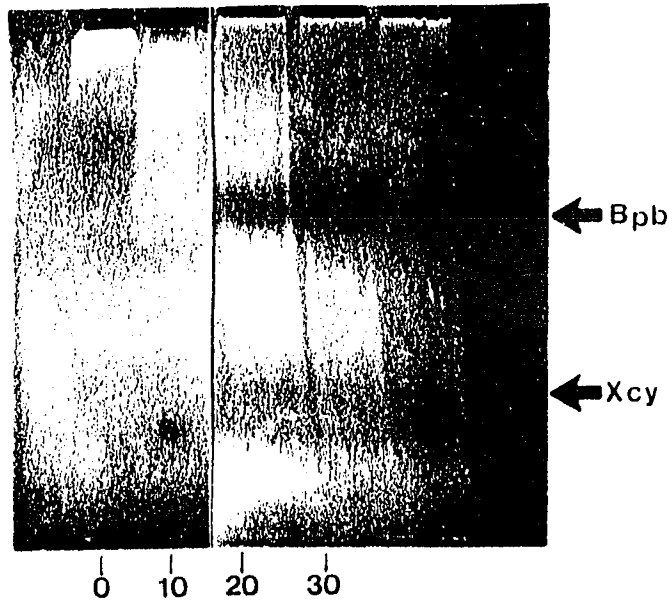


Figure 3: Ribosomal RNA (23s) hydrolysis visualized by acrylamide gel electrophoresis. The hydrolysis was of 1ug of isolated RNA at 0, 10, 20, to 30 minutes. The reaction was at 90 degrees (pH 9.5). Molecules degraded to about 100 nucleotides long migrate with the xylene cyanol dye marker (Xcy in the figure) in this 10% gel matrix. The bromophenol blue marker is denoted Bpb in the figure. The fragmented RNA reveals 5'- hydroxyl ends which are end labeled with ^{32}P by T4 polynucleotide kinase and γ-^{32}P-ATP by the method of Maizels (1977)

Figure 3



Minutes hydrolysis 23s rRNA

Gel is 10% acrylamide

Figure 4: End labelling kinetics of 23s rRNA with γ-^{32}P-ATP and T4 kinase. Gel purified sample of rRNA (lug) was labeled with ^{32}P at the 5' hydroxyl terminus according to Maizels (1977) as described in Methods. The γ ^{32}P- ATP was at 10uM concentration and the reaction volume was at 10 ul and ran at 37 degrees. At two to four minute intervals 1.0 ul aliquots were precipitated in 6% cold trichloroacetic acid in the presence of 100 ug/ml calf thymus DNA. Precipitated counts were collected on glass fiber filters and cpm was measured by liquid scintillation in BBOT (Methods).

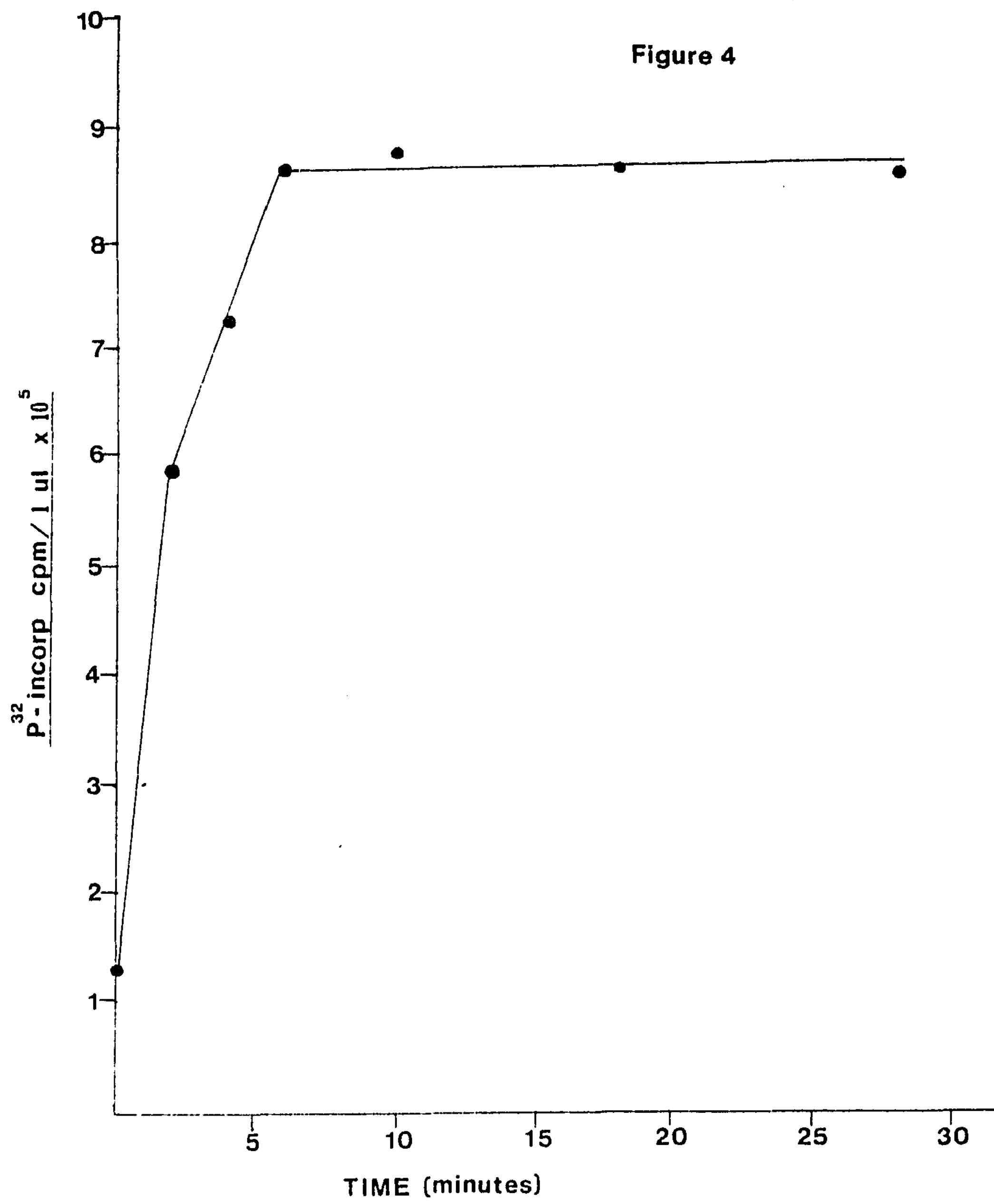


Figure 4
^{32}P - incorp cpm/ l ul x 10^5
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
TIME (minutes)

Figure 5: Sephadex G-50 exclusion chromatography of end labeled 23s rRNA. The product of an end labeled reaction such as depicted in figure 4, was loaded onto a 1ml column and eluted with buffer (Methods). Fractions were of 3.0 drops of which 0.5 ul aliquots were counted by liquid scintillation in Aquasol. Curve area was calculated by the summation of ^{32}P- cpm within each peak. Specific activity of product ranged from 1 to 3 X 10^7 cpm/ug.

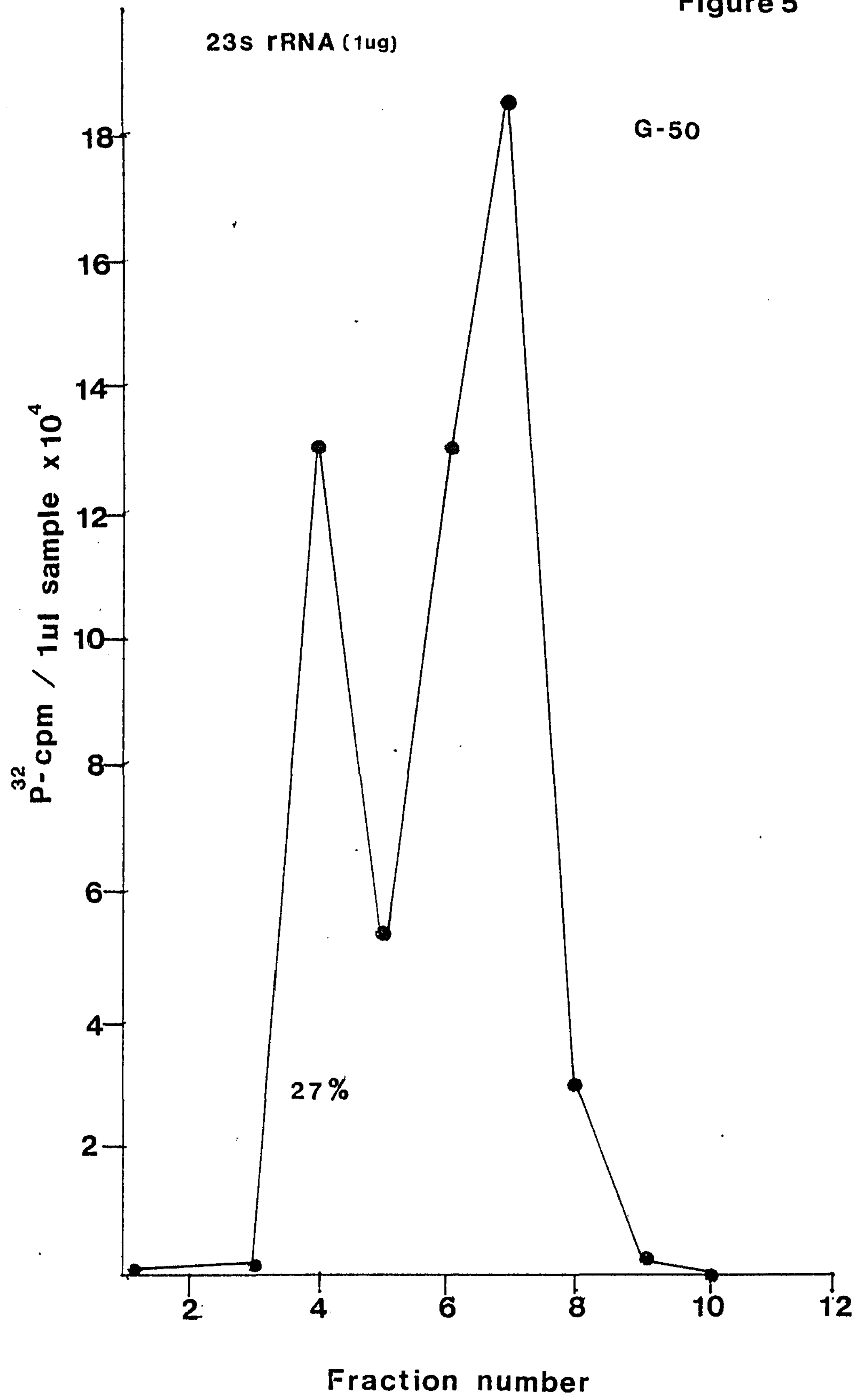


Figure 5
23S rRNA (1ug)
G-50
^{32}P-cpm / 1ul sample x10^4
18
16
14
12
10
8
6
4
2
27%
2
4
6
8
10
12
Fraction number

Figure 6: Generalized restriction map of *B. subtilis* rRNA gene sets. The map is as proposed by Stewart, Wilson, and Bott (1981). Ⓡ indicates the position of the EcoRl site not present in the rRNA gene sets of *B. globigii*, *B. pumilus*, and *B. amyloliquefaciens.* The five rDNA fragments from *B. subtilis* inserted into plasmids are represented beneath the restriction map. The solid portion of each line is homologous to the gene set, the broken line is DNA sequence beyond the rDNA boundary. Plasmid p14B1- 23s, 5s, 16s & 23s contains an insert that spans two rRNA gene sets. The arrows indicate the borders of the cloned chromosome fragment.

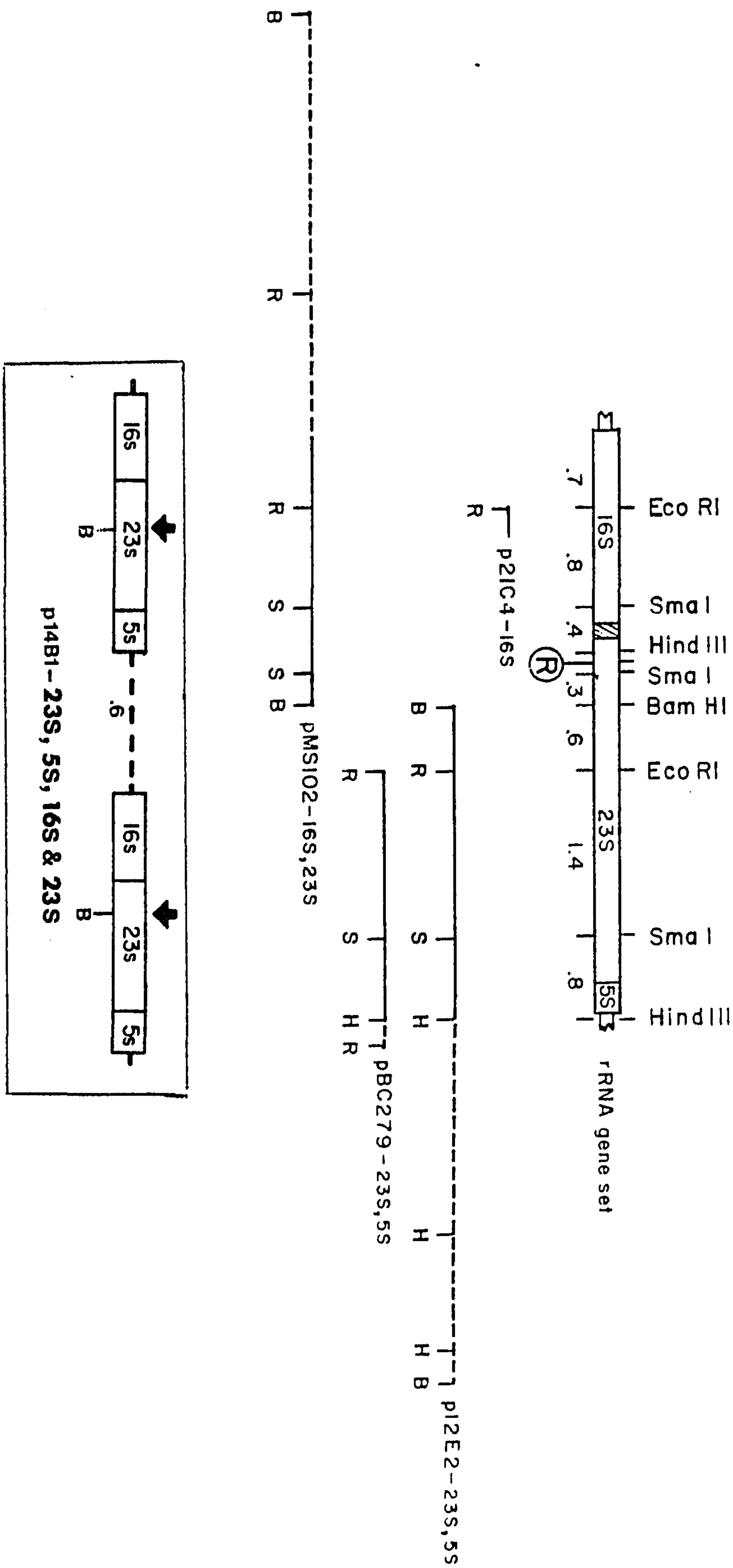


Figure 6

Figure 7: Northern blots of vegetative rRNA probed with cloned rDNA. Isolated ribosomal RNA was transferred from agarose gels to sheets of nitrocellulose as in Methods. Prior to electrophoresis the RNA was denatured by treatment with glyoxal. The cloned rDNA's were nick translated and used to probe 16s, 23s, and 5s rRNA species. Probe identification is:

A. p14B1- 16s, 23s, 5s

B. pBC279- 23s, 5s

C. p12E2, 23s, 5s

D. pMS102-B7'- 16s, 23s

E. p21C4-16s

F. Ethidium stained rRNA species prior to transfer to nitrocellulose.

Figure 7

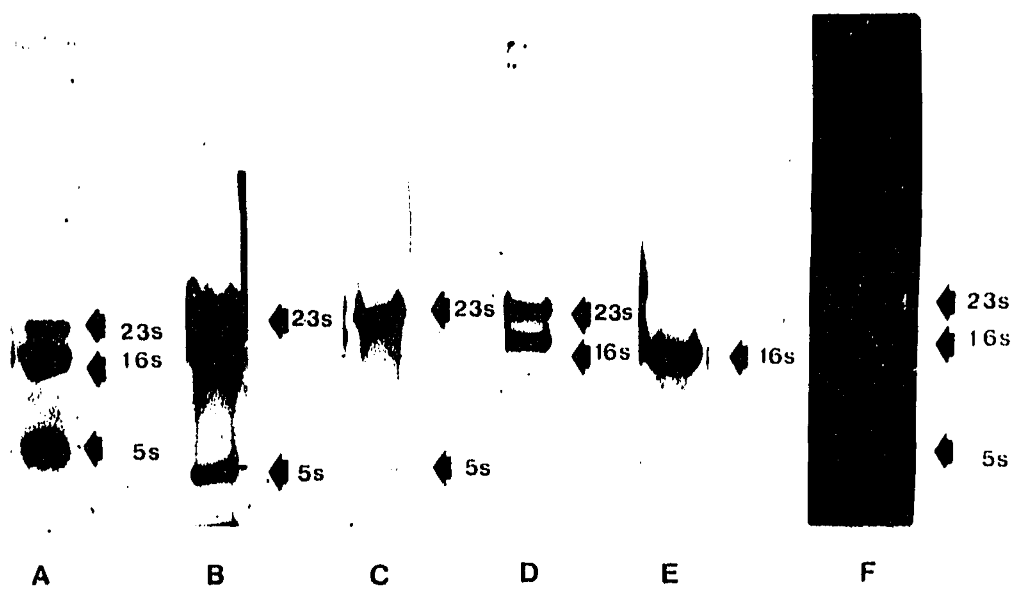


Figure 8: Nick translation kinetics of plasmid pUB110. Purified sample of plasmid (1 ug) was labelled with ^{3}H-(CH_3)- thymidine -5'- triphosphate (78.1 Ci/mM) according to Rigby et. al. (1977) and as described in Methods. Labeled TTP was at a concentration of 200pmoles and unlabeled dGTP, dCTP, and dATP were at 500pmoles. At 10 minute intervals after the addition of 0, 1, 5, or 10 units polI, aliquots (2.0ul) were withdrawn and reaction terminated by addition to 0.2ml stop solution (Methods). Acid insoluble counts were determined by collecting on glass fiber filters. The reaction temperature was 10 degrees. Reaction volume was 100ul.

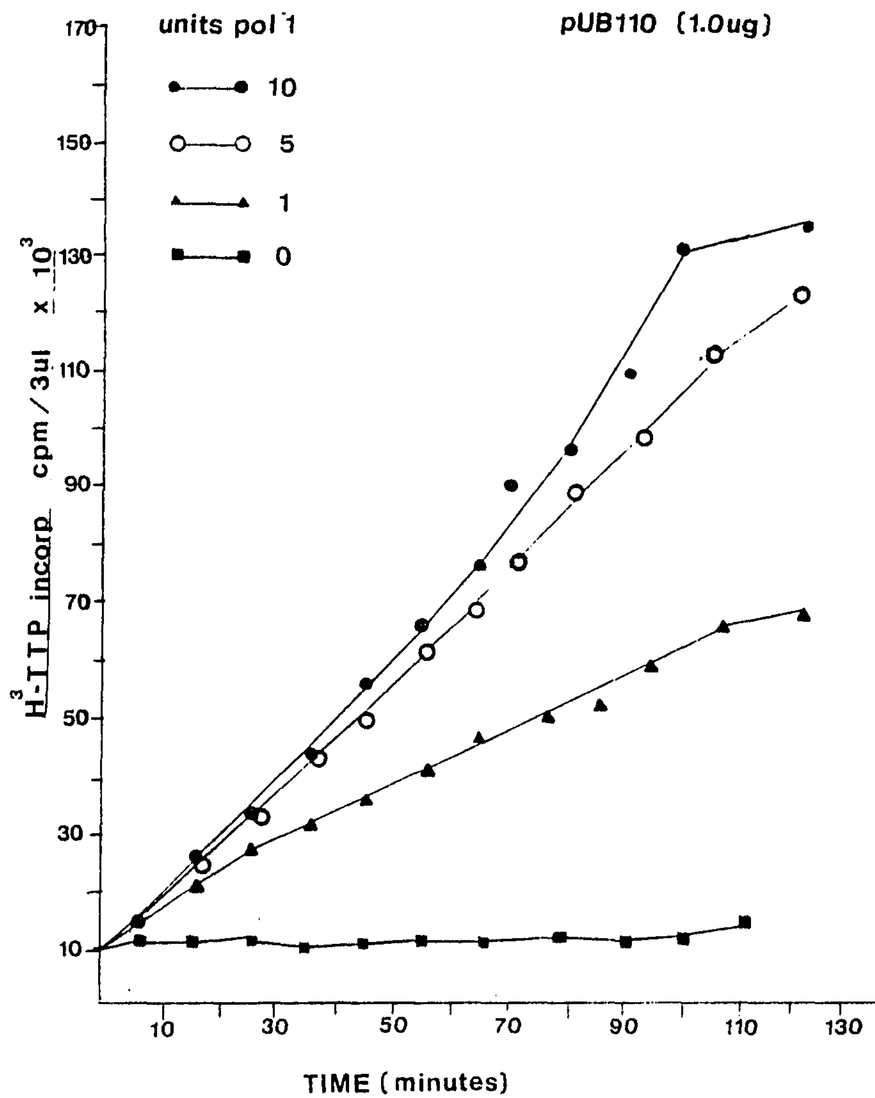


Figure 8
units pol 1
10
5
1
0
pUB110 (1.0ug)
^{3}H-TTP incorp cpm / 3ul x 10^{3}
170
150
130
110
90
70
50
30
10
10
30
50
70
90
110
130
TIME (minutes)

Figure 9: Sephadex G-50 exclusion chromatography of nick translated plasmid pUB110. The product of a 10 unit polI nick translation reaction as depicted in Figure 8 was loaded onto a 7 ml column and eluted with buffer (Methods). Radioactivity of each fraction (3 drops) was determined by the measure of cpm of 5 ul aliquots. Curve area was calculated by summation of counts from each peak. Specific activity of DNA labeled with α^{32}P- dATP and α^{32}P-dCTP for use as hybridization probes ranged from 0.5 to 2.0 X 10^9 cpm/ug, with above reaction conditions.

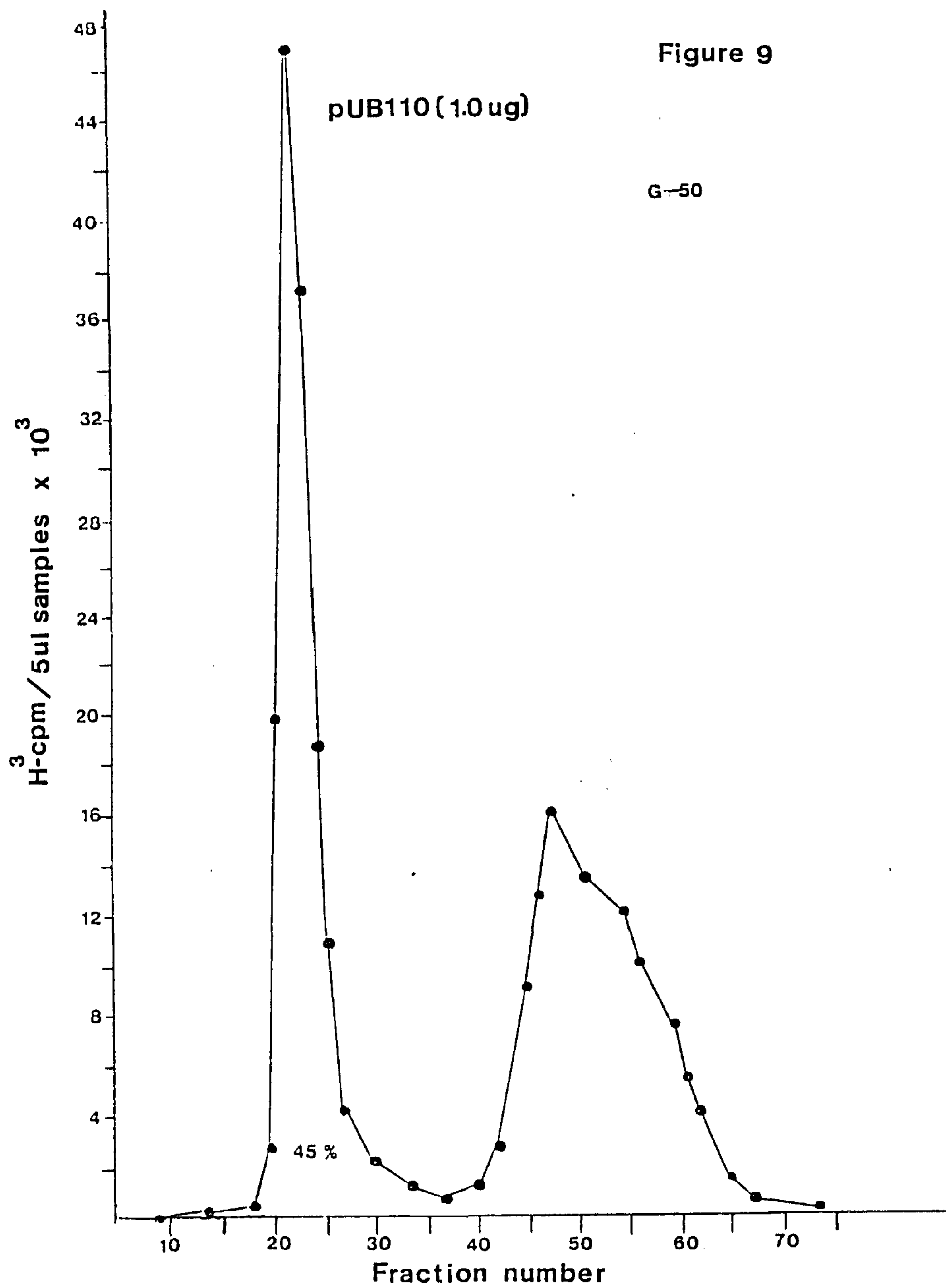


Figure 9
pUB110 (1.0 ug)
G-50
45 %
^{3}H-cpm/5ul samples x 10^{3}
Fraction number
48
44
40
36
32
28
24
20
16
12
8
4
10
20
30
40
50
60
70

Figure 10: Distribution of radioactivity in a lambda DNA HindIII digest labeled by nick translation. Label distribution following HindIII cleavage of lambda DNA (0.01 ug). DNA was labeled prior to endonuclease scission. The sample was electrophoresed in an 0.75% agarose gel. The gel was sliced into 1 mm sections dissolved in 0.2 ml NH_4OH at 80 degrees, followed by scintillation counting in Bray's solution. Specific activity of the DNA sample was about 4.0×10^5 cpm/ug.

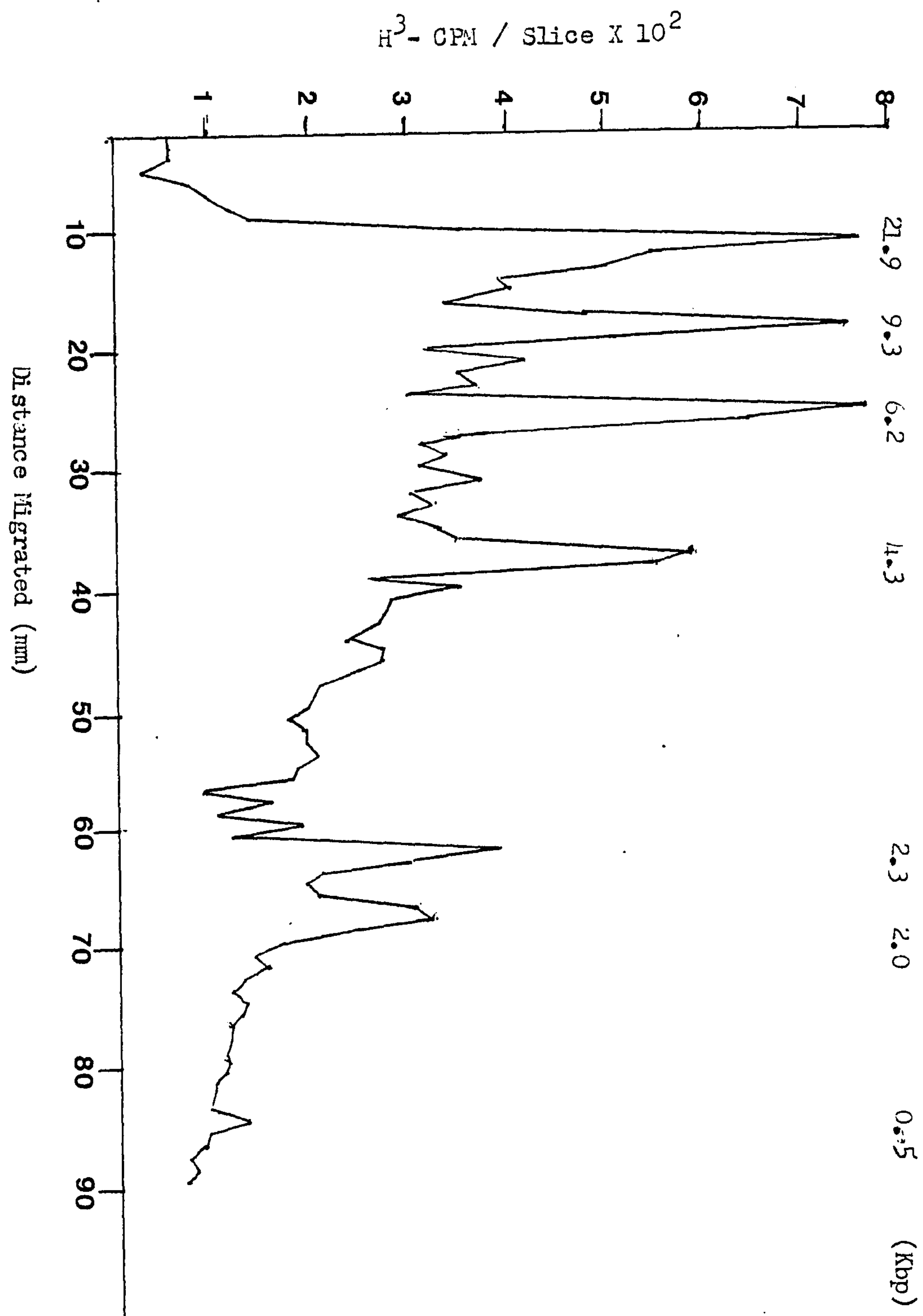


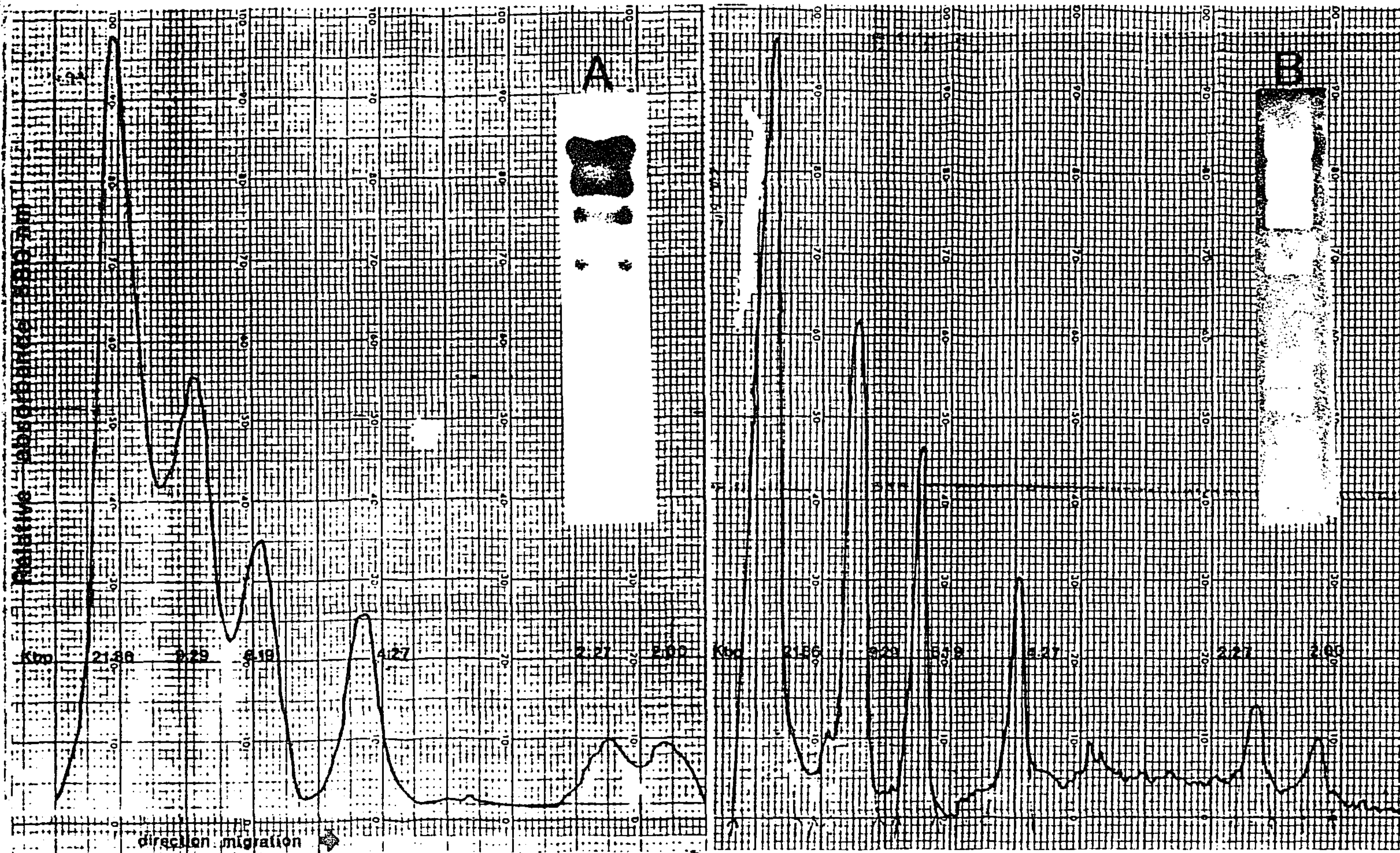
Figure 10

Figure 11: Densitometer scans of HindIII restricted lambda DNA after electrophoresis through a 0.75% agarose gel.

A. Gel was transferred to a nitrocellulose sheet and hybridized to a lambda DNA probe. The probe DNA had been labeled by nick translation with α-^{32}P-dATP and α-^{32}P-dCTP (Methods).

B. Gel was stained with ethidium bromide (0.2 ug/ml) and photographed by U.V. transillumination onto polaroid type 55 P/N film. The negative was then scanned.

Absorbance measurements were made at 580 nM.



Hybridization (dA,dC labeled probe)

Ethidium stained gel

Figure 11

Figure 12: Southern blot hybridization of Bacillus genomic DNA. ^{32}P nick translated rDNA or ^{32}P end labeled rRNA were used as probes. All genomic DNA was EcoRI digested (3 units enzyme/ug DNA). Two ug of restriction product were applied to each gel lane.

A. B. subtilis NCTC 3610 / 168M
probes used
1. 16s rRNA
2. pMS102-B7'-16s, 23s
3. 23s rRNA, pBC279- 23s, 5s; p12E2- 23s, 5s
4. 5s rRNA

B. B. subtilis W23
probes used
1. pMS102-B7'- 16s, 23s
2. 23s rRNA, pBC279- 23s, 5s; p12E2 -23s, 5s
3. 5s rRNA

C. B. licheniformis FD01
probes used
1. pMS102-B7'- 16s, 23s
2. 23s rRNA; pBC279- 23s,5s; p12E2- 23s,5s

D. B. licheniformis 8480
probes used
1. pMS102-B7'- 16s, 23s
2. 23s rRNA, pBC279- 23s, 5s; p12E2- 23s, 5s

E. lambda DNA HindIII digested

Figure 12

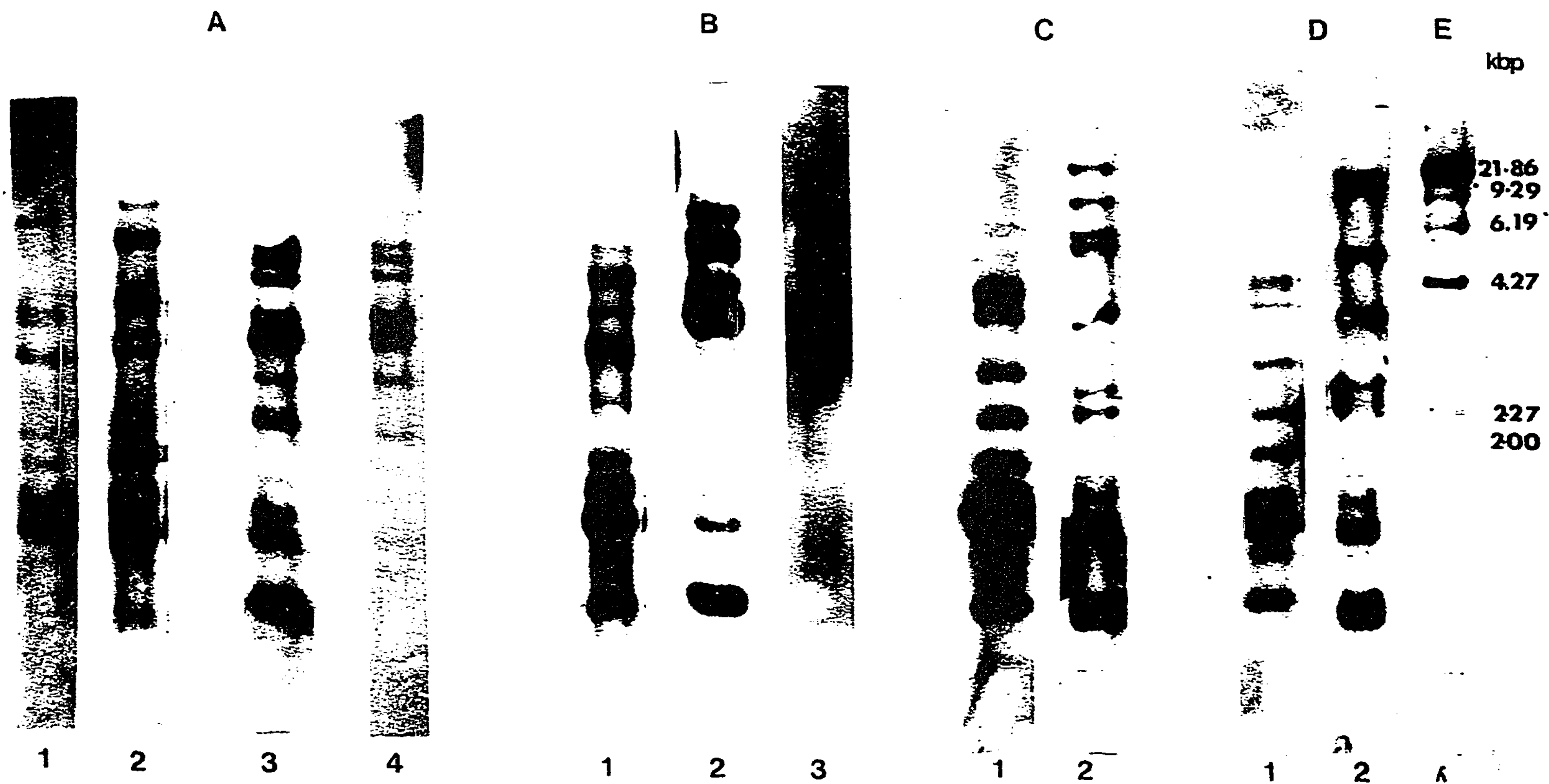


Figure 12: continued

F. B. globigii RUB562
probes used
1. pMS102-B7'- 16s, 23s
2. 23s rRNA, p12E2- 23s, 5s; pBC279- 23s, 5s
3. 5s rRNA

G. B. pumilus RUB502
probes used
1. pMS102-B7'- 16s, 23s
2. 23s rRNA, p12E2- 23s, 5s; pBC279- 23s, 5s
3. 5s rRNA

H. B. amyloliquefaciens H
probes used
1. pMS102-B7'- 16s, 23s
2. 23s rRNA, p12E2- 23s,5s; pBC279- 23s,5s

I. lambda DNA HindIII digested

Figure 12

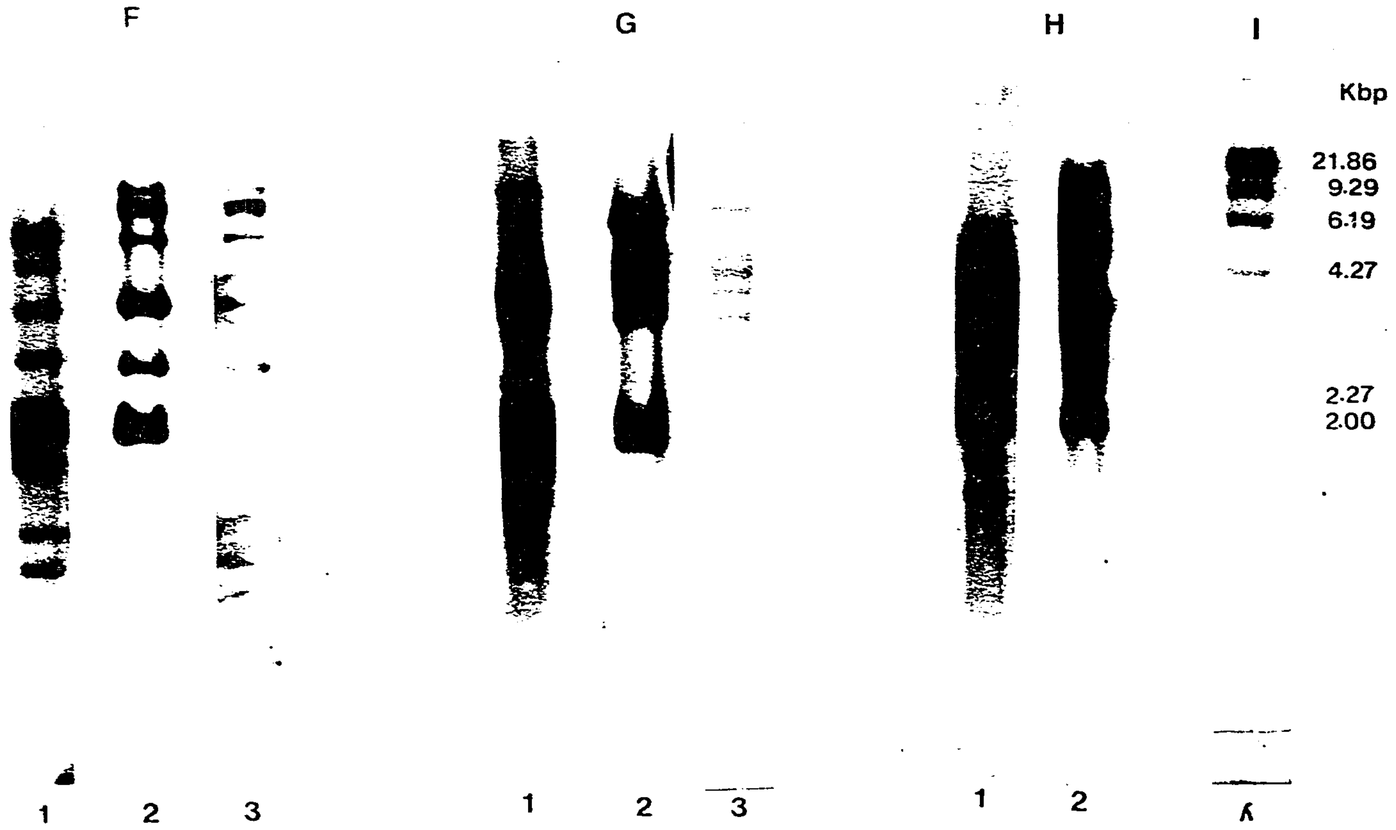


Figure 13: Southern blot hybridization of *Bacillus* genomic DNA. Hybridization was with rDNA specific probes. All genomic DNA was HindIII digested (3 units enzyme/ug DNA).

A. *B. subtilis* NCTC3610/168M
probes used
1. p21C4- 16s
2. 23s rRNA; pBC279- 23s, 5s; p12E2- 23s, 5s
3. 5s rRNA

B. *B. subtilis* W23
probes used
1. p21C4- 16s
2. 23s rRNA; pBC279- 23s, 5s; p12E2- 23s, 5s
3. 5s rRNA

C. *B. licheniformis* FD01
probes used
1. p21C4- 16s
2. 23s rRNA; pBC279- 23s, 5s; p12E2- 23s, 5s

D. *B. licheniformis* 8480
probes used
1. p21C4- 16s
2. 23s rRNA; pBC279- 23s, 5s; p12E2- 23s, 5s

E. lambda DNA HindIII digested

Figure 13

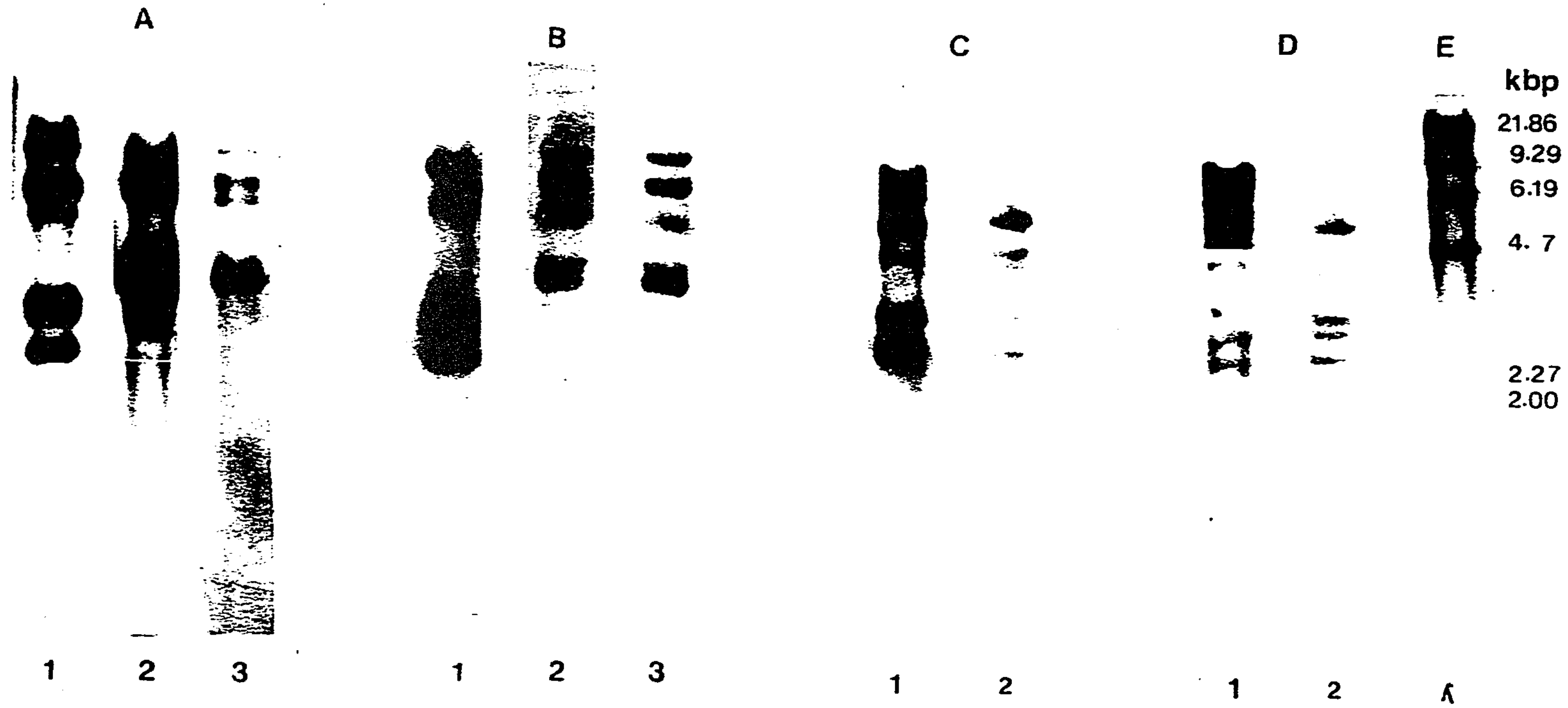


Figure 13: continued

F. *B. globigii* RUB562
probes used
1. p21C4- 16s
2. 23s rRNA; pBC279- 23s, 5s; p12E2- 23s, 5s

G. *B. pumilus* RUB502
probe used
1. p21C4- 16s

H. *B. amyloliquefaciens* H
probes used
1. p21C4- 16s
2. 23s rRNA; pBC279- 23s, 5s; p12E2- 23s, 5s

I. lambda DNA HindIII digested

Figure 13

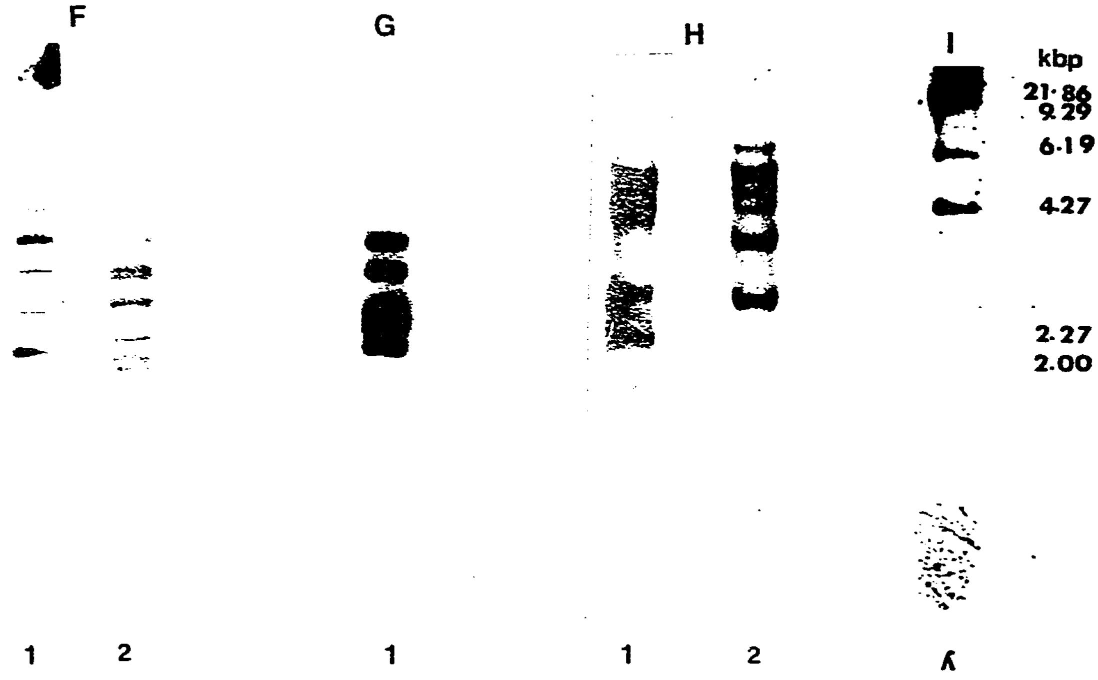


Figure 14: Southern blot hybridization of Bacillus genomic DNA. Hybridization was with rDNA specific probes. All genomic DNA was BamHI digested (3 units/ug DNA).

A. B. subtilis NCTC3610/168M
   probes used
   1. p21C4- 16s
   2. pBC279- 23s, 5s
   3. 5s rRNA

B. B. subtilis W23
   probes used
   1. p21C4- 16s
   2. pBC279- 23s, 5s
   3. 5s rRNA

C. B. licheniformis 8480
   probes used
   1. p21C4- 16s
   2. pBC279- 23s, 5s
   3. 5s rRNA

D. lambda DNA HindIII digested

Figure 14

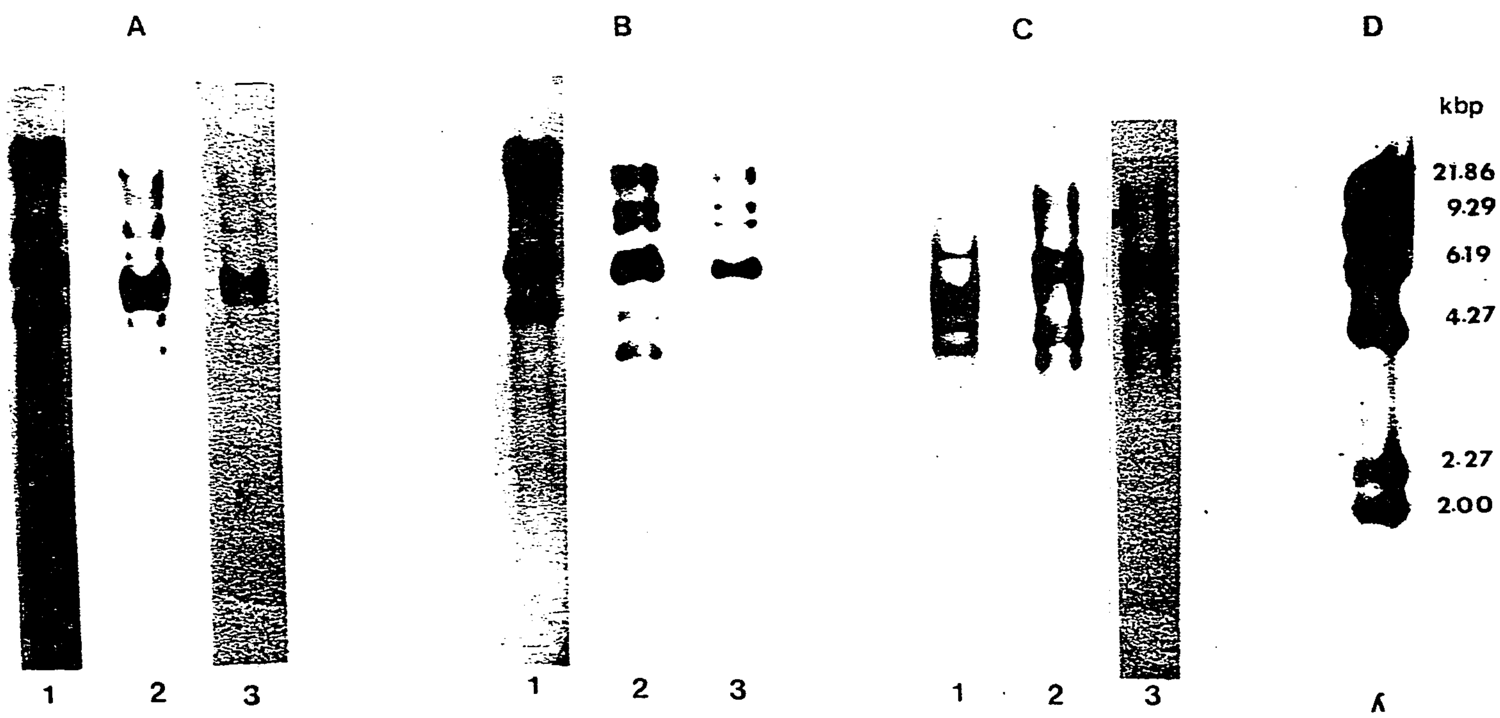


Figure 14: continued

E. B. pumilus RUB502
probes used
1. p21C4- 16s
2. pBC279- 23s, 5s
3. 5s rRNA

F. B. globigii RUB562
probes used
1. p21C4- 16s
2. pBC279- 23s, 5s
3. 5s rRNA

G. lambda DNA HindIII digested

Figure 14

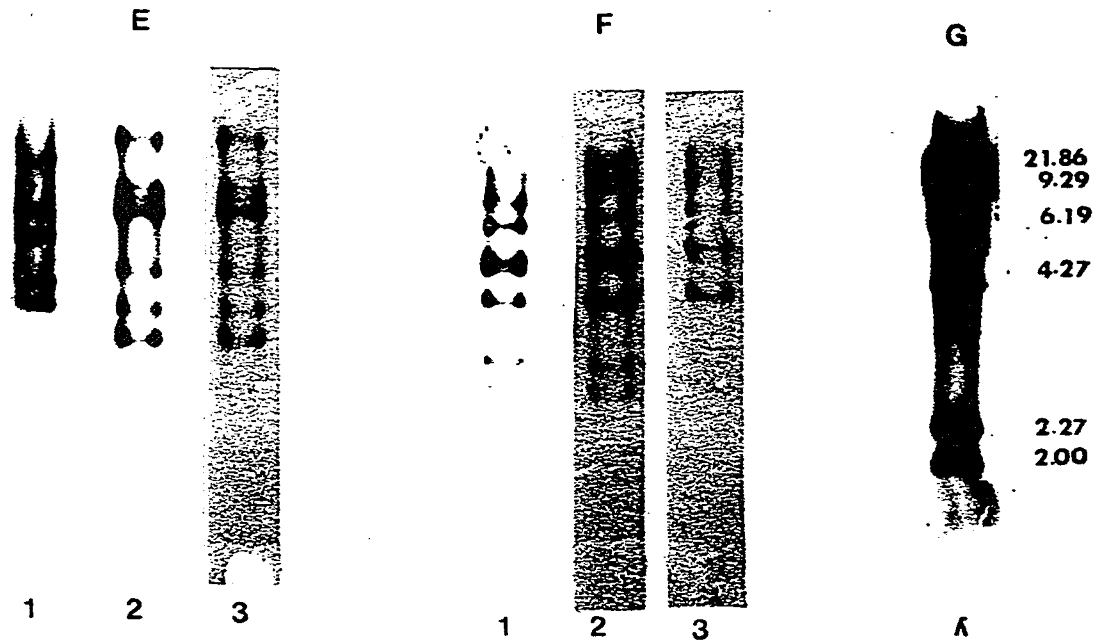


Figure 15: Southern blot hybridization of *Bacillus* genomic DNA. Hybridization was with rDNA specific probes. All genomic DNA was SmaI digested (3 units enzyme/ug DNA).

A. *B. subtilis* NCTC3610/168M
probes used
1. p21C4- 16s
2. pBC279- 23s, 5s
3. 5s rRNA

B. *B. subtilis* W23
probe used
1. p21C4- 16s

C. *B. globigii* RUB562
probe used
1. p21C4- 16s

D. *B. pumilus* RUB502
probe used
1. p21C4- 16s

E. lambda DNA HindIII digested

Figure 15

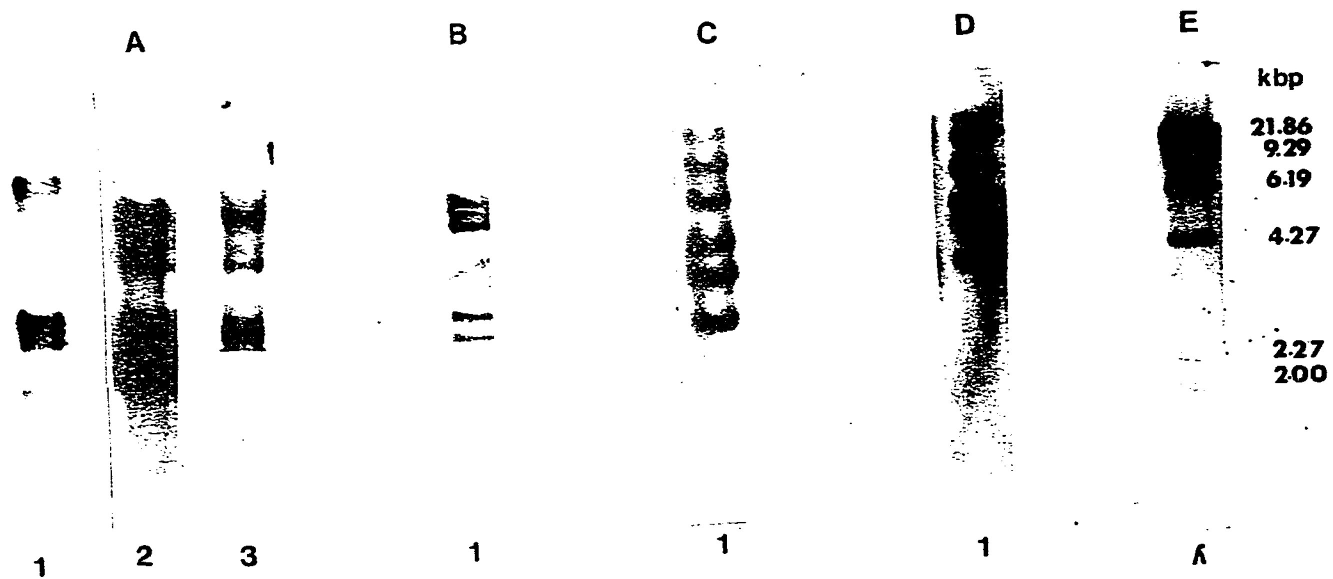


Figure 16: Southern hybridization of rRNA sequences in EcoRI digestions of Bacillus DNAs. The nick translated probes were cloned rDNA sequences.

A. probe is p21C4- 16s
1. B. subtilis 168T
2. B. amyloliquefaciens H
3. B. globigii RUB562
4. B. pumilus RUB502
5. B. licheniformis FD01
6. B. licheniformis 8480

B. probe is pMS102-B7'- 16s, 23S
1. B. licheniformis FD01
2. B. licheniformis 8480
3. B. pumilus RUB502
4. B. globigii RUB562
5. B. subtilis W23
6. B. subtilis 168T
7. B. subtilis NCTC 3610
8. B. amyloliquefaciens H

C. probe is p12E2- 23s, 5s
1. B. licheniformis FD01
2. B. licheniformis 8480
3. B. pumilus RUB502
4. B. globigii RUB562
5. B. subtilis W23
6. B. amyloliquefaciens H
7. B. subtilis GSY1269

D. probe is pBC279- 23s, 5s
1. B. licheniformis FD01
2. B. licheniformis 8480
3. B. pumilus RUB502
4. B. globigii RUB562
5. B. subtilis W23
6. B. subtilis NCTC 3610
7. B. subtilis 168T
8. lambda DNA HindIII digested

Figure 16

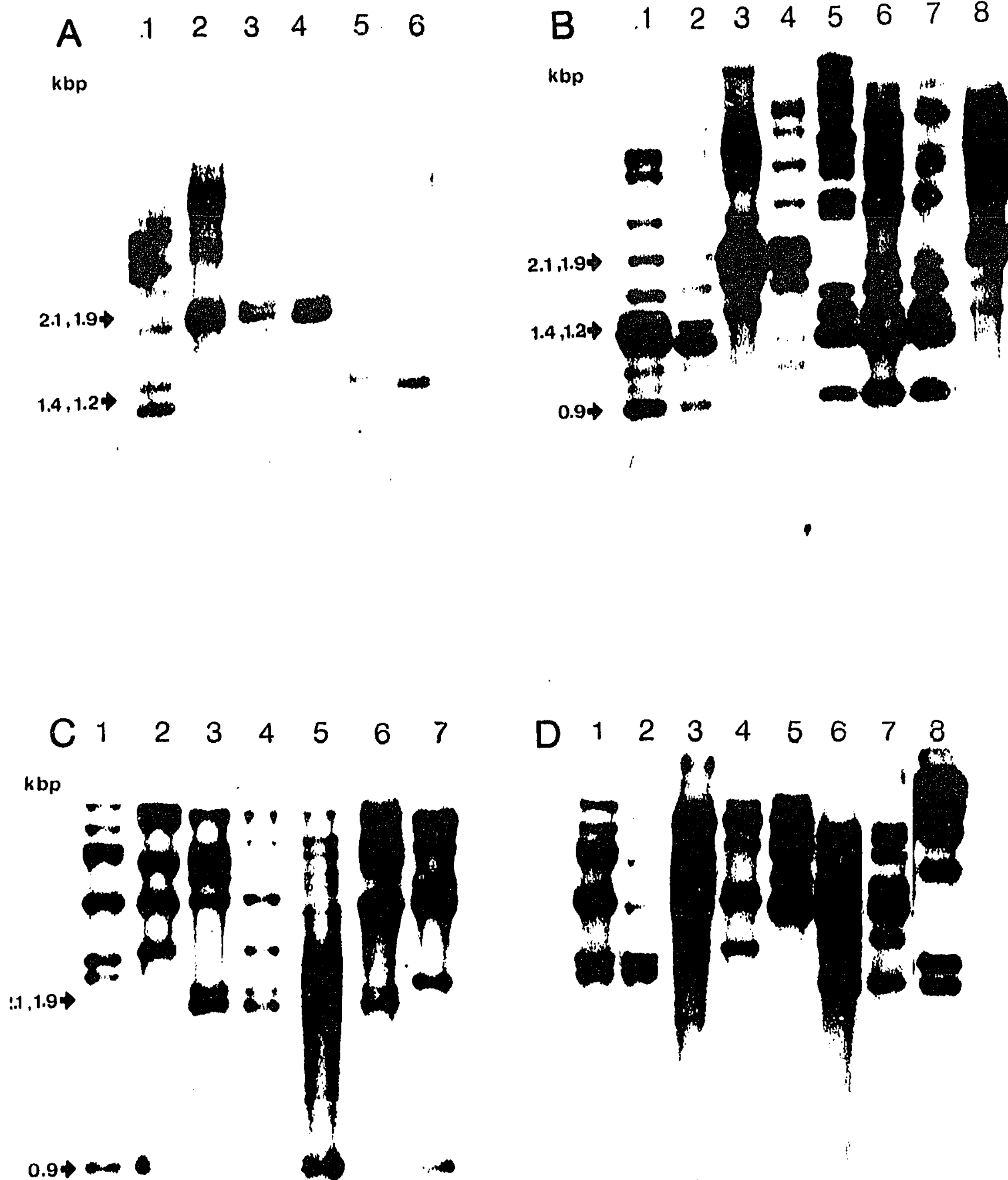


Figure 17: A comparison of homolog minimal sizes in SmaI chromosomal DNA digests. Chromosomal DNA (2 ug) was digested to completion and electrophoresed on an 0.75% agarose gel. Southern blot hybridizations used cloned rDNA as probes after labelling by nick translation.

A. probe p12E2- 23s, 5s or pBC279- 23s, 5s

1. *B. subtilis* 168
2. *B. globigii* RUB562
3. *B. pumilus* RUB502

B. probe pMS102-B7'- 16s, 23s

1. *B. subtilis* 168
2. *B. globigii* RUB562
3. *B. pumilus* RUB502

Figure 17

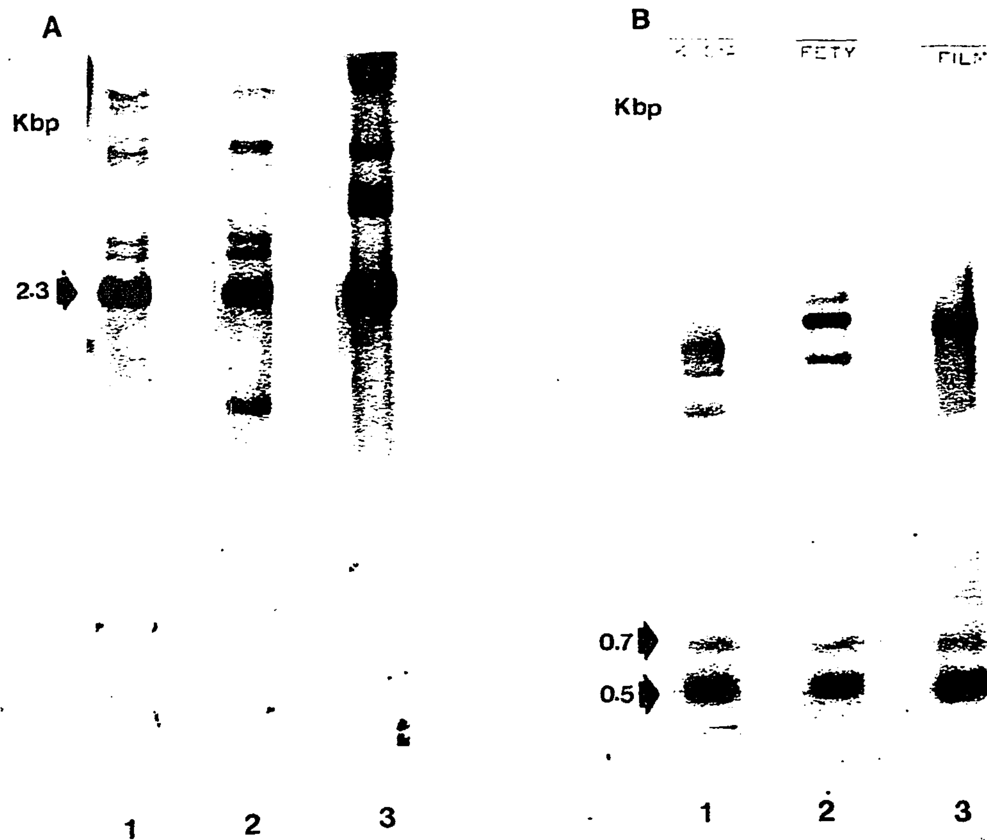
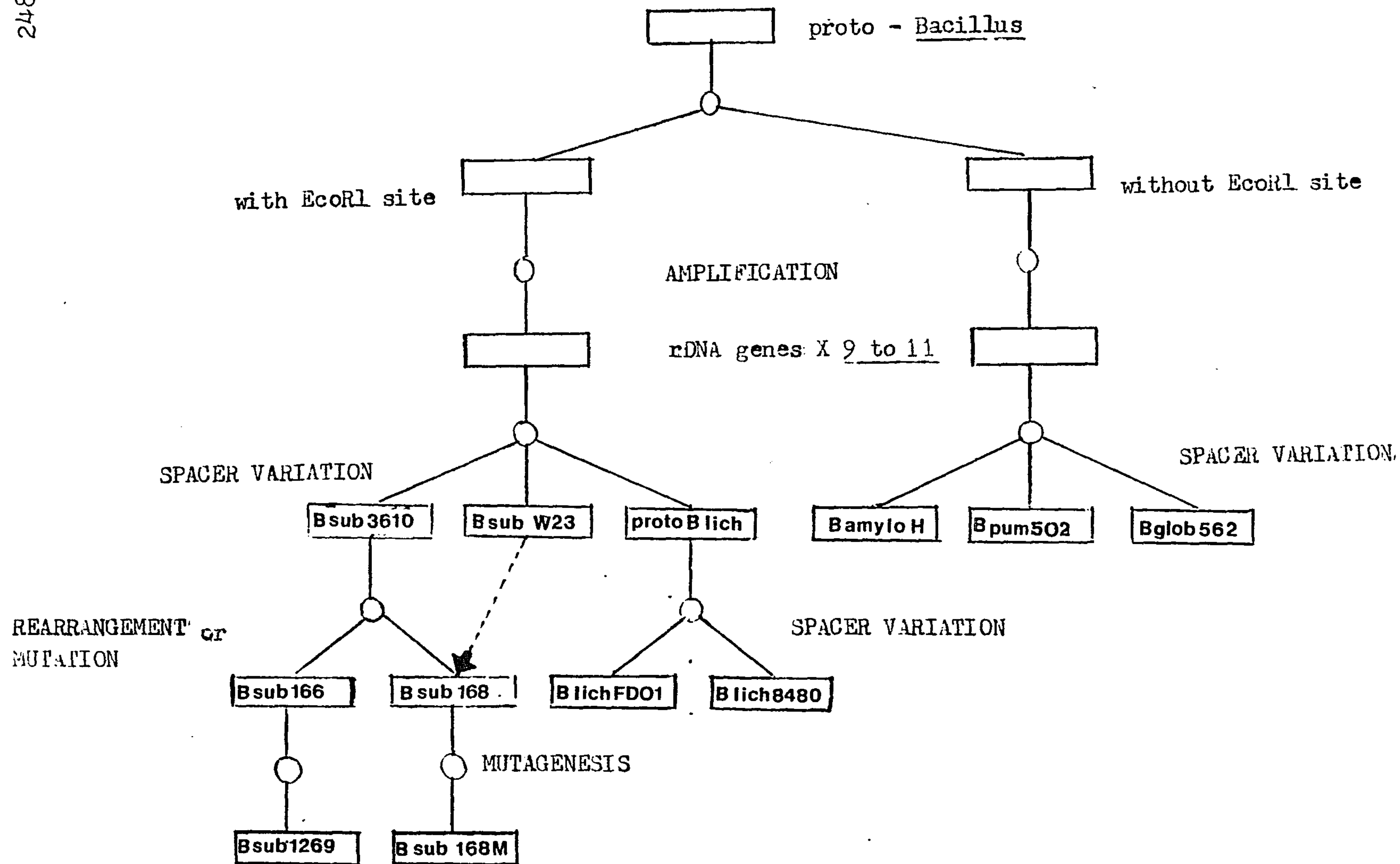


Figure 18: Dendrogram analysis of the relationship among eight species and strains of Bacillus. The Bacillus species/ strains examined in this study were divided into two lines of descent based upon the structure of their rRNA gene sets. B. subtilis and B. licheniformis have the type I rRNA genes possessing the 5', 23s sequence EcoRI site. B. amyloliquefaciens, B. pumilus, and B. globigii have the type II rRNA genes without the EcoRI site. The broken line between B. subtilis strains W23 and 168 represents the limited contribution of the former to the latter by transformation (see Results and discussion; The nonconserved genes of Bacillus).

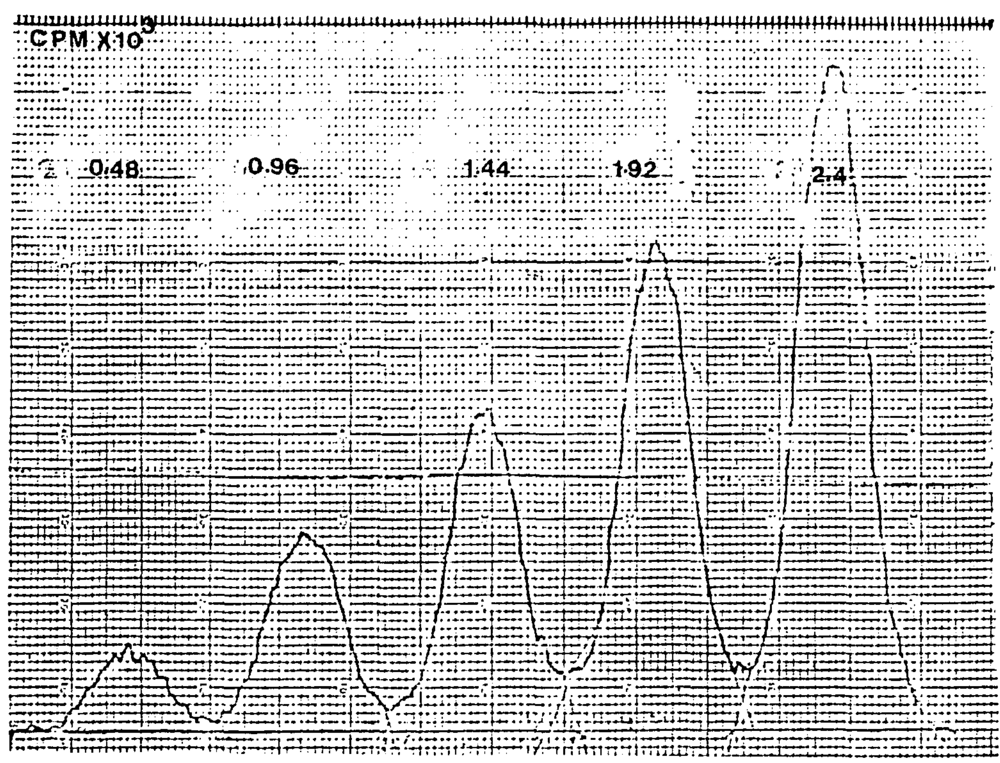


proto - Bacillus
with EcoRl site
without EcoRl site
AMPLIFICATION
rDNA genes X 9 to 11
SPACER VARIATION
SPACER VARIATION
Bsub3610
Bsub W23
proto B lich
B amylo H
B pum502
Bglob 562
REARRANGEMENT or
MUTATION
SPACER VARIATION
Bsub166
B sub 168
B lich FD01
B lich 8480
MUTAGENESIS
Bsub1269
B sub 168M

Figure 19: Densitometer scans of an autoradiogram exposed to quantitative amounts of radioactivity (^{32}P). ^{32}P isotope was spotted in a 2 ul volume onto a sheet of nitrocellulose. The spots were dried by an air blower and placed against Kodak X-Omat AR film with a Lightening Plus Cronex screen. Exposure was at room temperature for 10 hours. One ul of ^{32}P solution contains $1.2 X 10^3$ cpm. Appropriate dilutions of this stock were made to obtain the cpm that was applied to produce each peak.

A. The increase in radioactivity per peak follows an arithmetic relationship.

B. The increase in radioactivity per peak follows a geometric relationship.



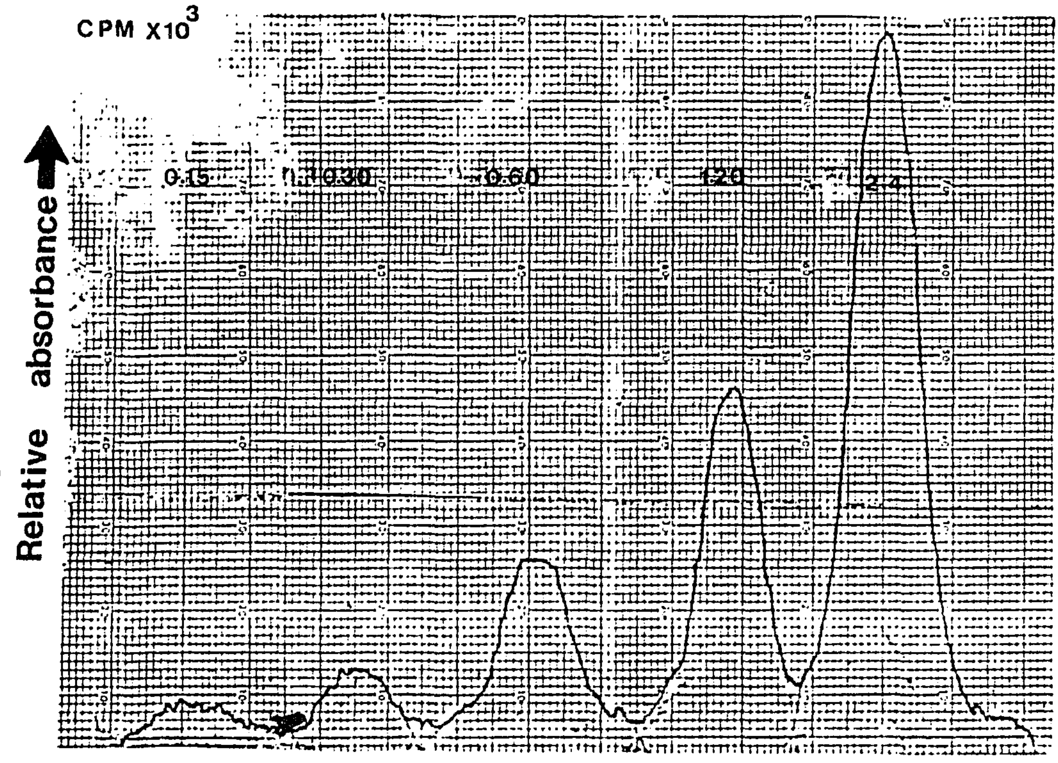


Figure 19

Figure 20: The linear range of densitometry tracings as a function of radioactivity. Peak areas of figure 19 A and B were determined by planometer measurements (Methods). These values are plotted vs. actual cpm added to each filter spot.

Figure 20

Autoradiogram linear responce range

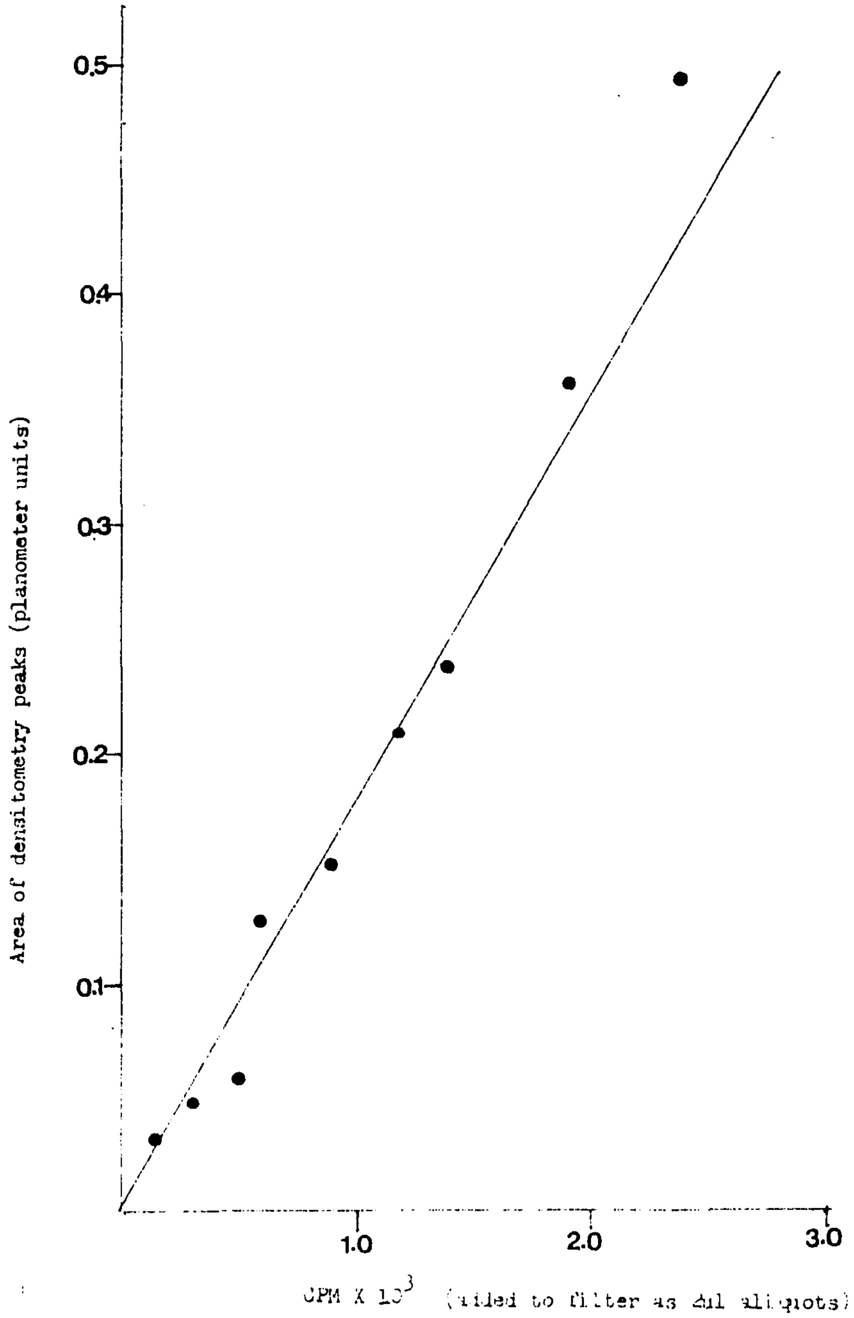


Figure 21: Densitometry tracings of autoradiograms of EcoRI, 23s rRNA homologs. Chromosomal DNA was restricted to completion with EcoRI endonuclease (3 units enzyme per ug DNA). Two ug of digest were added to each well of a 0.75% agarose gel. Southern blots were made of these gels and hybridized to ^{32}P labeled 23s rRNA (30 to 40 ng probe per gel lane). The hybridization reaction was for 12 hours by the conditions of Ostapchuk, Anilionis, and Riley (1980). The hybridized filter was filmed 10 hours at room temperature. The chart paper of the densitometer recorder ran at 20 cm/min, and the autoradiogram lane was scanned at 4 cm/min, (580 nM).

A. B. subtilis NCTC3610

B. B. subtilis W23

C. B. globigii RUB562

D. B. pumilus RUB502

E. B. amyloliquefaciens H

F. B. licheniformis 8480

G. B. licheniformis FD01

Molecular sizes printed over the peaks are in kilobase pairs.

Figure 21

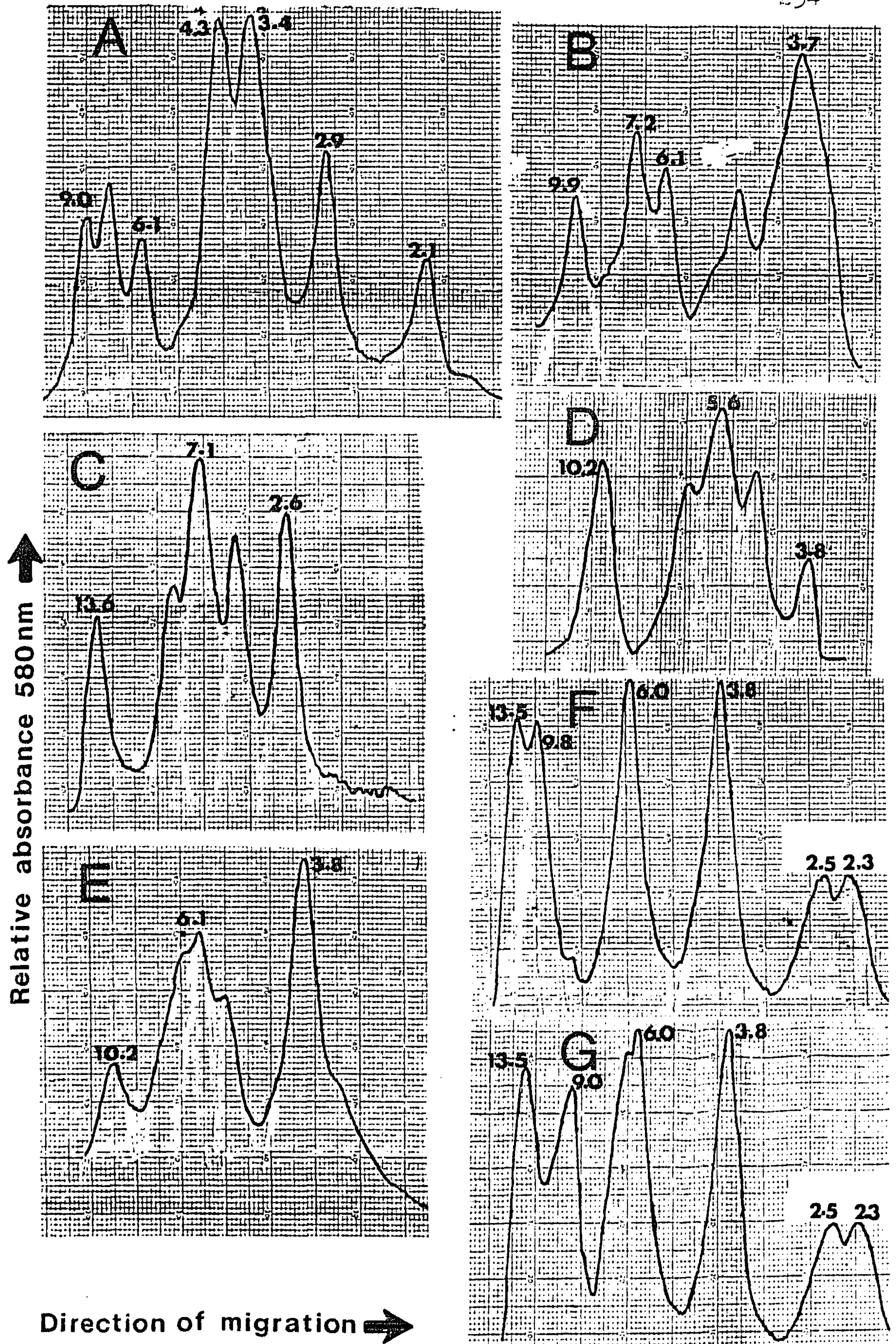


Figure 22: Densitometry tracings of autoradiograms of the two types of 16s, 23s abutmant fragments. Chromosomal DNA was restricted with EcoRI as described in Figure 21. Hybridization was with ^{32}P end labeled 23s rRNA. The region of the autoradiogram displaying the abutmant fragments was scanned at 1cm/min. and the chart recorder paper ran at 20cm/min.

A. Type I rDNA abutmant fragments (1.2 and 1.4 kbp) with the 23s determinant EcoRI site.

B. Type II rDNA abutmant fragments (1.9 and 2.1 kbp) without the 23s determinant EcoRI site.

Figure 22

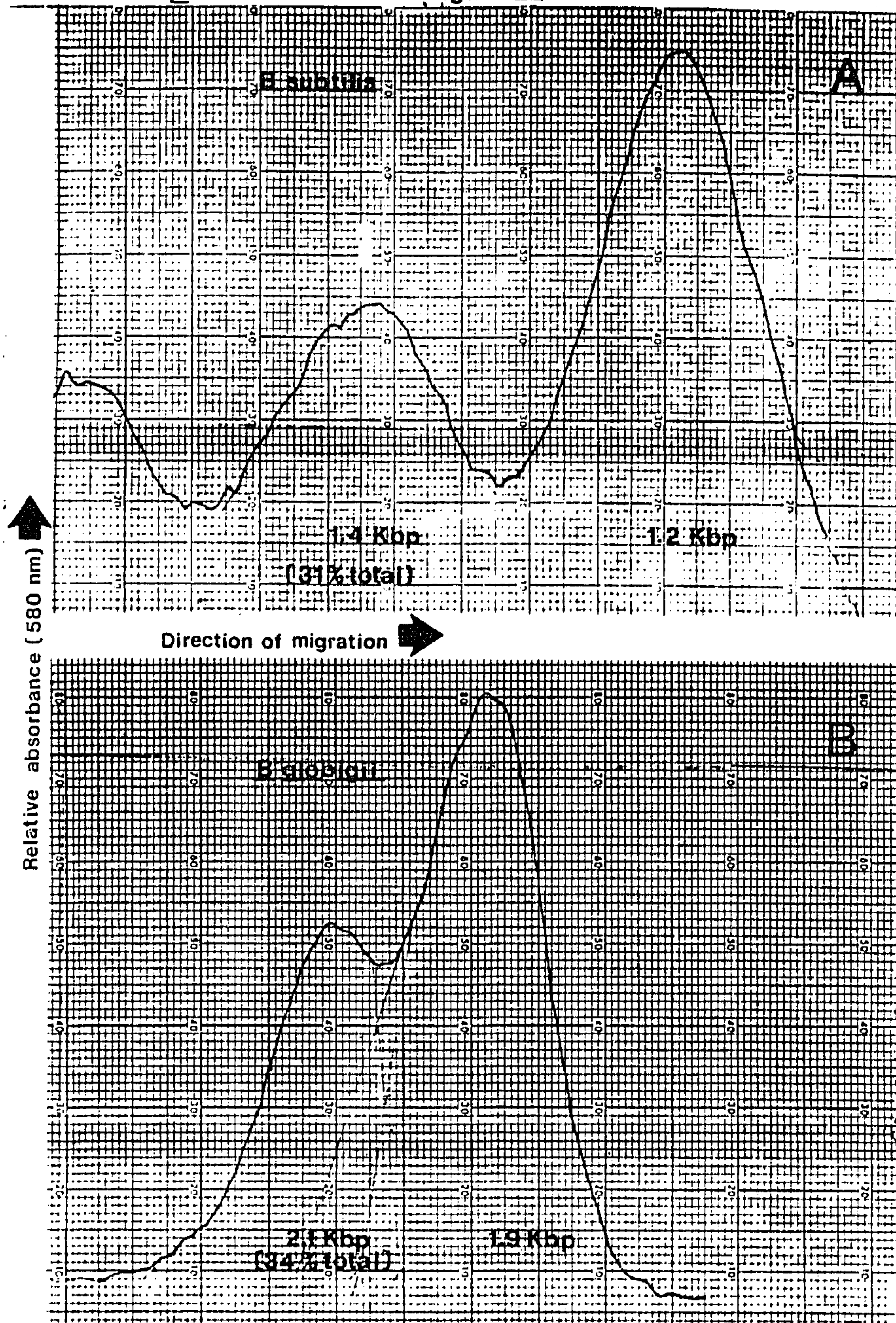
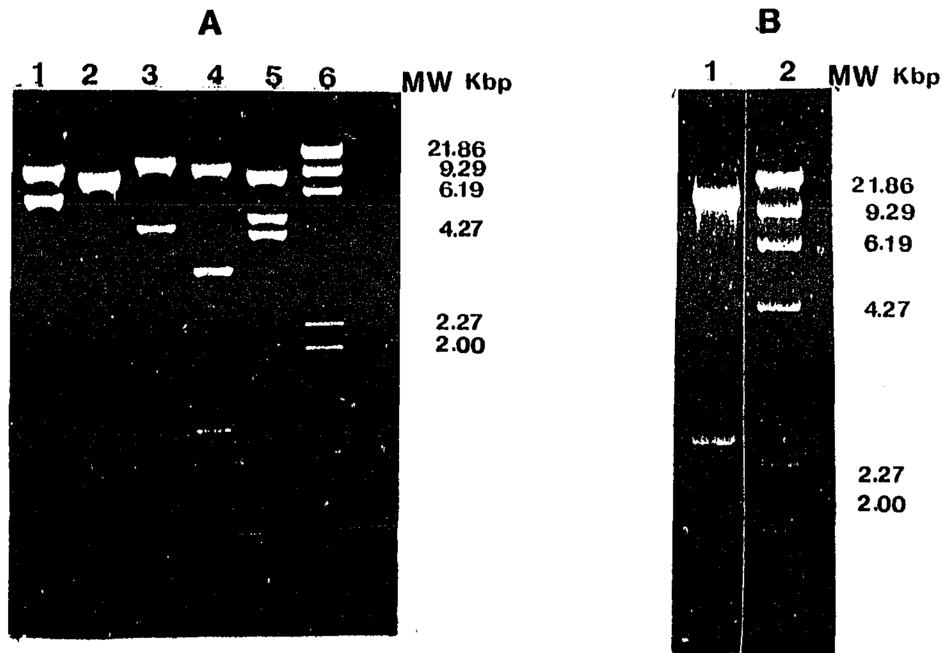


Figure 23



Restriction cleavage of plasmid RSF2124-B-leucine. The digestion fragments were analyzed on a 0.75% agarose gel.

Endonuclease digestions:

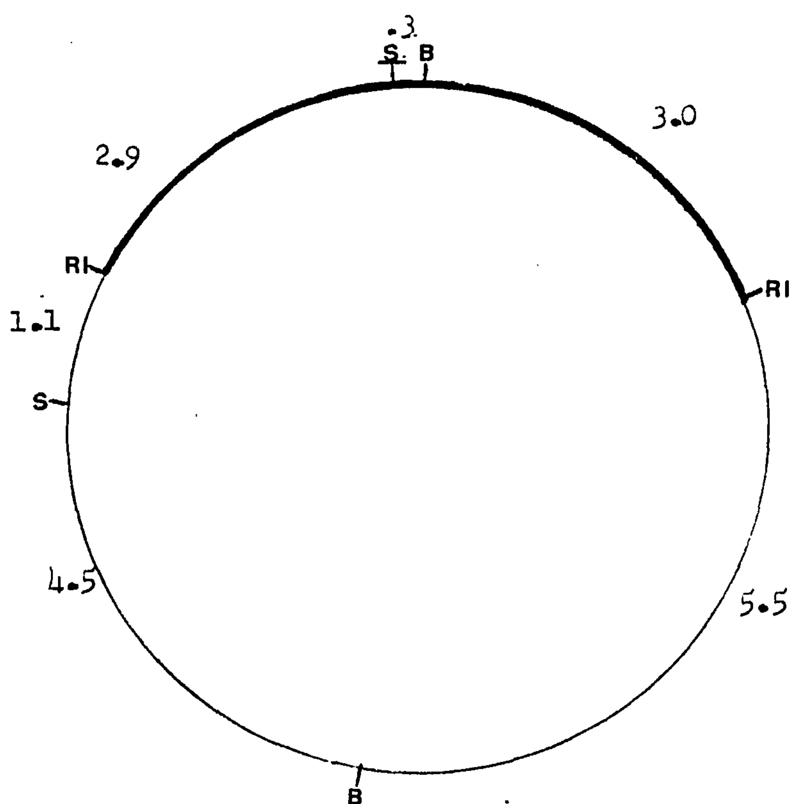
A

1. EcoR1
2. BamH1
3. Sma1
4. EcoR1/Sma1
5. BamH1/Sma1
6. lambda DNA Hind111 digestion

B

1. Hind111
2. lambda DNA Hind111 digestion

Figure 24



plasmid RSF2124-B, leucine

leucine insert

RI - Eco RI

S - Sma I

B - Bam HI

sizes are in kilobase pairs

Figure 25: Southern blot hybridization of Bacillus genomic DNA. Plasmid RSF2124.B- leucine was used as the probe.

A. EcoRI restricted
1. *B. subtilis* NCTC3610/168M
2. *B. subtilis* W23
3. *B. licheniformis* FD01
4. *B. licheniformis* 8480
5. *B. pumilus* RUB502
6. *B. globigii* RUB562

Figure 25A

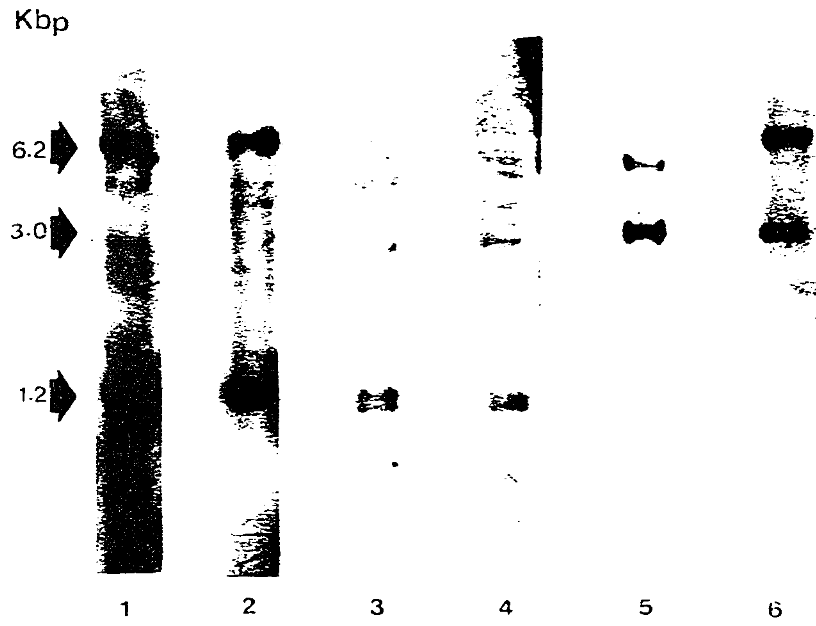


Figure 25: continued

B. HindIII restricted
1. *B. subtilis* NCTC3610 (probe sp. act. 1 to 2 X 10^9 cpm/ug)
2. *B. subtilis* NCTC3610/168M
3. *B. subtilis* W23
4. *B. licheniformis* FD01
5. *B. licheniformis* 8480
6. *B. pumilus* RUB502
7. *B. globigii* RUB562

C. SmaI restricted
1. *B. subtilis* NCTC3610/168M
2. *B. subtilis* W23
3. *B. globigii* RUB562
4. *B. amyloliquefaciens* H

Figure 25B

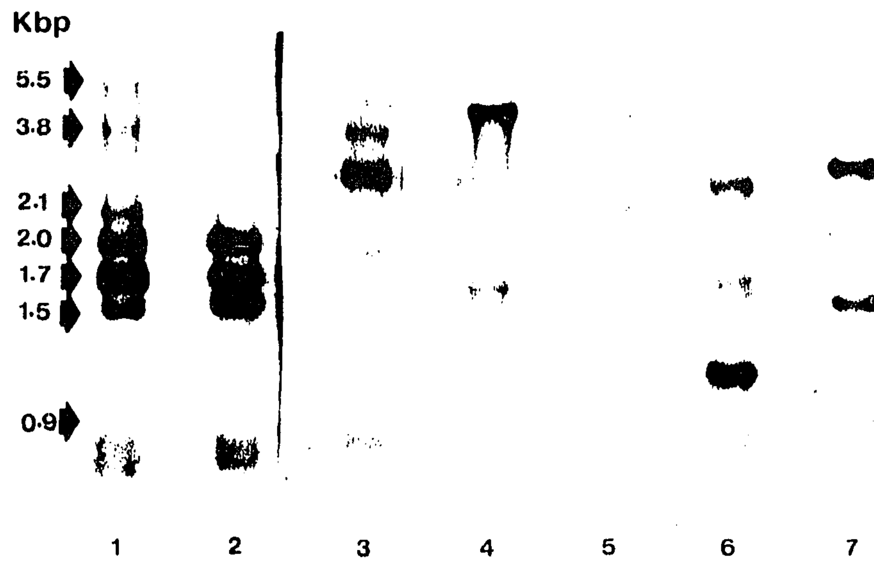
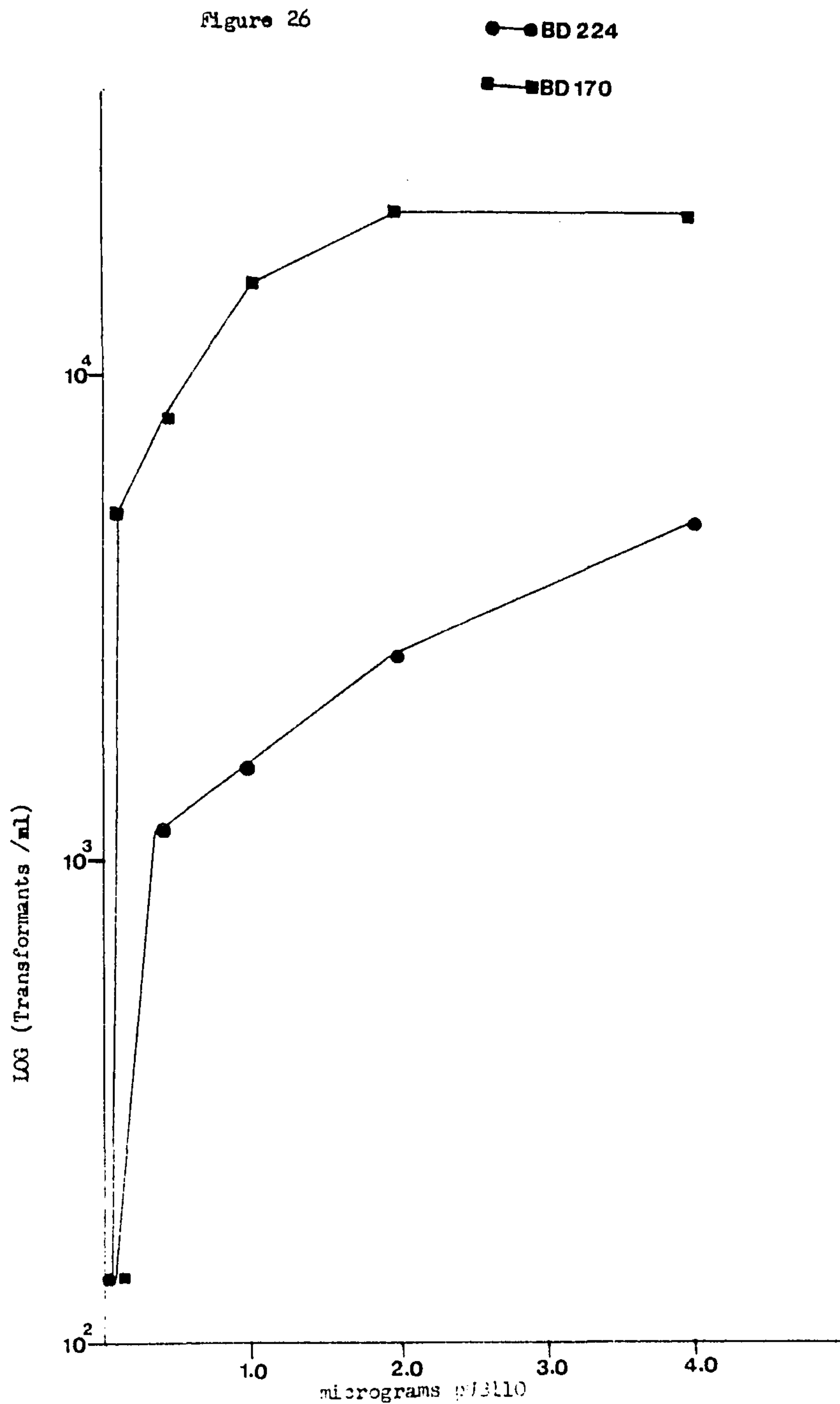
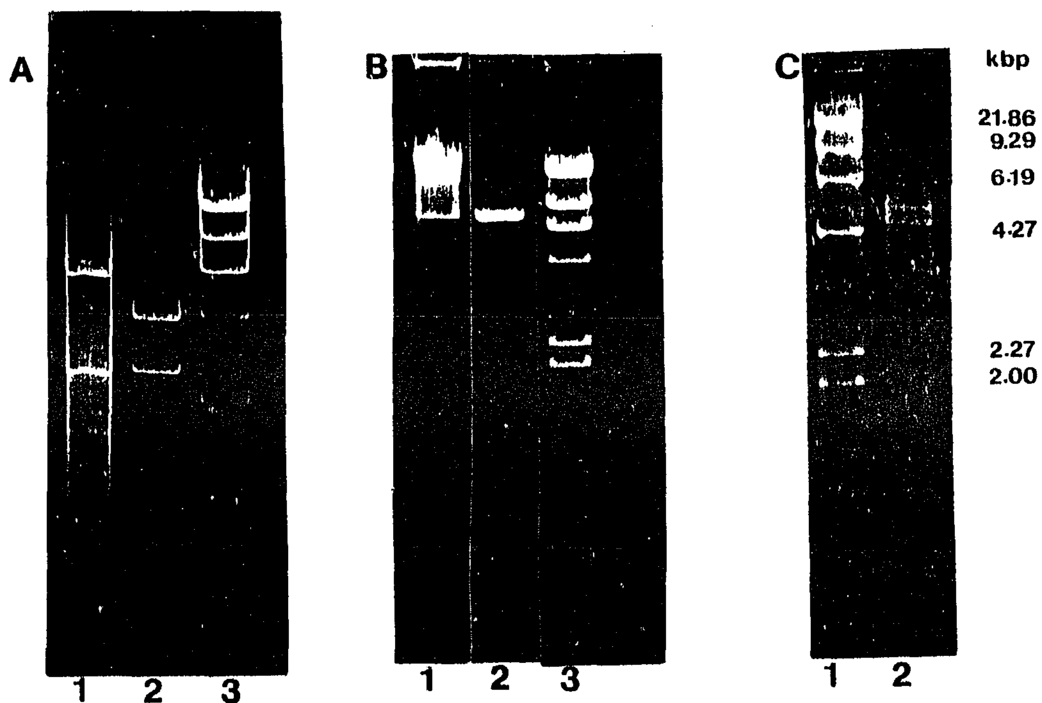


Figure 25C

Figure 26: Plasmid mediated transformation in *B. subtilis*. A CsCl-EthBr purified preparation of pUB110 was used to transform two strains of *B. subtilis* at a concentration of 0.1 to 4.0 ug/ml. The transformation reaction was in a 1 ml volume. Recipient cells were BD170(*trpC2*, *thr-5*, km^s, RecE) and BD224(*trpC2*, *thr-5*, km^s, *recE4*). Km resistant colonies were scored by overlay with 10ug/ml kanamycin sulfate following a 90 minute expression lag.

Figure 26





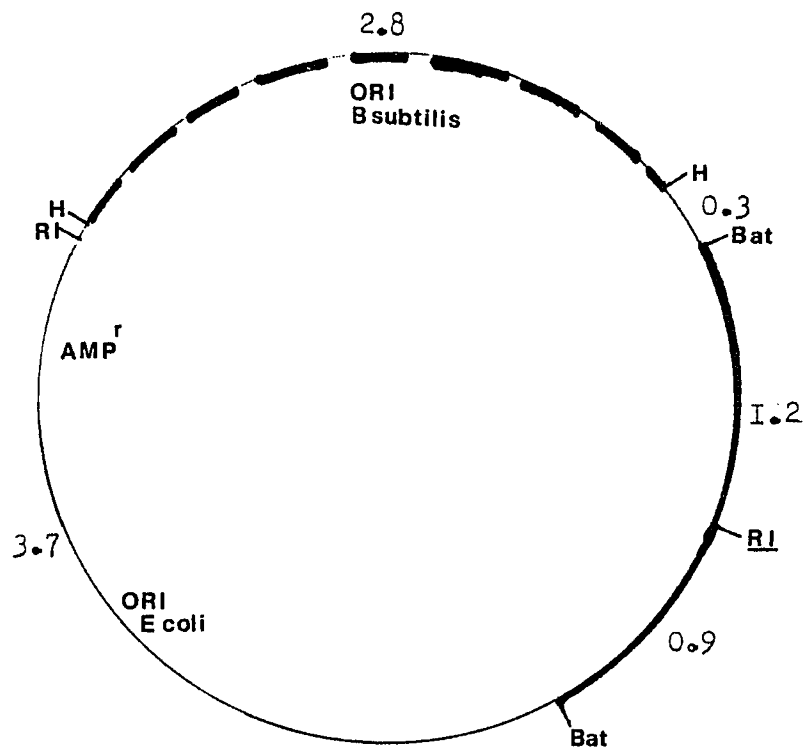
A. 1- pBSO2a-thr HIND III
   2- pHV33-vector HIND III
   3- lambda DNA HIND III

B. 1- pBSO2a-thr BAM HI
   2- pHV33-vector BAM HI
   3- lambda DNA HIND III

C. 1- lambda DNA HIND III
   2- pBSO2a-thr Eco RI

Restriction cleavage of plasmids pBSO2a-threonine and pHV33. Digestion fragments were analyzed on a 0.75% agarose gel.

Figure 28



plasmid p_302a - threonine

pJI94 - 3.

threonine insert

H - Hind III

RI - Eco RI

Bat - Bam HI , Poly (dT) addition

sizes are in kilobase pairs

ORI - origin of replication

AMP^{r}- determinant for ampicillan resistance

Figure 29

RESTRICTION ENDONUCLEASE CLEAVAGE SITE MAP OF pRR106

Figure 30: Southern blot hybridization of *Bacillus* genomic DNA. Nick translated pBS02a-threonine was used as the probe.

A. EcoRI digested
1. *B. subtilis* 168T
2. *B. subtilis* NCTC3610
3. *B. subtilis* W23
4. *B. licheniformis* FD01
5. *B. licheniformis* 8480
6. *B. pumilus* RUB502
7. *B. globigii* RUB562

B. HindIII digested
1. *B. subtilis* 168T
2. *B. subtilis* NCTC3610
3. *B. subtilis* W23
4. *B. licheniformis* FD01
5. *B. licheniformis* 8480
6. *B. pumilus* RUB502
7. *B. globigii* RUB562
8. *B. amyloliquefaciens* H

Figure 30A

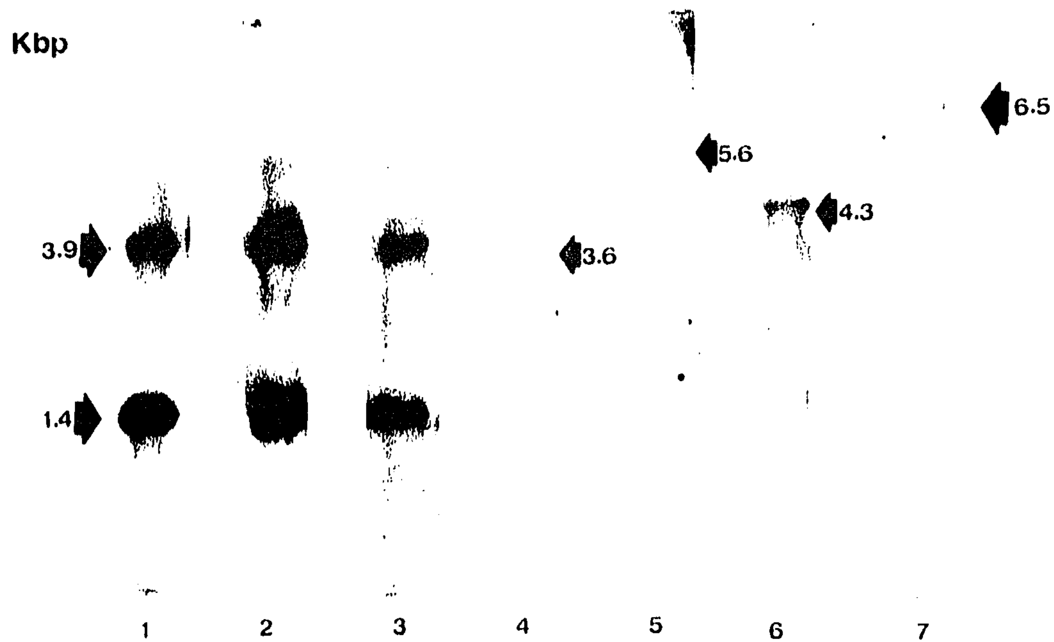


Figure 30B

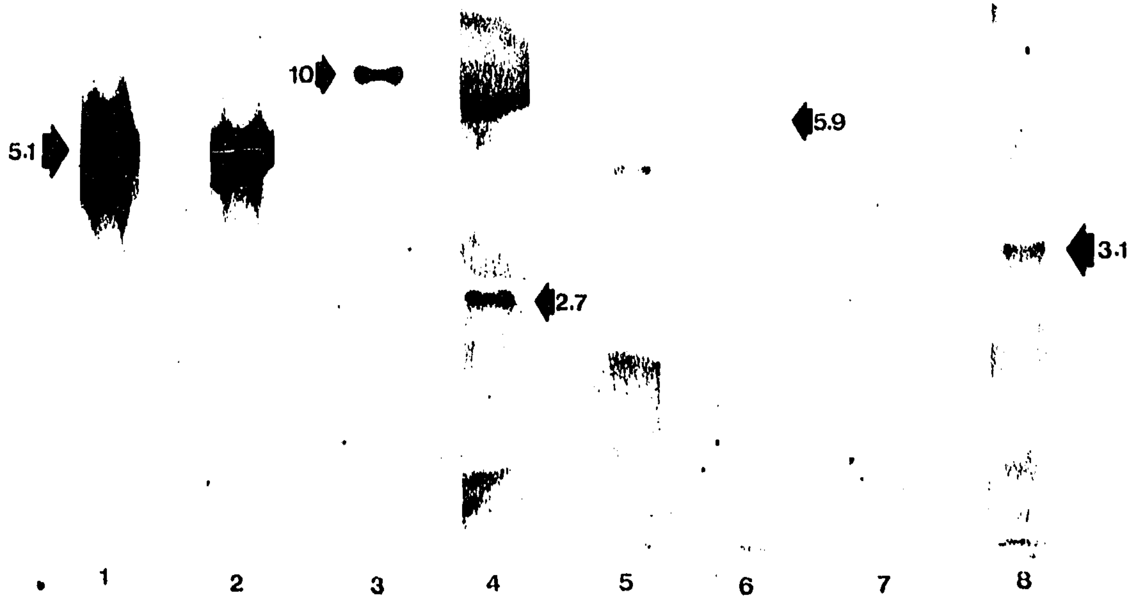


Figure 30: continued

C. SmaI digested
1. B. subtilis 168T
2. B. subtilis NCTC3610
3. B. subtilis W23
4. B. licheniformis FD01
5. B. pumilus RUB502
6. B. globigii RUB562
7. Lambda DNA HindIII digested

D. BamHI digested
1. B. subtilis 168T
2. B. subtilis NCTC3610
3. B. subtilis W23
4. Lambda HindIII digested

Figure 30C

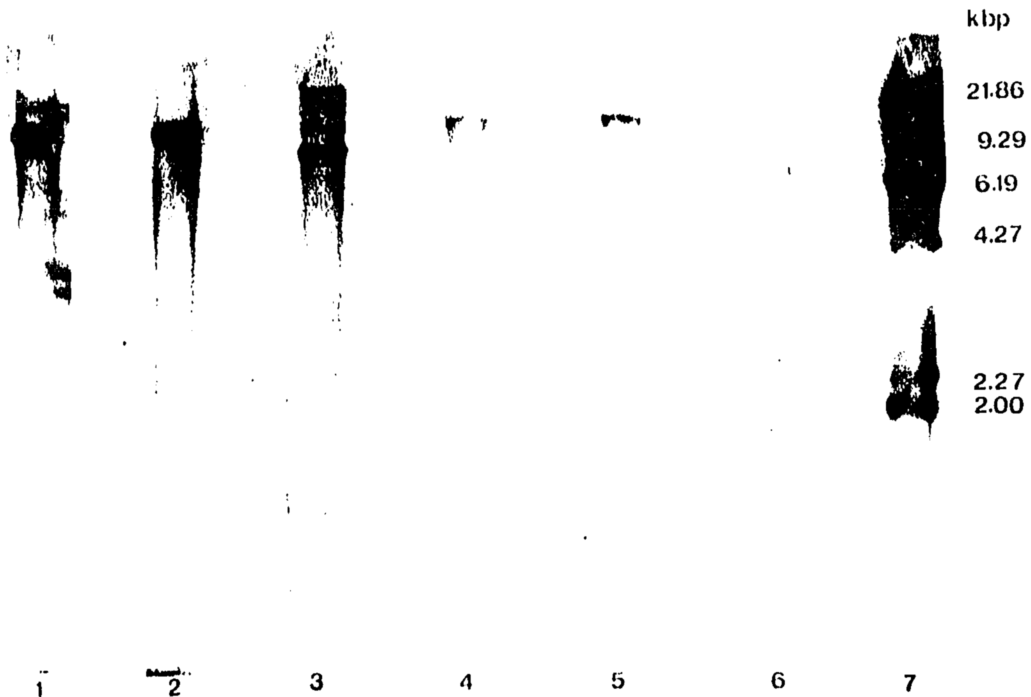


Figure 30 D

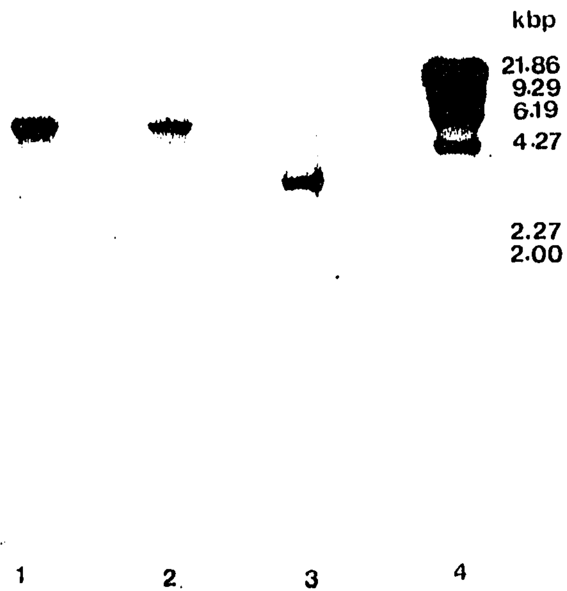


Figure 31: Southern blot hybridization of *Bacillus* genomic DNA. Nick translated pRR106-tryptophan was used as the probe. The *B. pumilus* EcoRI plasmid insert was isolated from a 1.0% low melting agarose gel prior to labelling (Methods).

A. EcoRI digested
1. *B. subtilis* W23
2. *B. subtilis* NCTC3610
3. *B. subtilis* SB25
4. *B. subtilis* BR151
5. *B. subtilis* BD170
6. *B. pumilus* RUB502
7. *B. licheniformis* FD01
8. lambda DNA HindIII digested

Figure 31 A

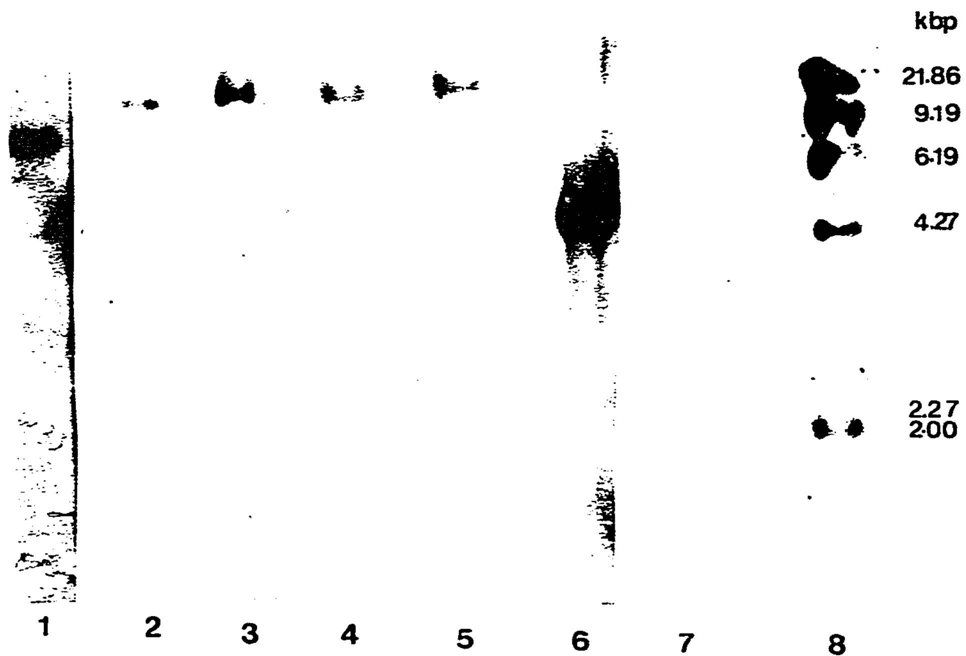


Figure 31: continued

B. HindIII digested
1. B. subtilis W23
2. B. subtilis NCTC3610
3. B. subtilis BR151
4. B. subtilis BD170
5. B. pumilus RUB502
6. B. licheniformis FD01
7. lambda DNA HindIII digested

Figure 31B

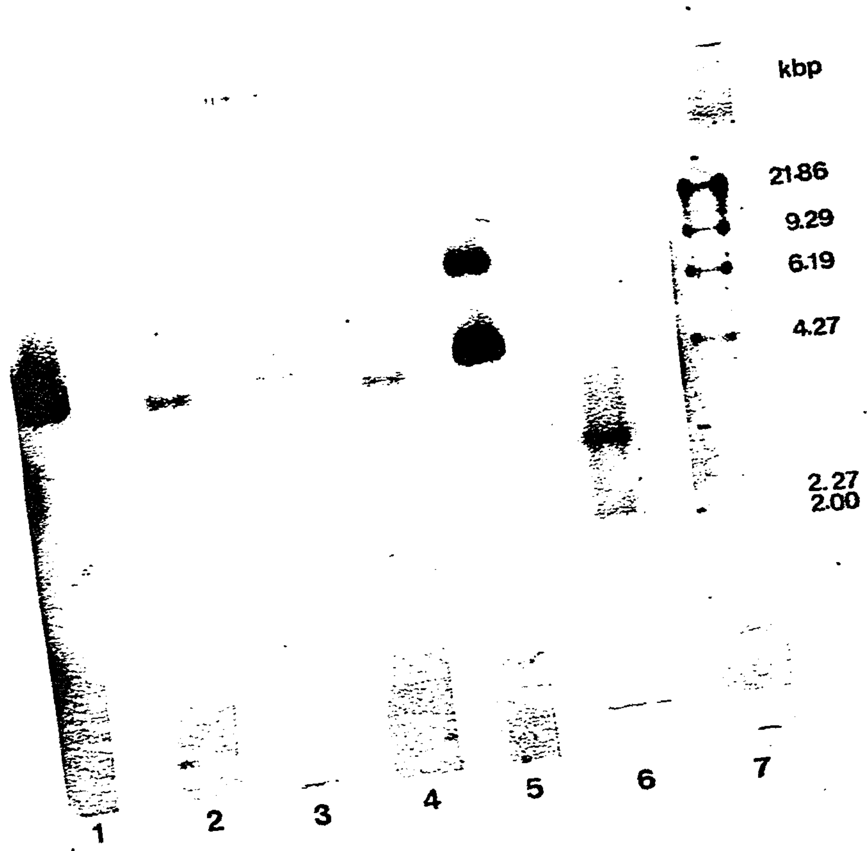


Figure 31: continued

C. EcoRI/HindIII digested

1. B. subtilis W23
2. B. subtilis NCTC3610
3. B. subtilis BR151
4. B. subtilis BD170
5. B. pumilus RUB502
6. B. licheniformis FD01
7. lambda DNA HindIII digested

Figure 31C

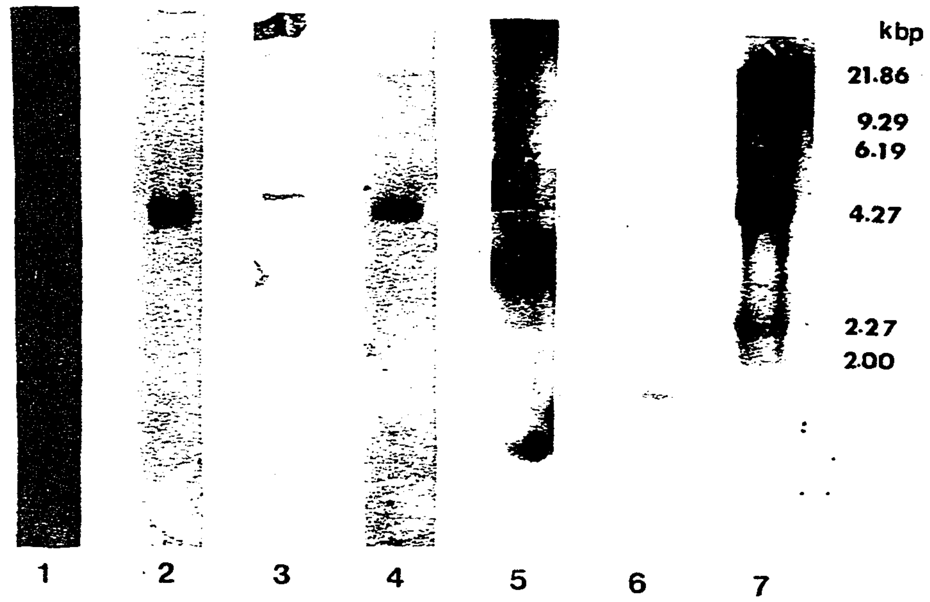


Figure A1: Densitometry tracings of Southern hybridizations of Bacillus genomic DNA. Chromosomal DNA was restricted with HindIII endonuclease. Two ug of this digest was applied to the well of an 0.75% agarose gel. The Southern blot made of this gel was hybridized with probe RSF2124.B-leucine (3 to 5 ng added per blot lane). Specific activity of the probe was $2.0X10^{8}$ cpm/ug. Autoradiogram scan speed is 4cm/min and the recorder chart speed is 20cm/min. Filming was at room temperature for ten hours. Peak areas were measured by planometer tracing (Methods).

FIGURE A1

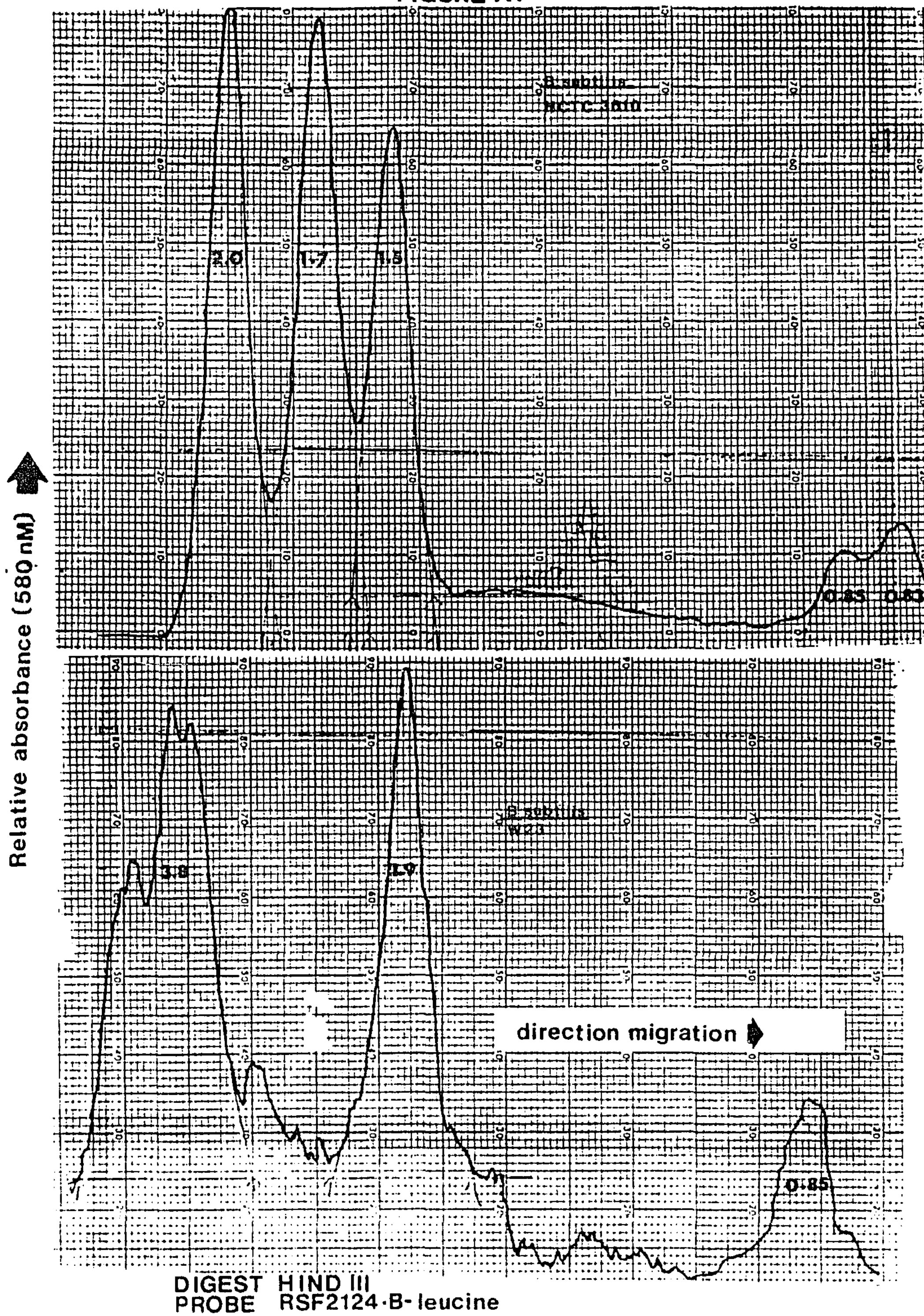


FIGURE A1 continued

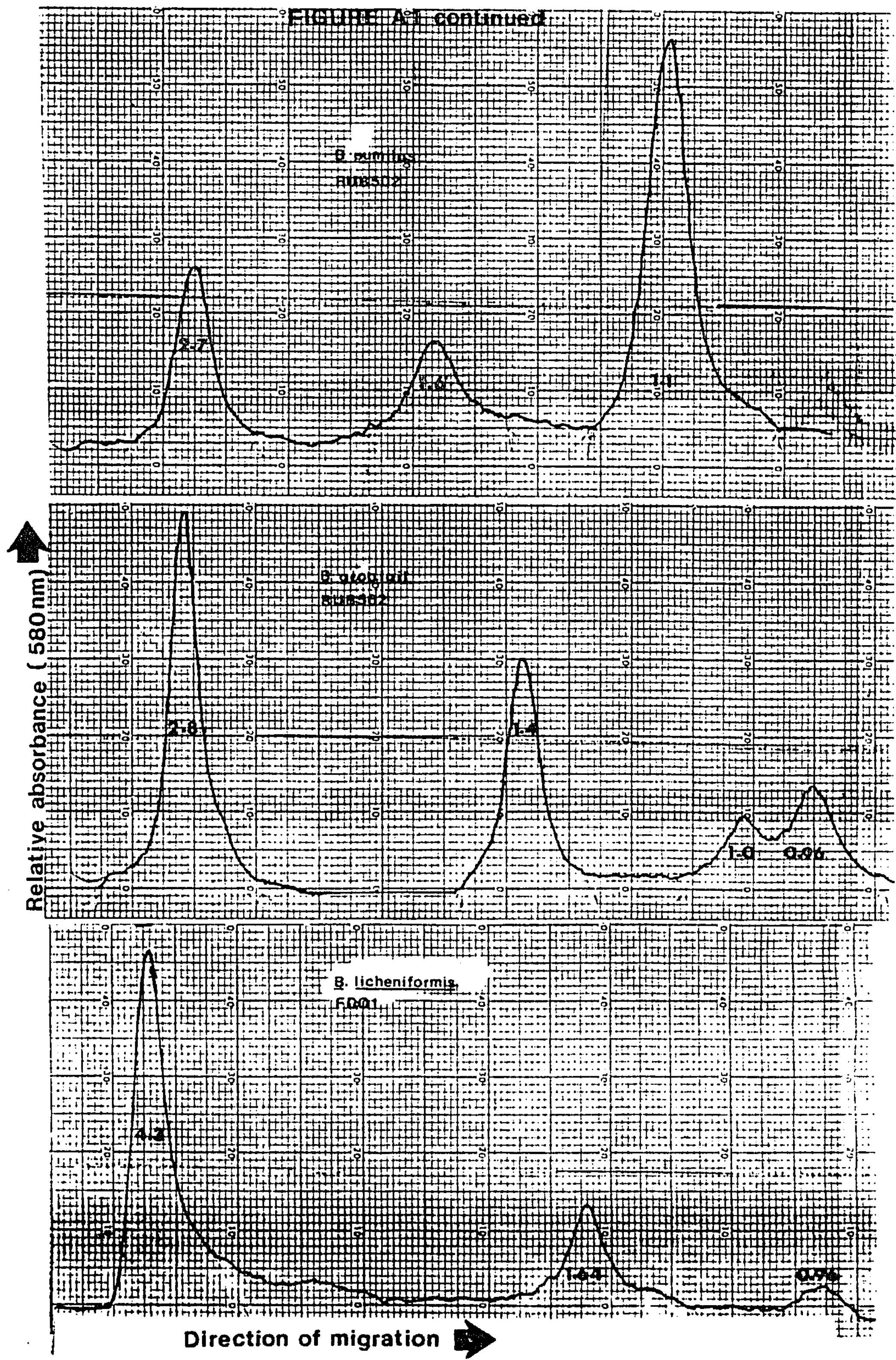
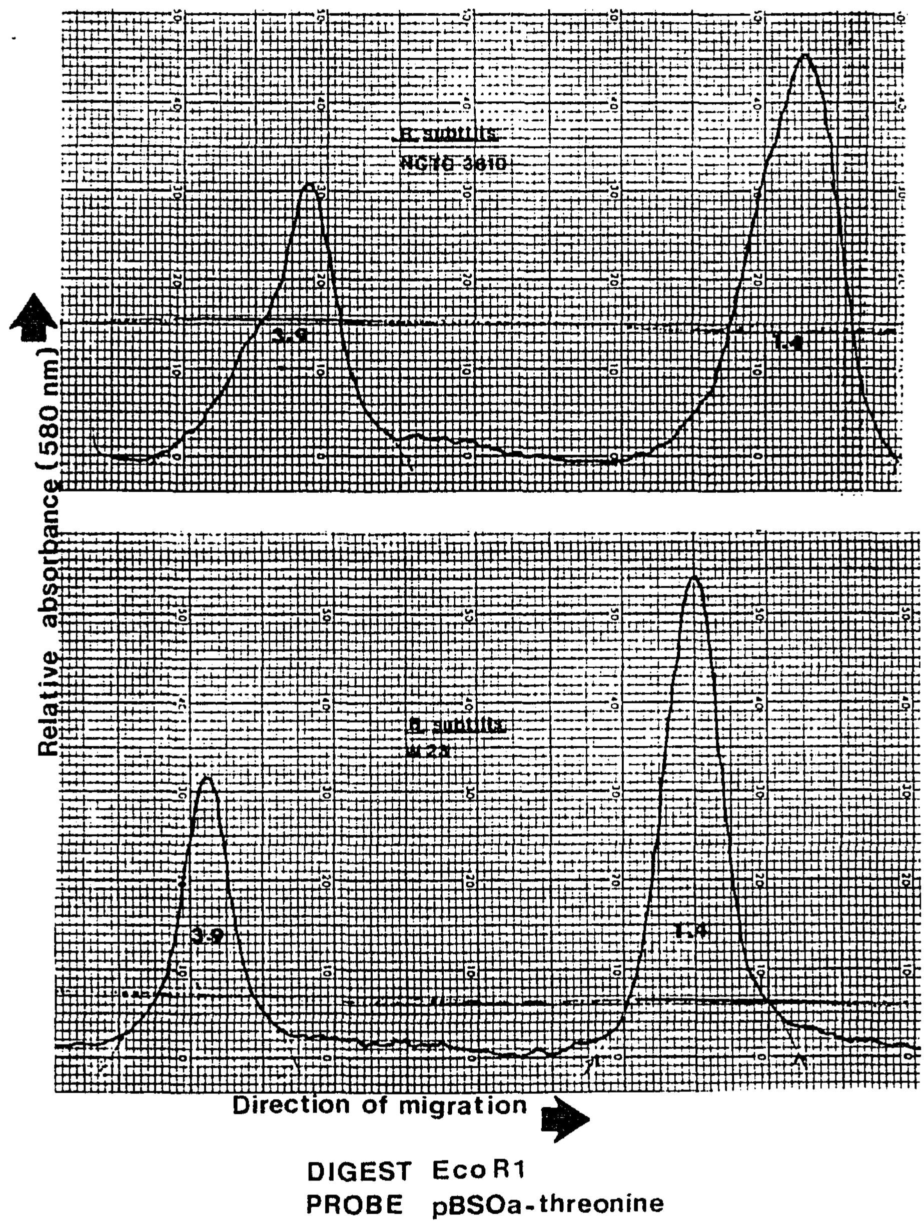


Figure A2: Densitometer tracings of Southern hybridizations of Bacillus genomic DNA. Chromosomal DNA is digested with EcoRI endonuclease. The probe is pBS02a-threonine (3 to 5 ng per blot lane). The specific activity of the probe is $2X10^8$ cpm/ug. Autoradiogram scan speed is 4cm/min and chart recorder speed is 20cm/min.

Figure A 2



DIGEST EcoR1
PROBE pBSOa-threonine

Figure A2 continued

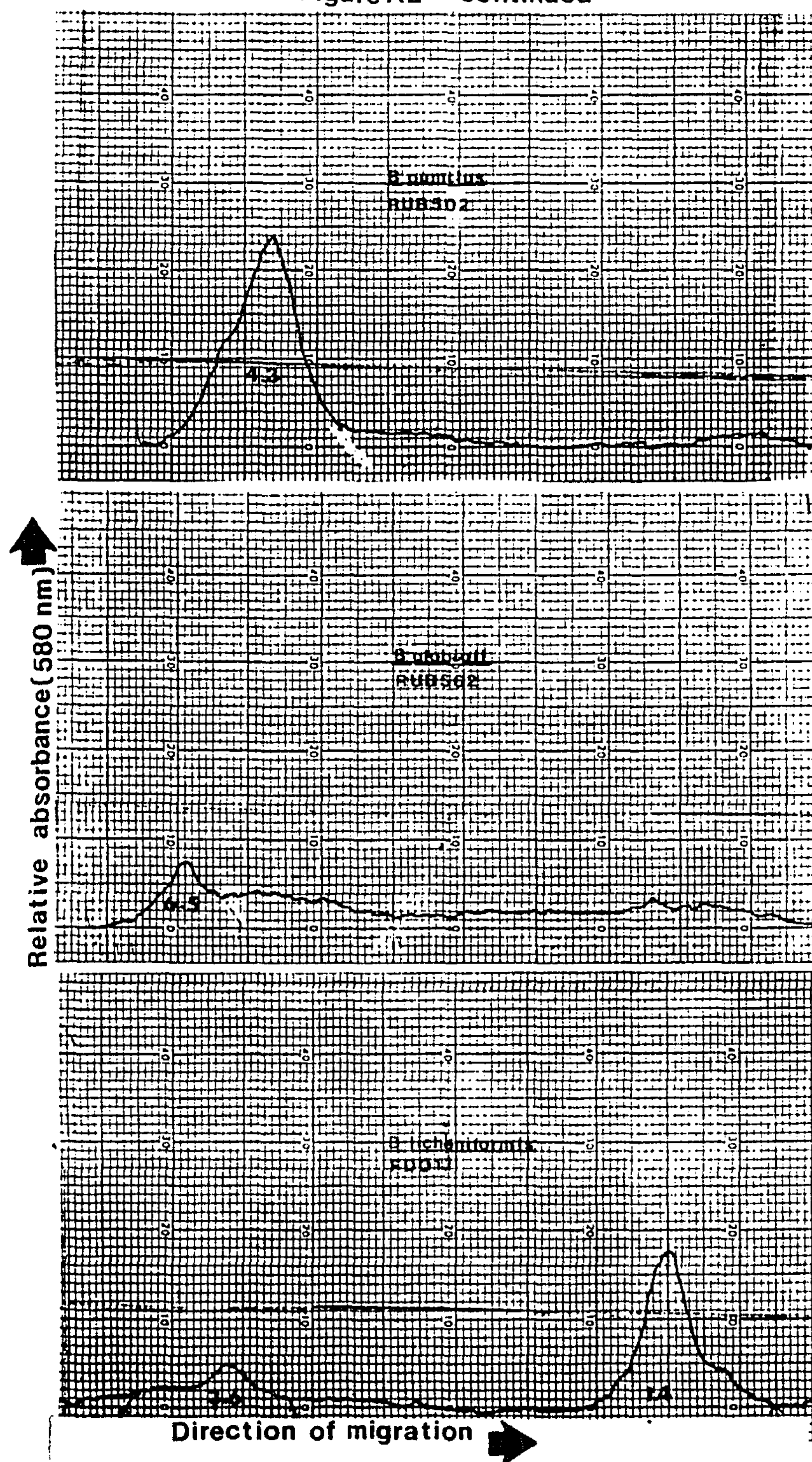


Figure 32: Southern blot hybridization of B. subtilis strains displaying loss or gain of rRNA gene sets.

A. probe is pBC279- 23s, 5s; EcoRI restricted chromosome
1. NCTC3610/168M
2. BD29
3. BD79
4. GSY 1269
5. lambda DNA HindIII restricted

B. probe is p21C4- 16s; HindIII restricted chromosome
1. NCTC3610/168M
2. BD29
3. BD79
4. BD73
5. GSY1269
6. 166
7. lambda DNA HindIII restricted

C. probe is pBC279- 23s, 5s; HindIII restricted chromosome
1. NCTC3610/168M
2. BD29
3. BD79
4. GSY1269

D. probe p21C4- 16s; SmaI restricted chromosome
1. NCTC3610/168M
2. BD29
3. BD79
4. BD73
5. lambda DNA HindIII restricted

E. probe is 5s rRNA; SmaI restricted chromosome
1. NCTC3610/168M
2. BD29
3. BD79

F. probe is 23s rRNA; SmaI restricted chromosome
1. NCTC3610/168M
2. BD29

G. probe is 23s rRNA; BamHI restricted chromosome
1. NCTC3610/168M
2. BD29
3. BD79
4. lambda DNA HindIII restricted

Figure 32

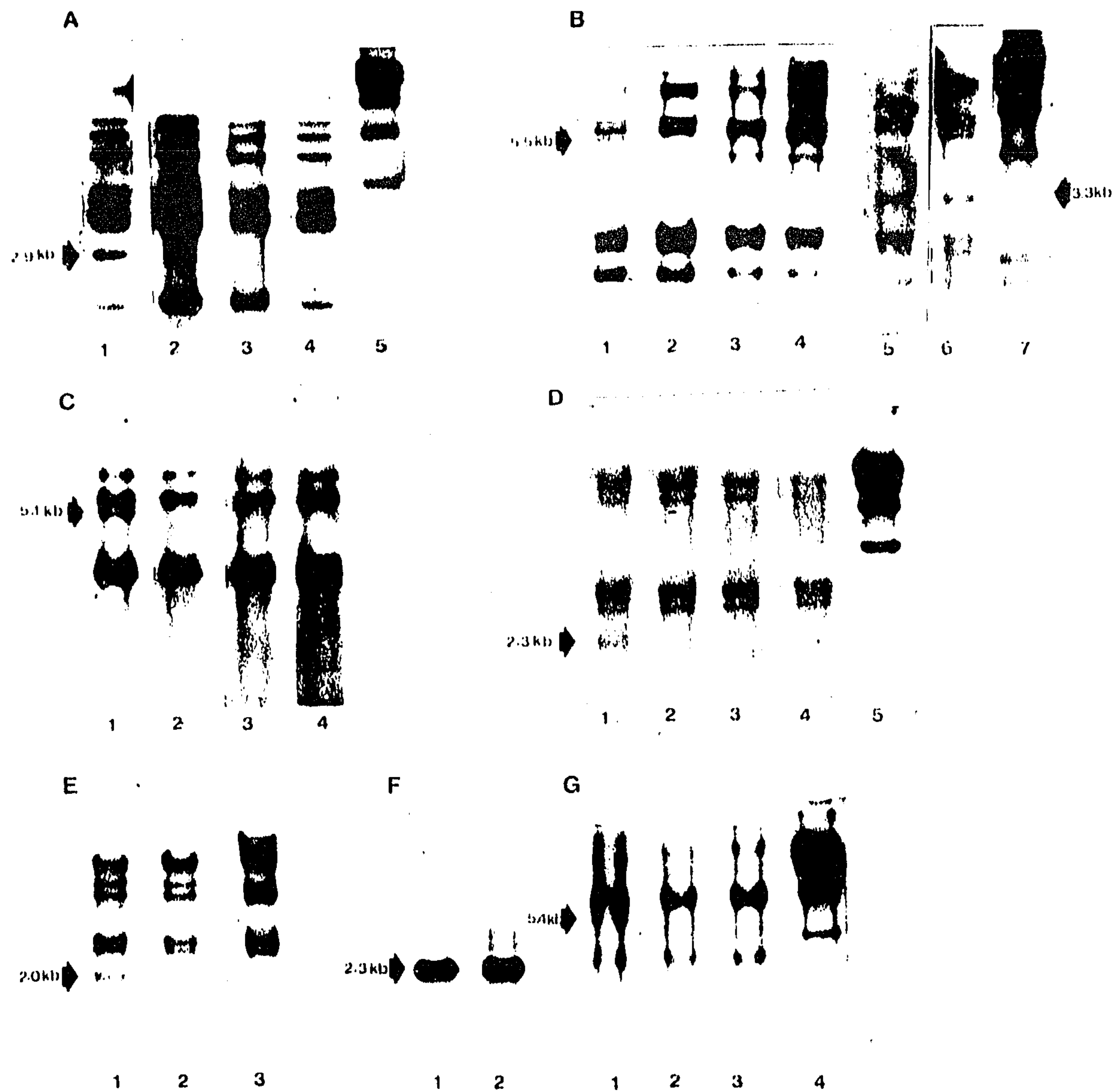


Figure 33: Southern hybridization of B. subtilis strains restricted with two endonucleases. The probe is 23s rRNA. Genomic DNA (2.0 ug) was restricted with EcoRI and HindIII (3.0 units ug DNA). In the cases where double restriction was performed initial digestion was with HindIII. At the completion of this first reaction the enzyme was deactivated at 65 degrees. Buffer conditions were adjusted to those of EcoRI and the second digest was performed.

A. 1.2, 1.4 kbp EcoRI fragments

B. 2.9 kbp EcoRI fragment

C. 5.1 kbp EcoRI fragment "trimmed" to 3.1 kbp by HindIII secondary restriction.

D. Position of the deleted 2.9 kbp fragment

E. Position of the deleted 5.1 kbp fragment

Figure 33

NCTC 3610
&168

BD 29 leuB1 argA2

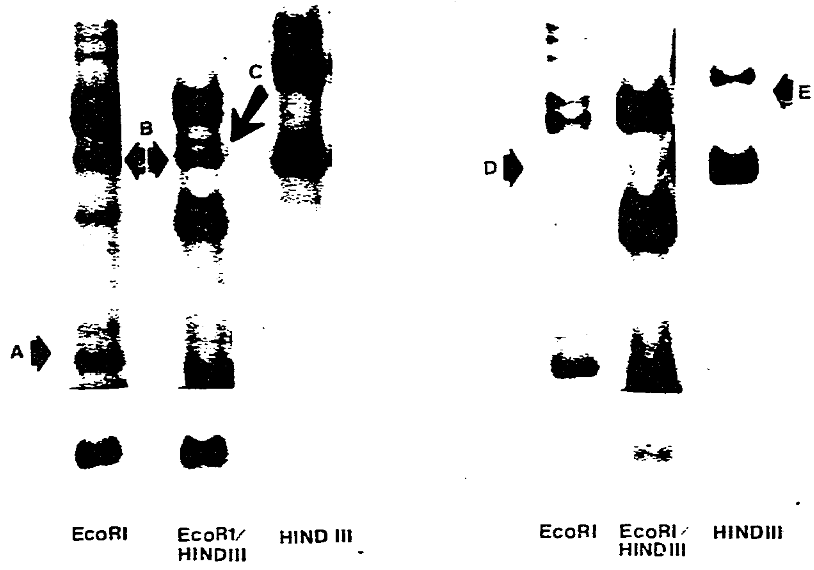


Figure 34: Southern hybridization of transformant strains of *B. subtilis*. Strain BD29(*leuB1*, *argA2*) was transformed by the method of Spizizen(1961), (see Methods). Donar DNAs are as indicated in table 3. The hybridization probe is plasmid RSF2124.B-leucine

A. SmaI restricted chromosome

1. NCTC3610/168M
2. W23
3. BD29 *leuB1*, *argA2*
4. BD29 Leu^{+W23}, *argA2*(1)
5. BD29 Leu^{+W23}, *argA2*(2)
6. BD29 Leu^{+W23}, *argA2*(3)
7. BD29 *leuB1*, Arg^{+W23}
8. lambda DNA HindIII restricted

B. HindIII restricted chromosome

1. NCTC3610/168M
2. W23
3. BD29 *leuB1*, *argA2*
4. BD29 Leu^{+W23}, *argA2*(1)
5. BD29 Leu^{+W23}, *argA2*(2)
6. BD29 Leu^{+W23}, *argA2*(3)
7. BD29 Leu^{+W23}, *argA2*(4)
8. BD29 Leu^{+3610}, *argA2*
9. BD29 *leuB1*, Arg^{+W23}
10. lambda DNA HindIII resticted

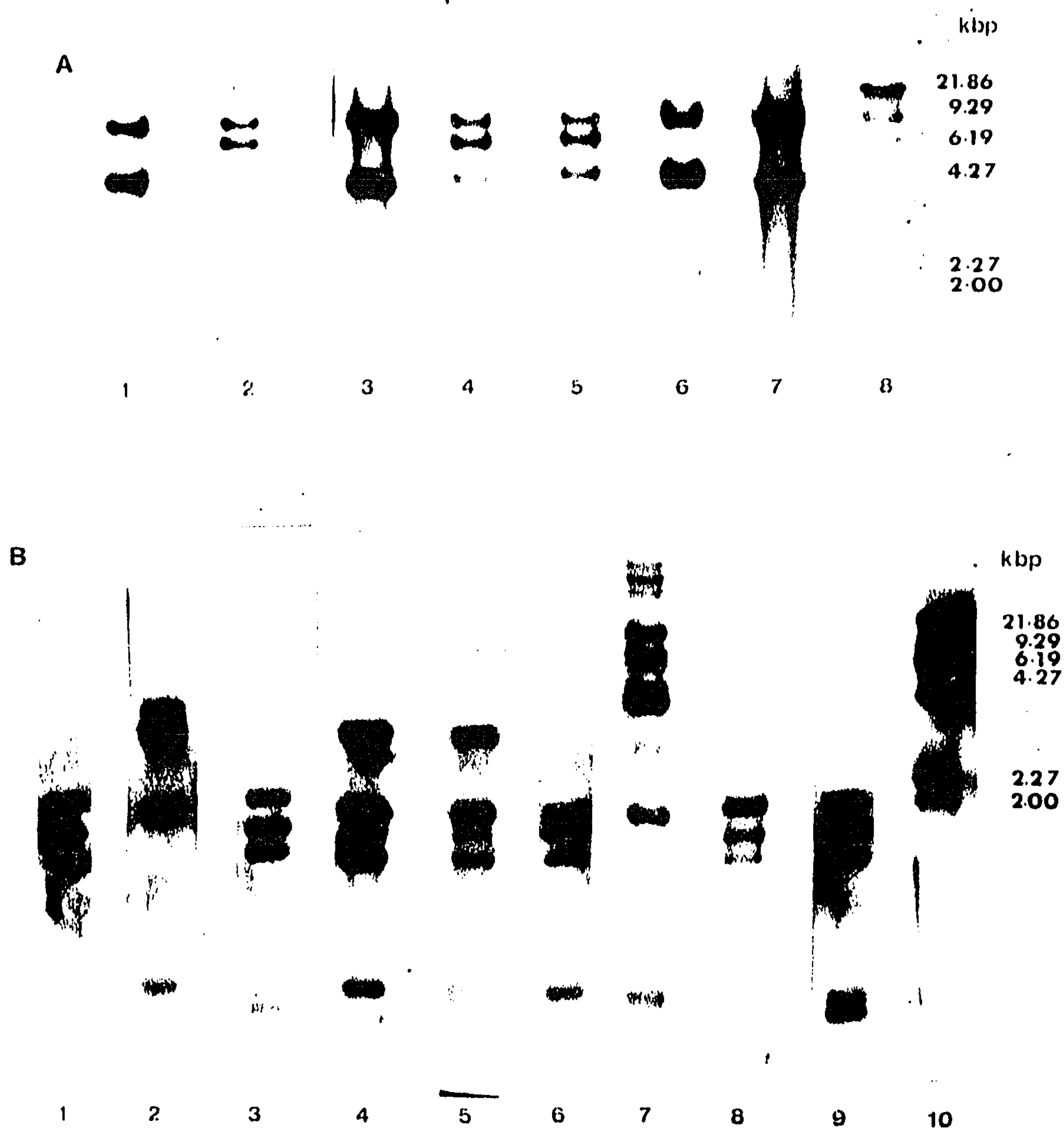


Figure 34
A
kbp
21.86
9.29
6.19
4.27
2.27
2.00
1
2
3
4
5
6
7
8
B
kbp
21.86
9.29
6.19
4.27
2.27
2.00
1
2
3
4
5
6
7
8
9
10

Figure 34: continued

C. HindIII restricted chromosome

1. NCTC 3610/168M
2. *B. pumilus* RUB502
3. BD29 Leu^{+502}, *argA2*
4. *B. globigii* RUB562
5. BD29 Leu^{+562}, *argA2*
6. BD29 *leuB1*, Arg^{+562}
7. lambda DNA HindIII restricted

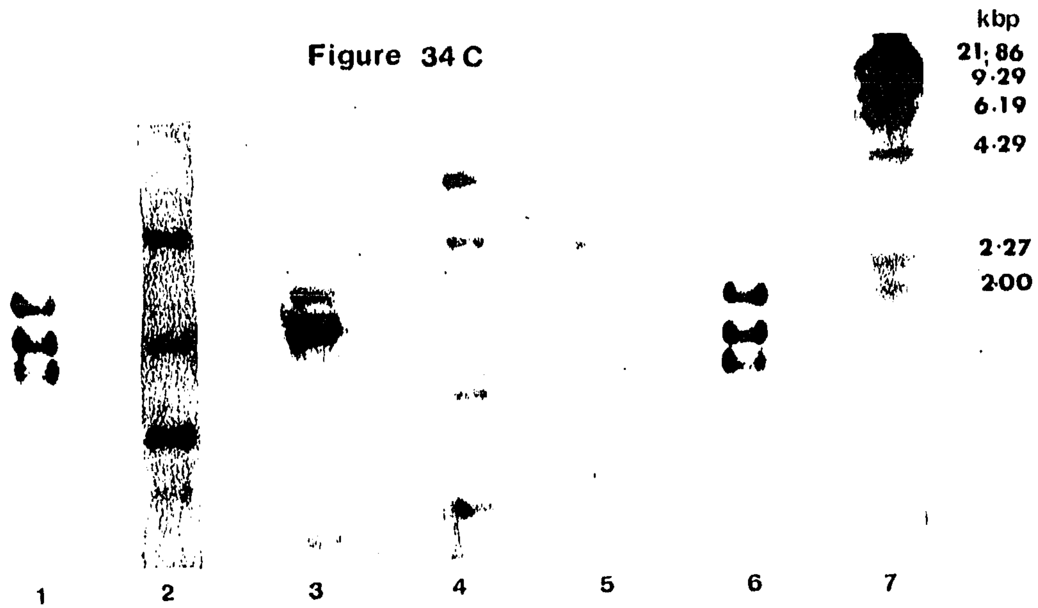


Figure 34 C
kbp
21.86
9·29
6.19
4·29
2·27
2·00
1
2
3
4
5
6
7

Figure 35: Densitometry tracings of Southern hybridizations of transformant strains of B. subtilis. The hybridization probe is RSF2124.B-leucine. Two ug of restricted chromosomal DNA was loaded into each well lane and transferred to the Southern filter after completion of electrophoresis. The autoradiogram scan rate was 4cm/min and the chart recorder ran at 20cm/min. Relative absorbance was at 580nM.

A. SmaI restricted chromosome

B. HindIII restricted chromosome

Transformant strains are described in table 3.

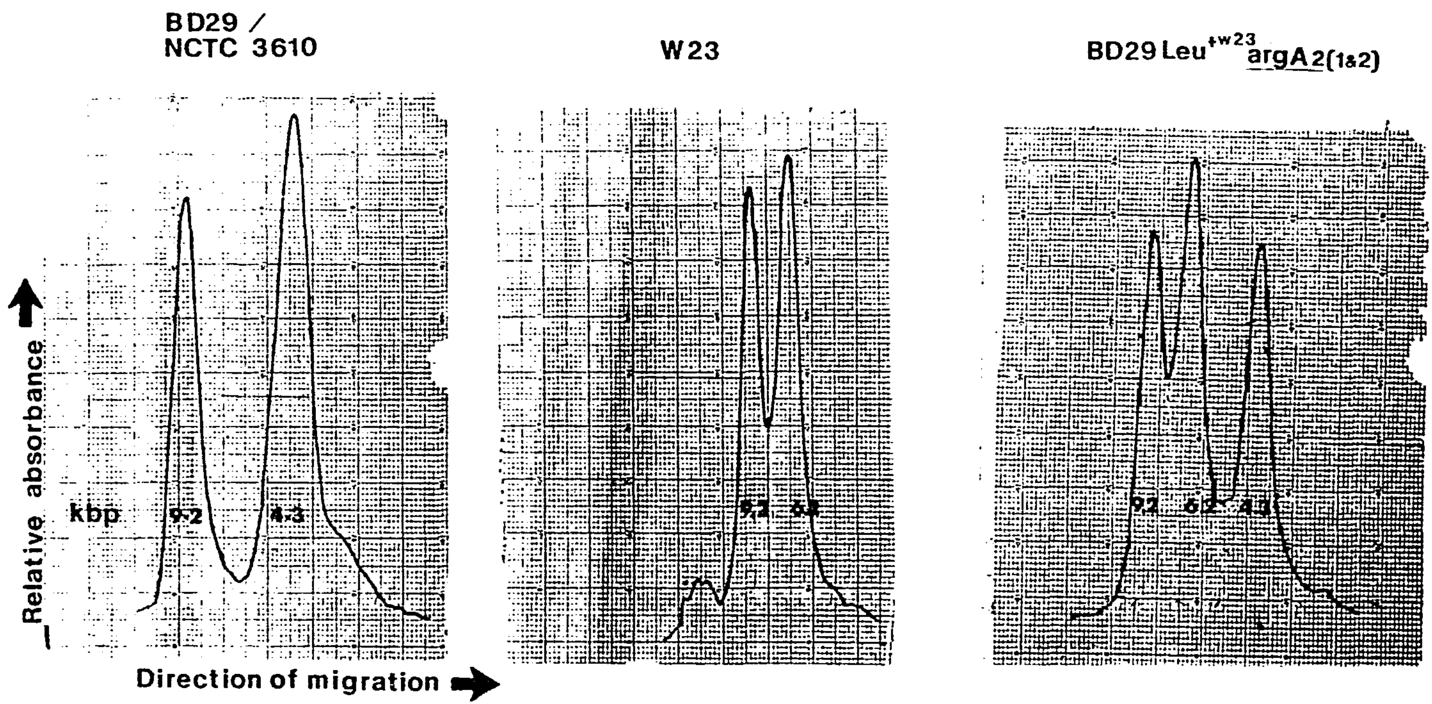
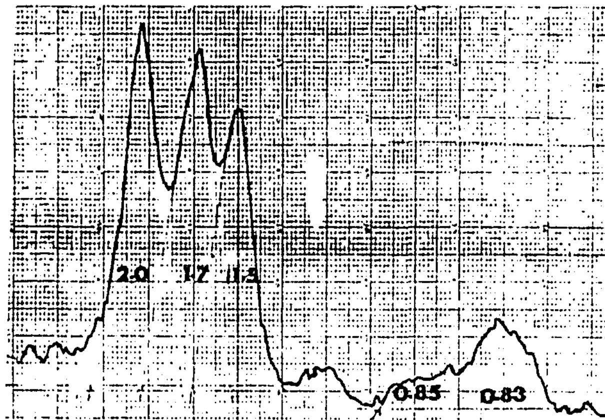
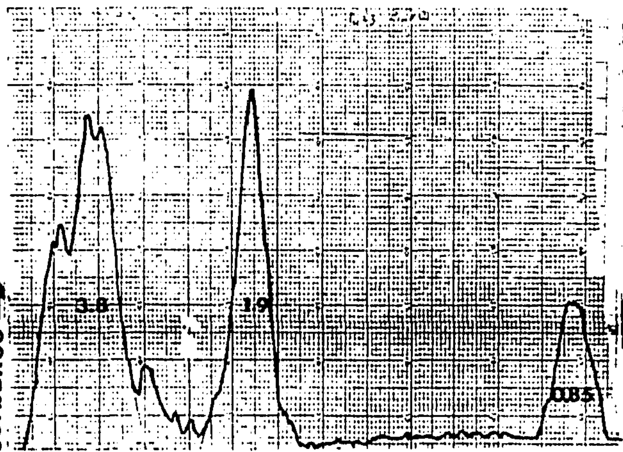


Figure 35 A

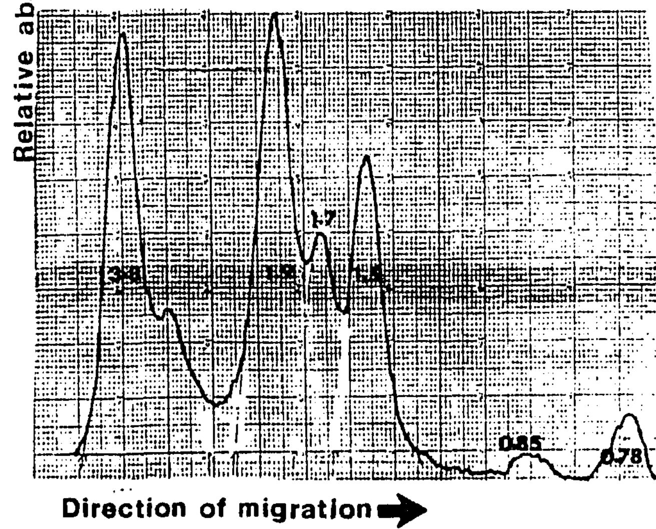
Figure 35 B



BD29 / NCTC 3610

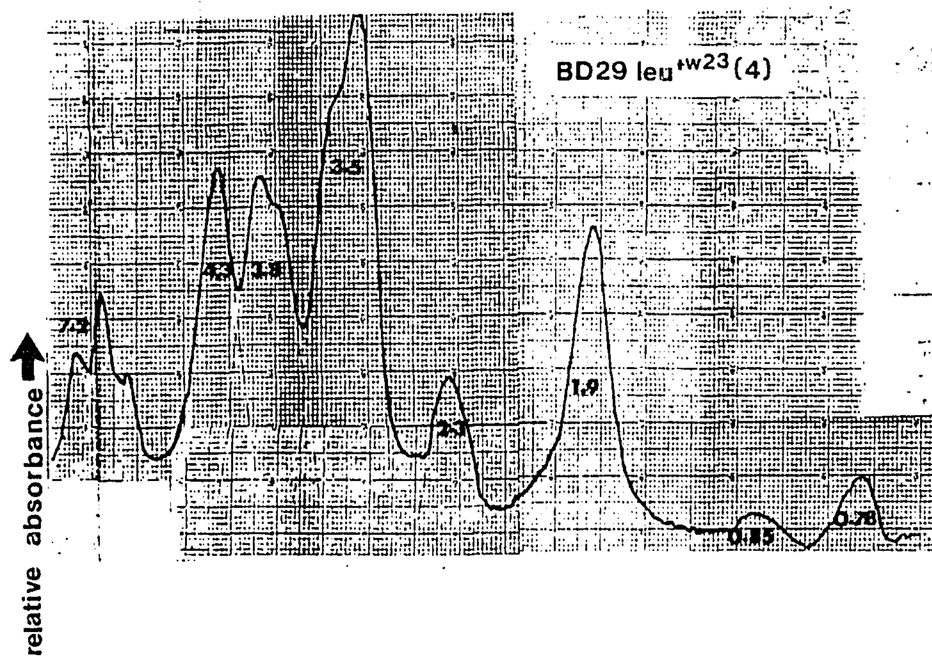


W 23



BD29 leu+W23 (1)&(2)

Figure 35 B continued



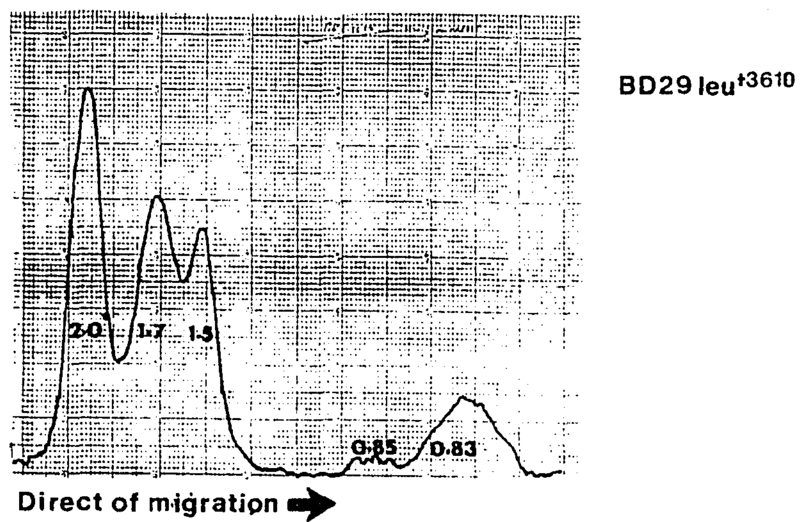


Figure 36: Southern hybridization of transformed strains of *B. subtilis*. The hybridization probe is pBC 279- 23s, 5s. All chromosomal DNA was restricted with EcoRI and electrophoresis was on a 0.75% agarose gel prior to transfer to the nitrocellulose sheets.

1. W23
2. NCTC 3610
3. BD29 *leuB1*, *argA2*
4. BD29 *leuB1*, Arg^{+W23}
5. BD29 Leu^{+W23}, *argA2*(3)
6. BD29 Leu^{+W23}, *argA2*(4)

Relevant band sizes are noted by an arrow with kilobase pairs.

Figure 36

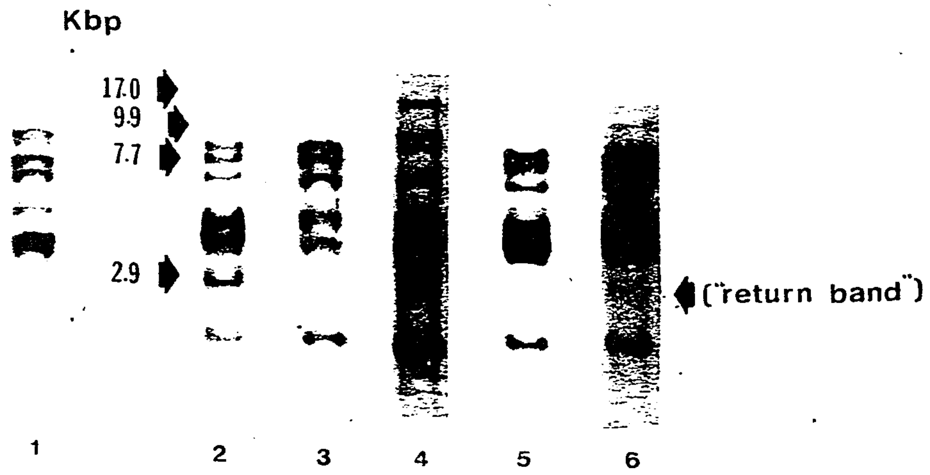


Figure 37: Densitometry tracings of Southern hybridizations of B. subtilis leucine transformant strains. The hybridization probe is pBC279- 23s, 5s. All chromosomal DNA was restricted with EcoRI endonuclease. Each gel had 2.0 ug of restricted DNA applied to it.

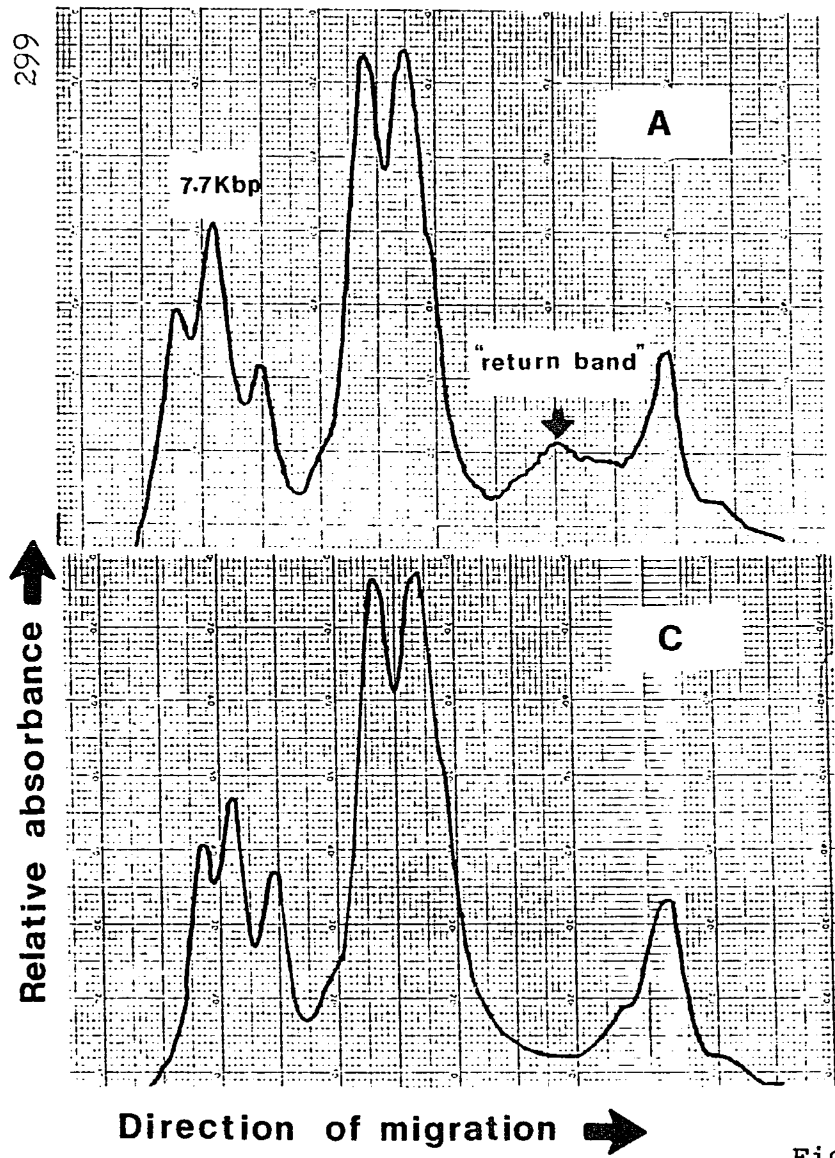
A. BD29 Leu^{+W23}, argA2(4)

B. BD29 Leu^{+W23}, argA2(3)

C. BD29 leuB1, argA2

D. NCTC3610

Autoradiogram scan speed is 4cm/min and the chart recorder speed is 20cm/min.



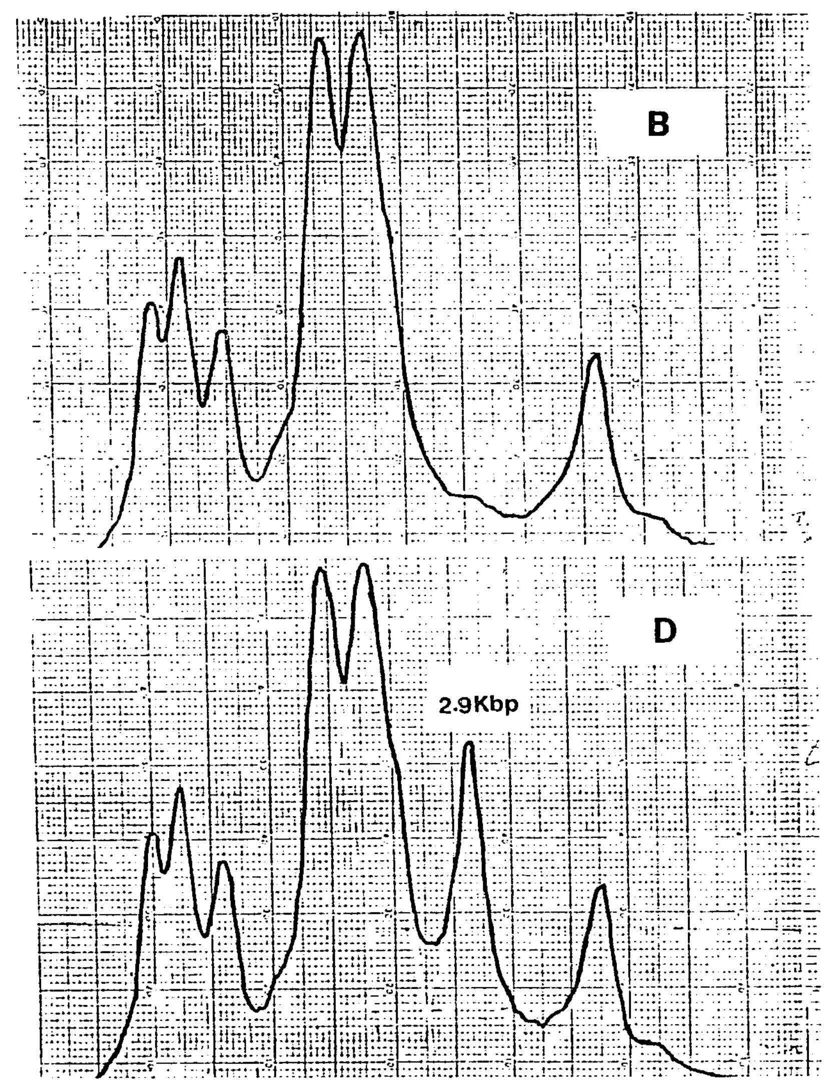
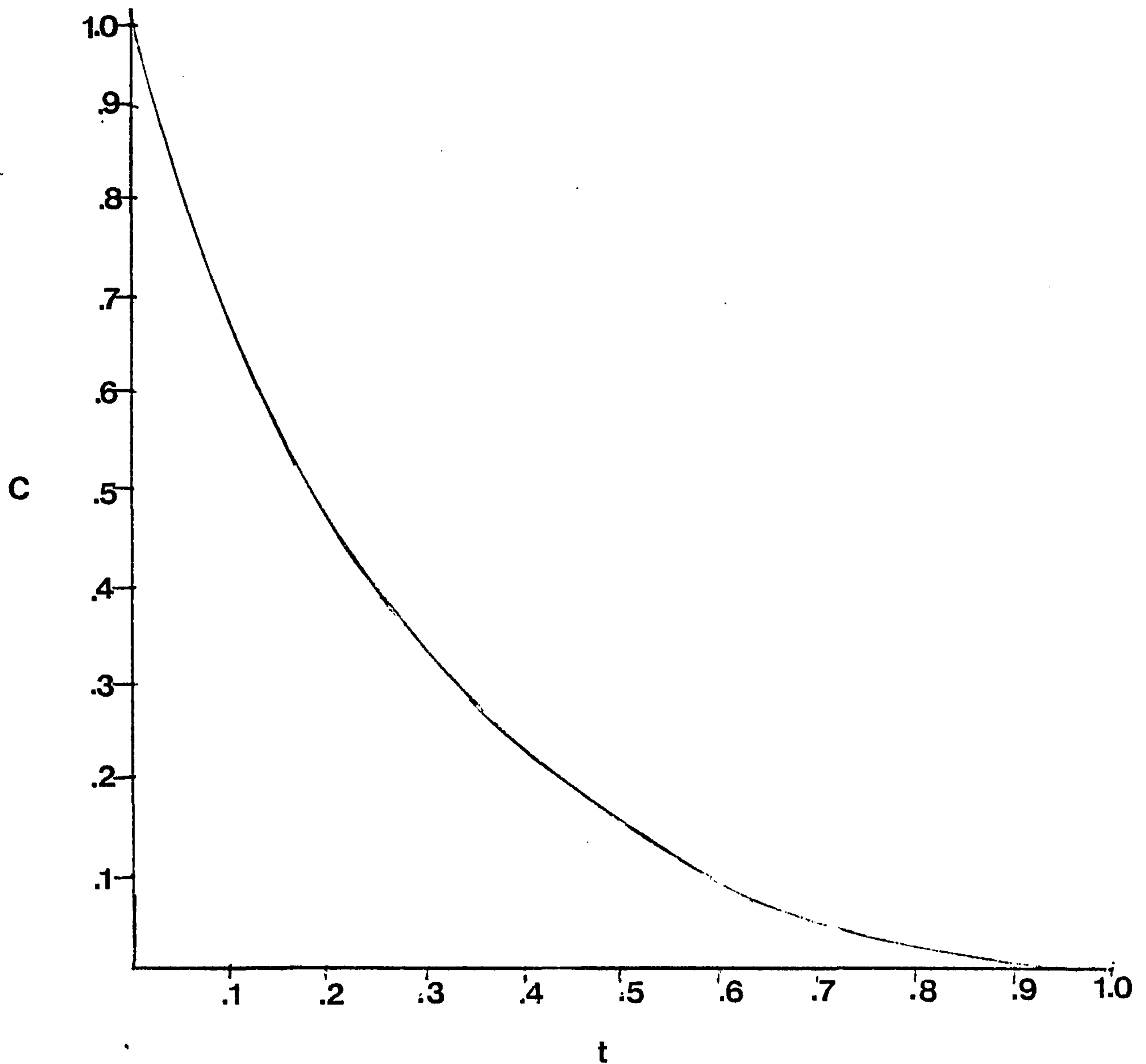


Figure 37

Figure 38

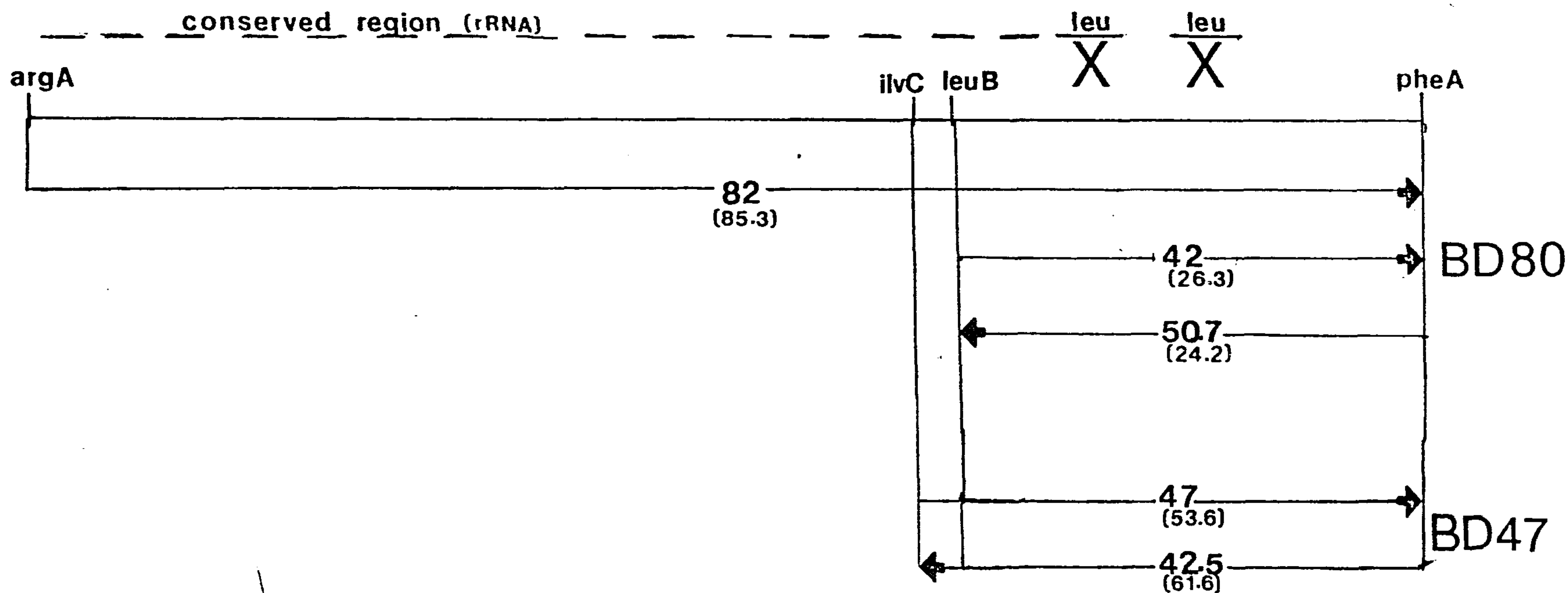


Standard curve based upon the equation of Kemper (1974).
$c = (1-t)+t(\ln t)$

c - the cotransduction index of two genetic markers
t - the fractional length of the transducing fragment separating the markers
This function relates the cotransduction index of two genetic markers to a physical distance on the DNA of a transducing particle.

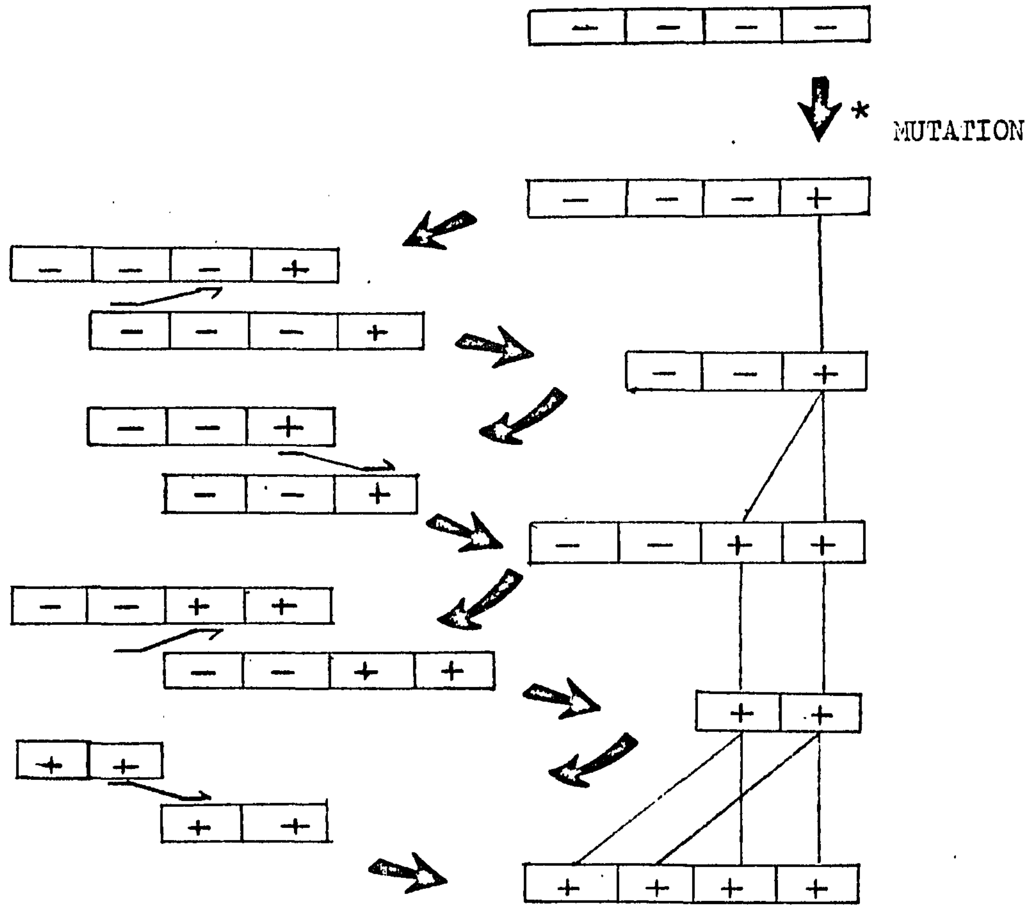
Figure 39

Linkage Relationships of the arg- ilv- leu- phe Region by AR-9 Transduction



Co-transduction frequencies are converted by the relationship 1-c to recombination units which are entered on the figure. Values in parenthesis are recombination units produced by transformant strain donars (see table 29). The symbol X marks the region of tandem Leu sequence integration.

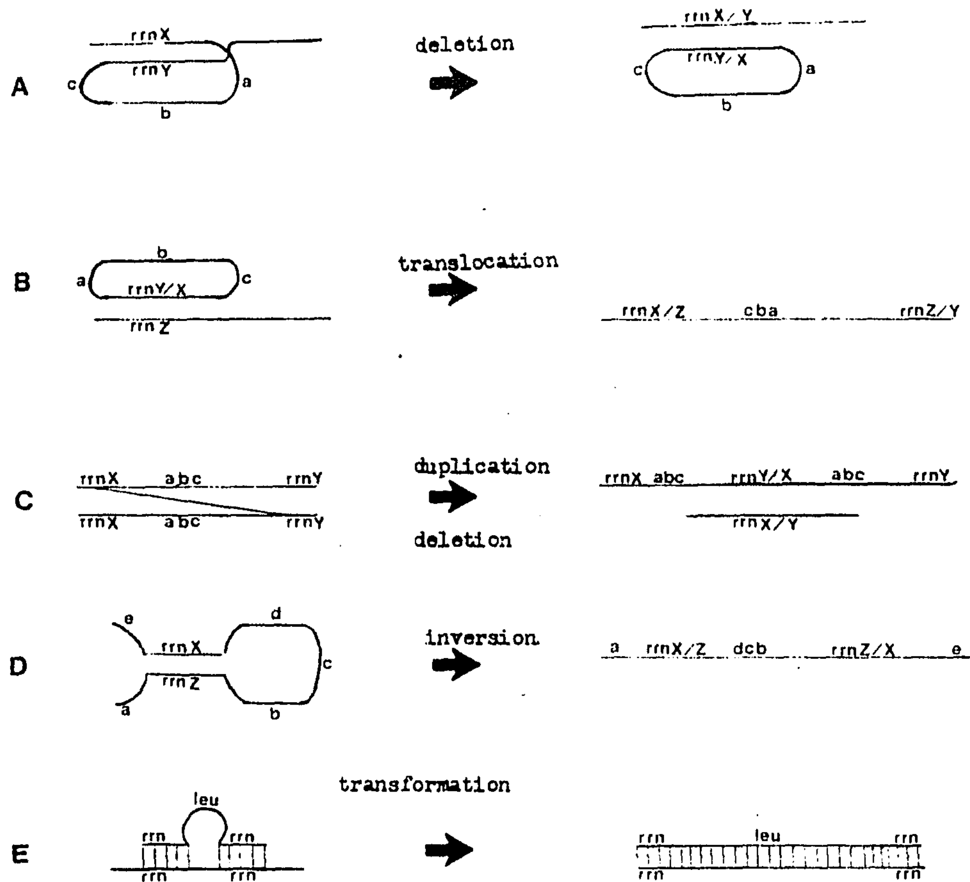
Figure 40 The Crossover Fixation Model



+ with the EcoRl site
— without the EcoRl site

Schematic illustration of crossover fixation. Each box represents an individual rRNA cistron. The crossover event occurs between two identical DNA molecules which are daughters of the same parental molecule. The cistrons are presented as being in a tandem array as observed in B. subtilis. This model is derived from that of Smith (1974). In this example an individual gene gains an EcoRl site ("MUTATION") which is then fixed as the only type in the chromosome.

Figure 41



Types of chromosomal rearrangements potentially produced by unequal recombination between rrn gene sets. Figures A to D are based on a model proposed by Lehner and Hill (1980). Figure E presents the integration of the leucine determinant via mediation by flanking rrn sequences. This transformation event involves single stranded homologous pair- of the rrn genes.