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Restriction Topography of the Bacillus Genome

by

Charles T. Tackney

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

1981

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

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Abstract

RESTRICTION TOPOGRAPHY OF THE BACILLUS GENOME

by

CHARLES T. TACKNEY

Advisor: Professor Rivka Rudner

The organization of the Bacillus subtilis genome was studied with respect to proximity of various loci to restriction nuclease palindrome recognition sites. By employing a transformation analysis with mutant recipient competent cells, the relative transforming activity (RTA) of prototrophic donor loci were determined with respect to efficiency, relative susceptibility, linkage relationships and strain variation and evolution. The RTA values of EcoRI or HindIII digested donor DNA fell into distinct class ranks. B. subtilis prototrophic strain W23 loci for trpC2, metB10, thr-5 represent high, medium and low RTA categories respectively following EcoRI cleavage. Loci for thr-5 and trpC2 give high and low RTA capacities in mutant rescue following Hind III digestion. Strain 3610 (ATCC) gave anomalously high EcoRI generated trpC2 survival (30% RTA) as compared to the same locus in W23 (7% RTA). In addition, a HindIII sensitive site is inferred in close

proximity to trpC2 of the W23 aromatic operon and is absent in this region of 3610 DNA. The entire trp aromatic cluster is intact following EcoRI cleavage of either W23 or 3610 DNA. Biological activity profiles of 168 donor trp information was similar to that of W23. Hind III digestion cleaves the W23 trp region such that the linkages trpE-hisB and trpC-hisB are interrupted while similar digestion of 3610 DNA leaves the entire trpD-A span intact. The linkage argA-leu-1 is interrupted by EcoRI cleavage in both 3610 and W23 with additional palindrome localization upstream and in proximity to arg and downstream distal to leu-1 sequences. Ligation generated repair (RTA L/R) was locus characteristic and distinctly inverted with low RTA members recovering biological activity to a much greater extent than high RTA representatives. In no case, however, could the trp-his linkage loss following Hind III digestion be improved. The relative susceptibilities of several loci to cleavage by EcoRI or Hind III were studied and a schematic organization relative to recognition palindromes presented.

A 2.4×10^6 M.W EcoRI fragment of B. pumilus trp⁺ genomic DNA was cloned in vector pUB110 and employed as a specific trp homologous probe. The chimera encompassed trp operon cistrons E, D, C, F and was used, following nick translation and Southern blot hybridization analysis, to identify and map the trp operon region of B. pumilus

chromosomal DNA as well as that of B. subtilis W23, 3610, 168 derivatives. A 5.7 and 9.8 Md. trp homologous Eco RI fragment was identified in B. subtilis W23 and 3610 respectively. Strain 168 derivatives had EcoRI fragment sizes resembling that of 3610. Hind III generated similar size fragments in both W23 and 3610 (3.0 Md). The W23 trp operon is interrupted by Hind III to generate a specific fragment of 3.0 while Eco/Hind double digestion gives 2.0 and 3.4 Md. fragments. Strain 3610 gives Hind III generated fragment sizes similar to W23. The RTA data and hybridization analysis allow the generation of organization schematics for the B. subtilis W23, 3610 and 168i⁻ genomes as well as that of B. pumilus. These data indicate that the 168 (i⁻) derivatives commonly used as recipients in transformation analysis arose from a 3610 (NTCC; ATCC 6051) progenitor. Furthermore, it appears that the trp prototrophic sequences that reside in proficient (trp⁺) 168i⁺ lines originated from strain W23 material. Subsequent mutation and drift of other loci have given rise to 168(i⁺) cells with strain variation of a microsequence nature. The history of the original Marburg organisms has been somewhat obscure and with the aid of biological and sequence specific probes, the "paragenotic" nature of W23 trp⁺ sequences residing in a laterally evolving 3610 background has been traced. In addition, the relative structure and organization of several loci relative to restriction palindromes, as well as the feasibility of availability for cloning, has been demonstrated.

I am deeply indebted to my sponsor and mentor of many years, Professor Rivka Rudner. Seldom does one encounter an individual whose love of ones work is so great. Her teaching and guidance have demonstrated to me that through hard work and perseverance, no matter how difficult the circumstances, one may succeed. Often success is a very personal event, and through her warm example I am now able to experience it.

To my wife Debra I express my love and gratitude for bearing with me and never failing to offer much needed encouragement. Her sacrifices have been great in order that my consuming scientific interests might be fulfilled. Without her love and consideration this document would not have been possible. For her support and that of my mother Anne, who first introduced me to science, I am truly grateful.

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INTRODUCTION

Alterations in the genetic constitution of many diverse organisms may be brought about by the uptake and expression of new, exogenously supplied information. This change is generated by a length of DNA proficient in the coding of some metabolic function and substituted, by re-combinational shuffling, for a deficient or variant site in the genome of the recipient organism. Originally identified as a nebulous "transforming principle" that altered the pathogenicity of Pneumococcus in mice (Griffith, 1928), the relatively obscure macromolecule DNA was shown by Avery, Macleod and McCarty (1944) to be responsible for heritable change by a mechanism "as yet undetermined". With the work of Hershey and Chase (1952) using bacteriophage, and the demonstration of transformation with purified DNA molecules in other microorganisms, the role of DNA as an "informational" entity was firmly established. The ability to alter the heritable constitution of populations of organisms by choosing which "marker" gene would be offered to a recipient cell,

and subsequently selecting novel combinations and capacities, foreshadowed the explosion of recombinant nucleic acid technology we experience today.

The involvement of restriction nucleases in the promotion and limitation of genetic exchange, as well as the possible role of DNA modification events during the Bacillus life cycle, make an understanding of genome topography crucial. The use of cloning technology to probe this developmentally significant genus points out the need to possess information relating the genetic and restriction "maps". The proximity to various restriction enzyme recognition sites will determine the relative availability of a coding region for cloning as an intact entity. Furthermore, the arrangement of diverse nuclease binding sites, both intra- and extragenic, may provide clues to the evolutionary history of the genus Bacillus and the relationship of its various members to one and other. In addition, analysis of specific cleavage palindromes within protein coding and non-coding sequences can shed light on the possible

role of these highly diverse sequences in recombination, genome fluidity and gene expression. Horizontal as well as vertical evolution from protogenomes may be indicated by the interspersal of extra-genus specific remnants within Bacillus DNA. This study attempts to relate, via transformation mediated exchange, the structural and functional maps of B. subtilis and some of its related members. Particular attention is called to certain domains which seem amenable to investigation with recombinant techniques from the point of view of sequence conservation and variation.

Transformation of suitable "competent" members of a population involves several steps: the recognition and binding of a naked DNA molecule by a receptive "competent" subpopulation, the processing and uptake of that molecule into the cell, the synaptic interaction of donor-recipient regions and the substitutional integration and phenotypic expression of the newly acquired genotype. The following is a discussion of these events as they occur in several genera.

1. Competency

The number of competent recipients in a population, as well as the time frame within which this transient state develops is dependent upon the organism and the metabolic conditions prevailing. In Bacillus subtilis (Spizizen, 1958), competence occurs at a critical cell density at the end of the logarithmic phase (Anagnostopoulos and Spizizen, 1961) and rises to its maximum level during the stationary growth period. In this organism, a minority (1-10%) of the cells become transiently competent (Nester and Stocker, 1963; Bott and Wilson, 1967) as opposed to Hemophilus sp. or Diplococcus sp. where a high proportion of the cells are transformable (90-100%) (Spencer and Herriott, 1965). Genetic transformation in E. coli, on the other hand, requires the exposure of the cell surface to CaCl_2 , and in addition, the recipient must lack the recBC enzyme system (Mandel and Higa, 1970; Cosloy and Oishi, 1973). In Pneumococcus, a competence inducing protein has been isolated and is responsible for the spread, via

diffusion, of the competent state from one cell to another (Tomasz, 1965). While these proteins exist in other genera, their role is less convincing. In common however is the obligatory requirement of protein, RNA synthesis, and energy metabolism, as opposed to net DNA synthesis (Notani and Setlow, 1974), for successful transformation. Competence development in B. subtilis is closely "linked" with sporulation capacity both genetically and metabolically although this relationship is not obligatory (Spizizen, 1965; Spizizen et al., 1966).

Transformation events have been demonstrated in Streptomyces (Bibb et al., 1980), algae (Shestakov and Khyen, 1970) and mammalian somatic cells (McBride and Ozer, 1973; Wigler et al., 1978). However, in these cases, very little information exists as to the nature of the mechanism. As in the above cases, the competent state is transient, nonheritable and coupled closely to metabolic and surface alterations in the recipient's cell cycle.

2. Binding

When a competent recipient collides with a fragment of DNA in solution, it must recognize and bind this macromolecule in a specific manner if successful uptake is to occur. While many cells may bind DNA molecules to their surfaces, this contact is short-lived and rapid dissociation occurs. While noncompetent cells fail to stably bind DNA, membrane vesicles from these cells may, albeit with different affinities. It is possible that a cell wall "unmasking" event is required to reveal these binding sites (Joenje et al., 1975). The possibility that DNA binding sites are associated with the mesosomal fractions is also indicated (Vermeulen and Venema, 1974). The number of sites at which DNA may be bound and taken into the cell varies depending on whether stable interactions or successful transformation events are scored. Nevertheless, the average values are consistent. In B. subtilis there are 20-50 sites (Singh, 1972) in Pneumococcus 30-80 and 2-8 in Hemophilus (Stachura et al., 1968).

The binding of denatured, non-duplex DNA is very low in transformable organisms but manipulations involving divalent cation chelation and pH alterations allow single stranded molecules to be utilized (Tevethia and Mandel, 1970). The evidence favoring some sort of enzymatic event during uptake is strong for B. subtilis, E. coli and H. influenzae. However, mutants exhibiting preferential binding of denatured DNA in Hemophilus may indicate discrete pathways of entry (Castor et al., 1970). The apparent transformation of auxotrophs by denatured or single strand DNA is largely attributable to inter-strand covalent cross-links (Alberts and Doty, 1968) or to self-annealing intrastrand sequences found in fractionated complementary strands (Rudner and Remeza, 1973; Galloway and Rudner, 1979).

The uptake of heterologous DNA is a confusing subject since binding, passage into the cell interior and genetic interaction must be considered separately. The level at which discrimination takes place may lead to disparate interpretations.

Synthetic polymers of poly dG:dC bound to competent B. subtilis cells but rG:rC or dG:rC did not. However, no firm evidence was presented of true "uptake" (Ciferri et al., 1970). Similarly, reports of heterologous uptake products resembling those formed during homologous processes in B. subtilis are explained on the basis of degradation and reutilization of heterologous (i.e. E. coli) donor material (Piechowska et al., 1975). The latter investigators report that B. subtilis is able to discriminate at the binding and transport stages between T6 DNA and its nonglycosylated form, the former being poorly adsorbed whereas the latter is transported and subsequently degraded.

Nonhomologous DNA from E. coli and calf thymus are known to compete with homologous DNA (probably at the stage of binding) and cause a transformation depression in Pneumococcus (Lerman and Tolmach, 1957) and B. subtilis (Bodmer and Ganesan, 1964). The highly pathogenic organism Neisseria gonorrhoeae on the other hand seems to have two discrete sets of DNA binding sites (Dougherty et al., 1979); one set binds to

foreign DNA strands and the other binds homologous duplex exclusively and transports it internally. Recently, a very interesting result was obtained (Sisco and Smith, 1979) that sheds light upon the discrimination of foreign DNA by Hemophilus influenzae (Scocca et al., 1974). These investigators show that DNA discriminating capacity resides in a membrane protein that recognizes two possible base sequences on interacting homologous duplex DNA. Using an 8.1 kb fragment of H. parainfluenzae DNA cloned onto the E. coli plasmid pBR322 it was shown that there are two unique sites, 120-140 base pairs long, separated by 3.8 kbp of chromosomal DNA, and that these sequences are responsible for binding and presumably specific uptake of DNA by Hemophilus cells. Hybridization competition analyses indicate that there are 600 copies of these sequences (1 copy per 4 kbp) per genome. Heterologous DNA, on the other hand, possesses only 1 copy per 300 kbp. Thus, the first barrier to heterologous discrimination may be the recognition of specific sequence signals interspersed along the genome and

the functioning of a receptor-pilot membrane protein in some species.

There seems to be no evidence that B. subtilis discriminates in the binding of linear duplex or circular DNA (Notani and Setlow, 1974; Contente and Dubnau, 1979) but it is of interest that while protoplasts of B. subtilis cannot be transformed with chromosomal DNA (Tichy and Landman, 1969), they can be transformed efficiently with plasmid DNA (Chang and Cohen, 1978). A report that duplex DNA containing overlapping single strand ends is disfavored in uptake (Williams and Green, 1972) is made less dramatic when it is considered that the "substrate" genome, phage $\phi 29$, required a specific binding protein attached to its single strand ends in order to circularize, concatenate and perhaps enter its productive infective cycle (Hirokawa, 1972), reflecting peculiar demands of replication in this phage. The lack of effect of the chemical nature of the duplex termini on residual transforming activity in enzymatically degraded DNA molecules is shown by Kopecka et al. (1973).

3. Processing and Uptake

Following the initial collisions of linear duplex DNA molecules and recipient competent cells, it may be shown that the incoming molecule assumes a state in which it is no longer shear sensitive. However, it still maintains its sensitivity to deoxyribonuclease (Dubnau and Cirigliano, 1972a). The incoming material is assumed to have passed from a linear, extended, end-on association with the cell to some conformation more resistant to hydrodynamic forces. Studying lag times in the expression of single and linked markers transformed into B. subtilis (Strauss, 1966), and the polarity of resistance to shear by markers on transfecting phage SP82G (McAllister, 1970), a temporal-sequential entry was suggested for this DNA, with a high degree of linear orientation. Although shear insensitive, the bound DNA fragments are still DNAase accessible and may be dissociated, suggesting non-covalent surface interactions (Morrison and Guild, 1973). As shown by Dubnau and Cirigliano (1972b) the surface DNA is fragmented at this point to a molecular weight of $10-20 \times 10^6$

daltons. Next ensues a phase in which the DNA is nuclease insensitive and continued energy expenditure is required of the system for transport of DNA fragments across the periplasmic space through the cell membrane (Strauss, 1970a). This fragmentation has been shown to be the result of random and limited nuclease attack. Each bound molecule receives a few incisions, and depending upon the initial size of the donor duplex, material (DSF) as large as 21×10^6 can accumulate (Dubnau and Cirigliano, 1974). Nucleases in B. subtilis (Scher and Dubnau, 1976) and Pneumococcus (end-noz locus) (Lacks et al., 1975) have been identified which may participate in this initial fragmentation. The noz nuclease may be vectorially oriented and serve a dual role of degradation and transport. As demonstrated by Piechowska and Fox (1971), high molecular weight single strand DNA of donor origin was isolable intracellularly following the DNAase insensitive stage. The appearance of this material corresponded with an "eclipse" phase during which active donor-specific genetic activity could not be recovered (Dubnau and

Cirigliano, 1972b). The appearance of internal single strand material was linked to the formation of extracellular exonucleolytic degradation products originating from those single strand lengths not transported (Davidoff-Abelson and Dubnau, 1973). The intracellular single strand donor fragments (SSF) were $3-5 \times 10^6$ daltons and were shown to rapidly associate with the host chromosome as a donor-recipient complex (DRC), covalently become continuous with it, and signal the end of the eclipse period. Using cell extracts of transformed recipients it is, however, possible to demonstrate the rescue of donor activity during the eclipse phase by in vitro ligation. Subsequent ligation beyond the eclipse period shows no further net improvement in activity as all potential DRC is assimilated and transformants maximized (Arwert and Venema, 1973). The integrated fragment size was estimated to be an average of 2.8×10^6 daltons (Dubnau and Cirigliano, 1972c) and involve clustered insertions of 2-4 independent SSF. Eisenstadt et al. (1975) have demonstrated a DNA

binding activity, not present in asporogenous/non-competent mutants, that may protect the obviously labile SSF in B. subtilis prior to its covalent restoration into the duplex state. As opposed to B. subtilis and Pneumococcus, Hemophilus sp. seems to take a double strand molecule across its cellular boundary and only after interaction with the recipient genome cast off one of its strands concomitant with integration of the other. This correlates with the absence of a donor-derived genetic eclipse period (Notani and Goodgal, 1966). In H. influenzae it may be shown that as much as 2% of the recipient genome itself is degraded during transformation (Steinbart and Herriot, 1968) perhaps as an obligatory result of strand displacement. At this point, perhaps via single strand regions, pairing and exchange may occur. Either strand seems capable of generating a transformant in the absence of any invocation of repair bias phenomena (Rudner et al., 1968; Strauss, 1970b) but there is evidence that some marker specific strand bias does

exist, That transient heteroduplexes are formed in B. subtilis (Dubnau and Davidoff-Abelson, 1971) is reinforced by the finding that co-transformation, with constructed artificial heteroduplexes, for linked markers occurs 1-2% when they are presented to the cell in the trans configuration (Strauss, 1970b). This represents a limit on the frequency of double strand incorporation.

As demanded by classical breakage-reunion theories, recombining strands must synapse, interrupt their covalent integrity and reform new physical and genetic combinations. According to Holloman et al. (1975) a single strand intermediate might be assimilated and substituted for a resident host strand through competition in locally "melted" regions induced by superhelical transients. However, in both B. subtilis and H. influenzae, there seems to be an accumulation of gaps in the resident genome that increase in parallel with the development of the competent state (Harris and arr, 1971; Sedgwick and Setlow, 1976). Using both buoyant density

shift and sensitivity to SI nuclease it was shown that as much as 5% of the Bacillus genome can become "gapped" and, in Hemophilus, the donor double strands are apparently gapped near their ends while the recipient molecule is variably interrupted. Puromycin will prevent this gap accumulation as will certain mutations in the transformation phenotype. The situation is unclear regarding the role of cellular polymerases in gap filling and the possible requirement of repair synthesis during integration. Inhibition of Pol III by 6-HPU did not prevent strand integration (Dubnau and Cirigliano, 1975) and neither did a mutation at the pol A locus. However, use of HPU in a Pol I deficient strain does have a negative effect. Polymerase III may have repair responsibilities imposed upon it in a pol A background. Whether gapping is necessary a priori for base-pairing interactions is not known.

The end product of successful transformation is a new homoduplex region, resolved by replication and continuous with the resident genome, endowed with novel and recombinant characteristics at one or more loci in the background of the host.

4. Chromosome Structure in B. subtilis

The chromosome of Bacillus subtilis has been estimated to have diverged from that of the gram negative enteric bacillus Escherichia coli about 1.5 billion years ago (Hori, 1976). Like other bacterial genomes, Bacillus was shown to possess a physically circular (Wake, 1974) chromosome structure of covalently closed topography (Worcel and Burgi, 1972; Dworsky, 1976) and significant superhelicity. From sedimentation-minimum measurements and binding of ethidium bromide it appears that this gram positive organism's DNA can be isolated, under suitable conditions, as a folded intact nuclear body. This structure contains small amounts of protein and lipid and can be sequentially unfolded by varying the ionic conditions of solution, the temperature, or by the introduction of single strand nicks, maximally at 30 per strand, similar to results in E. coli where superhelicity is lost at 60 breaks/strand (Worcel and Burgi, 1972). The ordered structure could not be refolded by restoration of environmental variables (Dworsky,

1977). Ribonuclease had no effect upon the native intact superstructure.

The molecular weight of the intact genome is primarily determined from sedimentation analysis which gives values of $2-3 \times 10^9$ daltons (Kavenoff, 1972; Dworsky, 1977) although compatible results are given by renaturation rates, and viscosity measurements (Klotz and Zimm, 1972). Elegant genetic experiments, measuring the change in recombination values between two loci when an addition or deletion of known size is located between them (Zahler et al., 1977; Henner and Hoch, 1980), give total genome size estimates that are genetically consistent with physical approximations.

Topologically, the genome of B. subtilis may be shown to be associated in some ordered way with its cell membrane. Early models of chromosome segregation considered this possibility (Jacob et al., 1963). Using membrane enrichment protocols (Ganesan and Lederberg, 1965), it was shown that membrane binding and marker enrichment were present for specific loci mapping

at the genetic origin, terminus and replication fork (Ivarie and Pene, 1973; Sueoka and Quinn, 1968). The work of Yamaguchi and Yoshikawa (1973) indicates that two origin proximal markers, pur A and ts 8132 may be attached to separate membrane binding points although they remain linked by a short putative replication initiation sequence in keeping with observations of bidirectional genome duplication. That many regions of the genetic map may find themselves associated with (mesosomal?) membrane sites, as well as the observation that stringently controlled plasmids preferentially associate with folded chromosomes (Kline and Miller, 1975), indicates that the B. subtilis genome may possess both structurally and functionally discrete domains. Possibly these have roles in genome control, repair, segregation or evolution.

Chemically, B. subtilis DNA has a buoyant density of between 1.703 and 1.705 g/cm³ depending upon whether the common strains W23 or 168 are examined respectively, and a T_m of 87°C. A guanine plus cytosine content (%G + C) of 42% has been noted (Deley, 1970). Although the

thermal transition is sharp about 87°C, a small amount of covalently crosslinked, non-denaturable duplex material may be isolated (Alberts, 1968). Nevertheless, the complementary strands of this DNA may, under certain specific conditions, be efficiently resolved (Rudner et al., 1968) on MAX columns into H and L strands, the L being more purine rich. Poly rG reacts preferentially with one of the separated strands (H) on the basis of an asymmetric cytosine distribution (Opara-Kubinska et al., 1964). Galloway and Rudner (1979) demonstrated that in addition to the H strand being the preferential vegetative coding template (Rudner and Remeza, 1973), it included considerable intrastrand self-complementary sequences. Moreover, these sequences appeared to be dispersed throughout the genome fairly ubiquitously (5-10%).

5. Genetic Organization

Spizizen's demonstration (1958) that B. subtilis could be transformed from auxotrophy to prototrophy marked the beginning of genetic analysis in this organism. Using mutant (auxotrophic)

recipients (Burkholder and Giles, 1947), four individual genes of the tryptophan synthetic pathway were shown to be clustered on a single molecule of transforming DNA (Anagnostopoulous and Crawford, 1961) and linked to the locus determining histidine anabolism (Ephrati-Elizur et al., 1961). This number was later expanded to six tryptophan loci and it was shown that as many as 13 individual loci concerned with aromatic amino acid synthesis could be co-transformed (Nester et al., 1963). In addition, other histidine synthesis genes are dispersed elsewhere, unlinked relative to this cluster, on other random fragments. The opportunity to analyze the spatial coding relationship between DNA and metabolic pathways was evident. As mutant recipients became available, it was clear that increasing numbers of loci could be grouped together on single fragments of donor DNA and that the possibility existed of demonstrating a single continuous linear array of genes.

The data of Yoshikawa and Sueoka (1963a) indicated an ordered cycle of genome replication

with a fixed origin and terminus of duplication. Using the relatively increased representation in a transforming DNA preparation of those genes near the postulated replication origin, it was shown that in exponential phase cells the ade locus was represented twice as frequently as the met locus. Normalized to stationary phase frequencies, met was placed at a putative terminus site distal to a growing fork. Increased gene frequencies in exponential cell DNA did not exceed twice those of stationary phase and as the former approached stationary phase, the ade/met ratio drifted from 2 to 1. Similarly, transferring stationary phase cells from a heavy (deuterated) H^2 medium to a light medium indicated that as replication began the ade marker was the first to assume a hybrid physical density followed sequentially by others and finally by met as semi-conservative replication progressed. This synchro-transfer technique (Yoshikawa and Sueoka, 1963b) was confirmed by synchronous spore outgrowth studies (Oishi et al., 1964). Dubnau and Goldthwaite (1967) greatly extended the existing linkage groups and demonstrated a

linear chromosome continuity from origin to terminus as presented in the map of Lepesant-Kejzlarova et al. (1975). The discovery of genome circularity and bi-directional replication (Gyurasits and Wake, 1973) together with the co-transduction data of O'Sullivan et al. (1975) completed the physical and genetic definition of the chromosome. In the latter study, the early replicating gltA14 locus was shown capable of co-transduction with late replicating citK1 in contrast to the synchro-transfer data.

An interesting outcome of these studies was the discovery that two closely related strains of B. subtilis imposed different controls upon their chromosome initiation and replication cycles. With strain W23 (Anagnostopoulos and Spizizen, 1961) DNA as prototrophic donor it was seen that although stationary growth phase DNA transferred various markers to auxotrophic recipients with equal frequency, the DNA of exponential growth cells possessed a frequency polarity when normalized to stationary transformation values. That is, certain genes were disproportionately represented relative to others

(Yoshikawa and Sueoka, 1963). An early replicating gene ade had a ratio of 2 relative to the late replicating met. This ratio approached unity as the donor strain neared stationary phase itself. This was interpreted to mean that replication was oriented and that in stationary phase, chromosomes were in a fully replicated resting state. On the other hand, strain 168 and its derivatives (Burkholder and Giles, 1947) gave more confusing results. Using marker frequency analysis with 168 stationary DNA as a control, no polarity was detected. It appeared as though exponential and stationary DNA were similar in that the replicating state seemed random, and the frequency of any one locus not necessarily a function of linear map position (Yoshikawa et al., 1964). When DNA from dormant 168 spores was used as the normalization control, a distinct polarity, identical to that in W23 was evinced. A less rigid control is placed upon 168 in the stationary phase regarding initiation and termination of rounds. Completed molecules are not accumulated. In the spore, regulation is as in stationary W23 cells. The apparent gene frequency non-polarity is not due to random

initiation but rather to differential control of re-initiation. Outgrowing spores however may exhibit dichotomous or multi-fork replication under certain circumstances and give ade/met ratios near 4 (Oishi et al., 1964). The isolation of chromosome initiation mutants in W23 (Upcroft et al., 1975) and 168 (Karamata and Gross, 1970) mapping near purB calls attention to this interesting region (Figure 1).

Many regions along the B. subtilis chromosome have been "mapped" by transformation or transduction (AR9, PBS1) analysis (Hoch and Henner, 1980). Working from the basic outline indicated by marker frequency and density transfer studies, several areas have been extensively examined. The region proximal to cysA14 has been shown to determine resistance to rifampicin and streptolydigin (Sonenshein et al., 1974)--phenotypes associated with altered RNA polymerase B and B' protein. This same replication-origin region encodes the multiple gene clusters responsible for ribosomal and transfer RNA (Dubnau et al., 1965; Oishi and Sueoka, 1965; Smith et al., 1968). Protein synthetic machinery also seems concentrated in this area

as 21 separate ribosomal proteins have been mapped in the origin interval cysA-attSP02 (Osawa and Tukvi, 1978). In addition, intriguing suppressor mutations of stage spo 0 spore mutants have been located here (Trowsdale et al., 1978). These loci, abrA and abrB may encode ribosomal protein modifying enzymes. The replication-origin region has been extensively mapped (Henner and Hoch, 1980) and defined by the ts 8132, nalA, and novA loci (Hara and Yoshikawa, 1973). Its center seems to be midway between purA16 and cysA14. In E. coli the analogous region has been cloned (Yasuda and Hirota, 1977; Diaz and Pritchord, 1978). Perhaps the B. subtilis origin will follow with emphasis directed at initiation events during division and germination.

In addition to the classical aromatic pathway cluster of tryptophan synthetic genes, a "super-operon" array has been proposed to explain the controls exerted upon tryptophan, histidine and tyrosine synthesis (Roth and Nester, 1971). In contrast, the arginine "operon" is dispersed although a single repressor still reacts with several distant operators (Maas and Clark, 1964) in a "regulon" model. At least 50 spore related loci,

many thought to be functioning in an operon-like manner have been shown to be widely scattered about the chromosome (Piggot and Coote, 1976). Some, closely linked to phe, cysA or lys have been cloned (Bonamy et al., 1976). A sporulation gene, clearly transcriptionally regulated, has been isolated (Segall and Losick, 1977) and shown to map near tms-26 at position 5° (Haldenwang et al., 1980) (Figure 1). Dubnau and Cirigliano (1974b) have identified and mapped 7 genes concerned with recombination and correlated these with the known events of transformation and transduction; the data of Mazza and Gallizi (1978) add 5 more. In the citK-gltA1 interval, defining the terminus of chromosome replication, the attachment site for phage SPB has been localized (Zahler et al., 1977). The same area contains att ϕ 3T, while the leuA region encompasses att ϕ 105 (Henner and Hoch, 1980). The region near pheA1 is linked to a locus tfm-1 which determines "transformability". This locus is very close to att ϕ 105 (Yasbin et al., 1976). A fairly ubiquitous phenotype in 168 strains, the killing activity exerted upon other strains, was shown to be determined by a resident prophage (PBSX) in the metC area (Garro et al., 1970),

whereas the region responsible for this prophage induction (ts1), at map position 50, also contains sequences coding for hsrM--the host restriction/modification phenotype displayed by some Marburg strains of B. subtilis towards phage (Saito et al., 1979). The leftward origin region (Fig. 1) contains additionally the determinant of BsuR, the restriction system (bsr) which acts against some phage and can limit PBS1 mediated transduction but not bacterial transformation (Trautner et al., 1974).

6. Homology Relationships

It was originally shown that unlinked genes are selectively inactivated at a critical temperature where strand separation, either on a gross or local scale, is known to occur. Linked pairs of genes, on the other hand, are simultaneously inactivated (Roger and Hotchkiss, 1961; Marmur and Lane, 1960). If heteroduplexes are constructed using isolated separated strands of complementary DNA, the thermal stability of various genes may be biologically determined; this is especially interesting if the strands originate from different relatives. Divergence of sequence may be estimated from the change in the thermal inactivation

transition point (Chilton and McCarthy, 1969) which is closely correlated with the T_m . It is estimated that a 1°C depression of T_m is equal to 1.5% base pair mismatch although complementary strands larger than 100 base pairs tend to approach the stability of longer duplex molecules (Laird et al., 1969). Enzymatic or spectroscopic measurements would seem more accurate in this situation. With B. subtilis strain 168 as auxotrophic recipient it was found that there was a patterned gradient of homologies when B. globigii DNA was the heterologous donor as measured by homologous/heterologous transformation frequency (Chilton and McCarthy, 1969). Relative homology was plotted versus gene map position and it was shown that str and leu were highly conserved regions among these bacilli. A gradient of conservation passed through other markers not linked to str such as ade-26. The locus ilvA was shown to be linked to leu but itself endowed with sequence conservation. Other markers, pur-6, cysB, metA, metB showed conservation ratios indicating partial to complete inhomology with B. globigii. Strain W23, related to 168, shows a similar but not as dramatic a conservation pattern around the genome.

When 168 cells are transformed by W23 or 168 DNA, a correlation of gradient ratio with map position is apparent although its range is quite small. However, when transduction is the vehicle, the homo/het ratios are dramatic, spanning 3 orders of magnitude. Homologous transduction shows no such marker discrimination (Dubnau et al., 1969). Rather than indicating separate paths of recombinant generation, it is suggested that this might be due to restriction exerted upon incoming heterologous double strand DNA as opposed to the single strand DNA vector in transformation (Hadden, 1977).

As shown by Dubnau et al. (1965, 1967) only str and mic were transformed by B. licheniformis DNA into B. subtilis. Only str was transferred from B. licheniformis to B. globigii. The transfer of the rif resistance gene from B. pumilus or B. amyloliquefaciens to B. subtilis occurred at a frequency 10^3 times that of prototrophic transformation (Wilson and Young, 1972). The existence of a conserved "core" of genetic sequences or a dosage effect due to increased numbers of linked ribosomal sequences (Oishi and Sueoka, 1965) could account for the high frequency transfer, intergenotically, of some genes and not others (Wilson and Young,

1972). According to Lovett and Young (1969), W23 and 168 (B. subtilis strains) are 89% related while B. licheniformis and 168 are 24%, B. pumilus and 168 are 16% and B. amyloliquefaciens and 168 are 11% related. As a comparison, E. coli is 4% related to B. subtilis 168. Chilton and McCarthy (1969) estimate that B. globigii and B. subtilis differ grossly by about 13-18% mismatch at the metB marker but have a homologous/heterologous exchange ratio of 10^4 ; the str locus with a 1.5% mismatch has a ratio of 40. Factors other than base sequence may be operating at synapsis such as favorable linkage to G + C rich rDNA. This would bias T_m values towards greater stability while integration would be governed by proximal homology. W23 and 168 differed by an average of 7% of their bases and had a small variance range (2-3 fold) for their T_m values. The conservation in the region of str, ade and leu can be due to the evolutionary demands of linkage to ribosomal cistrons (Smith et al., 1968), vital enzymes or proximity to the replication origin region. These results may reflect the differential mutation and fixation rates imposed along a chromosome (Chilton and McCarthy, 1969) during its evolution.

While some loci are clustered near the origin of B. subtilis there seems to be no grand organization to gene arrangement beyond a basic operon level. Developmental loci such as spo and genome maintenance loci such as rec are widely scattered (Fig. 1). There is however, as in E. coli, a lack of identified markers in the terminus region of the chromosome (Hoch and Henner, 1980). Whether this paucity represents a structural constraint of membrane association and transcriptional access (e.g. folding) or a selection problem is not known. The presence of genetically "silent" regions in general is not correlated with any supra-organization either structural or functional. Relative gene densities fail to form any axis of symmetry indicating gross genomic doubling from a proto-genomic ancestor (Bachman et al., 1976) nor any 90° or 180° axes as seen in E. coli. The only evidence of large scale chromosomal rearrangements in B. subtilis comes from unusual and rare mero-diploid strains (Audit and Anagnostopoulos, 1973) redundant in the aromatic region.

7. Effect of Size on Transformation

It is obvious that successful transformation involves a cascade of engineering events directed

at a random array of duplex fragments and ending in the precise integration of a single strand product many thousands of times smaller than that of the resident genome. The transition from DSF ($10-20 \times 10^6$) to SSF ($3-5 \times 10^6$) imposes requirements and limitations upon the incoming DNA (Dubnau and Cirigliano, 1972b; 1972c). In B. subtilis, Nester et al. (1963b) found that transformation was generally associated with higher than average DNA molecular weight in a donor cell lysate. Bodmer (1966) demonstrated that the efficiency of transformation was a function of DNA single strand molecular weight. Activity approached a maximum at $6-7 \times 10^6$ (ss) and declined rapidly below that value. As shown by Kopecka et al. (1973) for H. influenzae, this inactivation was related to the total number of bonds broken and not to the nature of the resulting molecular ends. Residual activity differences due to different enzymatic treatments were normalized when either the total number of breaks or the ss M.W were plotted versus activity.

When DNA is isolated by the Marmur (1961) procedure, the traditional protocol, average fragments (ds) of M.W, $10-30 \times 10^6$ are obtained.

There is no evidence that these fragments are generated by any unique or biased mechanism (Stewart, 1969), as fragments carrying any particular genetic determinant are heterogeneous with respect to molecular weight, buoyant density and thermal stability. This is different from the situation seen in *Pneumococcus* where unique break points are suggested to some extent (Roger et al., 1966).

Originally, a "critical size" model was proposed for transformation in *Pneumococcus* (Litt et al., 1958). This critical size was thought to encompass a region which, if intact, gave full transforming activity. Cato and Guild (1968) found that rather than an "all or nothing" critical relationship, there was a continuous size dependent function. From molecular weights of 10^7 to 3×10^5 (ds), biological activity declined across 4 orders of magnitude. More descriptively, this has been termed a "maybe or nothing" phenomenon (Notani and Setlow, 1974). A minimum active donor fragment size of approximately 3×10^5 (ds) was suggested and later an intracellular donor single strand molecular weight of 7.7×10^4 daltons was measured (Morrison and Guild, 1972a). Thus, models wherein

activity plummets to zero below a critical size are not supported. Critical sizes may more likely be reflective of differences in uptake between large and small duplexes, and demands for a minimum effective pairing or synaptic length rather than a critical genetically proficient size (Cato and Guild, 1968). Probability continua are more readily invoked than fixed mechanistic limitations. Lacks (1968) developed a theoretical prediction of the probability of marker integration based on his studies of the amylomaltase region of Pneumococcus. Measurements were made of the frequency of recombination between two markers in this region and a "switch frequency" of 0.8 crossovers per 10^6 daltons (ds) molecular weight was the closest fit. Integration outcomes were predicted for donor transforming DNA of varying (ds) molecular weight. As explained by Cato and Guild (1968), the theory predicts more activity at low M.W. than is actually observed, regardless of the "switch frequency" considered. If, however, the donor strand size were assumed to be 450 base pairs or 3×10^5 daltons shorter, the fit was very close to the theoretical. It appears as though recombination involves the shortening or non-availability of some

length of the recombining single strand intermediate (SSF) in Pneumococcus transformation.

This length could be exonucleolytically attacked or bound to some essential protein on one or both ends during synapsis. The "excluded" length is close to the minimum transforming size of Cato and Guild (1968, 1972), 3×10^5 d.s. M.W., and might be in the range of the minimum stable pairing length.

In B. subtilis, Morrison and Guild (1972b) extended the subject of "critical size" during transformation and found that transforming activity was very dependent on size below 6×10^6 and that biological activity could be detected as low as 1.2×10^6 daltons (ds) M.W. That no critical double strand length was involved was shown using highly purified size fractions of transforming DNA. Plots of M.W. versus transforming activity revealed a concave relationship, asymptotically approaching zero or insignificant values (when reversion levels were considered) as donor M.W. decreased. Clearly, below 1.2×10^6 daltons the biological activity was low but measurable. A much larger "excluded" length is indicated

(Guild et al., 1966) in this system. According to Dubnau (1972c), the efficiency of transfer from DSF to DRC is high even though one strand is actually degraded. Based on calculations with known donor fragment sizes, it was predicted that an insertion of 2.8×10^6 had occurred. This is demonstrated experimentally by alkaline sucrose and density displacement measurements. Since 64% of the exposed high M.W. d.s. DNA was converted to DRC it was estimated that a maximum of 28×10^6 daltons could actually be present. Experimentally, 2-3 inserted segments in a cluster, each 2.8×10^6 were found.

DNA activity sensitive to shearing forces is seen when B. subtilis is transformed to prototrophy by B. globigii for the aroB - aroE span. The resulting intergenote contains B. globigii material in a B. subtilis background. When this material is subsequently used to re-transform auxotrophic B. subtilis, it is extremely sensitive to size reduction, being selectively inactivated below $10-20 \times 10^6$ daltons. Homologous transformation does not show this extreme sensitivity (Harris-Warrick and Lederberg, 1978). It seems that flanking homologous sequences are necessary

to synapse the intergenotic material in a heterologous host and any size reduction jeopardizes this. Linkages in the intergenote length are unusually high (100%) if the sequences involved are non-conserved between donor and recipient. For conserved genetic loci participating in a heterologous exchange, this linkage increase and shear sensitivity need not appear, as synapsis within the recombining length is possible. A similar exaggeration of linkage and size reduction vulnerability is seen during transformation of deletions. The need for "splicing ends" of homologous material is evident.

The cumulative data indicate that the average transforming fragment is one tenth or less the size of the phage PBS1 transducing segment (Henner and Hoch, 1980). The transducing system of PBS1 can transmit as much as 10% of the Bacillus genome as a single linear entity, carrying approximately 2×10^8 daltons of DNA (Dubnau et al., 1969). Phage SP10 is smaller and most probably contains 5.9×10^7 daltons, somewhat more than a transforming fragment of average integrated size 2.8×10^6 . PBS1 mediated transfer demonstrates longer

linkages not seen with transformation (Dubnau et al., 1969). In B. licheniformis, arg and leu are cotransferred 1.5% by transformation, 7.3% by SP10 transduction and 42% by SP15, a phage slightly larger than PBS1 (Young and Wilson, 1975). H. influenzae loci str^r - nov^r are linked and survive 33% and 58% respectively after shearing stress while the linkage itself drops to 1.5% of its "native" value (Notani and Setlow, 1974). This effect was seen when the fragment reached 6×10^6 daltons. In the opposite manner, when the linkage between metB5 and KauA1 in B. subtilis was studied in a strain carrying prophage SP β (M.W. 6.2×10^7) the linkage was 18.1% while a cured strain had a PBS1 cotransduction of 77.7% (Henner and Hoch, 1980). To be cotransformed, two markers must be present on a single fragment of DNA. As the distance between them increases, the probability of a random scission between them rises. This is analogous to recombination theory where the probability of a crossover between two loci is proportional to their linear separation. If two loci are introduced to a recipient cell on different fragments, the probability of their being co-integrated is the product of their individual

recombination functions. Obviously, as DNA concentration rises so does this "congression" event. Other effects such as proximity to "excluded" recombinational regions will skew these otherwise random probability statements. There is no reason to expect a linear relationship between cotransformation and physical distance as this has not been found in transduction (Henner and Hoch, 1980); rather a curvilinear function is applicable to transformation if the average transforming fragment is 2×10^7 (ds) M.W. This holds even though the actual integrated single strand entity has a M.W. 2.8×10^6 (Dubnau and Cirigliano, 1972c).

8. Restriction Nucleases

Restriction enzymes take their name from the observation that phage grown on certain bacterial hosts are severely "restricted" from subsequently establishing their life cycle in other related hosts. It was shown that this limitation upon genetic exchange was due to specific "markings" or modifications placed upon the phage DNA at critical sites by the prior host. In another host, other susceptible sites would be "unmarked" and enzymatically cleaved, resulting in termination of phage function. Those phage that escaped

"restriction" could regrow on the new host with full efficiency because of new protective "marks" but not necessarily upon the old host (Smith, 1979; Arber, 1979). These phenomena were not heritable and indicated the presence of critical sites in the phage which could be cleaved by a host determined restriction nuclease or protected from cleavage by a host determined modification function. Thus, each subsequent host cell being exposed to an invading population of DNA molecules "born" in another host had an opportunity to restrict viral multiplication or to re-mark as its own and allow duplex replication in the new background.

It was found that the nucleases responsible for these limitations were of two types. Type I are large multimeric proteins having associated with them a methylating activity which could prevent the cleavage by the nuclease component. They require Mg^{++} , S-adenosylmethionine and ATP (Meselson and Yuan, 1968). Their cleavage however appeared to be some distance from the initial recognition site and they were thus difficult to characterize (Boyer, 1971). In this class are Eco B and Eco K. Type II enzymes on the other

hand are smaller, require only Mg^{++} for cutting action, are highly specific in recognition site cleavage (Smith, 1979) and do not have a methylase modification activity associated intimately with them. Examples of this group are Eco RI, Hind III and Bam HI. The prefix mnemonic indicates the genus, species and strain of the producer bacterium and the numerical designation is an indication of that enzyme's membership in a larger set. As predicted by Arber (Nobel Prize Lecture, 1978)(1979), many enzymes of this specificity were identified and their cleavage site sequences determined (see Table 2).

The nucleotide sequences recognized by these enzymes are small, often palindromic sites of 4-7 base pairs. Palindromic sites have the same nucleotide sequence on either strand when read in the 5' to 3' direction. That is, they possess two fold rotational symmetry (e.g. GATC) while others are degenerately symmetric (e.g. GTPyPuAC) and still others totally asymmetric (e.g. GATGC). Where two enzymes recognize the same site, they are termed isoschizomers (Roberts, 1980). Both Hae III and Bsu RI recognize the same sequence.

A recognition sequence for one enzyme may also appear within the prototype recognition site of another, Mbo is within the Bgl II recognition site and Hpa II is within Sma I. An unusual enzyme, designated as Type III (Kave and Piekarowicz, 1978) is Eco P1 which seems to be intermediate between types I and II. This enzyme cleaves 24-26 bases from the 3' end of its site (Smith, 1979--reprint of Nobel Prize Lecture, 1978). The enzyme Hga I cleaves in a unique manner and almost ensures the reuniting of original cohesive ends at reannealing. An interesting effect is seen when Eco RI activity is studied under reduced ionic conditions. Here, the canonical 6 base palindrome is recognized by altered affinity as an internal 4 base pair site (which may statistically exist elsewhere in isolation from the actual RI site). This reduced affinity "enzyme" activity is termed Eco RI* (Polisky et al., 1975), (Table 2). Similarly, Bsu I can recognize and cleave the subset 5'G C3' at low ionic conditions (Heininger et al., 1977). In the case of Dpn I, cleavage is initiated only if 6-methyladenine is in the sequence while the

isoschizomer Dpn II cuts only the unmethylated form. An additional isoschizomer Sau 3A cuts regardless of the sequence methylation state. This is most useful for determining adenine methylation at this particular site (Lui et al., 1979). Cleavage by site-specific enzymes may be blunt ended as in Hind II, staggered as in Pst I generating overhanging 3' cohesive termini, or 5' cohesive termini as in Hind III (Smith, 1979). In all cases, 3' hydroxyl and 5' phosphoryl termini are revealed. To prevent cleavage, a modification must occur at a particular nucleoside in the critical sequence. A methylation event generates 5-methylcytosine or 6-methyladenine. In Type I enzymes this is accomplished by an activity intrinsic to the cutting machinery. In Type II enzymes a separate molecule carries out this act.

The best studied example of Type II restriction and modification is that demonstrated by Eco RI nuclease (Yoshimori et al., 1972) and methylase respectively. Enzyme coding sequences are located on an R factor in host E. coli. The nuclease is a single polypeptide of M.W, 28, 500 $\pm$ 500 and active

in solution as dimers and tetramers (Modrich and Zabel, 1976). At 37°C, the enzyme makes two cuts within its recognition site (Hedgpeth et al., 1972) prior to dissociation. At 0°C, however, molecules with single strand interruptions may accumulate and are sealable by ligation. As the first break has a rate constant of 40 min⁻¹ and the second 14 min⁻¹, the rate limiting step seems to be release of the enzyme from its site substrate; at 30°C the turnover number is only 0.72 min⁻¹. The methylase enzyme (Rubin and Modrich, 1977) is a single polypeptide chain with a functional monomer M.W. 39,000. The enzyme requires Mg⁺⁺ as does the nuclease, but also the methyl donor (SAM) S-adenosyl-methionine and a reducing agent such as dithiothreitol, unlike the nuclease. The enzyme dissociates from its substrate, the N-6 of adenine, after each of two methylations within the palindrome. Unlike the situation in Type I modification (Vovis et al., 1979) by Eco B the second methylation is not more favored than the first although single methylated sites are immune to Eco RI cleavage. The methylase, as a monomer, possesses a specificity somewhat different than that of the nuclease which functions

as dimers or tetramers during its interaction with the rotationally symmetric binding site. The 2-amino group of deoxyguanine in the recognition site may be replaced by deoxyinosine which lacks this group and methylation is subsequently inhibited while restriction cleavage is not (Modrich and Rubin, 1977).

The measured K_m of Eco RI is $8 \times 10^{-9}M$ or $3 \times 10^{-8}M$ depending on whether the single Eco RI sites of ColE1 or SV40 DNA are used respectively as substrates. The K_m of the methylase for the Eco RI site of ColE1 is $1.3 \times 10^{-9}M$ while its K_m for SAM is $2.6 \times 10^{-7}M$. There is interesting evidence that the affinity of these enzymes for multiple sites on a single molecule is not constant. Thomas and Davis (1975) showed that phage lambda's 5 Eco RI sites were cut differentially by the nuclease. The site nearer the right hand end, fragment (F) M.W. 2.13×10^6 , is cut 10X more frequently than the others (Table 8, Figure 5). Using deletions and heteroduplex mapping, the site at 0.543 fractional length, separating fragments 3.02×10^6 and 3.48×10^6 , is cut least frequently. Similarly, hierarchical orders are seen with the "aberrant" Eco RI* activity

(Polisky et al., 1975) and there is evidence that RI* cleavages can occur in molecules methylated at the canonical RI sites though not necessarily within that site proper. The methylase also shows some site preference (Rubin and Modich, 1977) but this has not been fully investigated (Appendix figure). It is of interest however that methylation of the N6 group of adenine has profound effects not only upon enzyme susceptibility, but also upon regional DNA conformation and hydrogen bonding (Sternglanz and Bugg, 1973). Crystallography indicates that the most stable methylated structure would interfere with hydrogen bonding to thymine, a possible secondary structure perturbation signal to a restriction nuclease or a local melting. However, the problems imposed upon high fidelity replication and repair events are not dealt with and it is possible that in vivo structures are in other steric conformations. On the other hand if mismatch repair were less stringent or if restriction/modification sites were in non-essential regions, limited and local secondary structure changes might be tolerated.

Both restriction and modification, studied in vitro, show great specificity and discrimination.

The means by which molecules recognize sites of varied symmetry reveals the possibility that in vivo, recognition may be modulated or proceed through a hierarchy of recognition events. The presence of unusual periplasmic enzymes in transformable B. subtilis (Scher and Dubnau, 1976), the Eco RI induced in vivo recombination of plasmid DNA (Chang and Cohen, 1977), the appearance of specific methylases (Ganesan, 1979) during competency and the apparent induction of repair systems and restriction activities following ultraviolet irradiation or competency (Yasbin, 1977) indicate that restriction and modification may be more than a "line of defence" against the intrusion of foreign DNA. A system may exist wherein recombination is initiated, promoted or controlled by site specific enzymes such as those above.

9. Restriction during Transformation

It would seem logical to expect the control of genetic exchange between organisms to be both specific and flexible. Genotypic variation on an evolutionary time scale must be fluid and, at the same time, the static integrity of the genome

must be protected. B. subtilis is an organism which seems to readily undergo genetic exchange in nature (Graham and Stoch, 1979), excreting DNA during various phases of its life (competency, germination, autolysis) and recombining large blocks of genes in soil culture. While the ability to adsorb phage may be a cell surface receptor function, so in some transformation systems (Sisco and Smith, 1979) is the sequence specific ability to recognize and bind homologous DNA. B. subtilis is able to bind and transport heterologous DNA into the cell (Piechowska et al., 1975), but it is subsequently degraded and reutilized via salvage. While phage infection is clearly susceptible (Trautner et al., 1974) to host restriction-modification phenotypes, there is no firm evidence that transformation is similarly limited (Meselson and Yan, 1968; Boyer, 1971; Table 13). The ultimate recombining strand of DNA (SSF) is, as opposed to the phage genome, single stranded (Dubnau and Cirigliano, 1974b). The systems are intrinsically different in terms of the susceptible target and are under different biochemical and biological constraints. The restriction enzymes Eco RI, Bsu I and Hind III do not recognize and

cleave single strand substrates (Green et al., 1975; Bron et al., 1975). Although there have been reports (Horiuchi and Zinder, 1975) of the cleavage of single strand substrates by Hae II and Hpa II, it now appears as though these cleavages were spurious and due to transient intra-strand duplex formation (Blakesley et al., 1977).

Although somewhat specific double strand cleavages take place during the formation of the initial DSF (Scher and Dubnau, 1976), there is no firm restriction enzyme function during transformation. For instance, the Bsu I enzyme can cleave the linkage between trpC2-tyrA (Trautner et al., 1974) in vitro, but not in vivo when unmodified transforming DNA is offered to an auxotrophic recipient which is proficient for the restriction phenotype with phage substrates. If a B. subtilis strain, not of a restriction-modification phenotype ($r^{-}m^{-}$) is transformed to proficiency by a ($r^{+}m^{+}$) DNA, this new transformant will show an in vivo discrimination towards phage grown on a heterologous host but not towards transforming DNA. Transforming DNA from the original ($r^{-}m^{-}$) cell is equally efficient (Trautner et al., 1974)

on the donor (r^+m^+), the recipient (r^-m^-) and the transformant (r^+m^+). A similar situation is seen in Hemophilus where strains may produce enzymes that totally inactivate certain of each others genetic loci in vitro, but yet these strains readily exchange information reciprocally in vivo (Gromkova and Goodgal, 1974).

While the substrate profile presented to a possible restriction system is intrinsically different in transformation or transduction and phage infection, the effects of restriction-modification systems upon genetic exchange are not totally negated. When the leucine genes of one (r^-m^-) B. subtilis species were cloned onto a plasmid (pLS102) (Tanaka, 1979) and "transformed" via this plasmid vehicle into a leu⁻ (r^-m^-) cell, efficient prototrophic values were seen regardless of whether or not the cell was recombination proficient (recE⁺). If however the recipient was (r^+m^+) and recE⁻ the prototrophic values dropped precipitously. A recE⁺ (r^+m^+) recipient gave normal plasmid and chromosomal transformation levels. In a rec⁻ background, single strand transformation cannot occur, and only an independent plasmid replicon will propagate an inserted leu⁺ double strand element. In a rec⁺ system,

classical transformation to prototrophy by chromosomal integration of a single strand fragment is permitted. In the former case, restriction is demonstrated upon the leu genes but not in the latter. In this case, the plasmid vector itself was not a substrate for restriction.

Trautner et al. (1974) showed that some phage (ϕ 29, SP8, PBS1) were not affected by the (r^+) phenotype of the host while others (SPP1, ϕ 105) were. Eight other phage were not restricted by two different 168 strains. In this case, PBS1 was able to infect and transfect but its ability to promote double strand transduction was severely limited. Such was not seen when chromosomal DNA was passaged via transformation.

According to Ganesan (1979), competent cells may exhibit a cryptic methylase which acts on specific deoxycytidine residues (apparently at the Bsu I site) in both the resident chromosome and the incoming transforming strand. Such a system may also be induced by ultraviolet radiation (Yasbin, 1977). The induction of prophage may also occur in competent cells as well as irradiated ones. It is not known whether this corresponds to an inducible restriction-modification

phenotype or is reflective of modifications necessary to control "gapping" (Harris and Barr, 1971) and repair functions. In Hemophilus, com⁻ cells are negative for competence development and the (r^+m^+) phenotype (Gromkova and Goodgal, 1974). Infection with unmodified Ø105 phage into U.V. irradiated B. subtilis produces phage progeny with enhanced efficiency (modified?) on r^+m^+ strains (Yasbin, 1977). Competent infected cells show the same effect while vegetative phage infected cells do not. Chromosomal DNA however, modified through this means, had the same transforming efficiency as unmodified DNA on (r^+m^+) cells (Trautner, 1974; Yasbin, 1977).

Dubnau et al. (1969) originally proposed that there existed separate pathways of recombinant formation when transformation or transduction were the mediating paths. B. subtilis 168 and W23 (also 3610--this study) strains freely exchange genetic information via transformation. However, the transduction efficiency of these strains in heterologous (reciprocal) exchange was varied and reduced compared to homologous transduction. These effects were marker specific. In addition, the recA1 mutation greatly diminishes transformation and

heterologous transduction in 168 strains while slightly reducing homologous transduction. Path I and II were applied to the former and latter exchange systems respectively and they were thought to be limited and defined by the demands of short and long regions of sequence homology in the two paths. Hadden (1977) suggested that in a rec⁺ cell, DNA is transformed as a single strand, not susceptible to restriction because of configuration, biochemical latency or pairing with resident modified sequences. Heterologous transducing DNA could be inactivated because of the higher probability of this large fragment carrying restriction sensitive target sites. Intermediate levels of discrimination seen with phage of different carrying capacity (SP10 as opposed to PBS1) by Dubnau et al., (1969) would be correlated with this site frequency expectation. It is noted that, as a vector, PBS1 was one of the phage genomes demonstrated not to itself be susceptible to B. subtilis (Bsu I) inactivation (Trautner et al., 1974). The recA function may involve modification exerted upon incoming DNA (see Ganesan, 1979) or susceptibility of repaired/recombined regions to "restriction".

It may involve a tailoring step not required of ds homologous transduction exchange. The reverse may be true for transformation and heterologous transduction. Other rec mutations (Dubnau and Cirigliano, 1974b) more clearly separate the transformation and transduction modes.

A reasonable body of experimental evidence however supports the notion that, as far as transformation is concerned, sequence inhomology is the major barrier to effective recombination. Harris-Warrick and Lederberg (1978) demonstrated that high efficiency transformation into an intergenetic sequence could be obtained with heterologous DNA at levels several orders of magnitude higher than those achievable by heterologous transformation alone. Re-transformation into intergenetic B. globigii sequences residing in B. subtilis was 10^5 higher with B. globigii DNA than the primary recombining act or transformation outside the intergenetic region. The existence of loci of high and low sequence conservation has been demonstrated in related genomes (Chilton and McCarthy, 1969; Wilson and Young, 1972). While non-conserved sequences are transferred with low frequency, they involve obligatory en bloc high linkage status.

Conserved loci on the other hand do not show this unusually high linkage maintenance. This is explained as a need for stable linear lengths of homologous base pairing sequences for DRC formation as suggested by Dubnau et al. (1969) for transformation and transduction. Heterologous marker crosses, into intergenetic recipients, are usually shear sensitive as though critical flanking lengths must be intact. Since in vitro the two organisms have different susceptibilities to Bgl I and Bgl II enzyme, their restriction site patterns are distinct. Transformation by B. subtilis DNA into a heterologous intergenote region occurs at only 10% the level of B. globigii or intergenetic hybrid DNA donor. It appears that classical restriction is not a limitation brought to bear upon transforming DNA. Primarily, the configuration of the entering strand would prevent such interaction. Following synapsis, a heteroduplex would be formed in which any specific site would be hemi-methylated. This would not be recognized by the E. coli Type I or Type II systems but Trautner et al. (1974) found some evidence of in vivo cutting, although to a much lesser extent than with unmodified strands in Bacillus. This

phenomenon shows some strand bias; H strand modified heteroduplex phage being less sensitive to inactivation than the converse construction. However, these reconstructions were performed on manipulated strands which might already have significant secondary structure perturbations and signal repair bias.

Ganesan (1979) showed that incoming DNA was methylated during transformation in a specific manner. Single strand methylation appears to be possible for several other systems (Smith, 1979). Whether this is a rate favored step preventing subsequent restriction or an event involved with the considerable gap formation seen even in the recipient genome itself during competency is not known. Indeed, methylases are constitutively produced or induced during competency and repair-promoting events. The cumulative evidence would indicate that restriction-modification is clearly involved in double strand genetic interactions such as phage and plasmid infection and transduction. Transformation on the other hand is primarily limited by sequence homology and synaptic demands. The nature of the target molecule may have evolutionarily exerted pressure for the

development of an inactivating system against self-propagating replicons (double stranded in nature) where any scission leads to inviability, as opposed to single strand exchanges, where an elaborate recognition array is not necessary. Synapsing sequences may be accepted or rejected by an evolving genome based on homology and selection pressure. Restriction escape mechanisms might involve single strand protein binding (Eisenstadt, 1975) or in the case of the complex genome of phage T7, the synthesis of a restriction antagonizing protein (SAMase) (Studier, 1975). The former possibility is not clearly shown by experiment and may be a transport or melting protein while the latter would demand that every fragment of transforming DNA contain this gene. It is far more likely that restriction-modification acts primarily during infection and heterologous transduction and that any effects seen during transformation are secondary, pleiotropically involved with repair and recombination.

10. Specific Aims

This project was undertaken in order to examine the biological and physical response of B. subtilis transforming DNA to site specific

restriction cleavage. Analysis of native and restricted donor activity in a transformation system can shed light upon relative gene susceptibility, restriction palindrome location and clustering and the kinetics of donor activity loss. The B. subtilis transformation system is an excellent environment for analyses of size versus activity relationships following site specific cleavage. In addition, genes may be catalogued with respect to their "survivals" in a transformation assay following exposure to various restriction nucleases. This has important implications for future cloning attempts. The experiments presented herein examine the above phenomena and take advantage of genetic as well as recombinant biological techniques to probe the B. subtilis genome for restriction site organization as well as identifying specific loci which might be amenable to cloning and subsequent fine structure examination. Towards this end, we have restriction enzyme mapped several interesting loci with characteristic nuclease survival values. The relationship between several B. subtilis "strains" (e.g. W23, 168, 3610) is also of interest regarding their evolutionary history and subsequent divergence. The cloning of defined segments of

related genomes would provide a probe of otherwise indistinguishable sequence conservation and variation between related members of a genus. The correlation of the traditional functional genetic map with that of significant sequence "signposts" would represent a step towards a more complete understanding of genome organization, control and fluidity.

MATERIALS AND METHODS

Bacterial Strains

Mutant derivatives of B. subtilis 168 were laboratory strains obtained originally from D. Dubnau. Prototrophic strains W23 and 3610 were obtained from D. Dubnau and A. Sonenshein respectively. Strain Mu8u5u16, a 168 derivative was from N. Sueoka. B. pumilus RUB502 and B. licheniformis FDO-12 were from A. Garro. Strain RM125 was provided by M. Friedman. Various tryptophan requiring strains were obtained from S. Hoch. B. subtilis BD224, a rec E4 strain, was from D. Dubnau. Table 1 presents pertinent genotypic information on these and other organisms.

Nucleic Acids

Bacteriophage Ø105 DNA was kindly provided by A. Garro. Phage lambda cI857 S7 DNA was purchased from Miles Laboratories or New England Biolabs. Calf thymus DNA was from Worthington. Plasmid pUB110 (Km^r) was prepared from strain BD366 provided by D. Dubnau. Plasmid pBR322 was provided by D. Galloway and phage T7 was from the laboratory of J. Krakow.

Growth of Bacteria

Bacteria were routinely grown in Penassay broth (Difco). Large scale growth was in Veal Infusion broth (Difco) supplemented with 0.5% yeast extract (Difco). Minimal broth consisted of 1X Spizizen salt buffer (1958) supplemented with 0.5% glucose, 1% sodium glutamate and 100 µg/ml each of the required amino acids. Plating was performed on the same media to which 1.5% agar was added. Tryptose blood agar base containing an additional 0.5% agar and supplemented with 5.0 mM MgCl_2 , 0.02 mM MnCl_2 to enhance sporulation was used for storage streaks. Difco nutrient agar supplemented with 0.5% agar was used for routine streaking.

Buffers

1X Spizizen salts are 0.044 M KH_2PO_4 , 0.08 M K_2HPO_4 , 0.0034 M Na citrate, 0.001 M MgSO_4 . Electrophoresis buffer is 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.2. 1X SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0. Saline-EDTA is 0.15 M NaCl, 0.1 M EDTA, pH 8.0. Dilution saline is 0.15 M NaCl, 0.02 M K_2HPO_4 , pH 7.0. Buffer A for plasmids is 25% sucrose, 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5. Phenol buffer is 0.1 M Tris-HCl, pH 8.0. Dialysis

buffer for plasmids is 1.0 mM Tris-HCl, pH 7.5, 4 mM NaCl, 0.1 mM EDTA. Sephadex column buffer is 10.0 mM Tris-HCl, pH 8.0, 1.0 mM EDTA.

Preparation of Transforming DNA

a) Large scale growth of cells was in supplemented veal infusion broth in 250 ml volumes to a total of 4-5 liters, shaken 16 hours at 37°C in a large capacity gyratory shaker (New Brunswick Scientific Co.). An 8 hour Penassay broth culture was employed as the starter inoculum by adding 0.2 ml of a 10^{-2} dilution to the master flasks. DNA was isolated by the procedure of Marmur (1958) as specifically modified by Rudner *et al.* (1967). Phenol was prepared as a pH 7.0 buffer saturated solution with 0.1 M Tris-HCl, pH 8.0. Small amounts of phenol were prepared as needed and stored in the dark. High molecular weight DNA prepared by this method typically had 280/260 ratios approaching 0.5. Extensive dialysis was with 1X SSC and stock was stored over a drop of chloroform.

b) Small scale preparations of transforming DNA (200 ml) were prepared in the same manner except that several chloroform-isoamyl alcohol (24:1) extractions were substituted for the phenol step. All centrifugations were in a Sorvall RC-2B refrigerated

centrifuge in either an SS-34 or large capacity GSA rotor at 4°C.

Preparation of Plasmid DNA

a) Large scale preparation of plasmid DNA was as in Guerry et al. (1973) with modifications of Gryczan et al. (1978) for B. subtilis. A veal infusion broth (250 ml) supplemented with 0.5% yeast extract and 5.0 µg/ml Kanamycin was inoculated with 0.1 ml of a 10^{-2} dilution of an 8 hour Penassay/Kanamycin starter and shaken vigorously for 16 hours at 37°C. The solution was rapidly chilled in an ice-water slurry, centrifuged at 4°C and the pellet washed once with 0.1 volume of Buffer A. After pelleting, resuspension was in 0.1 volume of Buffer A containing 0.5 mg/ml lysozyme. This slurry was incubated without agitation for 15 min at 37°C. The lysate was moved to 25°C and 6 ml of 5 M NaCl, 1.5 ml of 0.5 M EDTA, pH 8.5, and 32.5 ml of 2% SDS-0.7 M NaCl was added/25 ml lysate without mixing. Two wrist inversions of the vessel were carried out and the viscous contents stored 18 hours on ice. The lysate was next centrifuged at 18K rpm at 4°C in a Spinco Ti30 rotor for 30 minutes. During this process, the solution must not be allowed to warm or "decrystallization" of high M.W. material will occur.

The supernatant or "cleared lysate" containing circular plasmid molecules and low molecular weight polynucleotide is made 0.3 M with sodium acetate on ice and added to 2 volumes of cold 95% ethanol, then stored at 0°C overnight. This is centrifuged at 5,000 rpm in a Sorvall GSA head and the pellet gently dissolved in 6.25 ml of TES buffer, treated 30 min at 37°C with 50 µg/ml pancreatic RNAase, 1 unit/ml ribonuclease T1. Pronase (autodigested 1 hour at 37°C) was added and the solution incubated at 37°C until clearing. If necessary, particulate matter was removed by low speed centrifugation. Aliquots of the treated lysate (usually 1-2 ml) were brought up to 6.0 ml with TES and added gently to 7.35g CsCl crystals. This was dissolved, and in subdued light 2 ml of ethidium bromide (1 mg/ml in water) was added. Centrifugation was in polyallomer tubes topped with heavy mineral oil and sealed with rim-gripping mechanical caps. A type 50 Ti rotor at 38,000 rpm, 15°C for 48 hours was employed.

b) Where minimally grown cells were required the medium was 250 ml of minimal growth broth supplemented with 100 µg/ml of the required amino acids (or lacking a specific one if selection is required) and 5 µg/ml of antibiotic. The starter culture is

as previously described but from a minimal environment. Here, the pellet is dissolved in as little as 10 ml of TES (rather than 25 ml as in enriched media) and the lysis ingredients scaled downward accordingly. Lysates prepared in this manner are most fragile and low temperature processing and speed are essential.

c) To obtain a total cellular lysate that is not "cleared", but rather reflects the total cellular DNA contents at gradient centrifugation, the initial pellet from a 25 ml minimal culture is not washed, suspended in 1-2 ml of Buffer A and 0.25 mg/ml lysozyme added. At 37°C lysis is rapid. An equal volume of 1.5% Sarkosyl, 200 µg/ml pronase is added and the mixture incubated 1-2 hours at 37°C. This solution is quite viscous depending on the initial cell mass and may be diluted with TES following lysis if necessary. When copy number was to be determined, the solution was vortexed at full speed for 15 seconds, 3 times. In this way, linear structures are preferentially sheared and mixing with the CsCl gradient is uniform. RNA in these preparations moves to the bottom of the gradient in a diffuse zone and does not interfere with subsequent processing.

Enzymes

DNAase I was Worthington Type DP dissolved in

0.2 M MgSO_4 to 100 $\mu\text{g/ml}$. RNAase A and RNAase T_1 were from Worthington. Pronase was from Calbiochem. Eco RI, Hind III, Hpa I, Hpa II, Bam HI were purchased from Miles. Hinc II, Sac I, Bcl I, Bgl II, T4 DNA ligase, Eco RI methylase were from New England Biolabs. RNAase A and pronase (autodigested) were heated to 80°C for 10 minutes to inactivate contaminating DNAase. Lysozyme was from Worthington.

Isotopes

Thymidine-5'-triphosphate (αP^{32}) (440-580 Ci/mmol), 5-adenosyl-L-methionine (methyl- H^3) (71.5 Ci/mmol), thymidine (methyl- H^3) (6.7 Ci/mmol) and thymidine-5'-triphosphate (methyl- H^3) (78.1 Ci/mM) were from New England Nuclear. Tracer was dried under high vacuum while held in dry ice to remove ethanol (or H_2SO_4 in the case of SAM) and reconstituted with cold material and the specific activity calculated.

Preparation of Competent Cells

A modification of the method of Anagnostopoulos and Spizizen (1961) was employed. A 5.0 ml Penassay culture of a single colony was incubated 37°C and allowed to grow for 8 hours. This culture was diluted 10^{-2} and 0.1 ml inoculated into 20.0 ml of Penassay broth. Following incubation overnight at 37°C in an NBS table-top gyratory shaker, 10.0 ml of the culture

was centrifuged at high speed and the supernatant discarded. The pellet was dissolved in 2.5 ml of Spizizen synthetic 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 µg/ml of each required amino acid. A 20 ml volume of medium I in a 250 ml side-arm flask was brought to a net Klett turbidity of 25 with the concentrated cell suspension and shaken vigorously by gyration at 37°. Growth was followed with a Klett-Summerson colorimeter with a 660 nm filter (red). Stationary phase growth was approached after 4-4.5 hours. At this point, the growing cells were diluted 1:10 into Medium II which consists of 1X Spizizen salt, 0.5% glucose, 0.01% vitamin-free casamino acids, 0.05% yeast extract, 2.5×10^{-3} M MgCl_2 , 1×10^{-3} M CaCl_2 , 5×10^{-4} M spermine tetrahydrochloride and 50 µg/ml each required amino acid. This culture was incubated with shaking for 90 min at which time maximum competence is obtained. On some occasions, the protocol is scaled upwards by a factor of 10 at the Medium II step, 200 ml being shaken 90 min followed by rapid centrifugation, 10-fold concentration and adjustment to 5% glycerol.

Rapid freezing in dry ice-ethanol followed by storage at -70°C enables these cells to be thawed and reconstituted with Medium II (warmed to 37°C) at a later time with no more than a 50% loss of viability. If reconstituted at double strength, the competency of this population is the same as freshly prepared cells.

Transformation Assay

To 0.9 ml of competent cells in a 12 X 100 mm Wasserman tube was added 0.1 ml of sterile DNA solution. The tube was incubated on a roller for 30 min at 37°C followed by termination of the uptake by the addition of 0.1 ml of DNAase I to a final concentration of 10 $\mu\text{g/ml}$. Sample tubes were appropriately diluted into saline, vigorously vortexed and either 0.1 or 0.2 ml aliquots plated on selective agar by spreading with an alcohol flamed rod. Incubation was for 48 hours. Appropriate sterility controls were included as were controls to monitor the genotype of the recipient cells.

Selection of Antibiotic Resistant Cells

Transformation was as above except that the transformed cell population was diluted and aliquots added to 5 ml of 1% nutrient agar held at 45°C . This was poured onto the surface of a bottom-layer nutrient agar dish and allowed to solidify. An

incubation period of 90 minutes at 37°C followed to allow for gene expression due to phenotypic lag and finally, 5 ml of top layer 1% nutrient agar containing 5 µg/ml Kanamycin sulfate or 25 µg/ml streptomycin sulfate was added over the cell layer. After hardening, plates were inverted and held at 37°C for 48 hours prior to scoring. Sterility of reagents and antibiotic sensitivity background of the recipient population were also assayed.

Scintillation Counting

Determination of isotope cpm was performed on a Beckman LS-800 counter operating with an automatic quench compensating program. All net cpm so obtained are corrected for sample quenching by external excitation from a radioactive cesium control source. Samples to be counted were either introduced into 5% ice cold trichloroacetic acid (TCA) or added in equal volume to cold 10% TCA. These were applied by suction washing to glass fiber discs (2.4 cm) (GF/C) by means of a Millipore chimney device and a table top vacuum source. Washing was accomplished by 3 successive 10 ml additions of ice cold 5% TCA followed by 2 X 2 ml washes of 95% ethanol. Filters were dried with a hair dryer and placed in borosilicate scintillation vials for counting. Samples applied

to paper discs (Whatman #1) were pencil labeled and washed en masse with 2 times 500 ml of 5% TCA, 2 X 500 ml 95% ethanol, dried and counted. Samples directly applied to GF/C discs were dried on pinboards and then individually processed as above. Liquid samples were counted in Aquasol (Beckman) while filter bound samples were counted in either Bray's or BBOT/toluene. Bray's (1960) is 60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 g absolute methanol, 20 ml ethylene glycol and brought to a liter with p-dioxone. BBOT/toluene was prepared at 4 mg/ml. An external tritium standard was provided by Dr. C. Ceccarini (S.A. = 1.7138×10^4 dpm/10 μ l) and a machine efficiency of 48.75% was calculated. Surface radiation (P^{32}) during flow from columns was monitored by means of a stand-mounted radiation survey meter with variable sensitivity controls.

Reagents

All growth media were from Difco. Amino acids (L) were from Calbiochem. Agarose (low electroendosmosis type) was from Miles. Low melting point agarose (low E.E.O.) was from Bethesda Research Lab. CsCl was from Pierce. Phenol was Mallinkrodt (guild label) and purified crystals were AnalaR grade from

BDH Chemicals, Poole, England. BBOT was from Packard. Kanamycin sulfate and streptomycin sulfate were from Sigma. Sephadex beads (G50) were from Pharmacia Fine Chemicals. Ethidium bromide was from Sigma. Unlabeled ribo- and deoxyribonucleoside triphosphates were from PL Biochemicals. Bovine serum albumin was fraction V obtained from Sigma. Unlabeled S-adenosyl-L-methionine was from Calbiochem as was 2'-deoxyadenosine. Density gradient grade sucrose was from Schwarz-Mann.

Spectrophotometry

A Gilford model 250 spectrophotometer equipped with a multiple sample cuvette carrier and a Gilson transferator device was used for optical measurements. An extinction of 1 O.D. at 260 nm was assumed for a native DNA solution of 50 µg/ml. DNA free of protein contamination gives an O.D. 280/260 ratio of 0.5. Solutions were prepared spectrally using a millimolar extinction of 13.8 at 256 nm (in 0.01 N H₂SO₄) for SAM, 9.1 at 271 nm (pH 7.0) for dCTP, 13.7 at 253 nm for dGTP, 9.6 at 267 nm for dTTP, 15.2 at 259 nm for dATP.

CsCl-EtBr Centrifugation

The procedure of Radloff et al. (1967) was used. Beckman 3.0 X 5/8 in polyallomer tubes were pre-treated

by boiling 10 minutes in 1 mM EDTA. All operations with ethidium bromide were carried out with subdued lighting. Visualization of intercalated chromophore was via exposure to oblique short wave length (UVL-21 Blak-Ray) ultraviolet illumination. The lower band (CCC) DNA was removed by puncturing the tube carefully with a 2.0 ml syringe and 18 gauge needle after removing the cap vent closure. With careful handling, an entire lower band may be removed without causing Schlieren motion and mixing with the upper band material. The ethidium bromide in the sample (total volume of 1.0-1.5 ml) is removed by extracting three times with isopropyl alcohol saturated with water/ CsCl ($\rho = 1.56 \text{ g/cm}^3$). The fourth and last extraction may be with a small amount of 100% isopropanol to reduce the aqueous volume if desired. Ethidium bromide rapidly partitions to the upper organic phase while the DNA is in the lower level. Failure to use the proper extraction procedure results in unacceptable volume loss and crystallization of the remaining CsCl . Ultracentrifugation is at 15°C to diminish convective disturbances following the spin. Where copy number analyses or radioactivity distributions were required, labeled substrate was processed as above and the entire contents of the tube pumped out

by means of a variable speed LKB peristaltic pump. Pumping was from the bottom to the top with a 50 μ l capillary tube as the collector, placed in the tube gently through the vent port and held in place by modeling clay. Five drop fractions were collected into 10 X 75 mm tubes and aliquots subsequently processed for scintillation counting. Copy number was calculated as fraction CCC times the ratio of chromosomal/plasmid (CCC) M.W. Area integration was by means of a Ti-58 programmable calculator.

Tritium-labeled DNA

Growth was followed by Klett readings and by precipitating 0.5 ml aliquots with 3.0 ml 5% TCA on ice. Prior to labeling, cells must be conditioned to minimal media by an overnight growth of a suitable starter inoculum. For prototrophic cell labeling (W23, 3610), minimal media supplemented with only 0.005% vitamin-free casamino acids and 50 μ g/ml tryptophan was employed. A minimal starter culture was used to inoculate a master culture containing 200 μ g/ml 2'-deoxyadenosine (filter sterilized) (Budman and Pardee, 1967). For efficient uptake of tritiated thymidine (2-5 μ Ci/ml) this nucleoside was essential. This substance caused an increased lag period and label was added at the first rise of turbidity. In

minimal media DNA preparations, great care and volume conservatism is required to ensure good phase partitioning and DNA spooling, as the cells are very fragile after this procedure. Cell isolation is carried out at the early stationary phase, as further turbidity increases do not elevate final cell number and spontaneous lysis may rapidly follow in handling. In these procedures the Buffer A used in plasmid preparations is brought to 0.1 M EDTA and substituted for the traditional NaCl-EDTA solution usually used in the Marmur procedure (1958).

Restriction Enzyme Digestion

DNA samples were digested with restriction nucleases in 1X digestion buffer in 6 X 50 mm tubes sealed with Parafilm paper and incubated in hemagglutination trays in a 37° water bath. DNA concentrations were such that 1 µg to 10 µg could be added to the reaction in small volumes in order that the final citrate concentration of the buffer was never more than 0.0015 M. On some occasions, DNA samples were dialyzed versus 1X dialysis buffer or 1X restriction buffer or 1X restriction buffer prior to reaction. In these cases, the restriction pattern was not altered relative to the dilution method. Reaction volumes were 5 µl and, at various times, the

reaction was halted by heating to 65°C for 10 minutes followed by the addition of 0.1 volume of 70% sucrose -0.001% bromophenol blue in water. For larger digestion volumes, reactions were run in conical 10 ml tubes. When many digestions were to be performed on a sample, solutions of 2.0 O.D. 260 were prepared in 1X dialysis buffer (or water if ion replacement followed soon after) and aliquots digested. A 50 µl sample of this solution was then suitable for gel loading. Time of reaction was varied where necessary but routine digestion was generally carried out over a 16 hr incubation period. Digestion enzyme levels were at a ratio of 3.0 units per microgram of DNA reacting. These volumes varied over a small range with the specific activity of the manufacturers product. Dialysis tubing was boiled for 30 minutes in 1.0 mM EDTA and 10 minutes in water and stored in the cold. All glassware was disposable or scrupulously cleaned with sulfuric acid-dichromate and extensive washing and baking. Some glassware was silicone treated with Siliclad to prevent loss of small volume samples. Where samples were to be treated with chloroform for sterilization this was omitted, generally on the large volume samples, as the silicone was leached from the tube. These

analyses required facility with extremely small sample volumes to ensure accuracy and reproducibility. A Gilson "Pipettman" unit was employed, while volumes smaller than 10 μ l or unusual volumes (e.g. 13 μ l) were delivered by means of an assortment of Drummond (Broomall, Pa.) "Microcap" pipettes. Corning pipettes were used for more routine work and for loading samples into electrophoresis wells. Buffer ingredients were prepared as a 10X stock according to manufacturers recommendations and will be quoted where required (e.g. Table 3, 4).

Gel Electrophoresis

A horizontal gel apparatus (Aqueboque, L.I.) (22 cm X 13 cm bed) with a U.V. transparent tray was routinely used. Two gel series could be run on the same apparatus and various combs were available to accomodate different sample numbers and volumes. Samples no larger than 50 μ l volume were loaded by means of sucrose (5%) and marker dye (0.001% bromophenol blue) into the submarine wells, 3 mm deep. The routine running buffer was of Greene et al. (1974) and is 89 mM Tris, 2 mM EDTA, 89 mM boric acid, final pH 8.2. The power source was either a Savant model HV-1000B or a Heathkit model HP-17 transformer. Gels were run from cathode to anode

at generally no more than 100 volts and at a current of no more than 35 milliamps on this apparatus to prevent coulombic heating. Very low milliamperage runs were conducted overnight and resulted in very good size separation. Gels were all pre-electrophoresed for 30 minutes at 10 mA. Following a run, the tracking dye had migrated about 10 cm into the matrix and the gel was gently removed, placed in 0.2 μ g/ml of ethidium bromide in water for 15 minutes, destained in water for 30 minutes and examined over a Model C-63 (Ultraviolet Products) U.V. transilluminator. The pattern was photographed with a Polaroid MP-4 system onto Polaroid type 57 film through Wratten 25A and 8 (Kodak) gelatin filters. When negatives were desired, type 55 P/N or Tri-X with an orange filter was used. Agarose power (low EEO) or low melting point agarose (low EEO) were made to the desired concentration in 1X running buffer and boiled until dissolved in a water bath. Gentle vacuum was used to degas the solution and it was allowed to cool to about 45°C, carefully poured and allowed to harden at room temperature. This was then covered with running buffer and allowed to "age" for several hours. In the case of low melting point material, the matrix is very fragile. After boiling, it was cooled to

37°C, poured, hardened and covered with buffer and held for a day in the cold to ensure proper gelation.

Determination of Molecular Weight

Molecular weights were calculated whenever possible by interpolation with known size restriction fragments in the same digestion ionicity. Slight differences are noticeable when samples are loaded in varying digestion buffers. Plots of mobility versus the logarithm of the molecular weight were employed although curvilinear relationships were noticeable at large and small fragment sizes (Fig. 5). To achieve greater accuracy and greater linearity, the graphic method of Bearden (1979) was found most convenient and satisfactory. This method employs a linear graphic display of the mobility versus the molecular weight raised to the -0.666 exponent. Although calculations are somewhat more cumbersome to convert in the opposite direction, the results are consistent and useful over a greater size range than the traditional logarithmic procedure to the base 10. This is particularly true of large fragments. Size standards are included at both edges or at the middle and an edge of the gel where possible to control for mobility differences along the width. Upper and lower gels, run simultaneously, contain

independent size standards. These are restricted plasmid, phage or viral fragments containing 1-2 μg per well. Measurements are taken as distance from the origin using photographs, or when the gel was to be subsequently sliced, eluted or blotted, directly from the fluorescing gel itself. Low melting point agarose is not reliable for the calculation of molecular weights and care must be exercised with this material.

Gel Extraction

In the case of regular agarose, DNA extraction is very difficult if reproducible yields are required. A method of crushing the agarose in a conical tube containing 1 ml of 1X SSC was employed. This tube was held at 45°C in a water bath for 24 hours followed by high speed centrifugation (15K rpm) at 4°C for one hour in a Sorvall SS-34 head. The supernatant contained biologically active material but the yield, based on radioactivity, was approximately 50%. Some samples were re-extracted with additional SSC and precipitated in the presence of 10 μg yeast tRNA as carrier (Sigma) and redissolved in a small volume of liquid. Methods such as electroelution proved better (80-100%) in terms of cpm recovery but were very cumbersome and volumes were difficult to control.

To determine radioactivity distributions, slices of gel prepared on a Mickle gel slicer were heated in sealed scintillation vials containing 0.2 to 0.5 ml of concentrated NH_4OH in an oven until the gel dissolved, cooled and counted in 5.0 ml of Bray's (1960) scintillant. Restriction fragments are more easily removed from gels by crushing and extraction due to their small size, but their low intrinsic biological activity makes comparative analyses difficult. In the case of low melting point agarose, physical recovery of cpm seems very good, at least 50-80% although there is some variability of this material in terms of biological activity and susceptibility to further enzymatic action. Extracted material is best "sized" on a regular matrix agarose slab for reliable M.W. determination. Extraction of low melting agarose is according to the manufacturers instructions (BRL). This procedure involves heating in a glass container until melted at 65°C , the addition of 0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA and transfer to 37°C . The volume of this buffer is somewhat empirically determined and is approximately 1.0 ml per 2.0 X 3.0 X 3.0 mm slice. Extraction is with 0.1 M Tris-HCl, pH 8.0, saturated phenol (volume equal to aqueous phase) at room temperature. High speed centrifugation in an Eppendorf 5412 Micro-fuge with plastic tubes

partitions the ethidium into the phenol phase and the agarose as an interfacial layer. Residual phenol is removed from the aqueous upper layer by 3 extractions with anhydrous ether. Sodium acetate is added to 0.3 M and precipitation is by the addition of 3 volumes of absolute ethanol and freezing in a dry ice-ethanol slurry for 30-60 minutes. Centrifugation at 15K rpm for 1 hour pellets the DNA which may then be dissolved in microliter volumes of water or buffer. Large extractions are scaled upwards accordingly in siliconized tubes.

Methylase Assay

Methylation (Rubin and Modrich, 1977) was carried out in 0.1 M Tris-HCl, pH 8.0, 2.5 mM dithiothreitol, 5 mM EDTA, 10 µg/ml bovine serum albumin, 1-2 µM S-adenosyl-L-methionine (H^3)(in 0.005 N H_2SO_4), and where possible, at least 1 nM DNA substrate sites. 0.5 - 2.0 units of Eco RI methylase were used in a 100 µl reaction. Reaction was halted by pipetting 5 µl samples into 0.2 ml of sonicated calf thymus DNA followed by 1.0 ml of cold 5% TCA. Precipitant was collected as described. This system is extremely labile due to decay of H^3 -SAM moiety.

Nick-translation

Labeling by the protocol of Rigby et al. (1977) was used as a guide for nucleotide replacement

synthesis. Reactions were carried out in a buffer of 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 50 µg/ml bovine serum albumin. DNAase I (Worthington) was stored in the freezer as a 1 mg/ml stock in 0.01 N HCl. At the time of use this is diluted 1:10 into 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mg/ml BSA and held on ice for 2 hours to reactivate. A further dilution to 0.1 µg/ml gives the working reagent. DNA, usually 0.5 to 2 µg, was added to a plastic reaction vessel in which approximately 200-300 picomoles of thymidine 5'-triphosphate (α P³² or methyl-H³) has been dried down. The unlabeled triphosphates are stored as 0.1 mM solutions in 1 mM Tris-HCl, pH 7.0, and 200-400 picomoles of each dATP, dGTP, dCTP are added and mixed. Sterile water is used to bring the reaction volume to 100 µl. Between 0.1 and 0.5 ng of activated DNAase I are added and the solution held at 25°C for 1 minute. Between 2 and 10 units of E. coli DNA polymerase I are added and the reaction incubated for 2 hours at 12°C. Reaction termination was by buffered phenol extraction, micro-fuge centrifugation and 3 ether separations. The time course of this reaction could be followed by removing 1-2 µl samples and adding to 0.2 ml of sonicated calf thymus DNA at 200 µg/ml

followed by precipitation with 1.0 ml of 1 N HCl containing 1% (w/v) NaH_2PO_4 and 1% (w/v) $\text{Na}_4\text{P}_2\text{O}_7$ (Rigby et al., 1977).

Sephadex Chromatography

A nick-translation product was "marked" with 1 μl of a 0.001% bromphenol blue tracking dye in water and applied to a 5.0 ml column of Sephadex G-50 (fine) in buffer which had been degassed by vacuum. The pipette column was packed over a wad of siliconized glass wool and washed with several volumes of buffer. A 100 μl product was loaded and elution was by gravity. By the time the dye marker had migrated 0.5 ml within the column, the first peak of activity, the excluded product duplex, was emerging. This was collected for approximately a 1.0 ml volume and when the dye approached the 1.5-2.0 ml mark, the second peak of unincorporated or low molecular weight material was exiting as a broad distribution. Where necessary, the entire column activity was collected in 2 drop fractions and analyzed for radioactivity distribution. The P^{32} activity was monitored with a mounted radiation survey to scan the column length and signal the approach of fractions of interest. The high molecular weight excluded peak was carried through 3

cycles of boil-freeze in an NaCl (saturated)-water bath to allow temperatures of 100°C without bumping, and quenched in a dry ice-ethanol slurry. Heating was for no more than 1 minute total.

Hybridization Procedure

Denatured nick-translation product was diluted with hybridization buffer to various volumes depending on the size of the reaction and the activity of the product. Buffer for this hybridization was Denhardt's solution in which filters had been previously prehybridized overnight. This is (Denhardt, 1966) 0.02% ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone in 6 X SSC. For situations where reasonable homology is expected, the hybridization was carried out at 65°-68°C for 24 hours in a volume of 4 ml per 10 lane filter area ($\sim 2 \times 10^6$ cpm/bag) (10 X 10 cm) in a heat resistant "Seal-A-Meal" polyethylene envelope. Calf thymus DNA, sonicated, was also present to prevent non-specific binding at 200 µg/envelope. Where less stringency was desired, the reaction was hybridized at lower temperatures and longer time periods. In some cases, 50% formamide, 60 mM NaCl, 40 mM NaH₂PO₄, pH 7.0, 4 mM EDTA, 0.3% SDS, 200 µg calf thymus DNA was employed at 42°-44°C for 24-36 hours. Hybridized filters were gently washed in

2 X 500 ml of hybridization buffer at the initial temperature (without carrier DNA) and 2 X 250 ml each of 1 X SSC and 0.1 X SSC at room temperature followed by slow drying with a hair dryer, transfer to Saran Wrap coverings and placed in a Picker X-ray cassette box with top and bottom Cronex Lightening-Plus intensifying screens. Exposure was from 2 hours to 2 weeks, generally at -70°C onto Kodak X-Omat type R film.

Blotting

Transfer of DNA from agarose slabs to nitrocellulose membranes was by the method of Southern (1975). Slabs were soaked in 0.5 M NaOH, 1.5 M NaCl for 60 minutes followed by 2 soaks in water, 5 minutes each and neutralization for 60 minutes in 3 M NaCl, 0.5 M Tris-HCl, pH 7.0. The gel is placed on a Whatman 3M wick support, making contact with chambers of 20 X SSC in a (BRL) Model H2/H3 blot transfer apparatus. A piece of nitrocellulose membrane (BA85, $0.45\text{ }\mu\text{m}$) from Schleicher and Schuell is carefully cut and soaked in distilled water. This is placed on top of the gel surface and air expelled by gently rolling a 0.1 ml pipette along its surface. Over this is placed approximately 30 sheets of chromatography grade paper (Whatman) and an even 160 gram pressure

applied by means of a plastic, weighted sheet. After 24 hours, with buffer replacement if necessary, the filter paper is discarded and the membrane removed, marked by nicking its edge for orientation and soaked "face" up in 100 ml of 5X SSC, 3 times in 100 ml of 1X SSC and 3 times in 100 ml of 0.1X SSC. Air drying on Saran Wrap followed by hot air drying and finally baking for 2 hours at 80°C (no vacuum, as accidental shattering of the membrane may occur on vacuum loss) on a glass sheet and stored at room temperature. The dried gel matrix is checked for complete DNA transfer by examination under a U.V. source and again after a very brief soaking in the fluorescent agent, ethidium bromide.

Ligation

Ligation was carried out with various concentrations (0.5 - 25 µg/ml) of DNA in 100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol, 0.2 - 5 mM ATP, 50 µg/ml BSA, 50 mM NaCl and 0.1 - 2.0 units of T₄ induced DNA ligase (as per supplier). For restricted samples, the restriction enzyme was denatured by heating at 65°C for 10 minutes. Depending on the original restriction enzyme environment, the reaction was supplemented with reaction

ingredients. Incubation was for 18-24 hours at 4°C. When samples were diluted and religated, reaction conditions were restored with concentrated stocks. ATP was stored at -20°C in 0.01 M sodium phosphate buffer, pH 7.0.

Polymerase I Assay

In attempting to fill in the cohesive ends resulting from Eco RI cleavage, the following reaction conditions were used. The polymerase I used in these studies was isolated in the laboratory of Dr. J. Krakow and contained 5.6 mg/ml of protein and 780 d(A-T) units/mg. Reactions were 150 µl and 66 mM Tris-HCl, pH 7.8, 33 µM dATP, 33 µM dTTP (methyl-H³) (specific activity 182,300 cpm/nM), 6.6 mM MgCl₂, 0.33 mM DTT, 50 µg/ml BSA. Approximately 1-4 units of Pol I were added and the reaction followed by removing 5 µl aliquots, adding to 0.1 ml calf thymus DNA (200 µg/ml) and precipitating with 5% TCA. To inhibit incorporation of label into internal nicked duplex regions, 120 mM NaCl was used in the reaction (Wu, 1968). Similarly, PMSF (phenyl methyl sulfonyl fluoride) was included at 1 mM to inhibit the 5'→3' exonuclease activity of Pol I (in 100% DMSO, diluted 1:1 with water). Reactions were run at 10°C and 37°C and where noted, with the addition of dCTP and dGTP to the Eco RI digested template. Thus, A-T cohesive

end directed synthesis could be checked versus the ability of the molecule to support nick translation.

RESULTS AND DISCUSSION

1. Transformation with Native and Restricted DNA Molecules

The decline of transforming activity seen when Bacillus subtilis is transformed by restriction nuclease treated DNA is highly gene characteristic. This decline may be best expressed by the values of RTA--defined as the relative transforming ability or the ratio of treated to untreated colony forming units as a percentage. This study examines many structural genes, located at various positions along the circular genome map (Figure 1) and investigates the nature of the observed gene survival patterns. The use of restriction nucleases allows a topography to be inferred with respect to gene organization, heterologous restriction site patterns and the potential availability of specific genomic fragments for cloning and amplification.

An examination of Table 3 shows the Eco RI nuclease generated gene survival "catalogue" obtained when high molecular weight B. subtilis DNA is exposed to this site specific nuclease (Table 2). These values are of course subject to the biological variability and complex aspects of competency

and recombination proficiency, but are nevertheless fairly reproducible when expressed as an RTA. They are very internally consistent when one gene of a prototrophic transforming set is compared relative to another. In Table 3, prototrophic strain W23 is the DNA donor and various 168 strain derivatives are the auxotrophic recipients (Table 1). The same donor molecule, exposed to complete digestion by nuclease Hind III yields the catalogue pattern shown in Table 4. The action of the low ionic strength recognition specificity of enzyme Eco RI, termed Eco RI*, gives the catalogue in Table 5. As a comparison, the DNA of the super-sporulating B. subtilis strain 3610, the putative progenitor of the 168 strains (Hemphill and Whiteley, 1975), gives its own distinctive survival pattern as seen in Table 6 for Eco RI digestion and Table 7 for Hind III digestion. B. subtilis W23 and 168 have been described in the introduction.

The data presented represent numerical averages of approximately ten individual determinations for each genetic locus. While the data may be analyzed via standard deviation statistics about the mean, it must be remembered that these are in vivo or at least quasi-in vivo determinations. As

such, they are inherently variant and subject to sometimes significant absolute fluctuation. With small sample size determinations of somewhat disperse measurements, the standard error of the mean is a more realistic measurement of sample variation from the population mean. However, these statistical evaluations have no useful predictive value in the catalogue sets and were not employed.. The strongest indication, of any utility, is that gene transforming ability following restriction enzyme exposure falls into classes or groups relative to other gene donating activities and that these classes may be very low, low, medium and high, with respect to RTA, depending upon the gene examined and the attacking enzyme specificity (see Tables 3-7). In some cases, as with strain 3610 and Eco RI, gene activity may fall into a very high RTA class (e.g. trpC2). The intuitive conclusion is that genes that are interrupted in the intragenic span are eliminated from donor capacity while those that are on fragments, extragenically cleaved, are reduced in their transforming proficiency as some complex non-linear function of their length. As will be discussed later, genes that are located proximal to a cut terminus, may or may not exhibit

prototrophic activity due to entry processing and recombinational "tailoring". More correctly, a particular coding length that is complementary to the recipient lesion must "survive" intact. That this sequence must reside on "critical" size classes is a demand of the exchange mechanism itself. That is, genes themselves need not "survive" restriction, but rather, in a transformation assay, specific auxotroph complementing stretches of polynucleotide must remain available for synapsis and substitutional integration (see Introduction). This is in contrast to the all or none mechanism of transfection. The relatively unclear origin of many B. subtilis mutations to auxotrophy also complicates the interpretation of fragment survival data. The first report of gene survivals were those of Harris_Warrick et al. (1975) while Dean and Halvorson (1976) were first to point out the complex nature of the relationship between donor molecule and recipient organisms regarding cleavage, size and regional homology. Although partial gene enrichments were obtained from restricted DNA size separated via electrophoresis, strain specific anomalies were noted in the RTA survival patterns. This study will attempt, in part, to extend these analyses.

Bacillus subtilis, as noted earlier, passes through a transient period of competence to take up and express new genetic information offered to it in the form of naked DNA fragments in solution (Spizizen, 1958). It was possible that there existed an initial temporal discrimination in the manner in which a competent cell interacted with DNA molecules of different size and structure. To determine whether or not the strict regimen used to induce competency was in any way predisposed against restricted DNA, the peak of competence development with time was studied. In Figure 2, cells auxotrophic for tryptophan and sensitive to Kanamycin are followed through the competency development period in Spizizen's II media with prototrophic native, restricted and sheared DNA. Additionally, circular (CCC) plasmid pUB110 molecules, bearing the Kanamycin-resistance determinant, are included to analyze the development of physiological receptiveness. The graph insert to this figure shows the traditional Klett turbidimetric (nephelometric) changes associated with the 4.5 hour Spizizen I protocol and the 90 minute Spizizen II nutritional step-down which precedes exposure to DNA. The 90 minute Spizizen II cells are taken uniformly in all

experiments for transformation. As seen, the competency period for all DNA species tested roughly coincide, although the absolute transforming values are reflective of the physical state of the molecules and the selective benefit with which they endow the recipient. Clearly, native and degraded DNA molecules may be compared without obvious bias of temporal discrimination against them. The levels of genetic activity in the survival catalogues (RTA) and those seen with plasmid vectors later in this report are true reflections of efficiency at least respective to an untreated moiety.

When competent cells are exposed to DNA, they take up and integrate that DNA and begin to express new information. The first interaction is between cell receptor sites and the duplex molecule. In B. subtilis about 30 such sites interact (Singh, 1972) and when sufficient numbers of fragments of DNA are present the system is saturated--no further increase in transformants occurs with additional substrate molecules. Receptor affinity and specificity will determine at what DNA input level the system saturates for different molecular substrates. With native DNA of average molecular weight $1-3 \times 10^7$ daltons, a competent population tends to saturate at

about 0.1-0.5 $\mu\text{g}/\text{ml}$. However, when restricted DNA is used, certain gene specific effects are noted. The tendency is for restricted DNA fragments to maintain a constant rather than a decreasing specific activity. That is, saturation seems to occur at a higher concentration than it does for the "native" samples. An example of this is seen in Figure 3. In terms of specific activity, native transformants/ $\mu\text{g}/\text{ml}$ tend to decline uniformly although to a small extent. Restricted DNA on the other hand shows a relatively constant specific activity and in some experiments it may even rise somewhat. This is the case for the metB10, trpC2 loci in strain BR151 with Eco RI digested W23 DNA as the donor. Simply, the RTA values of these loci may rise disproportionately as the native activity (the denominator) passes through an inflection. The rise is somewhat variable and obviously dependent upon physiologic competency and the quality of the DNA preparation. The lys-3 activity remains at a constant low value and may, interestingly, decrease as DNA weight concentration rises. This will be discussed more fully in later sections, but it is obvious that the cell is interacting in a specific manner with the elements determining prototrophy

for the respective loci when restricted DNA populations are offered to it. Dose response curves reveal this presumably surface interaction (Singh, 1972). This phenomenon is seen with another marker, argA2, in recipient BD-29 where specific activity declines with dose (Figure 3). Clearly, the cell handles these determinants uniquely from the point of view of size (Figure 19), be it through receptor affinity or recombinational discrimination. Dose response is defined somewhat by the loci's rank in the RTA catalogue. A sample of sheared DNA, degraded in a uniform process in which residual gene activity is approximately 10% of native values shows no significant shift in its specific gene activity profile (Fig. 3). Unique classes of transforming material are thus created by restriction cleavage and these no longer reflect the overall characteristics of the intact genome. An early report by Cato and Guild (1968) pointed out the effect of gross size alteration upon DNA fixation in Pneumococcus. Relative transforming activity was seen to shift depending on whether a preparation of DNA was assayed in the linear or plateau region of the dose response curve. Their conclusions, applicable here, were that size had little effect on

fixation when DNA was limiting, but that it was involved when DNA was in excess; bias being exerted against small helical material (i.e., lys3 and argA2 following Eco RI cleavage). The use of restriction nucleases enables one to create subpopulations or families of size fragments, unlike shearing, and to begin to infer gene activity to these populations. This report will attempt to describe the characteristics of these families, using as a "dictionary" their relative positions in the RTA survival catalogues. The problem is intriguing due to the ability of site specific nucleases to "tailor" genomic DNA and, at the same time, complicated due to the relatively "black box" aspects of competency and recombination.

2. Gel Electrophoresis and Size Determination

When B. subtilis, with a genome size of 2.0-2.5 X 10⁹ daltons (Henner and Hoch, 1980), is treated via the Marmur isolation process (1961) DNA is isolated whose mean molecular weight is 10-30 X 10⁶ (Stewart, 1969). If this DNA is exposed to restriction nuclease Eco RI, isolated from the gram negative coliform Escherichia coli (Yoshimori et al., 1972), the DNA is cleaved into

many fragments whose sizes range from 12×10^6 to well below 0.367×10^6 daltons. Figure 4 is a composite gel photograph taken with ethidium bromide staining and ultraviolet fluorescence. This intercalative planar molecule excites at 260 nm and emits enhanced fluorescence at around 600 nm. Electrophoresis is in the horizontal mode with the anode at the sample distal end. Examination reveals that the B. subtilis chromosome is cleaved into many fragments, almost a continuum of size span, with no apparent accumulation of any one member over the levels of the others. That is, there is no revealing of a true satellite group in this genus (or in any other prokaryote) as there is in higher representatives such as calf thymus DNA. Here, at least 3 bands representing the gathering in place of many identical members is obvious. These satellite species represent repeated polynucleotide stretches, many copies per genome, and have molecular weights of approximately 1.43 Md., 1.17 Md. and 1.06 Md., respectively (1 Md. = 1×10^6 d.). Other than 8 clustered ribosomal DNA cistrons, the prokaryotic organisms have no reiteration of genes (Dubnau et al., 1965). For comparison, viral standards are included to indicate approximate

molecular weight. Phage lambda is cleaved into 6 discrete fragments by Eco RI (Thomas and Davis, 1975) of which 5 are discernable. Hind III enzyme from Hemophilus sp. cuts lambda into 7 fragments with the 0.367 Md. band migrating rapidly through 1% agarose gels (Murray and Murray, 1975). Tables 2 and 8 show the enzyme recognition sequences and the standard cleavage maps of bacteriophage lambda and ϕ 105 molecular weight standards, respectively (see Fig. 5). The native W23 DNA sample is clearly of high molecular weight with little degradation obvious (see Fig. 4). This same DNA gives maximal transforming efficiency for the thr-5 gene in BD-170 of greater than 10^6 transformants/ μ g/ml of competent culture. Also included in this gel are samples of W23 cleaved with Hind III and Eco RI. In addition, a sample of mechanically sheared DNA as well as a plasmid sample both before and after restriction with Eco RI are included. In experiments where the electrophoretic run is not overloaded (less than 1 μ g/lane) and ethidium bromide staining is brief, the appearance of what seem to be several "bands" of defined molecular weight are noted for chromosomal DNA. These bands may seem numerous at times and the impulse is to attempt to

enumerate them. However, as Harris-Warrick et al. (1975) point out, these bands are not unique since the sum of their apparent sizes is only about a tenth that of the total chromosome. In addition, a computer simulation has shown that the gel fluorescence pattern is best fit by assuming random shear of the native genome during isolation to 120 kbp (7.9×10^7 d.) followed by site cleavage to yield size members of average molecular weight 6.6×10^6 d. (double strand) (Harris-Warrick et al., 1975). This type of distribution is clearly seen in the (Fig. 4) photograph. As long as the weight average molecular weight prior to restriction is at least 30 kbp, the "banding" pattern is evinced. Thus, the bands seen within the W23 X Eco RI lane are artifacts and not unique species of defined molecular weight after limit digestion. In no case, however, does the band pattern show disproportionate fluorescence intensity indicating a greater than molar representation of any size class.

Agarose gels separate on the basis of molecular weight and are not subject to subtle variations in base content as are polyacrylamide gels (Helling et al., 1974). They may, however, show mobility anomalies where gross conformation is concerned.

For instance, the native plasmid lane in Figure 4 shows 3 bands which, upon cleavage by an enzyme that recognizes a single site on this molecule, form a single band of greater intensity. The lowest of the 3 bands is the covalently closed circular form of plasmid pUB110 (CCC). The top band is the relaxed or open circular form (OC) and the middle is the fully linear form (L). The traditional method of determining molecular weight on electrophoretic patterns is to measure the mobility of several bands of known molecular weight and to plot this mobility versus the logarithm of the molecular weight. Interpolation of an unknown molecular weight is obtained by measurements of that species migration in the electric field. Normally, this method is very useful if good molecular weight standards are chosen within the range of the suspected unknown molecular weight. However, the best line joining the graphic points in this case takes on a distinct curvilinearity (Helling et al., 1974) at the higher weight range (Fig. 5 insert). That is, an agarose gel of given concentration possesses a narrow range of linearity relative to mobility. Outside this range, deviation occurs and calculations are not valid. In this report another procedure has been

employed (Bearden, 1979) and found most satisfactory with respect to range and visual accuracy. In this procedure molecular weights of restriction cleaved viral standards are converted to the negative two-thirds exponent function and plotted linearly against mobility. Figure 5 compares these two methods for the same data points. While more cumbersome mathematically, the linear method is more pleasing both visually and in terms of accuracy. This method is more amenable to large fragment sizes and allows a more comfortable interpolation of experimental points. Both procedures yield the same M.W. values in this report but the exponential method is preferred.

3. Preparation of Labeled Prototrophic DNA

To examine the relative susceptibilities of various genes to restriction cleavage by heterologous enzyme and the kinetic phenomena associated with this loss, transforming activity was followed by genetic and physical methods. In order to more accurately study the digestion process, (tritium) labeled DNA was required. In addition, this DNA must come from the same prototrophic W23 or 3610 cells used as the genetic transforming donors in the restriction catalogues. The problem of

uniformly labeling such DNA to reasonable specific activity was overcome by adding high concentrations of 2'-deoxyadenosine (200 $\mu\text{g/ml}$) to the growth media. This nucleoside can competitively inhibit the enzyme thymidine phosphorylase which cleaves thymidine to thymine and deoxyribose-1-phosphate by phosphorylation. Utilization of thymine must proceed by reformation, requiring a large pool of deoxyribose-phosphate, lacking in E. coli (Budman and Pardee, 1967) and presumably also in B. subtilis. Although it may additionally induce phosphorylase synthesis, 2'-deoxyadenosine can transfer deoxyribose-1-phosphate to intracellular thymine. The competitive and donating ability of this agent efficiently block thymidine hydrolyzing activity and in this study were essential for efficient tritium uptake and labeling of B. subtilis DNA with methyl- H^3 -thymidine. Figure 6 illustrates this effect clearly. Where prototrophic DNA was to be labeled, this procedure was employed successfully to give several DNA preparations of specific activity greater than 1×10^5 cpm/ μg even though the total cellular yield was somewhat depressed and the lag phase slightly lengthened by the inhibitor.

4. Kinetics of the Restriction Process

Knowing that different gene loci could be described by means of a restriction catalogue relative to one and other, an effort was made to further characterize the process by which these RTA's developed. That is, what are the relative marker susceptibilities? Where do "active" transforming fragments reside in the size pattern? Could subsequent gene purification be accomplished by exploiting this information? The possibility existed that there might be a bias as to which gene loci were located proximal or distal to a particular cutting site. There might be cleavage site clustering. A fragment defined by flanking Eco RI or Hind III specific sequences would not be expected to transform efficiently, if at all, if cleavage were intragenic; and if it were extragenic a complex nonlinear size dependent response might exist. Depending upon the nature and location of the recipient auxotrophic lesion, intragenically cleaved molecules might tenuously rescue a resident allele by recombination. As the DRC (donor-recipient complex) involves an integrating fragment estimated at 2.8×10^6 d., the prototrophic sequences would obviously be required to reside on as large a fragment

as possible. The trpC2, hisB2, argA2 and leu-1 loci arose via X-ray, U.V., HNO_2 and spontaneous origin, respectively (Table 1). Recent data show hisB to be variably revertible in different strains by MMS (suggesting uncharacterized genetic differences) while thr-5 is revertible by 2-AP; hisA, argA and thr-5 by MMS. TrpC2 and leu-1 are most likely small deletions rather than point mutations as they fail to revert (Rudner, 1980). That the situation involves more than just transforming efficiency being dependent upon size is hinted at in Figure 3. The reduction in size caused by restriction is a much more complex situation than that caused by random shearing. Each complementing activity reveals and maintains a characteristic profile. The bioassay imposes limitations and variables which make classical Michaelis-Menten analysis difficult if not impossible.

The kinetics of transforming activity loss with time were analyzed. In Figure 7, marker survival is plotted as a percent of its native value. For various markers or fragments bearing these markers, the ability to transform an auxotroph to prototrophy is distinct. All gene activities decline to characteristic levels with uniform, non-anomalous decay

kinetics. These levels reflect consistently the catalogue RTA values of limit digestion. Readdition of more nuclease causes no further decline in biological activity. The 50% survival levels yield initial decay kinetics ($K_{1/2}$) of 2-3 minutes uniformly. Physical studies (Modrich and Zabel, 1976) have shown that Eco RI operates with a turnover number of 3.8 double strand scissions per minute and that with ColEI substrate 98% of the CCC are converted to recBC nuclease sensitive linear molecules in 5 seconds. In the transformation assay, the marker loss indicates a common early target in the digestion process. It must be remembered that the "target" substrate of the enzyme in a biological transformation assay is not really the recognition palindrome recognized by the nuclease, but actually the genetic activity which might be located on a stretch of DNA flanked by these sequences or continuous with them. The primary target event may most reasonably be taken to be a size reduction whose effects are seen primarily in the recipient competent cells, reflective of the dependency of DSF upon initial duplex size and the ultimate formation of single strand fragment (SSF). The SSF being the immediate donor contribution to

recombination and to whose M.W. transformation is directly related. This interpretation is obviously distinct from one involving purely biochemical events of enzyme-substrate recognition. For comparison, Figure 8 shows the loss of donor activity when W23 DNA is mechanically sheared to reduce biological activity to 10% of native value. No matter what final base level of activity is generated, be it 25% or even 0.1%, no marker specific bias is seen. During restriction, as initial enzyme-recognition site collisions progress, individual genes begin to exhibit survival characteristics and present secondary targets due to the now relatively limited number and proximity of flanking cleavage sites. The bioassay at this point reveals subpopulations which a physical assay could not. In stark contrast, the exposure of a molecule such as plasmid pUB110, which determines resistance to neomycin or kanamycin, to Eco RI causes an immediate exponential drop of biological activity to zero at completion. This replicon cannot function in B. subtilis if it suffers even a single-strand interruption (Contente and Dubnau, 1978). Figure 9 is a representation of this. As a model, this system is somewhat analogous to the lethal scission of a potential

transforming gene or sequence intergenically since it is an all or nothing phenomenon. In the absence of a recognition site for Hind III in this replicon, biological activity remains unaltered.

5. The Distribution of Restricted DNA on Agarose Gels

Examination of Tables 3-7 indicates that the DNA of strains W23 and 3610 respond differently in some respects and very similarly in others to restriction cleavage. In an effort to reveal any gross differences in restriction products, gel profiles were analyzed for molecular weight distribution. Figure 10 shows 3610 and W23 DNA cleaved with Eco RI. Figure 11 shows W23 cleaved with either Eco RI or the less stringent specificity function of Eco RI*. Figures 12 and 14 show the distribution obtained when DNA is mechanically sheared to smaller size under conditions generating distinct size clusters. Also shown is the molecular weight approximated at a given point of mobility. Table 9 presents the weight average molecular weight obtained after the various digestions. This value is calculated by summing the amount of radioactive label at each molecular weight increment, multiplying by the respective molecular weight at that interval and dividing by the total cpm on the

electrophoretogram (Figures 10-13). Standard curves for these calculations were constructed for each individual gel. As illustrated, the Hind III digest of both W23 and 3610 show the same general distribution in Figure 13 with weight average molecular weight of about 2.4×10^6 daltons (Table 9). The gross distribution is however, extremely broad, encompassing ranges of 14×10^6 to well below 0.3×10^6 . The Eco RI digestion products of W23 and 3610 range from 16×10^6 to 1.3×10^6 daltons with smaller digestion products appearing as low as 0.367×10^6 daltons. Again, the gross distributions of W23 and 3610 are almost identical (see Appendix) with an average molecular weight of 6.1×10^6 d. Although both Eco RI and Hind III recognize 6 base pair sequences, the respective digestion patterns of M.W. vary from somewhat narrow to very broad. This is not reflected in fluorescence photographs with ethidium bromide since under these experimental conditions linear intensity is not achieved. It is obvious however that a significant proportion of the Hind III digested molecule is of a much lower molecular weight, reflecting either absolute recognition site frequency differences or a differential "patterning" in certain regions since higher molecular weight material still remains.

Figure 11 illustrates a situation where site frequency is significantly different. Eco RI enzyme "normally" recognizes (GAATTC) sequences with cleavage yielding a hydroxyl (3') terminated (G) and a 4 base (AATT) overhang. Under "abnormal" or low ionic conditions the enzyme (Eco RI*) recognizes the 4 base palindrome (AATT), both internal to the canonical site and elsewhere depending upon its frequency of appearance. Statistically, the 6 base palindrome will appear once for every 4000 bases while the degenerate site will be present every 250 bases (Appendix). In this case, the Eco RI* material of W23 ranges from small amounts of approximately 6×10^6 d., constantly increasing in amount as M.W. steadily drops to below 4×10^5 d., and probably much lower on a smaller sieve gel. The average weight is, in this case, 6.5×10^5 d. In Figure 12, shearing yields symmetrically distributed populations with a M.W. range average of 3.2×10^6 , for a general reduction of biological activity to 8-10% of native, and 1.45×10^6 for a reduction to 1% of native values. These values are consistent for all loci tested. Clearly, size reduction is correlated to loss of biological activity, most probably due to interference with the

proper generation of DSF (see introduction) and subsequent integrational loss. Figure 14 shows the restriction pattern of W23 compared to a series of controlled shear exposures. This analysis generates the data of Table 3 and Figures 10 and 12. The size versus biological activity profiles which are suggested indicate that the coding fragments from the restriction process are unique and give an indication of the marker position relative to enzyme recognition sites in the absence of any existing recombinant clone data for the system (Figure 19).

Analyses of the distribution of the myriad of palindromic sequences thus far identified in all organisms, including man, may eventually shed light on the origin and divergence of species and the development of the genetic code in a more general context. The evolution and conservation of specific sequences, recognized by proteins from vastly separated organisms, is an intriguing problem in information theory, recombination and gene development. In the case of B. subtilis in particular, we have detected the presence of at least 1.2×10^3 Eco RI specific (Appendix) palindromes. Surely, a great many of these must reside within the colinear context of structural and functional genes. The

differential "patterning" of totally heterologous and cross-genus specific sites must ultimately be related to protogenome mobilization and evolution.

6. Effect of Restriction on Genetic Activity

Originally demonstrated by Spizizen (1958), B. subtilis strains W23 and 168 are fully compatible with respect to donor activity. For certain loci (e.g. purB6 relative to metB5) higher relative transforming activity may be exhibited by native DNA. This is due to the fact that W23 strains complete chromosome synthetic rounds in stationary phase growth while 168 strains do not and accumulate incomplete replication fork structures (Yoshikawa and Sueoka, 1963a). The resulting marker frequency discrepancy relative to the stationary phase is most dramatic at the origin proximal initiation region (Yoshikawa et al., 1964) and declines both with linear distance from the origin and as the cell approaches stationary phase (see introduction). Vegetative 168 cells on the other hand give rise to DNA with unequal marker frequency differences (see introduction) unless they are normalized to a completed genome such as a 168 spore chromosome. The DNA isolated in the present study

is from very early stationary phase and might thus be expected to exhibit some limited marker frequency bias towards origin proximal loci of 168 derivative strains. However, as Dubnau points out (Dubnau et al., 1969), roughly equivalent levels of transforming activity are detected for all markers, except purB6, in late exponential 168 and W23 lysates. Analysis of the ratio of heterologous W23 to homologous 168 donor activity in 168 recipient auxotrophs showed that this value was close to unity for all markers with some fluctuation and recipient specific variation. When used as transforming donors for the recipients in Tables 3-7, native DNA fragments of W23, 3610 and 168 strains show (this study) the same relative transforming efficiency for all markers tested. With the exception of purA16 relative to metB5 in strain mu8u5u16 (Yoshikawa and Sueoka, 1963a) where an average 5.0% higher activity was noted (data not shown), the three donor frequencies were essentially identical in the native state. Analysis of the 168 donor samples was of course complicated by the myriad of donor-recipient combinations possible and the lack of certain genetic recipient combinations. Distinct

host variations were however noted for a given donor and these will be discussed later (Table 10). The conclusion of the above discussion is that native W23, 3610 and 168 DNAs are compatible in a transformation assay and possess, for the most part, identical transforming activity and genomic organization. That is, no general marker specific effects are noted with this mode of native exchange. This is in contrast to the comprehensive studies of Dubnau et al. (1964) on transduction exchange between 168 and W23 systems. While a few map correlated regions of higher relative activity existed for native W23 transforming DNA relative to 168 DNA in a 168 recipient (phe-12, hisB2), these differences are much more striking in a transduction recombination system. Whether they are due to separate modes of recombination as suggested by Dubnau or to restriction, exerted upon the double strand donor molecule or its phage vector as suggested by Hadden (1977), is still not clarified. Only in transduction systems is true marker variation demonstrated between 168 and W23 DNA. The loci leu-8, purA16 and hisA of Dubnau were severely limited in heterologous crosses while purB6, thr-5, hisB2 and trp-2 were not.

If differences exist between the different B. subtilis strains used in this study of restriction topography as suggested by the restriction generated RTA catalogues, these differences are of a micro-homology nature involving specific sequence variation rather than gross inhomology. The data of Hemphill and Whiteley (1975) however, call into question the original origin of the "168" cell as well as its trp coding sequences relative to 3610 and W23. Certainly, loss or gain of specific hexanucleotide sequences about the genome will lead to very subtle strain variations.

Hybrid duplexes of 168 and W23 possess lowered T_m values and hybridization of the respective DNA to pulse labeled RNA of the other is altered (Dubnau et al., 1969). Lovett and Young (1969) indicate that W23 and 168 are at least 89% related. The situation regarding the prototrophic donor 3610 is more complex, having been derived from the original Marburg bacillus and designated NCTC 3610. This strain is genetically and physically indistinguishable from W23 on many points (although they are separable on the basis of wall and phage range-- Hemphill and Whiteley, 1975). 3610 is endowed with an extremely efficient entry, in our hands,

into the sporulation pathway (Testa, 1977). In gross genetic terms it is as efficient a prototroph as W23 towards the classic 168 derivative recipients. Differences do arise however, most obviously, for certain regions of the 3610 chromosome and not others following site specific cleavage (Tables 3-7). The transformation data of native genomic fragments (Table 10) indicate that these differences do not manifest themselves under usual circumstances. Only when a restriction nuclease, recognizing an extremely specific cleavage site, interacts with these DNA's do hidden sequence organization patterns become obvious.

Rather than probe genetic relatedness on a genomic scale, site specific cleavage makes possible marker specific statements. The cleavage specific subpopulations of fragments generated take on an individual character with respect to donor origin and distinctly with respect to each other. This is not seen in a native or unrestricted DNA population which is inherently homogeneous no matter what random process is used to disrupt its integrity. Restriction offers a unique opportunity to study induced gene loss with respect to cleavage site proximity and to reveal palindrome topography as a preliminary to cloning.

7. Locus Specific Effects

The data of Tables 3-7 show that in all cases, restriction by either Eco RI or Hind III lowers biological activity to various levels depending on the locus selected. Both the origin of the donor DNA and the recipient background have significant effects (Table 10). While strict statistical variation treatments of the RTA values are possible, they are not reasonably predictive beyond the class hierarchies established in this complex system of in vitro digestion and in vivo assay. Where mechanical shear or deoxyribonuclease digestion may reduce biological activity uniformly (see Figure 8), it is nonspecific and processive, yielding no core of distinct biological products. Taken independently, each restricted genetic activity is classifiable into ranks of very low (e.g. lys-3), low (thr-5), medium (metB5) and high (trpC2) activity relative to a native standard (Table 3). Each gene of a donor group within a multi-auxotrophic recipient possesses an individual character. For example, in B. subtilis recipient BD-29 (argA2, leu-1) donor W23 DNA (X Eco RI) generates recombinant transformants prototrophic for arg with an RTA of 0.16% and leu of 3.8%. With BD-170 (trpC2,

thr-5) the values are 9.0% and 0.3% respectively, while for BR-151 (trpC2, metB10, lys-3) the RTA values are 1.7%, 0.3%, 0.7% respectively. In contrast, Hind III cleavage of W23 gives RTA's in BD-170 of 0.1% for trpC2 and 7.5% for thr-5. The argA2 and leu-1 loci of W23 have RTA's of 0.17% and 0.15% respectively after Hind III. Digestion with Eco RI* specific activity yields extremely low non-distinct marker values. Dramatic differences between various loci are seen with donor DNA isolated from strain 3610. Using this DNA with 168 recipient SB-25 (trpC2, hisB2) Eco RI high RTA's of 30.8% and 28.7% are obtained respectively. W23 trpC vs. Eco RI has an RTA of 7.3% by comparison (see Tables 3-7).

The lys-3 locus has an RTA of 0.43% with 3610 and only 0.07 with W23 or 168 (Tables 3, 6, 12). The hisA operon region, as compared to the distal hisB (hisH), gives RTA values of 0.45 and 0.09 for W23 with Eco RI and Hind III, while 3610 gives 9.48% and 7.18% respectively. The argA2 and leu-1 loci have 3610 Hind III RTA values of 2.45% and 12.4%.

TrpC2 from 3610 is 4.70% RTA while the same activity from W23 yields an RTA of only 0.05% after

Hind III. In BD-170 the trpC2 and thr-5 loci of W23 show a reverse pattern of Hind III survival; 0.1% and 7.4% respectively. With 3610 donor the activities are 4.7% and 3.8% (Figures 4 and 7).

That significant recipient specific effects may be present is shown in Table 10. Various 168 derivatives, all auxotrophic for the same mutant locus, respond to a common donor molecule with different levels of recombination as measured by the generation of prototrophic colonies. Samples of native DNA yielded the same levels of transformed prototrophs in each recipient host. Previously demonstrated by Dean and Halvorson (1976), this indicates that "localized nonhomologies" might exist and, although various recipient 168 derivatives may be genetically isogenic, this common organization breaks down at the level of sequence. Given the fact that transformation involves certain congressive aspects and that many mutant histories are obscure, it is not surprising that horizontal evolutionary drift may have taken place in what are termed "laboratory strains". Similar conclusions were reached by Rudner (1980) studying mutagenesis during transformation. In E. coli, this sequence drift has been shown by Anilionis and Riley (1980)

using hybridization analysis. As pointed out by Harris-Warrick et al. (1975), survival values for a given marker will be dependent upon the size of the DNA prior to and following digestion, the proximity of the coding length to the restriction site and therefore the proximity of the coding length to the fragment end. Superimposed upon these variables are the demands of transformation product (DSF, SSF, DRC) formation.

An investigation was conducted to determine the concentration of restriction nuclease Eco RI needed to bring a native sample of DNA (W23) to completion regarding its RTA for various genes. This was extremely important since it was hoped that these data would allow us to more confidently approach the problem of attempting to clone B. subtilis genes onto vector plasmids and study them in isolation from the chromosome. An intact gene sequence is necessary and partial digestion products are a possible danger. Thus, the stoichiometry of the RTA development was studied. In Figure 15, the decay of donor activity is plotted against enzyme dose. As can be clearly seen from this graph and the data of Tables 3 and 4, at low

concentrations of enzyme there is a distinct shoulder to the loss of trpC2 and hisB2 donor activity. This is also seen for the co-transfer of trpC2-hisB2 en bloc using Eco RI nuclease (Fig. 18). This multi-hit function assumes that there are many identical targets, each of which must be destroyed to inactivate the system. Inactivation is the achievement of the final plateau RTA value while 'target' destruction is the cleavage of many (GAATTC) palindromes signaled by the kinetics of approach to limit values. If the system possesses many identical targets (e.g. Eco RI or Hind III cleavage sites) the response to inactivation need not be linear as the system tends to become more "resistant" to "sub-lethal" inactivations (an asymptotic curve) depending on the "target" definition. The initial biochemical target is a 6 base pair palindrome, but as the reaction proceeds, populations of discrete fragments arise, their identity defined by the genetic selection. For these fragments, the reaction has ceased and they are no longer members of the reacting class. Their relative transforming efficiency will determine their rank in an RTA category.

Were the assay a pure "all or none" test, it would theoretically be possible to extrapolate back along the linear portions of the multihit curves to determine the extrapolation number at zero enzyme dose. This "hit number" (Hiatt, 1964) would reflect the number of single hit targets in the system depending upon its definition.

The data of Figure 15 indicate that trpC2 and hisB2 complementing information resides upon digestion products which are relatively more resistant to Eco RI effects than are argA2, leu-1 or lys-3. 3610 donor trpC2 activity (Table 6) is extremely resistant, 50% point reached at 8 units of enzyme, with a broad complex multihit appearance. It is not until approximately 4.5 and 3.0 Eco RI units act that W23 trpC2 and hisB2 exhibit 50% survival levels (assayed in SB-25). The en bloc co-transformation of the trpC2-hisB2 linkage indicates 2.0 units required. The argA2 and leu-1 activities give 50% loss effects at 2.9 and 4.2 units with argA2 exhibiting a much more precipitous decline in activity. On the other hand, lys-3 activity (in BR151) shows only a small shoulder, 50% survival being reached at 0.5 units. The thr-5 gene reaches 50% at 2.5 units followed by a rapid decline to

final RTA levels (Table 3). As a comparison, Figure 16 illustrates trpC2 and thr-5 activity decay following Hind III digestion of W23 DNA (Table 4). An inversion of response is clear; this enzyme interacts with the DNA in a different manner and required 3.5 units to bring thr-5 activity to 50% in one hour while only 1.0 unit is required for trpC2 activity decline. There is only a slight indication of a shoulder of trp activity. If the data of Figure 15 are transposed to a linear scale (Figure 17) it may be seen that argA2 declines linearly as an easily defined function of enzyme dose. The leu-1 locus, on the other hand, exhibits an initial more complex response. Not until the reaction has progressed somewhat is a linear response to dose seen.

Figures 15 and 17 indicate that while constant increments of target loss are not occurring, as would be the case if each target needed to be cleaved once to inactivate the biological response, the secondary recipient cell processing of argA2 and leu-1 (in BD-29) is perhaps very different. Considering the data of Figure 3, argA2 is assumed to reside on a relatively small fragment that is in close proximity, at least in one direction, to an

Eco RI palindrome. The approach to limit digestion is rapid as the biological assay indicates. The competent cell handles and expresses the progressive argA2 regional loss of efficiency in a characteristic manner for a given weight concentration of DNA. The leu-1 material is on a larger fragment, smaller than those of trpC2 or hisB2, and flanked more distantly, in the opposite direction to arg, by another Eco RI sequence (Fig. 18). This material is changing in size less dramatically as the cleavage reaction proceeds, and because of the complex demands of the in vivo assay, displays biphasic efficiency at the beginning and middle of the reaction course. That is, beyond a certain statistical point, the target of inactivation ceases to be Eco RI "sites" and becomes reflective of site proximity to fragments of specific genetic content. Similar arguments can be proposed to explain the hierarchy of W23 RTA values of trpC2, metB10 and lys-3 in BR151; purA16, metB5, leu-8 in mu8u5ul6 and most specifically, 3610 generated values for trpC2 which are unusually high. TrpC2 fragments in 168 strains would be similar to those of W23 based upon biological profile (Table 12), although strain microvariation may also be recognizable.

The argA2, thr-5 and lys-3 loci donated by restricted W23 DNA are all very low and presumably lie on a critical region of the size-response curve where, as Notani and Setlow (1974) point out, "all or maybe" phenomena may exist. Lys-3 is additionally placed very close to a restriction terminated end and may therefore be subject to additional expression difficulties. The Eco RI and Hind III data for trpC2 and thr-5 further indicate that a Hind III hexanucleotide is in extremely close proximity to or within trpC2 and fairly distal to thr-5 complementing sequences, the reverse of the Eco RI scheme (Fig. 19). The restriction site arrangement of 168 DNA about the trp cluster would seem to be more related to that of W23 while the genome of 3610 has specific regions where Eco RI, and perhaps, other sites are sparse or more distally flanking to certain gene activities. Those prototrophic sequences distal to cleavage sites are more resistant to activity loss even though restriction lowers the efficiency of all genes. As B. subtilis W23 and 3610 seem to possess similar Eco RI site frequencies (Appendix, Figure A-1), about 1200/genome, the relative location of a few can be inferred. ArgA2 is postulated to reside on a very small

(Figure 19) fragment after Eco RI cleavage, in proximity to, at least, one Eco RI site. Leu-1 is on a larger fragment, more distally flanked and within the size demands of transformation processing as its decay kinetics indicate (Figs. 15, 17, 19). Concave versus convex activity loss kinetics further support this notion for the arg-leu and trp-his regions respectively (Fig. 18).

8. Correlation of Biological Activity with Size

It is necessary to know the range of molecular weight within which a particular activity comes to reside to insure that we are examining a discrete molecular weight class in a transformation assay. Given that restriction recognition palindromes are distributed in some manner about the chromosome of B. subtilis, cleavage should generate families of fragments whose size is defined by the distance between two such cutting sites. Harris-Warrick et al. (1975) have demonstrated that banding patterns seen on electrophoresis of DNA are not unique. That is, these bands are polygenic and represent accumulations of similarly sized pieces with no one member disproportionately represented on a molar basis. The total size of the bands seen therefore does not represent the total size of the native genome.

Computer simulations (Harris-Warrick et al., 1975) have shown that such patterns (on EtBr staining of restricted DNA) are best described by random shear forces operating during isolation of the genome followed by specific cleavage. In addition, Stewart (1969) has shown that random fragmentation of the B. subtilis chromosome gives rise to a heterogeneous population wherein a particular genetic determinant may come to reside on fragments of varying size, melting temperature or buoyant density. Unique breakpoints are thus ruled out. To a small extent, an otherwise unique restriction fragment may suffer shear and be split either before or after enzyme attack. Furthermore, in any procedure to isolate individual members of the highly heterogeneous weight distribution that exists in restricted populations, considerable apparent error may arise, as the slope of activity versus size is very steep (Cato and Guild, 1968). Very small quantities of higher molecular weight material, originating from incomplete digestion products (Fig. 4) or from loss of gel linearity due to sample overload, may give rise to spurious results. The work of Goodgal and Gromkova (1973) using sucrose gradient separation of Hin and Hae (Table 2) generated H. influenzae

fragments shows that while markers exhibited a size peak of activity, there was still some (although small on a percent basis) present above and below the peak position. This "trailing" is unavoidable for technical reasons and arises from a continuous distribution of specific activity versus size. This is important to keep in mind since it can lead to false conclusions. The situation is more complex than that seen with shearing where size reduction is a continuous process. The appearance of biological activity at a weight that is higher than that of the major activity peak leads one to ask whether this is undigested material or is there in fact a "core" of restriction resistant material, perhaps generated by modification or mutation of recognition sequences. The latter argument would require that a population of B. subtilis cells, all arisen from a common progenitor, either have several copies of each gene of which a portion is modified to resist restriction, or that a monoploid population display a heterogeneous or leaky modification phenotype. Some recognition sequences flanking some genes, in some members of the population, would be required to be restriction resistant. It is highly unlikely, although interesting to speculate, that a cell

population would possess this complex mechanism to discriminate gene domains, specific binding sites and still maintain that same pattern in DNA's derived from many independently isolated clones. It is more likely then that the conditions of molecular sieving have artifacts built in since restriction fragment formation is a finite, length defined process, once all sites have been cleaved. This tends to be supported by Figure 7 and the kinetic profiles presented by different genes in Figure 15. The data of Harris-Warrick et al. (1975) would seem then to be a schematic representation of activity-peak versus size, much the same as seen in Figure 19 (legend). The need for large amounts of DNA, due to the intrinsically low colony forming activity of low RTA genes, and the low recoveries often experienced make this histogram presentation necessary. Activity is calculated as a percent of all activity recovered from the profile. The peak class is then the best reflection of the size member responsible for the population RTA of each specific locus. Molecular weights examined in this manner are very approximate but show obvious correlation with activity. Eco RI treated W23 DNA loci trpC2, trpC2-hisB2, leu-1 and thr-5 show this

correlation, as does Eco RI treated 3610 loci trpC2 and thr-5, and Hind III digested W23 loci thr-5 and leu-1. This profile would not be expected if a constant (reflected in RTA) fraction of the genes were "escaping". For this situation the population weight would fall but all activities would be expected to cluster about the "core" size and exhibit specific activities reflecting the gene's proportional representation in the "core".

9. Repair of Restricted DNA

If one argues that a particular RTA value is due to the inherent transforming efficiency of a cleavage fragment, then ligation of that product to higher molecular weight should alter the efficiency. As long as site cleavage is not within the prototrophic sequence, size increase via covalent rejoining and extension of fragments should cause biological activity to rise. A "gene" sequence cut intragenically might be expected to be effectively removed from repair options since the probability that any two particular fragments will specifically hydrogen bond at their tetranucleotide tails (generated by Eco RI or Hind III) is extremely small. Moreover, the probabilities become vanishing for successful restoration of native marker activities when one realizes that a fragment

generated by the above enzymes has identical ends and can therefore reanneal with another fragment in 4 unique orientations. However, as mutant marker rescue is determined by the integration of rather large sections of DNA (SSF) (Dubnau, 1972c) (approx. 2×10^6 ss.), auxotrophic areas (either point or deletion mutants) of recipient DNA could recombine with any suitable sub-cistron restriction fragment or overlapping set of prototrophic fragments encompassing the mutant allele. The relatively large en bloc transfer of transforming DNA from extracellular solution to intracellular integration (see introduction) makes it possible that sub-cistron sequences will be variably compatible with auxotroph rescue depending on the location of the lesion relative to the flanking restriction scissions in the prototrophic donor DNA. An uncut gene, on the other hand, will be informationally intact and subject to the synaptic constraints imposed by the biological system. Integration dependent upon size, relative to that required for "competent" uptake and expression may be evoked.

As shown in Table 14, biological activity rises noticeably for all the genes studied. Based on colony counts, DNA fragments repaired by T4 induced ligase

tend to approach a common transformation frequency. The recovery ratio is however distinctly inverted; high RTA survivors recover the least and low activity survivors recover the most on a relative basis following ligation. The tendency towards a common product level (CFU/ml) may reflect a change in affinity of the ligase for its progressively growing substrate and the diminishing net concentration of free ends. The Eco RI and Hind III "low" representative loci (Tables 3, 4, 14) show a more dramatic rise following ligation than those of the moderate RTA group and much more than those of the high RTA group. We were most interested in studying this gene specific response since it indicated further that gene activities were segregated onto specific size-ordered elements. Classification of survival would be of great help later when attempts were made to clone specific gene functions or overlapping sets of fragment. The repair ratios (RTA-L/RTA-R) for lys-3 and thr-5 could be shown to rise to as high as 113 and 73 respectively following Eco RI restriction and ATP dependent ligase repair. The trpC2, leu-1, argA2, metB10 activities gave fold increases of approximately 2, 4, 13, 9 respectively. When Hind III was employed and repaired by ligation, the

trpC2 element gave a different result consistent with its assumed greatly diminished size yet still intact coding integrity (at least as far as auxotroph lesion rescuing sequences are concerned). The putative smaller fragments appear to be better substrates for ligation and thus exhibit greater fold recovery increases. The same is seen for thr-5, a marker generating greatly improved colony counts after Eco RI cleavage and ligation and a lesser fold increase following Hind III digestion. That is, the coding elements responsible for trpC2 or thr-5 prototroph generation have different relationships to Hind III and Eco RI palindromes respectively. These flanking recognition sites determine the length of the intervening DNA backbone, and the use of several nucleases allows relative visualization of overlapping fragments sets defining gene domains.

As shown in Figure 14, a ligated sample of restriction nuclease generated DNA has a distribution of molecular weights that is shifted towards the higher size range. Obviously, each collision of nuclease generated "sticky ends" is as probable as any other and therefore the actual ligation event is inherently random. In general, size increase accomplished by this procedure may produce recovery

ratios of varying level depending on the limit-core digest activity. In this figure the ligated product has an average M.W. of 12×10^6 compared to its Eco RI restricted 6×10^6 size. The ligated material is still heterogeneous however. This is not the case with restricted and ligated plasmid where discrete size products are recognizable. Cleavage is an all or nothing event with regard to size loss but ligation is not, so long as free "ends" are still available. Each gene activity of a given sample satisfies a multi-auxotrophic requirement in a given recipient in a very characteristic manner. For example, in BD-29 argA2 and leu-1 Eco RI survivals are low and moderate respectively but ligation restoration of activity is high and low respectively (as measured by RTA L/R). Similarly in BR151, Eco RI digestion of W23 DNA yields trpC2, metB10 and lys-3 activities that are moderate, low and very low compared to each other but ligation generates low, moderate and high recoveries respectively. This result is survival-specific, since Hind III digestion of W23 DNA gives very low trpC2 and high thr-5 survival while ligation generates moderate and low marker restoration ratios respectively. Mechanically sheared DNA (Table 14) shows

little marker improvement with the conditions employed here since covalent breakage is non-specific (Stewart, 1969) with regard to position and resultant molecular termini, which the ligase has no specificity towards.

Both Eco RI and Hind III produce self-complementary 4 nucleotide single strand "ends" from a canonical hexanucleotide recognition site (Table 2). The collision frequency between termini will determine how efficient the concatameric joining of DNA fragments will be. Furthermore, depending upon how long any individual fragment is, there is a finite possibility of that fragment colliding with its own complementary end and rejoining to form a covalently closed circle. Thus, the situation is more complex than a mere size increase from a physical point of view and extremely complex from a genetic standpoint. With a given molar concentration of DNA molecules, restriction by Eco RI or Hind III will generate many fragments of highly heterogeneous size and representation. One may calculate the number of "ends" revealed in a homogeneous phage system (Dugaiczky et al., 1975) knowing the molar concentration of the sample fragment. For both Eco RI and Hind III Bacillus chromosomal digests, this

value (termed i) is not directly applicable since many size fragments of unknown concentration are formed. That is, no one particular fragment can be directly identified and quantitated on a molar basis (unlike a phage system where individual fragment products can be isolated and measured). In the abstract sense however, one may visualize an individual restriction fragment within the population as having a definable size and molar concentration. At the same time, another value (termed j) is operating. This factor is the local concentration of any given end, in the neighborhood or rotational domain of the other end, on the same fragment. The value (j) is a function of length and measures the tendency of a fragment to fold back and circularize with itself whereas (i) measures the tendency to concatamerization in the processive linear sense. Circular products will be favored when $j \gg i$, and when $j = i$ equal frequencies of linear n -mers and circles will be expected; when $i > j$, circles will be disfavored. Simply, (i) is a function of total fragment concentration while (j) is independent of this and varies only with contour length (Dugaiczky *et al.*, 1975). Physically, a ligation mixture will contain a myriad of products in various stages of

reaction. Genetically, the situation is even more intriguing since restriction generates discrete size populations, and the experimental evidence presented here indicates that gene complementing activities may be assigned to relative size subclasses. Since a given genetic function may reside on a homogeneous fragment length (as opposed to shearing) following restriction, each locus will recover activity gene specifically depending upon its (i) value and unique (j) function. Given that fragments of assigned function are equally represented on a molar basis (no reiteration) then (i) values for any selected fragment become equivalent to any other fragment at a given concentration or molar basis. The (j) values remain unique and will independently determine the ratio j/i for a selected locus rather than for the population in general. Since (j) is related to contour length (Dugaiczky et al., 1975) small-fragment activity will experience a depressed j/i ratio compared to a larger restriction fragment where j/i will be relatively higher due to an increase in the (j) function. The competition between n-mer concatenation and circularization will favor extension as j/i decreases. Thus, low activity members (e.g. argA2 cleaved with Eco RI and trpC2

with Hind III) exhibit greater fold restoration of biological activity than higher survivors due to their unique locations on small size subsets.

It was necessary to determine whether or not any circular products did indeed accumulate in the ligation reaction of Table 14 and whether these were responsible in any way for the observed data. Figure 20 shows a cesium chloride-ethidium bromide gradient profile of a representative ligation mixture. This procedure (Radloff et al., 1967) causes linear molecules to rotate, perpendicular to their central axis, as intercalation drives unwinding. Such an alteration causes molecules free to rotate to assume a less dense position in a salt gradient compared to covalently closed circular species not free to unwind and extend. These molecules assume a more dense position in the gradient. It is possible to calculate the integrated area under each curve and measure the amounts of circular and linear product generated by ligation. A restricted sample, unligated, of course contains no such circular species. The more dense products form between 8% and 20% of the total yield when Eco RI cleaved W23 DNA (20 µg/ml) is ligated with 2.0 Units/ml of T4 induced DNA ligase (methods). Ligation was carried

out at 4°C for 18 hours to favor hydrogen bonding of the enzyme generated ends (T_m 5 - 6°C) (Mertz and Davis, 1972). Hind III ligation involves slightly more stable hydrogen bonds (dG·dC). In any reaction, linear monomers of a size class will disappear with time as dimers etc. or circles form. At the above concentration, n-mers will be favored initially until the total concentration of ends declines sufficiently to favor a higher j/i ratio for the population (or unique genetic fragment class) when circularization will be favored. Polymeric closed structures, once formed, are no longer substrates for further ligation and represent terminal products. The rest of the population will continue engaging in thermal collision until, theoretically, all ends are engaged. It is difficult to otherwise demonstrate the circular products due to their low representation and sensitivity but their sizes do vary considerably in each experiment. The smallest size fragment not shown to circularize was the Ø 80 Eco RI 1.4×10^5 dalton fragment (225 bp) (Dugaiczky et al., 1975) which is most probably subject to the steric hindrance of a minimum rigid length.

We wished to know whether or not gene representation in the circular population was at all biased.

To determine which ligation product group, the circular or linear n-mers generated the observed recovery ratios, we isolated DNA ligation products from a density gradient (Fig. 20) and analyzed their genetic capacity. Table 15 shows such an experiment. All recovered activity appears in the less dense linear concatameric population. No gene bias is seen regarding which activity comes to reside in the more dense closed circular (LB) region on the basis of comparative colony counts. As these transformations were done at a normalized DNA concentration of approximately 1 $\mu\text{g/ml}$, the possibility arises the B. subtilis may discriminate against circular topological forms as opposed to linear structures. The circular forms herein created by ligation and quasi-cloning are not themselves independent replicons, subject to orders of dependence greater than unity as is the case for transfection. It seems reasonable to postulate that while all genes examined have a statistical likelihood of being represented in a circular ligation product, their transforming efficiencies are lower due to molecular weight-reducing scissions at the cell surface (DSF production for instance) where the absence of a native "end" may allow unusually frequent

covalent cleavage prior to uptake and eclipse (see introduction). This "uncoupling" of DSF generating cleavages and concomitant processing via the transforming cascade may cause these relatively sensitive circular structures to exhibit lowered frequencies of transformation because of interruption of coding spans and inordinate size reduction. This is obviously speculation as the actual M.W. of the CCC fraction is difficult to measure, being an assortment of heterogeneously sized molecules varying from experiment to experiment. This is not unexpected as ligation of total populations is itself inherently random. The CsCl-ethidium bromide purification and the DNA concentration normalization indicate, however, that these molecules do not contribute to the observed recovery ratio phenomena (Table 15, Fig. 20) for the mutant loci studied.

10. Effects on Linkage

The ability of W23 DNA to co-transform the double auxotrophs SB-25 (Table 11) and BD-29 following Eco RI digestion is shown in Figure 18. At 1 µg of DNA, W23 shows a native co-transformation of trpC2-hisB2 of approximately 65% prior to Eco RI restriction and about 3.0% for the co-transfer of argA2-leu-1, reflecting the greater map distance spanned between these two gene sets respectively.

Following EcoRI action these values drop to 40-50% of native for the aromatic linkage and precipitously approach zero for argA2-leu-1 (Table 11). This places an Eco RI palindrome between arg-leu in W23 and places one or more to the exterior flanks of the trp-his cluster (Fig. 18). A Hind III site is, however, located extremely near or within the trpC2 region (Table 16) as trpC2-hisB2 cotransfer is lost. The trpE-hisB linkage is also affected by specific cleavage. In strain W23, trpE activity has a Hind III RTA of 4% while hisB is greatly diminished (0.2%). Co-transfer is not seen in this case either. These data are presented in Tables 11 and 16. Ligation restores biological activity to the respective trp and his Hind III generated fragments but fails to restore a linkage relationship (Table 14) as predicted by the vanishingly small probability of any two specific fragments reannealing to restore a native configuration. Eco RI treated samples, however, show the expected recovery of cotransfer activity as expected for an intact trpE-hisB or trpC-hisB region, as any randomly generated size increase causes an increase in net biological activity. Note however, that strain 3610 fails to show a dramatic loss of activity within members of the

trp operon (Table 16) (as in trpC of W23) which indicates that loss of co-transfer frequency after Hind III cleavage is from a process somewhat different in relative topography from that seen in strain W23 (or 168). Diagrammatically, this may be "mapped" as in Figure 32a&b.

That host specified marker specific restriction is not operating, in any apparent sense, is shown by Table 13 where strain RM125 arg15, leu-8, r⁻m⁻ (Bsu) (Table 2) is used as both a DNA donor and recipient. This cell lacks any Bsu I specific base modification and fails to produce any identified restriction nuclease activity (Tanaka and Sakaguchi, 1978). This, along with the configurational constraints imposed (see introduction) on any restriction of incoming single strand DNA make it more certain that the gene catalogues faithfully reflect restriction fragment efficiencies and not the superimposition of recipient restriction processes. DNA from different B. subtilis representatives (e.g. 168, W23, 3610) serve as donors for prototroph generation in strain RM125 and show similar relative efficiency values for argA2 and leu-8 loci (see Tables 3, 6) in terms of RTA catalogue hierarchy. The strain BD-29 (argA2, leu-1) gives similar results. As a donor, the DNA of RM125 is much the

same as that of W23 in restoring prototrophic activity to recipient BD-170 (trpC2, thr-5). Thus, obvious restriction/modification events do not seem to be taking place in these intragenus (yet inter-strain) exchanges.

11. Plasmid pUB110 as a Cloning Vehicle

In the course of these studies, several antibiotic resistance plasmids became available which were capable of autonomous replication in B. subtilis (Ehrlich, 1977). These plasmids, originally resident in Staphylococcus aureus, were introduced into competent Bacillus sp. by transformation and transduction (via PBS1 or SP10) and appropriately expressed their respective antibiotic resistance determinants. One of these plasmids, pUB110 (Ehrlich, 1978; Keggin et al., 1978) codes for a 37,000 M.W. inactivating-Kanamycin aminoglycoside nucleotidyl transferase activity located near the Bgl II site and may be stably maintained extra-chromosomally in B. subtilis cells (Gryczan et al., 1980). It has been demonstrated that this 3.0 megadalton plasmid (Fig. 24) replicates in both recE proficient and recE4 deficient recipients (Dubnau and Cirigliano, 1974) to a level of approximately 50 plasmid copies per chromosome complement and,

although not under true "relaxed" replication control, may be amplified under certain conditions to several hundred copies per cell (Shivakumar and Dubnau, 1978). The pUB110 replicon possesses a single Eco RI sensitive palindrome (see Fig. 24) at about 82° on a 100° circular map with a single Bam HI site located at 0° . The distance between the Eco RI and Bam HI sites is 0.52 Mdal. (Gzyczan et al., 1978).

The original work of Harris-Warrick et al. (1975) indicated that restriction cleavage directed against a complex genome could give information relevant to gene localization and purification. We have been interested for some time in the observed chemical asymmetry found in bacterial DNA (Rudner et al., 1968), i.e. the asymmetric distribution of pyrimidine isostichs (Rudner et al., 1972) in the genome, the shift to specific L strand transcription events (strand-switching) during morphogenesis (Margulies et al., 1978) and the presence of intra-strand self-complementary sequences that are in part transcribed (Galloway and Rudner, 1979). This led us to "comb" the chromosomal domains of B. subtilis for gene representatives which might be amenable to isolation and amplification. The cloning of

such regions, by selecting prototrophic transformants, required substantial knowledge of the response of the genome to various cleavage specificities. Once purified, a given gene might be analyzed for its hybridization to H or L strand specific transcripts and for the presence of unique promoter recognition sequences. The recent isolation of a discrete sporulation specific RNA species, and the identification of a 0.4 kb DNA fragment under morphogenetic control in the purA-cysA region (Segall and Losick, 1977; Haldenwang et al., 1980) has provided the first opportunity to confirm coding specificity with L or H specific transcription probes. The isolation of other genome regions, and the determination of strand bias during transcription is of vital importance in gene recognition and modulation.

Plasmid pUB110 seemed ideal for approaching the above task, and we began to pursue the isolation and eventual cloning of members of our restriction catalogue survey. Successful isolation and amplification of homogeneous genome fragments demands that they be under the controlled replication of a selectable vector or vehicle, and that they be prevented from engaging in recombinational integration with the host

genome. The use of selectable antibiotic resistance vehicles such as plasmid pUB110 ($\text{km}^{\text{r}}/\text{neo}^{\text{r}}$) meets the first requirement. The second demand is accomplished under conditions that eliminate synapsis and integration. The *B. subtilis* *recE*⁴ lesion (Dubnau and Cirigliano, 1974b) at (Fig. 1) map position 150° (Henner and Hoch, 1980) causes the loss of an ATP-dependent nuclease and DRC fails to organize. As a result, chromosomal transformation and transduction are eliminated while plasmid transformation (Contente and Dubnau, 1978) and replication still occur. Duplex molecules integrated into the plasmid structure replicate in unison under extrachromosomal direction in a *recE* host. In addition, if the integrated material carried by the plasmid chimera is sufficiently heterologous with the *B. subtilis* genome, it may be stably maintained and replicated extrachromosomally in a *rec*⁺ proficient host. Such a condition is met by DNA from *B. subtilis* relatives such as *B. pumilus*, *B. licheniformis* and *B. amyloliquefaciens* which share a similar arrangement of chromosomal genes (Lovett and Young, 1969; 1971) and limited sequence homology with *B. subtilis* W23 or 168 strains (21% and 18% respectively for *B. pumilus*) (Young and

Wilson, 1972). These organisms transform B. subtilis to prototrophy at vanishingly low frequency except for certain "conserved" loci regions (e.g. rif, str) located near evolutionarily constant genome "cores" (Chilton and McCarthy, 1969; Dubnau et al., 1969; Wilson and Young, 1972). Harris-Warrick and Lederberg (1978) demonstrate that the inability of loci outside this constant core (see introduction) to transform B. subtilis is due to sequence mismatch and not restriction action.

Plasmid pUB110 was isolated from strain BD366 (see Materials and Methods) by dye-buoyant density partition in ethidium bromide-cesium chloride gradients (Radloff et al., 1967) following a cleared lysate extraction of B. subtilis cells selected on media containing the antibiotic kanamycin (Gryczan et al., 1978). A profile of a representative gradient isolation is given in Figure 21. Using tritium labeled thymidine in the presence of 2' deoxyadenosine (Materials and Methods), the covalently closed circular structures segregate to a more dense position in the salt gradient as they resist the torsional unwinding forces induced by the planar intercalative dye. Molecules which are linear such as chromosomal fragments and nicked circular plasmid

are free to rotate about a single covalent bond as intercalation progresses and assume a relatively less dense configuration. This is a classical technique for demonstrating the presence of plasmid replicons in cells and was utilized here also to demonstrate the presence of interesting chromosomal ligation products (Fig. 20). Cells devoid of circular elements will fail to show this more dense circular structure. This is seen when strain BD-170 (trpC2, thr-5) is analyzed versus its plasmid bearing (Fig. 21) isogenic counterpart BD-366 (trpC2, thr-5, pUB110). Unlike circular in vitro ligation products isolated in such a system, the plasmid molecules are independently replicating and may be present in the cell in varying amounts or "copies" relative to the genome of the host cell. Such a value is termed the copy number and may be calculated from the ratio of the molecular weight of chromosomal DNA relative to that of the plasmid genome times the fraction of the total DNA (from uncleared lysates) in the more dense CCC region. In "stringently" replicating plasmid systems this number is small as the plasmid and chromosome replicate in approximate unison. In "relaxed" systems the plasmid replicon multiplies itself hundreds of

times more frequently than that of the host. The latter may be amplified to thousands of plasmid copies per cell if host DNA replication is selectively inhibited. In the case of pUB110, the plasmid normally exists at 50 copies/cell (Keggins *et al.*, 1978) and is not readily amplifiable although Shivakumar and Dubnau (1978) have demonstrated limited amplification to 260 copies/cell using hydroxyurea to specifically interfere with host genome synthesis. In the present study, plasmid pUB110 is consistently found (Fig. 21) to be present at between 100 and 150 copies when allowed to replicate in BD-170 (trpC2, thr-5) or BR-151 (trpC2, metB10, lys-3). As shown in Figure 4 the isolated CCC DNA is present after handling as a mixture of a predominant class of high mobility superhelical circles, a small quantity of fully linear monomers and a fraction of OC or once-nicked relaxed circles. In some native plasmid preparations a family of higher molecular weight bands can be seen which represent polymeric, tandem repeats of the basic 3.0 Md. structure in circular or linear array. Cleavage of this family by a nuclease like Eco RI generates one band of high intensity at the position of the initial linear species with the

concomitant loss of the other bands. Cleaving pUB110 at its single EcoRI sequence followed by ligation gives rise to an even more complex electrophoretic pattern (Fig. 14) as n-mer tandem linear and circular species develop. In many cases, while cleavage destroys biological activity, or as shown by Contente and Dubnau (1978) even a single strand nick, biological activity can often be returned to above 100% following ligation as these n-mer circular products are created (Table 14). Perhaps this mimics the in vivo replicating molecule pool. Interestingly, the concatameric members are responsible in great part for the observed biological activity of unfractionated plasmid populations such as those in the dense region of Figure 21 (Mottes et al., 1979). This has critical implications for successful cloning in B. subtilis with this vector, as multimers must be favored; this is a reflection of the pUB110 replicating and infecting pool (Gryczan et al., 1980).

To prepare potential hybrid molecules or chimeras, pUB110 was cleaved with EcoRI to linearize and provide cohesive termini. A five-fold excess of similarly restricted chromosomal DNA from B. subtilis, B. pumilus and B. licheniformis

was added to separate reactions and, after thermally inactivating the restriction nuclease at 65°C for 5 minutes, the mixture was annealed at 5-10°C for 18 hours with approximately 1 Weiss Unit of T4 induced polynucleotide ligase per 10 µg DNA (Materials and Methods). The low temperatures, while not being optimum for enzymatic activity, stabilize the (AATT) cohesive ends which have a T_m of only 5-6°C (Mertz and Davis, 1972). The ligated mixture (L-M) was inactivated and sterilized by the addition of chloroform and added to competent B. subtilis auxotrophic recipients. Table 17 shows the results of one such experiment employing B. pumilus donor sequences.

12. Cloning of an Eco RI Tryptophan Fragment

The ligation reaction was efficient at restoring transforming activity to the vector and characteristically to a control W23 chromosomal digest. The activity of the plasmid rises to 106% after ligation as presumptive high efficiency circular concatamers are generated in the original native population, participate in net sealing (Mottes et al., 1979; Fig. 14, Table 14). The trp prototrophic locus, donated by Eco RI digested W23, rises from 1.8% RTA to 8.9% in auxotroph BR151 following ligation. Similarly, the metB10 and

lys-3 rescuing loci rise from 0.4% to 3.9 and 0.05 to 3.2%, respectively. In strain BD170, the trpC2 and thr-5 loci give ligation recoveries of 2.1% to 4.3% and 0.1% to 5.2% respectively for W23 prototrophic DNA as donor (see Table 14). A recE4 deficient recipient BD224 (Table 1) was incapable of generating prototrophic recombinants in this experiment although it was transformable with pUB110 for km^r to a level very similar to that of the rec⁺ hosts. DNA from B. pumilus was also incapable of generating prototrophic colonies for the reasons described. However, trp⁺ colonies did appear from plasmid-chromosome co-ligated mixtures after 48 hours incubation in recipients BD170 and BR151 as did many km^r transformants. All trp⁺ colonies were tested directly by replica plating to media containing 10 µg/ml kanamycin sulfate for drug resistance linkage. Several hundred of the primary km^r colonies were picked from the agar overlay and established on a master nutrient plate with sterile toothpicks and replica plated for tryptophan independence. The lys-3 and metB10 prototrophs failed to show any drug resistance linkage in strain BR151 and these colonies are presumed due to chromosomal contamination within the plasmid population,

originating from the native pUB110 host BD366 (trpC2, thr-5) which is met⁺, lys⁺. In addition, these pseudo-recombinants failed to demonstrate the presence of circular elements and therefore must be chromosomal transformants. This is verified by the fact that isolated high molecular weight DNA is newly endowed with prototrophy for these genes in a subsequent transformation assay. However, a few hybrid trp⁺ clones were found to be stably maintained, exhibiting total linkage between the trp⁺ character and km^r from the ligated mixture containing B. pumilus RUB502 DNA. One of these BR151 transformants was designated pRR-6 and grown for plasmid isolation as described in Materials and Methods (Table 17).

It was not immediately clear as to why so many initial recombinants were scored on primary selection and failed to faithfully replica plate on secondary plating. It may be that the putative cloned hybrid is unstable and fails to efficiently partition at cell division or efficiently initiate transcription and is rapidly lost by segregation. The direction of selection pressure may itself play a role in this phenomenon as the initial isolation, while not statistically meaningful, was from a cell

grown without drug pressure in the absence of tryptophan. Covalently closed circular (CCC) DNA was isolated from pRR-6 grown under semi-relaxed nutritional (VY) conditions (veal infusion and kanamycin) or more stringent nutritional selection conditions in minimal media (mm) containing antibiotic (see Materials). In Table 18, CCC DNA was isolated and used to transform BD224 (trpC2, thr-5, recE4) and its isogenic counterpart strain BD170 (trpC2, thr-5). Resultant colonies (i.e. km^r or trp⁺) were checked for the linked phenotype and the ratios in parentheses represent these relationships. In the recE4 recipient, plasmid transformation with pRR-6 occurred at a frequency of 10-50% that of the isogenic rec⁺ cell. There was a consistent disparity in the number of trp⁺ transformants versus km^r in the recE4 recipient. This difference may be due to the selection regimen because transformation of drug resistance is done after a 90 minute expression lag period while prototrophs are selected without this phenotype delay (Materials). Under different growth regimes, there was a rapid segregation of the characters when initial growth was in less stringent (VY) conditions and plating selection was for km^r. As shown, among the km^r colonies from VY, the trp⁺

gene was present 40% and 54% respectively in recE4 and rec⁺ strains. Segregational loss may be due to the imposition of pressures at the transcriptional level in that initial selection in VY favors vector replication and drug inactivating synthesis as exogenous tryptophan is present. Intra-molecular re-combinational events may select against an inserted "passenger" molecule and when selection is for drug survival the added burden of trp⁺ specifying sequence may be cast off. However, when trp⁺ colonies are selected, they are all of necessity km^r linked since this is their only means of replication. In conditions of greater pressure (MM), selection for km^r and trp⁺ is simultaneous and transcriptional events may be selected such that initiation of trp⁺ information is through a more efficient and fidelic B. pumilus promoter rather than read through from pUB110 promoters. The decline to approximately 64% of km^r among trp⁺ in the recE4 host may be due to an inherent instability in this background as already seen in the failure to generate a primary recombinant trp⁺ clone in Table 17. Tanaka (1979) has demonstrated that in a recE4 background the ability to transform a leu⁻ cell to prototrophy with a chimeric plasmid was reduced if the cell had a

restricting phenotype whereas if the cell were rec⁺ the colony efficiency was unaltered regardless of the restriction phenotype. This may account for the initial plating failure on BD224 since double strand substrates for restriction are available as opposed to the single-strand structure selected via a chromosomal transformation pathway (see introduction) which is insensitive to restriction. Second generation growth on BD224 may be possible, in part, because of a modification marking imposed by primary growth on BD170 (this would imply an incomplete strain isogenicity for these cells). Incomplete linkage may also be due to instability of the cloned structure because of disturbances in concatamer formation (Gryczan et al., 1980) and intra- and inter-molecular recombination events (e.g. plasmid rescue) in the cellular plasmid pool in a recE4 background. Selection for trp⁺ in recE4 but not rec⁺ may induce rearrangements not as efficient in generating km^r activity as those seen with pUB110 primary expression (Mottes et al., 1979). Figure 22 is a CsCl-ethidium bromide gradient of tritiated pRR-6 chimera from an uncleared total lysate. By integrating the curve areas and using a value of 2×10^9 d. for the B. subtilis genome (Introduction) and 5.4×10^6 d.

(Figure 23, Table 20) for the recombinant clone, a copy number of 30 molecules per chromosome is achieved (Fig. 22). This value is significantly below the copy number, approx. 100/genome (Fig. 22), of the vector pUB110. Both analyses were performed from minimal media grown cells and it is clear that even under this efficient phenotypic pressure there are many fewer copies of the larger recombinant than the vector. Transcriptional and configurational difficulties together with the possible inability of the system to replicate a larger circular structure (3×10^6 d. compared to 5.4×10^6) may contribute to the observed copy and plating bias.

13. Genetic Content of the Cloned *B. pumilus* Fragment

A similar cloned tryptophan activity from *B. pumilus* NRRL B-3275 (Lovett and Young, 1969) was reported by Keggin et al. (1978). Although physically unmapped, these investigations demonstrated that a recombinant pUB110 molecule of 5×10^6 d. could complement trp E, D, C, F activities but not aroB or trpB, A or hisB2 (see Fig. 1). They additionally generated recombinants from *B. licheniformis* that complement trp C, F, B, A, and from *B. subtilis* 168 that complement trp C and F. This

represented the first time nutritional markers had been transferred between these two species (Young and Wilson, 1972). Recently, a report from Gryczan et al. (1980), employing a somewhat different approach, exploited the demand for concatameric replicons by favoring recombination with a resident plasmid via intracellular "marker rescue" of damaged molecules.

Our gene catalogue of relative transforming activity (RTA) following specific cleavage has shown that the trp region (Tables 3, 4, 6, 7) exhibits interesting responses and that the operon itself and its linkages (Tables 11, 16) can be probed in a crude but meaningful way. For instance, co-transfer of loci trpC2-hisB2 and trpE46-hisB2 as well as individual operon constituent survivals, indicate that strain W23 (Fig. 32) is organized such that the extreme loci trpE and hisB are flanked by Eco RI palindromes. Hind III cleavage drastically depresses the RTA of trpC and hisB as well as aroE. In addition, cotransfer of trpE-hisB or trpC-hisB is totally eliminated. In strain 3610 the RTA survival values point towards a much larger Eco RI flanked domain. Hind III cleavage in this case seems to be occurring in the

vicinity of hisB as opposed to the drastic trpC locus effects seen in W23. Thus, the cotransfer loss upon Hind III cleavage is via a slightly different topography. The 168 strains exhibit (Tables 10, 12) RTA values approximating those of W23, albeit with strain variations, for Eco RI cleavage within the tryptophan operon, and are consistent with both 3610 and W23 for Hind III elimination of cotransfer events. The strains behave similarly for loci argA2 and leu-1 (Table 11, Fig. 18). Our hopes were to utilize our cloned molecule, isolated from B. pumilus RUB 502, and perhaps identical to that of Keggins et al. (1978), as a model of Bacillus trp gene organization and as a probe of the W23, 168 and 3610 genomes. Our first steps were to elucidate the complementing activities of our pRR-6. Table 19 presents such data. Complementation is seen for trpE, D, C, F but not for aroB nor hisB in the B. subtilis recipients. These data should be compared with that of Table 16 showing the ability of restricted W23 or 3610 DNA to complement the various members of the operon cluster. Here the entire W23 and 3610 operons seem intact following Eco RI cleavage, the same digestion applied to the original

B. pumilus DNA that generated the recombinant clone. To date only pBR-6 and the Keggins et al. (1978) plasmid contain trpE. The latter seems to be maintained at 50 copies per cell although direct copy number calculations were not made available. The cellular enzyme levels of these amplified genes are relatively low (Keggins et al., 1979); similar "slow growth" was reported for a B. licheniformis trp D, C, F, B, A insert (Keggins et al., 1978) and by Gryczan et al. (1980) where several days were required for colony growth. Our clone, however, had a growth rate in medium lacking tryptophan that was identical to that of a prototrophic isogenic trp⁺ strain.

14. Map of the Cloned Insert

Figure 23 shows the electrophoresis gel pattern of restricted pRR-6. As seen, plasmid pUB110, compared to standard Eco RI and Hind III fragments of phage λ and ϕ 105 (Tables 8, 9; Fig. 5) has a single Eco RI site to generate a 3.0 Mdal. linear molecule. No Hind III site is present as shown by Gryczan et al. (1978) (Figs. 9, 24). The B. pumilus cloned fragment however introduces a new Hind III site, as cleavage generates a single 5.4×10^6 d. molecule. This was not unexpected since our catalogue had indicated

that Hind III cleavage was within the W23 trp operon (Tables 4, 7) and in proximity, at least, to that of 3610. Although not generally homologous, the aromatic operons of members of the genus Bacillus might be sequence conserved. The plasmid was also cleaved once by Bcl I (Table 2) and twice by Sac I (L. Enea, unpublished observations). These sites are not found in pUB110. As shown in Table 20, various size fragments are produced by pRR-6 following enzymatic cleavage. Utilizing the pUB110 map of Gryczan et al. (1978) as a guide, an additional Bgl II and approximately 6 Hpa II and Hae III sites are noted. Clearly, the Eco RI B. pumilus insert is positioned within the original vector palindrome at 82° (Figs. 14, 24, 25); re-cutting with this enzyme liberates two fragments 3.0×10^6 and 2.4×10^6 d., respectively. Hind III liberates a single 5.4 Mdal. piece consistent with the assignment of a Hind III palindrome to the trp fragment. Three fragments are found after Eco RI/Hind III double digestion of pRR-6. One fragment represents an Eco RI pUB110 molecule while the other two, 1.70 and 0.70, represent molecules of B. pumilus DNA flanked by cleaved Eco-Hind recognition sequences. At present, work in progress is directed towards a detailed map of these fragments with particular attention to the

trpE region of the operon presumed to lie to one end of our fragment. A 0.3 Md. Eco RI-Bcl I (Tables 2 and 20; Fig. 23) is particularly important as our tentative orientation within pRR-6 (Fig. 25) indicates that this may be the leader-promoter region. RNA polymerase binding capacity and direct sequencing could be employed to clarify this.

The novel Hind III site of our plasmid makes it potentially useful as a second generation cloning vehicle for the specific insertion of Hind III digested sequences. The thr-5 fragment survives well (Tables 4, 3, 7, 6) following Hind III as opposed to Eco RI digestion and is a good candidate for selection. Since this study, Keggins et al. (1979) have inferred a Hind III site in close proximity to trpC based upon the polar loss of enzyme production when cells were infected with recombinant plasmids containing foreign inserts in their Hind III sensitive sites. Our map, assuming identity of the B. pumilus sequences, clearly demonstrates this physically.

To orient the B. pumilus 2.4 Md. insert relative to the plasmid map of pUB110 at its Eco RI site, plasmid pBD64 is currently being used (Rudner, in progress). Obtained from D. Dubnau (Gryczan et al., 1980), this is a constructed 3.2 Mdal. km^r and

chloramphenicol (cm^R) hybrid (which has undergone a spontaneous deletion) of pUB110 (km^R) and pCM194 (cm^R) (2.0 Mdal. M.W.). Both pUB110 and pBD64 contain a single Bgl II site located in the km^R specifying region (Fig. 24). A second Bgl II site is assigned by our investigations to the cloned Bacillus insert. Note that Table 20 reveals the presence of 2.4 Mdal., and 3.0 Mdal. Bgl II cleavage products from pRR-6. These fragments are generated as depicted in Figures 24 and 25. Digestion of pBD64 and pRR-6 with Bgl II, followed by co-ligation, generates second generation trp⁺ colonies that are cm^R km^S when selected. Although this has not been studied extensively, to date the only prototrophic activities we have recovered linked to the cm^R recombinant are trpE and trpD. Our proposal is consistent with these experiments and places the orientation of the cloned B. pumilus trp operon as trp E, D, C, F clockwise relative to pUB110 tentatively as in Figures 23, 25. The growth rate of the plasmid is consistent with a system in which an efficient promoter (trp E?) is operating. Whether in fact the trp E and D activities lie upon the 3.0 Mdal. or the 2.4 Mdal. Bgl II fragments of this new recombinant can only be confirmed by further mapping and cloning fragments specifically into pBD64

or some other vector and isolating surviving activity as above. Similarly, the 1.62 Mdal. Eco RI-Bgl II (Fig. 25) may be pursued. As we have not attempted this (work in progress) from isolated pRR-6 Bgl II fragments, nor mapped our putative trp E, D, cm^R clone we can only speculate schematically as to the precise operon orientation relative to the pUB110 clockwise map. Either orientation may actually exist. The fact that our plasmid subclone seems to grow readily would imply at least that transcription within the vector pUB110 or pBD64 and the trp E → F functions are compatible and probably in the same direction (Figs. 24 and 25).

15. Nick-translation of the B. pumilus trp Fragment

A correlation was made between the B. pumilus chromosomal trp region and that in our clone. This was necessary since we wished to study and compare the organization of that region compared to W23, 3610, and 168 DNA from B. subtilis. The degree of relatedness in terms of nucleotide differences and restriction fragment size patterns between members of the genus Bacillus for this vital and ubiquitous operon is worthy of investigation. In addition, the possible use of the plasmid chimera pRR-6 as a specific hybridization probe of the B. subtilis genome was an exciting possibility. Our failure to

demonstrate B. subtilis specific fragment clones (Table 17) even in a recE recipient is an example of the difficulty of successful cloning in this system. A plasmid chimera reported from Lovett's group (Keggins et al., 1978; Keggins et al., 1979) contains B. subtilis trp C and F activity and is inferred to have a Hind III sensitive trp C activity. Our genetic data (Tables 3, 4, 6, 7) indicate that the B. subtilis trp operon is intact, up to and including hisB2, following Eco RI digestion and that a topography may be inferred with respect to Hind III sensitive sites and linkage relations. Clearly, the relation between W23, 3610 and 168 and other Bacillus fragments would be of interest. Working from the assumption that although B. pumilus and B. subtilis share only limited sequence homology (Introduction), the aromatic region of their chromosomes may possess sufficient sequence micro-homology, once isolated from the genome, to allow hybridization to occur. The presence of continuous, although not complete, regions of base pair nucleation might allow stable hybridization interactions (Laird et al., 1969). If a suitably radioactive probe of trp specific sequences were available, then perhaps we might map the cloned plasmid sequences onto the

B. pumilus donor genome itself and, more importantly, identify via gel hybridization, the Eco RI or Hind III digestion products specific for B. subtilis trp information. In Figures 26 and 27, we exploited the ability of E. coli DNA polymerase I to carry out nick-translation by in vitro substitution of radioactive $P^{32}(\alpha)$ -labeled dNTP's for nucleotides in the template via the procedures of Rigby et al. (1977). As described in the Methods section, samples of chromosomal DNA isolated from Marmur (1961) protocols, plasmid isolated from cleared lysates (Guerry et al., 1973) or from gel purified restriction fragments supported substitutive nucleotide incorporation via nick-translation.

Measuring tritium incorporation (dTTP-methyl H^3) into plasmid pUB110 and chromosomal DNA from B. subtilis it is demonstrated that the plasmid template, isolated via a cleared lysis and density purification is an excellent template for E. coli Pol I driven labeling. Of course, endonuclease activity associated with the Pol I preparation may itself contribute somewhat to primer termini formation. The plasmid template seems to be relatively free of nicks that would reveal internal 3'OH termini. As seen in Figure 26, the system is readily responsive to the

introduction of these nicks which provide primer termini for 3' extension and simultaneous 5'→3' hydrolysis by the polymerase and nuclease activities of Pol I respectively. The endonuclease DNase I generates these internal covalent interruptions and as more nicks are provided, the reaction rate rises as compared to a "native" molecule where naturally occurring 3'OH termini or those provided as artifacts of the isolation procedure are the only templates available. In contrast, in Figure 27 the bacterial chromosomal template is less responsive, in terms of reaction rate, to exogenously generated nicking by DNase I. Most probably this system possesses significant endogenous single strand interruptions which serve as natural primer termini. The addition of more nicks (even at 0.5 ng DNase I/reaction) does not significantly raise the reaction rate or the final plateau value. The effect of excess DNase I action is also shown in Figure 27. While the 0.1 ng DNase generated nicks are suitable for enzyme binding and coupled polymerization-translation along the chain, excess nicking is degradative in a net sense (Fig. 8). Available enzyme may be unprofitably bound by the abundance of free (OH) groups as the DNA backbone is progressively cleaved.

The accumulation of staggered ss interruptions would eventually lead to ds breakage. In the absence of sufficient dNTP, the enzyme may revert to a more degradative mode if 5'→3' exo activity is favored. There is a tendency in these figures for pUB110 kinetics to approach plateau later than those of chromosomal DNA. Under normal preparative conditions (approx. 5-10 units Pol I and 0.5 ng DNase I/ reaction) (Materials and Methods), two hours is generally sufficient for 1 µg template completion. The apparent difference between the plasmid and chromosomal DNA is most probably due to size effects rather than gross conformation as Eco RI cleavage of the templates prior to labeling does not alter the observed reaction path (data not shown). The radioactive nick-translation product (generally 1.0 µg of DNA) is purified, by Sephadex G-50 exclusion chromatography from unincorporated dNTP's, and prepared for hybridization analysis. A typical G-50 distribution is given in Figure 28. The excluded high molecular weight material is calculated by area integration to constitute 28% of the total cpm available during the reaction. The second peak is unincorporated nucleotide. The excluded volume is used as the hybridization probe following denaturation.

Under our conditions, where a single radioactive dTTP pool has been provided, specific activity is generally greater than 1×10^7 cpm/ μ g assuming complete recovery from the column.

16. Hybridization to *B. pumilus* Genome Fragments

To identify the specific *B. pumilus* chromosomal fragments that originally gave rise to our clone pRR-6, we isolated specifically the 2.4 Md. fragment from an Eco RI digest of the chimera. This is the lower band seen in the Eco RI lane of Figure 29. The purity of the fragment was confirmed by secondary digestion of the material with Hind III to achieve the pattern seen in the Eco-Hind double digest lane of Figure 23 where the lower bands (1.70 Md. and 0.70 Md.) (Table 20) represent *B. pumilus* sequences cloned into the pUB110 molecule; the 3.0 Md. band is pUB110. Digestion products from Eco RI and Eco RI/Hind III of pRR-6 were purified in batch from low melting point agarose gels. Re-gelling and cleavage of this material confirmed its purity and integrity. These fragments were used as substrates for nick-translation and hybridization to enzymatically cleaved chromosomal DNA via the procedure of Southern (1975) (Materials and Methods). With the specific activities we achieve and the lack of total homology between the isolated

clone fragments and various Bacillus genomes other than B. pumilus itself, the hybridization process is not very efficient. Often, long exposure times are required along with the use of enhancing screens to discern bands of homology on X-ray film. Nevertheless, interesting results can be gathered. Examination of Figure 29 shows the result of a Southern "blot" autoradiogram of Eco RI, Hind III and doubly digested B. pumilus DNA when the pRR-6 Eco RI 2.4 Md. fragment and the 1.70 and 0.70 Md. Eco-Hind products are individually used as probes against the chromosomal progenitor of our trp⁺ chimera. Digestion of B. pumilus DNA with Eco RI indicates a single band of hybridization of 2.4 Md. M.W. when the pRR-6 2.4 Md. insert is the probe (Table 21). Since the original cloning protocol employed RI digested material, this fragment is identical to the cloned insert. Small differences in M.W. are most probably due to artifacts introduced by the gel blotting procedure that appear, reflected in M.W. calculation, when the gel matrix is pressed down by the nitro-cellulose binding sheet and the absorbant blotting material. However, it cannot be ruled out that very small deletions or rearrangements have occurred within the self-replicating chimera. It is also

important to recall that our clone contains sequences (Table 19, Fig. 23) genetically proficient in only the trp E $\rightarrow$ F genes. Any chromosomal material containing trp information distal to F would not appear on this autoradiogram (trp B, A). A Hind III digest of B. pumilus DNA gives rise to two distinct bands of homology, however, at 5.5 and 3.2 Md. Clearly, the probe is able to identify homologous material of Bacillus origin within the chromosomal milieu which is inclusive of at least trp E $\rightarrow$ F sequences and interrupted by Hind III cleavage. When chromosomal DNA from parental B. pumilus is doubly digested with Eco RI and Hind III (compare to plasmid pRR-6 pattern in Figs. 23, 25; Table 20) two different homologous fragments are revealed at 1.70 and 0.70 Md. M.W. These fragments are identical and consistent with those of the isolated cloned insert of pRR-6 itself. An even more intriguing result is obtained if gel purified plasmid double restriction products are utilized as fine structure probes (Fig. 23) of trp fragment organization. With purified 1.70 Md. cloned Eco-Hind fragment, Eco RI digested B. pumilus gives one band as expected (data not shown). The Hind III digest, however, gives only one band of homology at 3.23 Md. M.W. The 5.5 Md. sequences are

not seen. Lack of sequence complementarity between these sequences and the 1.70 Md. piece must be assumed. When the 0.70 Md. clone segment is purified, labeled and hybridized to Hind III cut chromosomal DNA on the other hand, the higher M.W. 5.5 Md. band "lights up", although weakly. The 3.23 Md. band fails to register hybridization. A complete double digest of chromosomal DNA versus isolated clone trp subfragments shows bona fide register at the respective M.W. of the fragments themselves.

We propose a schematic such as that in Figure 30 to explain this pattern of hybridization for B. pumilus. The Eco RI sensitive sites have been schematically placed, by means of the genetic complementing data, somewhere to the left of trpE (presumably inclusive of transcription modulating sequences) and downstream of trpF, the last gene expressed by the clone. We determine this fragment size to be about 2.4 Md. M.W. Hind III cleavage generates a chromosomal fragment of 3.2 Md. which hybridizes only with a plasmid fragment of 1.7 Md. and a 5.5 Md. piece which encompasses the 0.7 Md. related sequences. Double digestion cuts the trp specific sequences from the chromosomal background to reveal two fragments identical to the pRR-6

insert. No other physical orientation of restriction sites meets these results since inversion of the entire operon within the chromosome, as an alternative, gives rise to a situation wherein the 0.7 Md. plasmid fragment would be expected to hybridize exclusively with the 3.2 Md. chromosomal fragment. This is not seen. The growth characteristics of our clone, our genetic data, and the trpC inactivation (with Hind III) data of Keggins et al. (1979) lead us to superimpose the gene functions upon the physical map as shown in Figure 30. We further propose that the 0.7 Md. plasmid fragment may be expected to contain trpF sequences while the 1.7 Md. piece contains chromosomal material perhaps upstream of trpE and including D. Cleavage through trpC by Hind III is not certain at this point as we prefer to interpret cutting in great proximity to C on either side as also inactivating its function. According to Figure 29, then, the Eco RI 2.4 Md. chromosomal fragment excludes trpB and A which are included on a 5.5 Md. Hind III fragment identifiable only by virtue of trpF specific sequences in the clone and the genome. Specific B and A sequences must therefore reside upon a postulated 4.8 Md. (5.5 minus 0.7 Md.) Eco-Hind fragment within the

chromosome (Fig. 30). This piece is not detected because we lack a probe for this region specifically. If a molecular weight of 236,000 is applied cumulatively (Table 16) to the trp operon products (Henner and Hoch, 1980; Crawford, 1975), 6000 bp of DNA would be required (assuming an amino acid M.W. 120) or about 4×10^6 d. double strand M.W. including a 200 nucleotide "leader" stretch. A 2.4×10^6 cloned fragment could accomodate Eco RI delimited trp E $\rightarrow$ F activities by a molecular weight of 2.6×10^6 using the operon product weights of Hoch and Crawford (1973). The trpB and A functions would require an additional 1.8×10^6 d. with trpF alone requiring 4.0×10^5 d. of double strand M.W. (our proposed trpF "segment" is 7.0×10^5 d.). These site demands are within the range of our cloned map proposals for both isolated trp E $\rightarrow$ F sequences and the chromosomal arrangement in the B. pumilus genome (Fig. 30). Discrepancies are inevitable, however, since the data of Hoch and Crawford (1973) represent average values in a large range and are generated from various Bacillus species. Each product has not been independently isolated, sized and sequenced (Crawford, 1975). In addition, normalizing the molecular weights of the trp E $\rightarrow$ F

products of B. pumilus (the B. subtilis sizes are slightly larger) to that of trpE yields the size coding ratio (legend, Fig. 25) of 1:0.68:0.32:0.36. Clearly, examination of schematic Figures 25 and 30 makes assignment of a Hind III site within trpC reasonable when restriction fragment size and relative gene coding length are considered.

17. Identification of Specific trp Homologs in Bacillus Species

The behavior of various genes in response to restriction cleavage in our gene catalogues indicates that the possibility exists of identifying potential candidates for successful cloning as well as inferring restriction site topography. Of particular interest was the tryptophan operon. Originally, Harris-Warrick et al. (1975) proposed a trp specific fragment in the size range 1.5×10^7 d. for trpE → trpA (Fig. 1) based on comparative survival data alone. They further postulate that hisB and aroE but not aroB resided on this rather huge Eco RI fragment. To test the size of the trp specific homologs in the strains of B. subtilis with which we were most familiar, we blotted digests of W23, 3610 and 168 onto nitrocellulose supports and hybridized these to nick-translated

trp clone sequences of B. pumilus after gel purification. By employing hybridization conditions which are less stringent than those used for the homologous plasmid-B. pumilus hybridization (Methods), we were able to demonstrate B. subtilis and B. licheniformis trp specific fragments hybridizing with B. pumilus cloned probe. Figure 31 shows the results obtained when total 2.4 Md. trp Eco RI fragment is extensively purified on agarose gels, extracted and nick-translated as probe. Table 21 tabulates the data when different DNA's are specifically cleaved and hybridized. The correlation between fragment size and trp gene survival in the catalogue (Tables 3, 4, 6, 7) is excellent for W23 and 3610 DNA where fragments hybridizing to trp information are 5.8 and 9.8 Md., respectively, following Eco RI digestion. Hind III digestion gives a 3.0 Md. fragment for both W23 and 3610. Strain 168, previously described, is the traditional recipient in the transformation assay and gives an Eco RI fragment of 10.03 Md. Figure 32 is a representation of W23, 3610 and 168 DNA organization with respect to restriction site location. This figure is based on biological activity

(Tables 3, 4, 6, 7, 21 and Fig. 31) and hybridization profile in the vicinity of the trp operon and is, of course, only approximate with respect to precise nuclease cleavage within a particular cistron. However, other 168 derivatives give Eco RI generated trp homologs of 10.5 Md. and 12.8 Md. for BR151 and BD170, respectively. Strain SB-25 has the same 10.03 Md. fragment as prototype 168i⁻. The Hind III patterns among the 168 derivatives are more varies, with homologs at 2.75 Md. and 4.64 Md. for BD170 and BR151, respectively. In some cases, double digestion of the genomic DNA with Eco RI and Hind III causes a shortening of the homolog indicating the presence of respective cleavage sites in proximity to one and other. That material, cleaved beyond the flanks of trp sequences in of course not seen in the autoradiograms since it is not homologous to trp specific probe. Importantly, however, it must be remembered that any sequences belonging to trpB or A might not be visualized by our procedure as the probe does not encompass these regions. An interesting pattern is seen with B. subtilis strain 166 carrying mutant allele trpE26 (Audit and Anagnostopoulos, 1972). This unusual strain is capable of becoming

merodiploid upon transformation of the phenotype to prototrophy although we have not pursued this. Large areas in the genome of these fairly unstable cells may be duplicated, carrying trpE26⁺/trpE26 sequences. As seen in Table 21, three Eco RI generated trp homologs and one Hind III product are seen. The Hind III sites must flank an 11.0 Md. area with four Eco RI sites situated internally on the segment. Double digestion yields the same pattern as seen with Eco RI alone; 0.75 Md. of sequence not detectible due to inhomology.

The number and size of the trp homologs varied considerably among the Bacillus species. The Eco RI product homologs varied from 2.5 to 12.8 Md., although we could not demonstrate trp sequences in B. licheniformis due to the very low M.W. products generated by Eco RI. B. pumilus and B. licheniformis Hind III homologs gave two bands while B. subtilis strains yielded only one. The common average 3.0 Md. Hind III homolog indicates that at least within the trp segment some sites have been conserved among species.

Sequence conservation has recently been seen in E. coli and related Enterobacteriacia (Anilionis and Riley, 1980) for trp, lac and lambda related

sequences. The intensity of the autoradiogram bands is in some cases very weak, indicating that we are not operating at relaxed enough hybridization conditions to achieve greater grain density, or that we are at the limits of sensitivity of this probe for heterologous trp sequences. Band intensity progressively decreased in the order B. pumilus, B. subtilis W23, 3610, 168, B. licheniformis. The trp chromosomal domain has evolved differently in Bacillus members. The differences are greatest outside the operon and less between individual trp loci. This is correlated by means of genetic survival data and the size variation of various restriction products. The size of the 168 Eco RI trp homologs is fairly large, 10-12 Md., yet expression of information presumably residing within this segment is not anomalously high, resembling W23 rather than 3610 genetic patterns. One can only conclude that factors other than size play a role in productive integration during transformation such as general strain background effects (Table 10), critical micro-homology and horizontal evolution relationships within the selected sequences. The history of B. subtilis strains has been complex as pointed out by Hemphill and Whiteley (1975).

The archetype 1681⁻ indole requiring bacillus of Burkholder and Giles (1947) seems to have arisen by X-ray mutagenesis of a line of 3610 to yield the trpC2 strain. This has subsequently been manipulated by various means, some quite traumatic in terms of mutagenesis, to yield a myriad of mutant combinations. In addition, trp⁺ sequences in 168 derivatives may have arisen via subsequent transformation to prototrophy with W23 DNA (Nester et al., 1963a). Clear distinctions can thus not be drawn relative to the degree of homology between various strains on the micro-sequence level. The evolution of many "lab strains" calls attention to the need to express relatedness on the cistron rather than genome level. The size of the 168 (i.e., BD29, SB25, BR151) trp⁺ homologs versus those of W23 and 3610 may signal the evolutionary relatedness between these three bacilli. The biological activity (Tables 3, 6, 12) of cleaved 168 DNA may be more related to the intrinsic trp⁺ uptake and incorporation efficiency of a quasi-W23 sequence while the Eco RI pattern of cleavage may be a 3610 remnant, at least in the vicinity of the "trp" sequence, whatever its primal origin. The

168 strains would then seem to be related on different levels, physical and biological, to both 3610 and, at least in the vicinity of the tryptophan operon, to W23, respectively. This is both indicated and affirmed by the RTA, genetic and hybridization data presented. Without specific sequence probe, this information would have remained less clear.

APPENDIX

Statistical Expectation of Recognition Sequence
Frequency

a) There are many possible ways in which a sample of size x may be randomly drawn from an infinitely large set of n different members. In the case of nucleic acids there are four possible member nucleotides which generate the primary structure of DNA. In general, n^x different groups may be chosen of size x . For the Eco RI and Hind III recognition sequences (GAATTC) and (AAGCTT), respectively, the frequency expectation is identical, once per every $(4)^6$. That is, these particular hexonucleotides may be expected once for every 4096 bases. B. subtilis has a M.W. of 2.5×10^9 or 4.2×10^6 bp. As the palindrome defines its own sequence in the $5' \rightarrow 3'$ direction on the opposite strand, the base paired hexanucleotide palindrome may be statistically expected $4.2 \times 10^6 / 4096$ or 10^3 times or every 2.5×10^6 double strand M.W. In the case of the less stringent RI* specificity (AATT), the palindrome might be expected once every 256 bases (basepairs) or 1.64×10^4 times. A purely random distribution would place this sequence once every 1.5×10^5 M.W. (ds). Of course, the expectation may not reflect

the biological reality under pressure of millions of years of evolution. Phage T7 (M.W. 2.5×10^7) has no Eco RI sensitive sites although it is expected by the above reasoning to contain ten. Phage lambda (M.W. 3.2×10^7) contains five sites where it might, on a random basis be expected to contain 13.

b) If one considers the frequency with which a particular base appears adjacent to another base in the DNA backbone, then more sequence specific calculations may be performed. Such data are available from "nearest neighbor" frequency studies (1). Use of a modification of Bayes conditional decision theory (2) allows the sequence (GAATTC) to be analyzed for appearance in the B. subtilis genome. The operation is given by:

$$\text{Frequency} = p_G \times \frac{p_{GA}}{p_G} \times \frac{p_{AA}}{p_A} \times \frac{p_{AT}}{p_A} \times \frac{p_{TT}}{p_T} \times \frac{p_{TC}}{p_T}$$

where p is the mole percent of nearest neighbor frequencies of, for example G and GA, respectively.

Substituting values from the literature (1), the Eco RI palindrome should appear with a frequency of about 5×10^{-4} or once per 2 kbp. Using 4.2×10^6 bp, the site should be present (statistically) 2000 times in B. subtilis, on the average once every 1.25×10^6 ds M.W. The Hind III site is approximated by essentially the same probability.

c) Aside from the purely random appearance of particular palindromic sites, the biological reality may be approximated. By taking advantage of the enzyme Eco RI methylase to add a CH_3 group to adenine at the 6 position within the canonical Eco RI sequence, the actual number of such sequences may be estimated. Covalent filling of Eco RI generated cohesive ends was not successful in our hands with Pol I from *E. coli*. This made incorporation of A/T residues into the AATT overhang impossible to measure as an adjunct to the methylase data analysis. Most probably, this template-primer is not sufficient for the E. coli enzyme to initiate and carry out net synthesis. An enzyme such as the RSV or AMV DNA polymerase might have been more suitable in the utilization of this limited substrate site. Figure 1A shows a time course for methyl- H^3 -SAM labeling of Eco RI sites in W23, 3610, pBR322 plasmid, phage T7 and phage lambda. Conversion of TCA precipitable cpm ($\text{H}^3\text{-CH}_3$) to moles of CH_3 /genome divided by 2 (each RI sequence can interact with 2 CH_3 groups) gives the number of such palindromic sites. For pBR322 and lambda the values calculate to one and five sites precisely. Phage T7, which contains no sensitive sites, as well as Eco RI digested phage lambda, contain no sequences receptive to methyl

uptake as expected. Chromosomal B. subtilis DNA behaves in an extremely complex kinetic manner, probably reflecting affinity variation within the domains of individual hexanucleotide sequences as discussed in the introduction. Nevertheless, both W23 and 3610 appear (although completion of the reaction cannot be verified) to have 1298 and 1188 Eco RI sensitive cleavage sites respectively per genome at the plateau. This is very close to theoretical random expectation. Phage lambda has five Eco RI sites, while nearest neighbor calculations predict as many as 19 such sites randomly. Hind III predictions based upon nearest neighbor frequencies indicate 15 such sites whereas six are actually found. Of course, these random base content calculations do not necessarily have any in vivo meaning, but it is interesting to speculate that phage lambda has evolved fewer Eco RI palindromes than expected because of its history in the coliform-bacilli genus where its normal host is restriction nuclease producing E. coli.

- 1) Josse, J., Keiser, A., Kornberg, A. Enzymatic Synthesis of DNA. VIII Frequencies of nearest neighbor base sequences in DNA. J. Biol. Chem. 236, 864-875 (1961).
- 2) Hoel, P. ed. Elementary Statistics. John Wiley and Sons, Inc. N.Y., 1966, pp. 63-66.

- Alberts, B., P. Doty. Characterization of a naturally occurring, crosslinked fraction of DNA. *J. Mol. Biol.* 32: 379-403, 1968.
- Anagnostopoulos, C., I. Crawford. Transformation studies on the linkage of markers in the tryptophan pathway in *B. subtilis*. *Proc. Nat. Acad. Sci.* 47: 378-390, 1961.
- Anagnostopoulos, C., J. Spizizen. Requirements for transformation in *B. subtilis*. *J. Bact.* 81: 741-746, 1961.
- Anilionis, A., M. Riley. Conservation and variation of nucleotide sequences within related bacterial genomes: *E. coli* strains. *J. Bact.* 143, 355-365, 1980.
- Arber, W. Promotion and limitation of genetic exchange. *Science* 205: 361-365, 1979.
- Arwert, F., G. Venema. Evidence for a non-covalently bound intermediate in recombination during transformation of *B. subtilis*. In: *Bacterial Transformation* (L. Archer, ed.), Academic Press, N.Y., pp. 203-214, 1973.
- Audit, C., C. Anagnostopoulos. Genetic studies relating to the production of transformed clones diploid in the tryptophan region of the *B. subtilis* genome. *J. Bact.* 114: 18-27, 1973.
- Avery, O., C. Macleod, M. McCarty. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. I. Induction of transformation by a DNA fraction isolated from pneumococcus type III. *J. Exp. Med.* 79: 137-158, 1944.
- Bachmann, B., K. Low, A. Taylor. Recalibrated linkage map of *E. coli* K-12. *Bact. Rev.* 40, 116-167, 1976.
- Bearden, J. Electrophoretic mobility of high molecular weight double strand DNA on agarose gels. *Gene* 6, 221-234, 1979.
- Bertrand, K., L. Korn, F. Lee, T. Platt, C. L. Squires, C. Squires, C. Yanofsky. New features of the regulation of the tryptophan operon. *Science* 189, 22-26, 1975.

- Bibb, M., J. Schottel, S. Cohen. A DNA cloning system for interspecies gene transfer in antibiotic-producing Streptomyces. Nature 284: 526-531, 1980.
- Blakesley, R., J. Dodson, I. Nes, R. Wells. Duplex regions in "single strand" ϕ X174 DNA are cleaved by a restriction endonuclease from H. aegyptus. J. Biol. Chem. 252: 7300-7306, 1977.
- Bodmer, W. Integration of DNase treated DNA in B. subtilis transformation. J. Gen. Phys. 49, no. 6, part 2: 233-258, 1966.
- Bonamy, C., E. Keryer, J. Szulmajster. Cloning of B. subtilis DNA fragments carrying spore genes. In: Amer. Soc. Micro. Spores VII. (G. Chambliss and J. Vary, eds.) pp. 139-143, 1978.
- Bott, K., G. Wilson. Development of competence in the B. subtilis transformation system. J. Bact. 94: 562-570, 1967.
- Boyer, H. DNA restriction and modification systems in bacteria. Ann. Rev. Micro. 25, 153-176.
- Bray, G. A simple efficient liquid scintillator for counting aqueous samples. Anal. Bioch. 1: 279-285, 1960.
- Bron, S., K. Murray, T. Trautner. Restriction and modification in B. subtilis. Purification and general properties of a restriction endonuclease from strain R. Mol. Gen. Genet. 143, 13-24, 1975.
- Brown, L., C. Jones. Random and unique B. subtilis DNA fragments carrying a portion of the RNA polymerase genes. In: RNA Polymerase. Cold Spring Harbor Series (R. Losick and M. Chamberlin, eds.) pp. 553-565, 1976.
- Budman, D., A. Pardee. Thymidine and thymine incorporation into DNA. Inhibition and repression by uridine of thymidine phosphorylase of E. coli. J. Bact. 94: 1546-1550, 1967.
- Burkholder, P., N. Giles. Induced biochemical mutants in B. subtilis. Amer. J. Bot. 34: 345-348, 1947.

- Cato, A., W. Guild. Transformation and DNA size. I. Activity of fragments of defined size and a fit to a random cross-over model. *J. Mol. Biol.* 37: 157-178, 1968.
- Chang, S., S. Cohen. *In vivo* site specific generation of recombinants promoted by *Eco* RI endonuclease. *Proc. Nat. Acad. Sci. USA* 74: 4811-4815, 1977.
- Chang, S., S. Cohen. High frequency transformation of *B. subtilis* protoplasts by plasmid DNA. *Mol. Gen. Genet.* 166: 259-267, 1978.
- Chilton, M., B. McCarthy. Genetic and base sequence homologies in *Bacilli*. *Genetics* 62: 697-710, 1969.
- Ciferri, O., S. Barlati, J. Lederberg. Uptake of synthetic polynucleotides by competent cells of *B. subtilis*. *J. Bact.* 104: 684-688, 1970.
- Contente, S., D. Dubnau. Characteristics of plasmid transformation in *B. subtilis*: kinetic properties and the effect of DNA conformation. *Mol. Gen. Genet.* 167: 251-258, 1978.
- Cosloy, S., M. Oishi. Genetic transformation in *E. coli* K-12. *Proc. Nat. Acad. Sci. USA* 70: 84-87, 1973.
- Crawford, I. Gene rearrangement in the evolution of the tryptophan synthetic pathway. *Bact. Rev.* 39: 87-120, 1975.
- Davidoff-Abelson, R., D. Dubnau. Kinetic analysis of the products of donor DNA in transformed cells of *B. subtilis*. *J. Bact.* 116: 154-162, 1973.
- Dean, D., H. Halvorson. Isolation of polygenic fragments of the *Bacillus* chromosome: Transformation activity of *endo* R-*Eco* RI fragmented DNA. *Amer. Soc. Micro. (D. Schlessinger, ed.)* pp. 380-387, 1976.
- DeLey, J. Reexamination of the association between melting point, buoyant density and chemical base composition of DNA. *J. Bact.* 101: 738-754, 1970.

- Denhardt, D. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23: 641-646, 1966.
- Diaz, R., R. Pritchard. Cloning of replication origins from *E. coli* K-12 chromosome. *Nature* (London) 275: 561-564, 1978.
- Dougherty, T., A. Asmus, A. Tomasz. Specificity of DNA uptake in genetic transformation of *Gonococci*. *Biochem. Biophys. Res. Commun.* 86: 97-104, 1979.
- Dubnau, D., C. Cirigliano. Fate of transforming DNA following uptake by competent *B. subtilis*. IV. The endwise attachment and uptake of transforming DNA. *J. Mol. Biol.* 64: 31-46, 1972a.
- Dubnau, D., C. Cirigliano. Fate of transforming DNA following uptake by competent *B. subtilis*. III. Formation and properties of products isolated from transformed cells which are derived entirely from donor DNA. *J. Mol. Biol.* 64: 31-46, 1972b.
- Dubnau, D., C. Cirigliano. Fate of transforming DNA after uptake by competent *B. subtilis*: Size and distribution of the integrated donor segments. *J. Bact.* 111: 488-494, 1972c.
- Dubnau, D., C. Cirigliano. Uptake and integration of transforming DNA in *B. subtilis*. In: *Mechanisms in Recombination* (R. Grell, ed.) Plenum Press, New York, pp. 167-178, 1974a.
- Dubnau, D., C. Cirigliano. Genetic characterization of recombination deficient mutants of *B. subtilis*. *J. Bact.* 117: 488-493, 1974b.
- Dubnau, D., C. Cirigliano. Requirement of DNA polymerase III for the integration of transforming DNA in *B. subtilis*. *Molec. Gen. Genet.* 1975.
- Dubnau, D., R. Davidoff-Abelson, I. Smith. Transformation and transduction in *B. subtilis*: Evidence for separate modes of recombinant formation. *J. Mol. Biol.* 45: 155-179, 1969.
- Dubnau, D., R. Davidoff-Abelson. Fate of transforming DNA following uptake by competent *B. subtilis*. I. Formation and properties of the donor recipient complex. *J. Mol. Biol.* 56: 209-221, 1971.

- Dubnau, D., C. Goldthwaite, I. Smith, J. Marmur. Genetic mapping in B. subtilis. J. Mol. Biol. 27: 163-185, 1967.
- Dubnau, D., I. Smith, P. Morell, J. Marmur. Gene conservation in Bacillus species. I. The location of genes concerned with synthesis of ribosomal components and soluble RNA. Proc. Nat. Acad. Sci. USA 54: 491-498, 1965.
- Dworsky, P. Comparative studies on membrane associated, folded chromosomes from E. coli. J. Bact. 126: 64-71, 1976.
- Dworsky, P. Isolation of folded DNA from B. subtilis. FEMS Letters 1: 95-98, 1977.
- Ehrlich, S. Replication and expression of plasmids from S. aureus in B. subtilis. Proc. Nat. Acad. Sci. USA 74: 1680-1682, 1977.
- Ehrlich, S. DNA cloning in B. subtilis. Proc. Nat. Acad. Sci. USA 75: 1433-1436, 1978.
- Eisenstadt, E., R. Lange, K. Willecke. Competent B. subtilis cultures synthesize a denatured DNA binding activity. Proc. Nat. Acad. Sci. USA 72: 323-327, 1975.
- Ephrati-Elizur, E., P. Srinivasan, S. Zamenhof. Genetic analysis, by means of transformation, of histidine linkage groups in B. subtilis. Proc. Nat. Acad. Sci. USA 47: 56-63, 1961.
- Fox, M., R. Hotchkiss. Initiation of bacterial transformation. Nature (London) 179: 1322-1324, 1957.
- Galloway, D., R. Rudner. Intrastrand self-complementary sequences in B. subtilis DNA. J. Gen. Micro. 111: 353-361.
- Ganesan, A. Genetic recombination during transformation in B. subtilis: Appearance of a DNA methylase. J. Bact. 139: 270-279, 1979.
- Ganesan, A., J. Lederberg. A cell membrane bound fraction of bacterial DNA. Biochem. Biophys. Res. Commun. 18: 824-835, 1965.

- Garro, A., H. Leffert, J. Marmur. Genetic mapping of a defective bacteriophage on the chromosome of B. subtilis 168. J. Virol. 6: 340-343, 1970.
- Goodgal, S., R. Gromkova. Separation of specific segments of transforming DNA after treatment with endodeoxyribonuclease. Proc. Nat. Acad. Sci. USA 70: 503-506, 1973.
- Graham, J., C. Stock. Genetic exchange and natural selection cause B. subtilis to evolve in soil culture. Science 204: 637-638, 1979.
- Greene, P., M. Betloch, H. Goodman, H. Boyer. The Eco RI restriction endonuclease. In: Methods in Molecular Biology Series, DNA replication and biosynthesis, Vol. 7 (R. Wickner, ed.) Marcel Dekker, Inc., New York, pp. 87-111, 1974.
- Greene, P., M. Poonian, A. Nussbaum, L. Tobias, D. Garfin, H. Boyer, H. Goodman. Restriction and modification of self-complementary octanucleotide containing the Eco RI substrate. J. Mol. Biol. 99: 237-261, 1975.
- Griffith, F. Significance of pneumococcal types. J. Hygiene 27: 113-159, 1928.
- Gromkova, R., S. Goodgal. On the role of restriction enzymes of Hemophilus in transformation and transfection. In: Mechanisms of Recombination (R. Grell, ed.) Plenum Press, New York, pp. 209-215, 1974.
- Gryczan, T., S. Contente, D. Dubnau. Characterization of S. aureus plasmids introduced by transformation into B. subtilis. J. Bact. 134: 318-329, 1978.
- Gryczan, T., S. Contente, D. Dubnau. Molecular cloning of heterologous chromosomal DNA by recombination between a plasmid vector and a homologous resident plasmid in B. subtilis. Molec. Gen. Genet. 177: 459-467, 1980.
- Guerry, P., D. LeBlanc, S. Falkow. General method for the isolation of plasmid DNA. J. Bact. 116: 1064-1066, 1973.

- Guild, W., A. Cato, S. Lacks. Transformation and DNA size: two controlling parameters and the efficiency of the single strand intermediate. Cold Spring Harbor Symp. Quant. Biol. 33: 643-645, 1968.
- Gyurasits, E., R. Wake. Bidirectional chromosome replication in B. subtilis. J. Mol. Biol. 73: 55-63, 1973.
- Hadden, C. Restriction-like phenomena in transformation of B. subtilis rec A. J. Bact. 132: 847-855, 1977.
- Haldenwang, W., C. Banner, J. Ollington, R. Losick, J. Hoch, M. O'Connor, A. Sonenshein. Mapping a cloned gene under sporulation control by insertion of a drug resistance marker into B. subtilis chromosome. J. Bact. 142: 90-98.
- Hara, H., H. Yoshikawa. Asymmetric bidirectional replication of B. subtilis chromosome. Nature (London) New Biology 244: 200-203.
- Harris-Warrick, R., Y. Eklana, S. Ehrlich, J. Lederberg. Electrophoretic separation of B. subtilis genes. Proc. Nat. Acad. Sci. USA 72: 2207-2211, 1975.
- Harris-Warrick, R., J. Lederberg. Interspecies transformation in Bacillus: Mechanism of heterologous intergenate transformation. J. Bact. 133: 1246-1253, 1978.
- Hedgpeth, J., H. Goodman, H. Boyer. DNA nucleotide sequence recognized by RI endonuclease. Proc. Nat. Acad. Sci. USA 69: 3448-3452, 1972.
- Heininger, K., W. Horz, H. Zachau. Specificity of cleavage by a restriction nuclease from B. subtilis. Gene 1: 291-303, 1977.
- Helling, R., H. Goodman, H. Boyer. Analysis of Eco RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. 14: 1235-1244.
- Hemphill, E., H. Whiteley. Bacteriophages of Bacillus subtilis. Bact. Rev. 39: 257-315, 1975.

- Henner, D., J. Hoch. The Bacillus subtilis chromosome. Micro. Rev. 44: 57-82, 1980.
- Hershey, A., M. Chase. Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol. 26: 36-56, 1952.
- Hiatt, C. Kinetics of the inactivation of viruses. Bact. Rev. 28: 150-163, 1964.
- Hirokawa, H. Transfecting DNA of Bacillus bacteriophage ϕ 29 that is protease sensitive. Proc. Nat. Acad. Sci. USA 69: 1555-1559.
- Hoch, S., I. Crawford. Enzymes of tryptophan pathway in three Bacillus species. J. Bact. 116: 685-693, 1973.
- Holloman, W., R. Wiegand, C. Radding. Uptake of homologous single strand fragments by superhelical DNA: A possible mechanism for initiation of genetic recombination. Proc. Nat. Acad. Sci. USA 72: 2394-2398, 1975.
- Hori, H. Molecular evolution of 5S RNA. Mol. Gen. Genet. 145: 119-123, 1976.
- Horiuchi, K., N. Zinder. Site specific cleavage of a single strand DNA by a Hemophilus restriction enzyme. Proc. Nat. Acad. Sci. USA 72: 2555-2558, 1975.
- Ivarie, R., J. Pene. Association of many regions of the B. subtilis chromosome with the cell membrane. J. Bact. 114, 571-576, 1973.
- Jacob, F., Brenner, S., Cuzin, F. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28: 329-384, 1963.
- Joenje, H., W. Konings, G. Venema. Interactions between exogenous DNA and membrane vesicles isolated from competent and noncompetent B. subtilis. J. Bact. 121: 771-776, 1975.
- Karamata, D., J. Gross. Isolation and genetic analysis of temperature sensitive mutants of B. subtilis defective in DNA synthesis. Mol. Gen. Genet. 108: 277-287, 1970.

- Kauc, L., A. Piekarowicz. Purification and properties of a new restriction endonuclease from H. influenzae R_f. Eur. J. Biochem. 92: 417-426, 1978.
- Kavenoff, R. Characteristics of the W23 genome by sedimentation analysis. J. Mol. Biol. 72: 801-806, 1972.
- Keggins, K., P. Lovett, E. Duvall. Molecular cloning of genetically active fragments of Bacillus DNA in B. subtilis and properties of the vector plasmid pUB110. Proc. Nat. Acad. Sci. USA 75: 1423-1427, 1978.
- Keggins, K., P. Lovett, R. R. Marrero, S. Hoch. Insertional inactivation of trpC in cloned Bacillus trp segments: Evidence for a polar effect on trp F. J. Bact. 139: 1001-1006, 1979.
- Kline, B., J. Miller. Detection of nonintegrated plasmid DNA in the folded chromosome of E. coli: Physiochemical approach to studying the unit of segregation. J. Bact. 121: 165-172, 1975.
- Klotz, L., B. Zimm. Size of DNA determined by viscoelastic measurements: results on phage, B. subtilis and E. coli. J. Mol. Biol. 72: 779-800, 1972.
- Kopecka, H., M. Chevallier, A. Prunell, G. Bernardi. Inactivation of transforming Hemophilus influenza DNA by deoxyribonucleases. In: Bacterial Transformation (Luis Archer, ed.) Academic Press, New York, pp. 173-182, 1973.
- Lacks, S. Theoretical relationship between probability of marker integration and length of donor DNA in Pneumococcus transformation. J. Mol. Biol. 37: 179-180 (appendix), 1968.
- Lacks, S., B. Greenberg, M. Neuberger. Identification of a DNase implicated in genetic transformation in D. pneumoniae. J. Bact. 123: 222-232, 1975.
- Laird, C., B. McConaughy, B. McCarthy. On the rate of fixation of nucleotide substitutions in evolution. Nature 224: 149-154, 1969.

- Lee, F., K. Bertrand, G. Bennett, C. Yanofsky.
Comparison of the nucleotide sequences of the
initial transcribed regions of the tryptophan
operons of E. coli and S. typhimurium. J. Mol.
Biol. 121: 193-217.
- Lepesant-Kejzlarova, J., J. Lepesant, J. Walle, A.
Billault, R. Dedonder. Revision of the linkage
map of B. subtilis 168: indications for circu-
larity of the chromosome. J. Bact. 121: 823-834,
1975.
- Lerman, L., L. Tolmach. Genetic transformation.
Cellular incorporation of DNA accompanying trans-
formation in Pneumococcus. Biochim. Biophys. Acta
26: 68-82, 1957.
- Litt, M., J. Marmur, H. Ephrussi-Taylor, P. Doty.
The dependence of Pneumococcal transformation on
the molecular weight of DNA. Proc. Nat. Acad.
Sci. USA 44: 144-152, 1958.
- Lovett, P., F. Young. Identification of B. subtilis
NRRL B-3275 as a strain of B. pumilus. J. Bact.
100: 658-661, 1969.
- Lovett, F., F. Young. Linkage groups in B. pumilus
determined by bacteriophage PBS1 mediated trans-
duction. J. Bact. 106: 697-699, 1971.
- Lui, A., B. McBride, G. Vovis, M. Smith. Site
specific endonuclease from Fusobacterium nuclea-
tum. Nucl. Acids Res. 6: 1-15, 1979.
- Maas, W., A. Clark. Studies on the mechanism of re-
pression of arginine biosynthesis in E. coli. J.
Mol. Biol. 8: 365-370, 1964.
- Mandel, M., A. Higa. Calcium dependent phage DNA
infection. J. Mol. Biol. 53: 159-162, 1970.
- Margolin, P. Regulation of tryptophan synthesis.
In: Metabolic Regulation. Volume 5 (H. Vogel,
ed.) Academic Press, New York, pp. 389-446, 1971.
- Margulies, L., Y. Setoguchi, R. Rudner. Asymmetric
transcription during post-germinative development
of B. subtilis spores. I. Hybridization patterns.
Biochim. Biophys. Acta 521, 708-718, 1978.

- Marmur, J. A procedure for the isolation of DNA from microorganisms. *J. Mol. Biol.* 3: 208-218, 1961.
- Marmur, J., D. Lane. Strand separation and specific recombination in DNA: Biological studies. *Proc. Nat. Acad. Sci. USA* 46: 453-461, 1960.
- Mazza, G., A. Gallizi. The genetics of DNA replication, repair and recombination in *B. subtilis*. *Microbiologica* 1: 111-135, 1978.
- McAllister, W. Bacteriophage infection: Which end of the SP82G genome goes in first? *J. Virol.* 5: 194-198, 1970.
- McBride, O., H. Ozer. Transfer of genetic information by purified metaphase chromosomes. *Proc. Nat. Acad. Sci.* 70: 1258-1262, 1973.
- Mertz, J., R. Davis. Cleavage of DNA by RI restriction endonuclease generates cohesive ends. *Proc. Nat. Acad. Sci.* 69: 3370-3374, 1972.
- Meselson, M., R. Yuan. DNA restriction enzyme from *E. coli*. *Nature* 217: 1110-1114, 1968.
- Modrich, P., R. Rubin. Role of the 2-amino group of deoxyguanine in sequence recognition by *Eco* RI restriction and modification enzymes. *J. Biol. Chem.* 252: 7273-7278, 1977.
- Modrich, P., D. Zabel. *Eco* RI endonuclease. Physical and catalytic properties of the homogeneous enzyme. *J. Biol. Chem.* 251: 5866-5874, 1976.
- Morrison, D., W. Guild. Transformation and DNA size: Extent of degradation on entry varies with size of donor. *J. Bact.* 112: 1157-1168, 1972a.
- Morrison, D., W. Guild. Activity of DNA fragments of defined size in *B. subtilis* transformation. *J. Bact.* 112: 220-223, 1972b.
- Morrison, D., W. Guild. Breakage prior to entry of donor DNA in *Pneumococcus* transformation. *Biochim. Biophys. Acta* 299: 545-556, 1973.
- Mottes, M., G. Grandi, V. Sgaramella, U. Canosi, G. Morelli, T. Trautner. Different specific activities of monomeric and oligomeric forms of plasmid

- DNA in transformation of B. subtilis and E. coli.
Mol. Gen. Genet. 174: 281-286, 1979.
- Murray, K., N. Murray. Phage lambda receptor chromosomes for DNA fragments made with restriction nuclease Hind III and endonuclease I of E. coli.
J. Mol. Biol. 98: 551-564, 1975.
- Nester, E., A. Ganesan, J. Lederberg. Effects of mechanical shearing on genetic activity of B. subtilis DNA. Proc. Nat. Acad. Sci. USA 49: 61-68, 1963b.
- Nester, E., Schaefer, M., Lederberg, J. Gene linkage in DNA transfer: A cluster of genes concerned with aromatic biosynthesis in B. subtilis. Genetics 48: 529-551, 1963a.
- Nester, E., B. Stocker. Biosynthetic latency in the early stages of DNA transformation in B. subtilis. J. Bact. 86: 785-796, 1963.
- Notani, N., S. Goodgal. On the nature of recombinants formed during transformation in H. influenzae. J. Gen. Physiol. 49, suppl.: 197-209, 1966.
- Notani, N., J. Setlow. Mechanism of bacterial transformation and transfection. In: Progress in Nucleic Acid Research and Molecular Biology, Vol. 14 (W. Cohn, ed.) Academic Press, New York, pp. 39-100, 1974.
- Oishi, M., N. Sueoka. Location of genetic loci of rRNA on the B. subtilis chromosome. Proc. Nat. Acad. Sci. USA 54: 483-491, 1965.
- Oishi, M., H. Yoshikawa, N. Sueoka. Synchronous and dichotomous replications of the B. subtilis chromosome during spore germination. Nature 204: 1069-1073, 1964.
- Opara-Kubinska, Z., H. Kubinski, W. Szybalski. Interaction between denatured DNA, polyribonucleotides and ribosomal RNA: Attempts at preparative separation of the complementary DNA strands. Proc. Nat. Acad. Sci. USA 52: 923-930, 1964.
- Osawa, S., A. Tukai. Mapping by interspecies transformation experiments of several ribosomal protein genes near the replication origin of B. subtilis chromosome. Mol. Gen. Genet. 164: 113-129, 1978.

- O'Sullivan, A., K. Howard, N. Sueoka. Localization of a unique replication terminus and genetic evidence for partial bidirectional replication in the B. subtilis chromosome. J. Mol. Biol. 91: 15-38, 1975.
- Piechowska, M., M. Fox. Fate of transforming DNA in B. subtilis. J. Bact. 108: 680-689, 1971.
- Piggot, P., J. Coote. Genetic aspects of bacterial endospore formation. Bact. Rev. 40: 908-962, 1976.
- Piechowska, M., A. Soltyk, D. Shugar. Fate of heterologous DNA in B. subtilis. J. Bact. 122: 610-622, 1975.
- Polisky, B., P. Greene, D. Garfin, B. McCarthy, H. Goodman, H. Boyer. Specificity of substrate recognition by the Eco RI restriction nuclease. Proc. Nat. Acad. Sci. USA 72: 3310-3314, 1975.
- Radloff, R., W. Bauer, J. Vinograd. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Nat. Acad. Sci. USA 57: 1514-1521, 1967.
- Rigby, P., M. Dieckmann, C. Rhodes, P. Berg. Labeling DNA to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237-251, 1977.
- Roberts, R. Restriction and modification enzymes and their recognition sequences. Nucl. Acids Res. 8: r63-80, 1980.
- Roger, M., C. Beckman, R. Hotchkiss. Separation of native and denatured fractions from partially denatured pneumococcal DNA. J. Mol. Biol. 18: 156-173, 1966.
- Roger, M., R. Hotchkiss. Selective heat inactivation of Pneumococcal transforming DNA. Proc. Nat. Acad. Sci. USA 47: 653-669, 1961.
- Roth, C., E. Nester. Coordinate control of tryptophan, histidine and tyrosine enzyme synthesis in B. subtilis. J. Mol. Biol. 62: 577-589, 1971.
- Rubin, R., P. Modrich. Eco RI methylase. Physical and catalytic properties of the homogeneous enzyme. J. Biol. Chem. 252: 7265-7272, 1977.

- Rudner, R., H. Lin, S. Hoffman, E. Chargaff. Studies on the loss and restoration of transforming activity of the DNA of B. subtilis. Biochim. Biophys. Acta 149: 199-219, 1967.
- Rudner, R., Karkas, J., Chargaff, E. Separation of B. subtilis DNA into complementary strands. I. Biological properties. Proc. Nat. Acad. Sci. USA 60: 630-635, 1968.
- Rudner, R., M. LeDoux, A. Mazelis. Distribution of pyrimidine oligonucleotides in strands L and H of B. subtilis DNA. Proc. Nat. Acad. Sci. USA 69: 2745-2749, 1972.
- Rudner, R., V. Remeza. Chromatographic fractionation of complementary strands of B. subtilis DNA: biological properties. J. Bact. 113: 739-753, 1973.
- Rudner, R. Mutagenesis during transformation of B. subtilis: I. An increase in "selfing" resulting from hybrid donor DNA. Mut. Res. (in press) 1980.
- Saito, H., T. Shibata, T. Ando. Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in B. subtilis Marburg. Mol. Gen. Genet. 170: 117-122, 1979.
- Scher, B., D. Dubnau. Purification and properties of a Mn^{++} stimulated endonuclease from B. subtilis. J. Bact. 126: 429-438, 1976.
- Scher, B., M. Law, A. Garro. Correlated genetic and Eco RI cleavage map of B. subtilis phage ϕ 105 DNA. J. Virol. 28: 395-402, 1978.
- Schroeder, J., F. Blattner. Least squares method for restriction mapping. Gene 4: 167-174, 1978.
- Scocca, J., R. Poland, H. Zoon. Specificity of DNA uptake by transformable H. influenzae. J. Bact. 118: 369-373, 1974.
- Sedgwick, B., Setlow, J. Single strand regions in transforming DNA after uptake by competent H. influenzae. J. Bact. 125: 588-594, 1976.

- Segall, J., R. Losick. Cloned B. subtilis DNA containing a gene that is activated early during sporulation. Cell 11: 751-761, 1977.
- Shestakov, S., N. Khyen. Evidence for genetic transformation in the blue-green alga Anacystis nidulans. Mol. Gen. Genet. 107: 372, 1970.
- Shivakuman, A., D. Dubnau. Differential effect of hydroxyurea on the replication of plasmid and chromosomal DNA in B. subtilis. J. Bact. 136: 1205-1207, 1978.
- Singh, R. The number of DNA uptake sites in competent cells of B. subtilis. J. Bact. 110: 266-272, 1972.
- Sisco, K., H. Smith. Sequence-specific DNA uptake in Haemophilus transformation. Proc. Nat. Acad. Sci. USA 76: 972-976, 1979.
- Smith, I., D. Dubnau, P. Morell, J. Marmur. Chromosomal location of base sequences complementary to tRNA, 5S, 16S, 23S rRNA in B. subtilis. J. Mol. Biol. 33: 123-140, 1968.
- Smith, H. Nucleotide specificity of restriction endonucleases. Science 205: 455-462, 1979.
- Sonenshein, A., B. Cami, J. Brevet, R. Cote. Isolation and characterization of rifampicin-resistant mutants of B. subtilis with altered sporulation properties. J. Bact. 120: 253-265, 1974.
- Southern, E. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517, 1975.
- Spencer, H., R. Herriott. Development of competence of H. influenzae. J. Bact. 90: 911-920, 1965.
- Spizizen, S. Transformation of a biochemically deficient strain of B. subtilis by deoxyribonuclease. Proc. Nat. Acad. Sci. USA 71: 4787-4791, 1958.
- Spizizen, J. Analysis of asporogenic mutants in B. subtilis by genetic transformation. In: Spores III. (L. Campbell and H. Halvorson, eds.) American Society for Microbiology, Ann Arbor, Mich., pp. 125-137, 1965.

- Spizizen, J., B. Reilly, A. Evans. Microbial transformation and transfection. *Ann. Rev. Microbiol.* 20: 371, 1966.
- Stachura, I., F. McKinley, G. Leidy, H. Alexander. Electron microscopy of *H. influenzae* DNA. *Pediat. Res.* 2: 410, 1968.
- Steinhart, W., R. Herriot. Fate of recipient DNA during transformation in *H. influenzae*. *J. Bact.* 96: 1718-1724, 1968.
- Sternglanz, H., C. Bugg. Conformation of N6-methyladenine, a base involved in DNA modification: restriction processes. *Science* 23: 833-834, 1973.
- Stewart, C. Physical heterogeneity among *B. subtilis* DNA molecules carrying particular genetic markers. *J. Bact.* 98: 1239-1247, 1969.
- Strauss, N. Further evidence concerning the configuration of transforming DNA during entry into *B. subtilis*. *J. Bact.* 91: 702-708, 1966.
- Strauss, N. Early energy dependent step in the entry of transforming DNA. *J. Bact.* 101: 35-37, 1970a.
- Strauss, N. Transformation of *B. subtilis* using hybrid DNA molecules constructed by annealing resolved complementary strands. *Genetics* 66: 583-593, 1970b.
- Studier, F. Gene 0.3 of phage T7 acts to overcome DNA restriction system of the host. *J. Mol. Biol.* 94: 283-295, 1975.
- Sueoka, N., W. Quinn. Membrane attachment of the chromosome replication origin in *B. subtilis*. *Cold Spring Harbor Symp. Quant. Biol.* 33: 695-705, 1968.
- Tanaka, T. Restriction of plasmid mediated transformation in *B. subtilis* 168. *Mol. Gen. Genet.* 175: 235-237, 1979.
- Tanaka, T., K. Sakaguchi. Construction of a recombinant plasmid composed of *B. subtilis* leucine genes and a *B. subtilis* (natto) plasmid: Its use as a cloning vehicle in *B. subtilis* 168. *Mol. Gen. Genet.* 165: 269-276, 1978.

- Testa, D. Ph.D. thesis, Hunter College of the City University of New York, 1977.
- Tevethia, M., M. Mandel. Nature of the EDTA requirement for transformation of B. subtilis with single strand DNA. J. Bact. 101: 844-850, 1970.
- Thomas, M., R. Davis. Studies on the cleavage of phage lambda DNA with Eco RI restriction endonuclease. J. Mol. Biol. 91: 315-328, 1975.
- Tichy, P., O. Landman. Transformation in quasi-spheroplasts of B. subtilis. J. Bact. 97: 42-51, 1969.
- Tomasz, A. Control of the competent state of Pneumococcus by a hormone-like cell product: an example of a new type of regulatory mechanism in bacteria. Nature 208: 155-159, 1965.
- Trautner, T., B. Pawlek, S. Bron, C. Anagnostopoulos. Restriction and modification in B. subtilis. Biological aspects. Mol. Gen. Genet. 131: 181-191, 1974.
- Trowsdale, J., M. Shiflett, J. Hoch. New cluster of ribosomal genes in B. subtilis with regulatory role in sporulation. Nature (London) 272: 179-180, 1978.
- Upcroft, P., H. Dyson, R. Wake. Characteristics of a B. subtilis W23 mutant temperature sensitive for initiation of chromosome replication. J. Bact. 121: 121-127, 1975.
- Vermeulen, C., G. Venema. Electron microscope and autoradiographic study of ultrastructural aspects of competence and DNA absorption in B. subtilis. J. Bact. 118: 334-341, 1974.
- Vovis, G., K. Horiuchi, N. Zinder. Kinetics of methylation of DNA by a restriction endonuclease from E. coli B. Proc. Nat. Acad. Sci. USA 71: 3810-3813, 1974.
- Wake, R. Termination of B. subtilis chromosomes by autoradiography. J. Mol. Biol. 86: 223-231, 1974.

- Wigler, M., A. Pellicer, S. Silverstein, R. Axel. Biochemical transformation of single copy eukaryotic genes using DNA as donor. *Cell* 14: 725-731, 1978.
- Williams, G., D. Green. Early extracellular events in infection of competent *B. subtilis* by DNA of bacteriophage SP82G. *Proc. Nat. Acad. Sci. USA* 69: 1545, 1972.
- Wilson, G., F. Young. Intergenetic transformation of the *B. subtilis* genospecies. *J. Bact.* 111, 705-716, 1972.
- Worcel, A., E. Burgi. On the structure of the folded chromosome of *E. coli*. *J. Mol. Biol.* 71: 127-147, 1972.
- Yamaguchi, K., H. Yoshikawa. Topography of chromosome membrane junction in *B. subtilis*. *Nature New Biol.* 244: 204-206, 1973.
- Yasbin, R. DNA repair in *B. subtilis*. II. Activity of the inducible system in competent cells. *Mol. Gen. Genet.* 153: 219-225, 1977.
- Yasbin, R., G. Wilson, F. Young. Transformation and transfection in lysogenic strains of *B. subtilis*. Evidence for selective induction of prophage in competent cells. *J. Bact.* 121: 296-304, 1975.
- Yasuda, S., Y. Hirota. Cloning and mapping of the replication origin in *E. coli*. *Proc. Nat. Acad. Sci. USA* 74: 5458-5462, 1977.
- Yoshikawa, H., N. Sueoka. Sequential replication of the *B. subtilis* chromosome. I. Comparison of marker frequency analysis in exponential and stationary growth phases. *Proc. Nat. Acad. Sci. USA* 49: 559-566, 1963a.
- Yoshikawa, H., N. Sueoka. Sequential replication of the *B. subtilis* chromosome. II. Isotope transfer experiments. *Proc. Nat. Acad. Sci. USA* 49: 806-813, 1963b.
- Yoshikawa, H., A. O'Sullivan, N. Sueoka. Sequential replication of the *B. subtilis* chromosome. III. Regulation of initiation. *Genetics* 52: 973-980, 1964.

- Yoshimori, R., D. Roulland-Dussoix, H. Boyer. R. factor controlled restriction and modification of DNA: restriction mutants. *J. Bact.* 112, 1275-1279, 1972.
- Young, F., G. Wilson. Genetics of B. subtilis and other gram positive sporulating bacilli. In: Spores V (H. O. Halvorson, R. Hanson, L. Campbell, eds.) *Am. Soc. Micro.*, Washington, D.C., pp. 77-106, 1972.
- Zahler, S., R. Zorman, R. Rosenthal, H. Hemphill. B. subtilis phage SPB: localization of the prophage attachment site, and specialized transduction. *J. Bact.* 129: 556-558, 1977.

Table 1

Bacterial Strains

<u>B. subtilis</u>	<u>Marker</u>	<u>Map Location</u>
W23	prototroph (str ^r ,ery ^r ,mic ^r)	10
3610 (NTCC)	prototroph (ATCC 6051)	-
168i ⁻	trpC	205
mu8u5u16	purA16, leu-8, metB5	355, 250, 200
SB 25	trpC2, hisB2	205
BD 170	trpC2, thr-5, hisA1	205, 290, 305
BD 392	trpC2, thr-5, cysA14	205, 290, 10
BD 29	argA2, leu-1	260, 250
BD 170	trpC2, thr-5	205, 290
BR 151	trpC2, metB10, lys-3	205, 200, 210
BD 193	aroE1	210
BD 194	aroF5	210
BD 224	trpC2, thr-5, recE4	205, 290, 150
BD 366	trpC2, thr-5, pUB110	205, 290, 150
E 88	trpE46, hisB2	205
DB 83	trpD2	205
T 12	trpF12	205
DB 82	trpB4	205
DB 81	trpA4	205
DB 84	trpE26	205
rml25	arg 15, leu-8, r ⁻ m ⁻ (Bsu)	260, 250, 194
<u>B. pumilus</u> RUB502	rif ^r , str ^r , ery ^r , bio ⁻	10, 270
<u>B. licheniformis</u> FDO-12	str ^r	10

Map location data is as cited in Henner and Hoch (1980) (Fig.1)

Table 2
Restriction Enzymes

<u>Nuclease</u>	<u>Source</u>	<u>Recognition Site</u>	<u>λ</u>
EcoRI	<u>E. coli</u> RY13	G A $\dot{A}$ ATTC	5
Hind II	<u>H. influenzae</u>	GTPy Pu $\dot{A}$ C	34
Hind III	"	A AGCTT	6
Eco B	<u>E. coli</u> B	TG $\dot{A}$ (N) ₈ TGCT	-
Eco K	" K	AAC(N) ₆ GTGC	-
BamHI	<u>B. amyloliquefaciens</u>	G GAT $\dot{C}$ C	5
Hae III	<u>H. aegyptus</u>	GG $\dot{C}$ C	> 50
Bsu I	<u>B. subtilis</u>	GG $\dot{C}$ C	> 50
Mbo I	<u>M. bovis</u>	GATC	> 50
Hpa II	<u>H. parainfluenzae</u>	C $\dot{C}$ GG	> 50
Sma I	<u>S. marcescens</u>	CCC GGG	3
Eco P1	<u>E. coli</u> P1	AG $\dot{A}$ CC	-
Hga I	<u>H. gallinarum</u>	GACGCNNNNN	> 50
Eco RI *	<u>E. coli</u>	A $\dot{A}$ TT	-
Dpn I	<u>D. pneumoniae</u>	G $\dot{A}$ TC	> 50
Dpn II	"	GATC	> 50
Sau 3A	<u>S. aureus</u>	GATC	> 50
Pst I	<u>P. stuartii</u>	CTGCA G	18
Bgl II	<u>B. globigii</u>	A GATCT	6

Restriction nucleases were obtained as in Materials and Methods. Specificity data and reaction conditions were as cited by manufacturer and in Roberts, 1980.

Table 3

Marker survival following Eco RI digestion of
B. subtilis W23 DNA

<u>Marker</u>	<u>Recipient</u>	<u>RTA(%)</u>
purA16	mu8u5u16	0.10
leu-8	mu8u5u16	1.2
metB5	mu8u5u16	1.0
trpC2	SB25	7.3
hisA1	BD99	0.47
hisB2(H2)	SB25	6.5
leu-1	BD29	3.8
thr-5	BD170	0.12
metB10	BR151	0.52
lys-3	BR151	0.07
cysA14	BD392	0.07
argA2	BD29	0.16
aroE1	BD193	2.4
aroF5	BD194	0.41
str-1	BD170	4.3
argA2-leu1	BD29	0.2 (3.0%)
trpC2-hisB2	SB25	25 (65%)
trpE-hisB2	E88	10 (35%)

RTA is the number of transformants/ml. remaining after digestion divided by the number obtained with untreated DNA, expressed as a percent. Assays were performed at 1.0 ug/ml. DNA/rx. Cleavage was with 3 Units/ug. overnight in 100mM Tris-HCl, pH7.5; 50mM NaCl; 5mM MgCl₂. Native CFU/ml were 5-10 x 10⁵ uniformly/ug. DNA. () are native cotransfer levels. RTA values are means of ten determinations.

Table 4

Marker Survival Following HindIII Digestion of
B. subtilis W23 DNA

<u>Marker</u>	<u>RTA(%)</u>
purA16	0.17
leu-8	0.33
metB5	0.32
trpC2	0.05
hisA1	0.09
hisB2	0.05
leu-1	0.15
thr-5	7.5
metB10	0.06
lys-3	0.06
cys A14	0.27
arg A2	0.17
aro E1	0.04
aro F5	0.03
str-1	0.09
argA2-leu-1	0
trpC2-hisB2	0
trpE-hisB2	0

RTA is defined as in Table 3. Digestion was overnight (3.0 units/ug. DNA in 60mM NaCl; 7mM MgCl₂; 7mM Tris-HCl, pH 7.4. Assay was at 1.0 ug. DNA/reaction. Native transformant CFU/ml. were uniformly in the range 5-10 x 10⁵/ug. DNA.

Table 5

Marker Survival Following EcoRI* Digestion of
W23 DNA

<u>Marker</u>	<u>RTA</u> (%)
purA16	0
leu-8	0.02
metB5	0.02
trpC2	0.06
hisA1	0.03
leu-1	0.07
thr-5	0.02
metB10	0.03
lys-3	0.04
cysA14	0
argA2	0.08
aroE1	0.15
aroF5	0

Digestion with EcoRI* activity was in 25mM Tris-HCl, pH 8.5;
2mM MgCl₂.

Table 6

Marker Survival Following EcoRI Digestion of
B. subtilis 3610

<u>Marker</u>	<u>RTA(%)</u>
pur A16	0.07
leu-8	7.5
met B5	7.2
trp C2	30.8
his A1	9.5
his B2	28.7
leu-1	12.4
thr-5	0.28
met B10	2.0
lys-3	0.43
cys A14	0.44
arg A2	2.5
aro E1	43.0
aro F5	0.46
argA2-leu-1	0.50 (3.6%)
trpC2-hisB2	27.0 (70%)
trpE-hisB2	12.0 (30%)

Digestion was as in Fig.3. () is the native % cotransfer of the respective genes prior to treatment. Transformation assay is at 1.0 ug./rx.

Table 7

Marker Survival Following HindIII Digestion of 3610 DNA

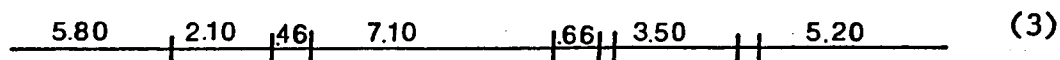
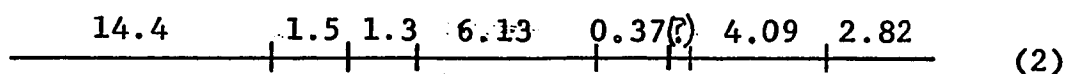
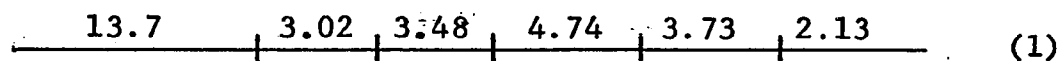
<u>Marker</u>	<u>RTA(%)</u>
purA16	0.56
leu-8	1.3
metB5	3.5
trpC2	4.7
hisA1	7.2
hisB2	0.40
leu-1	0.05
thr-5	3.8
metB10	1.3
lys-3	0.60
cysA14	0.79
argA2	0.08
aroE1	2.1
aroF5	0.20
argA-leu-1	0
trpC-hisB	0
trpE-hisB	0

Digestion was as previously described.

Table 8

Restriction Fragment- Molecular Weight Gel Standards

$\lambda(\text{EcoRI}) \times 10^6 \text{d.}$	$\lambda(\text{HindIII})$	$\phi 105(\text{EcoRI})$
13.7	14.4	11.0 (*)
4.74	6.13	7.10
3.73 (1)	4.09 (2)	5.80 (3)
3.48	2.82	5.20
3.02	1.50	3.50
2.13	1.32	2.10
	0.37	0.66
	(?)	0.46



(*) cohesive product of the 5.80 and 5.20 fragments.
Two smaller fragments, 2×10^5 were not used here.

(?) another low molecular weight product may be present.

1. Thomas and Davis, 1975; Helling et al, 1974.
2. Schroeder and Blattner, 1978.
3. Scher et al, 1978.

Table 9

Molecular Weight of Cleavage Products

<u>Treatment</u>	<u>W23 DNA</u>	<u>3610 DNA</u>
<u>EcoRI</u>	6.1×10^6 d.	6.1×10^6 d.
<u>HindIII</u>	2.4×10^6	2.4×10^6
<u>EcoRI*</u>	6.5×10^5	n.d.
Shear 1'	3.2×10^6	n.d.
Shear 15'	1.4×10^6	n.d.

Molecular weights are expressed as weight average from tritiated gel distributions. Biological activity remaining is presented in Tables 3-7. Shearing was at setting 5 of a Sorvall Ommimixer; biological activity reduction was to 10% and 1.0% of native for the 1' and 15' samples respectively. Transformation was measured in strain BR151 at 1.0 ug. DNA/rx.

Table 10

Strain Variation Among Mutant Recipients

<u>Donor DNA</u>	<u>Recipient Strain</u>	<u>Locus</u>	<u>EcoRI</u>	<u>HindIII</u>
W23	BD 170	<u>trpC2</u>	9.5%	0.30%
	SB 25		14.1	0.43
	BR 151		1.7	0.05
3610	BD 170	<u>trpC2</u>	21.5%	1.1%
	SB 25		41.1	4.1
	BR 151		18.5	0.9
168	BD 170	<u>trpC2</u>	10.3%	0.1%
	SB 25		15.0	0.01
	BR 151		3.2	0.15

Transformation was conducted at 1.0 ug./rx. and RTA%(N/R) calculated. 168 strain is, in this case, BD-29(argA2, leu-1). Native transformants/ml. were approximately 1.0×10^6 /ml. The viable CFU/ml. was 3.8×10^8 .

Table 11

The Effect of Restriction Cleavage on Cotransformation

<u>DNA</u>	<u>Recipient linkage</u>	<u>Native</u>	<u>EcoRI</u>	<u>HindIII</u>
W23	trpC2-hisB2	65.0%	25.0%	0% (*)
	trpE46-hisB2	40.0	10.0	0 (#)
	argA2-leu-1	3.0	0.2	0
3610	trpC2-hisB2	70.0%	27.0%	0%
	trpE46-hisB2	42.0	15.3	0
	argA2-leu-1	3.6	0.5	0
168	trpC2-hisB2	39.0	20.0%	0%
	trpE46-hisB2	35.0	12.2	0
	argA2-leu-1	1.7	0.1	0

Transformation assay was at 1.0ug. DNA/rx. before and after digestion. Cotransformation is calculated as $a^+b^+ / 0.5(a^+b + ab^+)$ by direct plating. The 168 donor is BD-29, except for arg-leu, where it is BD-170. In each case, digestion is with approx. 3 enzyme units/ug. DNA overnight. Recipients SB-25, E-88, and BD-29 were employed. (*, #)- Ligation(see Table 14) failed to demonstrate improvement in HindIII generated cotransfer loss while individual genes showed net RTA_L/RTA_H of 32.5 and 50.0 for trpC2 and hisB2; 55.4 and 15.6 for trpE46 and hisB2 respectively.

Table 12

Restricted DNA Activity in 168 Donors

<u>Donor DNA</u>	<u>Recipient</u>	<u>Locus</u>	<u>EcoRI</u>	<u>HindIII</u>
BD 29	SB 25	trpC2	15.0%	0.01%
		hisB2	7.5	0.08
BR 151	BD 99	thr-5	0.1%	16.9%
		hisA	1.5	0.16
BD 29	BD 170	trpC2	10.3	0.10
		thr-5	0.1	5.6
BD 29	E 88	trpE46	1.5	nd.
		hisB2	7.9	nd.

Transformation assays were conducted at 1.0ug. DNA/rx. before and after enzyme cleavage, and RTA (N/R) calculated. Native CFU/ml. was consistently in the range of 5-10 x10⁵/ml. while total viable count was approx. 3.0 x10⁸ /ml.

Table 13

Transforming Activity in a Nonrestricting Host

<u>Donor DNA</u>		<u>Locus (CFU/ml.)</u>		<u>RTA (%)</u>	
		<u>arg 15</u>	<u>leu-8</u>	<u>arg 15</u>	<u>leu-8</u>
W23	native	4.0x10 ⁵	4.3x10 ⁵	100	100
	<u>EcoRI</u>	3.6x10 ²	9.0x10 ³	0.09	2.1
3610	native	6.0x10 ⁵	6.1x10 ⁵	100	100
	<u>EcoRI</u>	1.8x10 ⁴	8.3x10 ⁴	3.0	13.6
168	native	4.9x10 ⁵	4.7x10 ⁵	100	100
	<u>EcoRI</u>	4.9x10 ²	1.9x10 ⁴	0.10	4.1
		<u>trpC2</u>	<u>thr-5</u>	<u>trpC2</u>	<u>thr-5</u>
rml25	native	5.6x10 ⁵	8.6x10 ⁵	100	100
	<u>EcoRI</u>	3.3x10 ⁴	4.6x10 ²	5.9	0.05

Transformation was as previously described. Strain rml25 is (arg15, leu-8 r⁻m⁻) a nonrestricting B. subtilis, presumably deficient in the Bsu enzyme system, used as a recipient in this study. 168 donor is BD170 DNA, while the recipient of rml25 DNA is BD170 cells. Control RTA's of BD29(168 derivative) DNA transformed into BD170(trpC2, thr-5) were 10.3 and 0.10 for trp and thr respectively following EcoRI cleavage, the same relative gene survival relation seen with rml25 donor; RTA's of 3.2% and 0.08% were obtained with W23 DNA respectively. See leu-8 locus of mu8u5u16 as compared to rml25: no significant variation.

Table 14

Ligation ~ Repair of Restriction Cleaved B. subtilis DNA

(A)						
<u>Locus</u>	<u>Native</u> <u>CFU/ml.</u>	<u>Restricted</u> <u>CFU/ml.</u>	<u>Ligated</u> <u>CFU/ml.</u>	<u>(R)</u> <u>RTA</u>	<u>(L)</u> <u>RTA</u>	<u>RTA</u> <u>L/R</u>
trpC2 (*)	7.1×10^5	2.3×10^4	5.0×10^4	3.2	7.0	2.2
metB10	1.3×10^6	5.0×10^3	4.4×10^4	0.39	3.4	8.7
lys-3	1.7×10^6	5.0×10^2	5.7×10^4	0.03	3.4	113
argA2	9.6×10^5	2.0×10^3	2.6×10^4	0.21	2.7	12.9
leu-1	8.2×10^5	9.8×10^3	3.9×10^4	1.2	4.8	4.0
thr-5	5.7×10^5	4.5×10^2	3.3×10^4	0.08	5.8	73.3
pUB110-km	1.2×10^5	0	1.4×10^5	0	116	-
(B)						
trpC2 (#)	1.1×10^6	9.0×10^2	4.2×10^4	0.09	4.2	46.7
thr-5	7.3×10^5	1.3×10^4	5.5×10^4	4.7	7.6	1.6
(C)						
		<u>Sheared</u>	<u>Ligated</u>	<u>RTA/S</u>	<u>RTA/L</u>	<u>L/S</u>
trpC2	8.0×10^5	9.5×10^4	1.1×10^5	12.2	13.8	1.13
thr-5	7.2×10^5	5.9×10^4	6.3×10^4	8.3	9.0	1.08

All transformation assay was at 1.0 ug. DNA/rx. DNA from pUB110 was at 2.0ug/rx. and selected as km^r colonies in BD-170 with 5.0ug./ml. kanamycin sulfate after a 90' expression lag at 25°C. Shearing was in EcoRI buffer (Table 3), ligation was as in Methods. (A) samples versus EcoRI enzyme, (B) HindIII enzyme, (C) shear. (*, #) - see Table 11.

Table 15

Biological Activity of Linear and Circular Ligation Products

<u>Marker</u>	<u>Native</u> <u>CFU/ml.</u>	<u>Restricted</u> <u>CFU/ml.</u>	<u>(R)%</u> <u>RTA</u>	<u>Ligated</u> <u>CFU/ml.</u>	<u>(L)%</u> <u>RTA</u>	<u>RTA</u> <u>L/R</u>
trpC2	7.1×10^5	2.3×10^4	3.24	5.0×10^4	7.04	2.17
thr-5	5.7×10^5	4.5×10^2	0.08	3.3×10^4	5.79	73.3
(A) lys-3	5.5×10^5	1.6×10^2	0.03	1.4×10^4	2.70	86.8
metB10	8.0×10^5	4.9×10^3	0.61	2.4×10^4	3.00	4.92
trpC2	CsCl-UB -	-	-	6.4×10^4	9.01	2.78
	LB -	-	-	3.7×10^3	0.52	0.16
thr-5	" UB -	-	-	2.3×10^4	4.04	51.1
(B)	LB -	-	-	1.5×10^2	0.03	0.16
lys-3	" UB -	-	-	1.4×10^4	2.70	87.5
	LB -	-	-	7.6×10^2	0.15	4.80
metB10	" UB -	-	-	1.9×10^4	2.38	3.90
	LB -	-	-	2.0×10^2	0.03	0.05

Transformation of recipients BR151 and BD170(thr-5) was with EtBr CsCl gradient fractionated chromosomal DNA. Restriction and ligation were as described in Methods. EcoRI cleaved and ligated total reaction(A) and isolated linear and circular(B) products were normalized to a DNA conc. of 1.0ug/assay. RTA L/R reflects fold improvement following repair of 20ug/ml. cleaved DNA with 5.0 Weiss units T4 ligase (total vol. 2.5ml.) Approx. 50ug DNA were loaded/8ml. run. All handling of samples prior to removal of dye was in subdued light. UB and LB refer to upper and lower CsCl/EtBr gradient bands.

Table 16

Marker Survival Following EcoRI and HindIII Cleavage of the B. subtilis trp region of strains W23 and 3610 DNA

<u>Recipient</u>		<u>RTA(%)</u>			
		<u>W23 DNA</u>		<u>3610 DNA</u>	
		<u>EcoRI</u>	<u>HindIII</u>	<u>EcoRI</u>	<u>HindIII</u>
E88	trpE46	3.3	4.2	3.8	0.10
	hisB2(H)	13.2	0.08	49.3	0.06
DB83	trpD2	16.2	2.7	25.1	3.4
BD170	trpC2	13.9	0.04	41.1	2.2
	thr-5	0.24	8.6	0.4	4.4
SB25	trpC2	18.6	0.05	47.6	2.8
	hisB2	14.0	0.06	49.3	0.67
BR151	trpC2	3.5	0.02	20.8	3.2
	metB10	1.8	0.10	3.4	1.1
	lys-3	0.08	0.10	0.52	0.92
T12	trpF12	7.2	0.53	47.1	5.2
DB82	trpB4	17.4	0.93	46.7	2.6
DB81	trpA4	9.1	0.53	46.7	2.6

trpE= anthranilate synthetase - 84,000 d.
 D= anthranilate phosphoribosyl transferase - 57,000
 C= indole-glycerole-phosphate synthetase - 27,000
 F= anthranilate isomerase - 30,500
 B= tryptophan synthase α - 82,000
 A= tryptophan synthase β - 26,000

Transformation was as previously described with 1.0 ug. DNA per assay. Cleavage was as described in Methods with 3 units nuclease/ug. DNA. RTA is expressed as R/N as a %. M.W. of operon products(E $\rightarrow$ A) is from Crawford(1975). Operon organization is presented in Figures 25, 26, 32.

Table 17

Transformation B. subtilis by Plasmid, Homologous and Heterologous Chromosomal DNA and Ligated Mixtures

<u>Recipient</u>	<u>Donor DNA</u>	<u>Km^r</u>	<u>Selected Marker (CFU/ml.)</u>			
			<u>trpC2</u>	<u>thr-5</u>	<u>lys-3</u>	<u>metB10</u>
BD170	pUB110	6.2x10 ⁴	0	0	-	-
<u>trpC2, thr-5, rec⁺</u>	W23	0	1.1x10 ⁶	7.6x10 ⁵	-	-
	<u>B. pumilus</u>	0	92	22	-	-
BD224	pUB110	1.1x10 ⁵	0	0	-	-
<u>trpC2, thr-5, recE4</u>	W23	0	0	0	-	-
	<u>B. pumilus</u>	0	0	0	-	0
BR151	pUB110	8.4x10 ⁴	0	-	3.0x10 ³	8.5x10 ³
<u>trpC2, metB10, lys3</u>	W23	0	1.3x10 ⁶	-	6.8x10 ⁵	6.9x10 ⁵
	<u>B. pumilus</u>	0	50	-	45	50
BD170	L-M (*)	7.8x10 ³	1.3x10 ³	1.1x10 ³	-	-
BD224	L-M	1.4x10 ⁴	0	0	-	-
BR151	L-M	4.4x10 ⁴	1.1x10 ³	-	1.9x10 ³	2.9x10 ³

(*) B. pumilus-pUB110 ligated mixture.

Table 18

Hybrid Plasmid Transformation (pRR-6) into recE4
and rec⁺ Recipient Strains of B. subtilis

<u>Recipient</u>	<u>Growth Medium</u>	<u>DNA</u>	<u>Transformants/ml.</u>		
			<u>Km</u> ⁺	<u>trp</u> ⁺	<u>thr</u> ⁺
BD224	-	W23	0	0	0
	VY	CCC	1.6x10 ⁴ (88/218)	5.6x10 ³ (249/390)	0
<u>trpC2, thr-5</u> <u>recE4</u>	MM	CCC	1.2x10 ⁵ (108/108)	2.3x10 ⁴ (119/186)	0
BD170	-	W23	0	1.7x10 ⁶	2.1x10 ⁶
<u>trpC2, thr-5</u> <u>rec</u>	VY	CCC	1.3x10 ⁵ (58/108)	1.8x10 ⁵ (283/283)	3.6x10 ⁴
	MM	CCC	2.2x10 ⁵ (108/108)	2.4x10 ⁵ (246/246)	1.4x10 ⁵

Plasmid DNA from pRR-6(trp⁺) isolated from among L-M of Table 17, the B. pumilus-pUB110 mixture in BR151, was isolated from overnight VY or MM cultures (Methods) in the presence of 10ug/ml. kanamycin. Transformation was at ug/ml plasmid DNA. Numbers in parenthesis indicate how many clones of the selected class gave rise to the unselected phenotype upon replica plating to the reciprocal selection. W23 DNA is Marmur isolated while the CCC is obtained via CsCl-EtBr gradient cent.

Table 19

Chromosomal and Plasmid Transformation in
trp Mutants of B. subtilis

<u>Recipient</u>	<u>DNA</u>	<u>Transformants/ml</u>		<u>RTA(%)</u>	
		<u>Km^r</u>	<u>trp⁺</u>	<u>EcoRI</u>	<u>HindIII</u>
E88(<u>trp</u> E46, <u>his</u> B2)	W23	0	5.2x10 ⁴	3.3	4.2
	pUB110	5.4x10 ³	0		
	pRR-6	1.1x10 ⁴	3.5x10 ⁴		
DB83(<u>trp</u> D2)	W23	0	3.3x10 ⁵	16.2	2.7
	pUB110	1.3x10 ⁴	0		
	pRR-6	5.9x10 ⁴	6.5x10 ⁴		
BD170(<u>trp</u> C2, <u>thr</u> -5)	W23	0	9.8x10 ⁵	13.9	0.04
	pUB110	8.2x10 ⁴	0		
	pRR-6	1.3x10 ⁵	1.5x10 ⁵		
T12(<u>trp</u> F)	W23	0	1.2x10 ⁶	7.2	0.53
	pUB110	3.0x10 ⁴	0		
	pRR-6	2.9x10 ⁴	4.2x10 ⁴		
DB82(<u>trp</u> B4)	W23	0	3.7x10 ⁵	17.4	0.93
	pUB110	2.3x10 ⁴	0		
	pRR-6	1.8x10 ⁴	0		
DB81(<u>trp</u> A5)	W23	0	2.8x10 ⁵	9.1	0.53
	pUB110	1.3x10 ⁴	0		
	pRR-6	4.8x10 ⁴	0		

Transformation of W23, pUB110, pRR-6 was with 1.0 ug., 2.0 ug., and 2.0ug. DNA respectively. RTA (N/R) is as previously described (Table 16). pRR-6 is a selected recombinant of EcoRI digested B. pumilis DNA and pUB110 as in L/M of Table 17 selected in BR151. The chimera genotype is trp⁺(E,D,C,F), Km^r.

Table 20

Fragments Produced by Restriction Cleavage of Clone pRR-6

<u>Enzyme</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>	<u>G</u>	<u>H</u>	<u>Sum(Mdal.)</u>
<u>EcoRI</u>	3.00	2.40							5.40
<u>HindIII</u>	5.40								5.40
<u>EcoRI/HindIII</u>	3.00	1.70	0.7						5.40
<u>BglII</u>	3.00	2.40							5.40
<u>BglIII/EcoRI</u>	1.62	1.50	1.44	0.90					5.46
<u>HpaII</u>	1.50	1.02	0.51	0.48	(x2) 0.42	0.39	0.21	0.15	5.10
<u>HaeIII</u>	1.56	1.44	0.60	0.36	0.30	0.28	0.19	0.15	4.88
<u>SacI/EcoRI</u>	3.00	1.74	0.54						5.28
<u>SacI,EcoRI/HindIII</u>	3.00	1.62	0.54	0.15	0.09				5.40
<u>BglIII/BamHI</u>	2.40	2.22	0.90						5.52
<u>SacI/BglIII</u>	3.00	1.50	0.54	0.27					5.31

Enzymatic cleavage of 1.0-3.0ug. of density purified plasmid DNA was in a volume of 50ul. in buffer of composition according to manufacturers specifications. Digestion was at 37°C for 3 hours with 3-10 units of activity per reaction. Reactions were terminated by heating to 65°C for 10 min., followed by electrophoresis with tracking dye (Materials and Methods). M.W. is by interpolation with known standards. Dr. L. DiGiovanna-Enea carried out many of these accurate M.W. calculations and analyses.

Table 21

Size of trp Homologous Restriction Fragments
in Bacillus Chromosomal DNA

<u>DNA</u>	<u>EcoRI</u>	<u>Mol. Wt. (Mdal.)</u>		
		<u>HindIII</u>	<u>EcoRI/HindIII</u>	
<u>B. pumilus</u>	2.40	a) 5.50 b) 3.23	a) 1.70 b) 0.70	
<u>B. licheniformis</u>	n.d.*	a) 1.80 b) 1.31	(1.23)	
<u>B. subtilis</u>				
a) W23	5.77	3.03	1.90(*)	
b) 3610	9.79	3.00	2.84	
c) 168i ⁻	10.03	n.d.	n.d.	
d) BR151	10.50	4.64	3.16	
e) BD170	12.77	2.75	2.75	
f) SB25	10.03	n.d.	n.d.	
<u>B. subtilis</u> E26	a)4.94 b)2.97 c)2.52	a)11.20	a)4.94 b)2.97 c)2.52	
	<u>BamHI</u>	<u>BglIII</u>	<u>BglIII/HindIII</u>	<u>BglIII/EcoRI</u>
<u>B. pumilus</u>	6.7	a) 12.6 b) 2.2	a)5.6 b)2.2	a) 1.67 b) 1.08

(*) an additional 3.0Md. fragment is variably detected.

Density gradient isolated and electrophoretically purified pRR-6 EcoRI or EcoRI/HindIII digested fragments (1.7 and 0.7Md.) were nick translated with E. coli DNA Pol I using (α) P³²-dTTP. Probe was hybridized to suitably digested chromosomal fragments bound to nitrocellulose by the method of Southern. Autoradiograms were developed on intensified X-ray film and molecular weights interpolated as in Methods. These values represent averages of four to eight individual determinations. In each case, the integrity of the pRR-6 probe fragments were tested against blotted pUB110 and pRR-6 fragments as a control. (*)-digestion products were of very low M.W. and failed to suitably blot(bind) to filter. Sequences beyond the assumed trp E→F (*) content of the probe, or those residing on fragments of low homology might not be detected. See also Fig.23,25,30,32.

Figure 1

The genetic organization of the B. subtilis genome is, in most part, from the data of Henner and Hoch, (1980). Various structural and functional genes and gene clusters are positioned on the outer circle while the approximate map locations are expressed, based upon a 360° circle, on the inner ring. The replication origin is at $0^{\circ}/360^{\circ}$, proceeding bidirectionally to the terminus (approx. 180°) near citK.

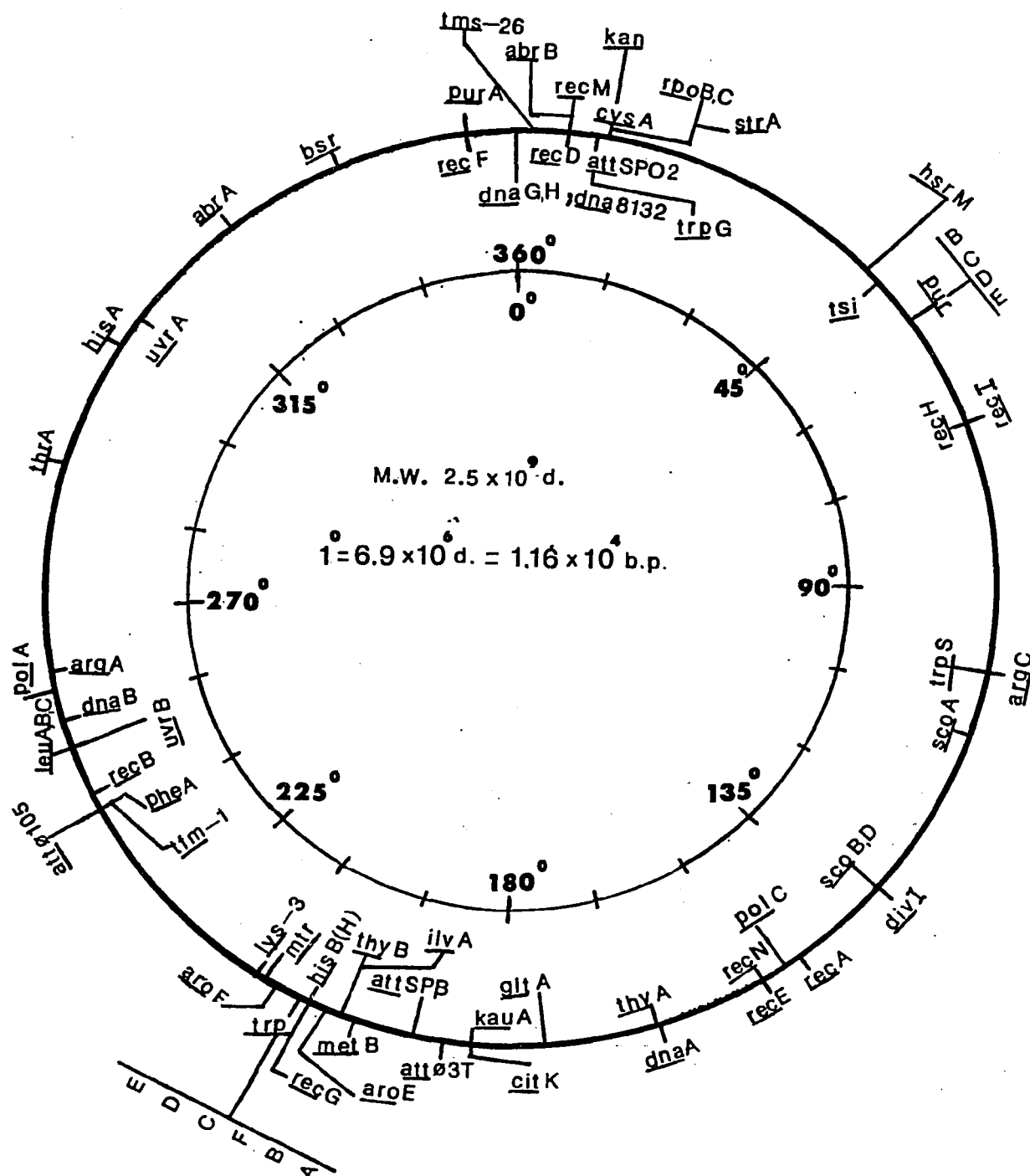
The Bacillus subtilis Genome

Figure 2.

Determination of peak of competency period. B. subtilis cells were carried through the competency regime of Spizizen (1958) as modified by Rudner and Remeza (1973). The arrow indicates the transfer of cells (see insert) to the step-down conditions followed by exposure to 1 ug/reaction DNA. Peak transformation is reached 90 min. after introduction to medium II, and used uniformly. Drug (km^R) resistance is scored by overlay with 10 ug/ml kanamycin following a 90' expression lag. Recipients were present in competent culture at 2.5×10^8 cells/ml. Prototrophs are expressed as trans/CFU/ml $\times 10^{-3}$. Recipient was BD170 (trpC2, thr-5, km^S) scored for native trp⁺ ($\blacktriangle$ — $\blacktriangle$); Eco RI residual trp⁺ ($\bullet$ — $\bullet$); shear (1') residual trp⁺ ($\triangle$ — $\triangle$); km^R colonies ($\circ$ — $\circ$). Prototrophic native DNA is W23 and drug genes are from plasmid pUB110. Approximately 5% of the viable cells were competent in this analysis based on transfer of unlinked genes.

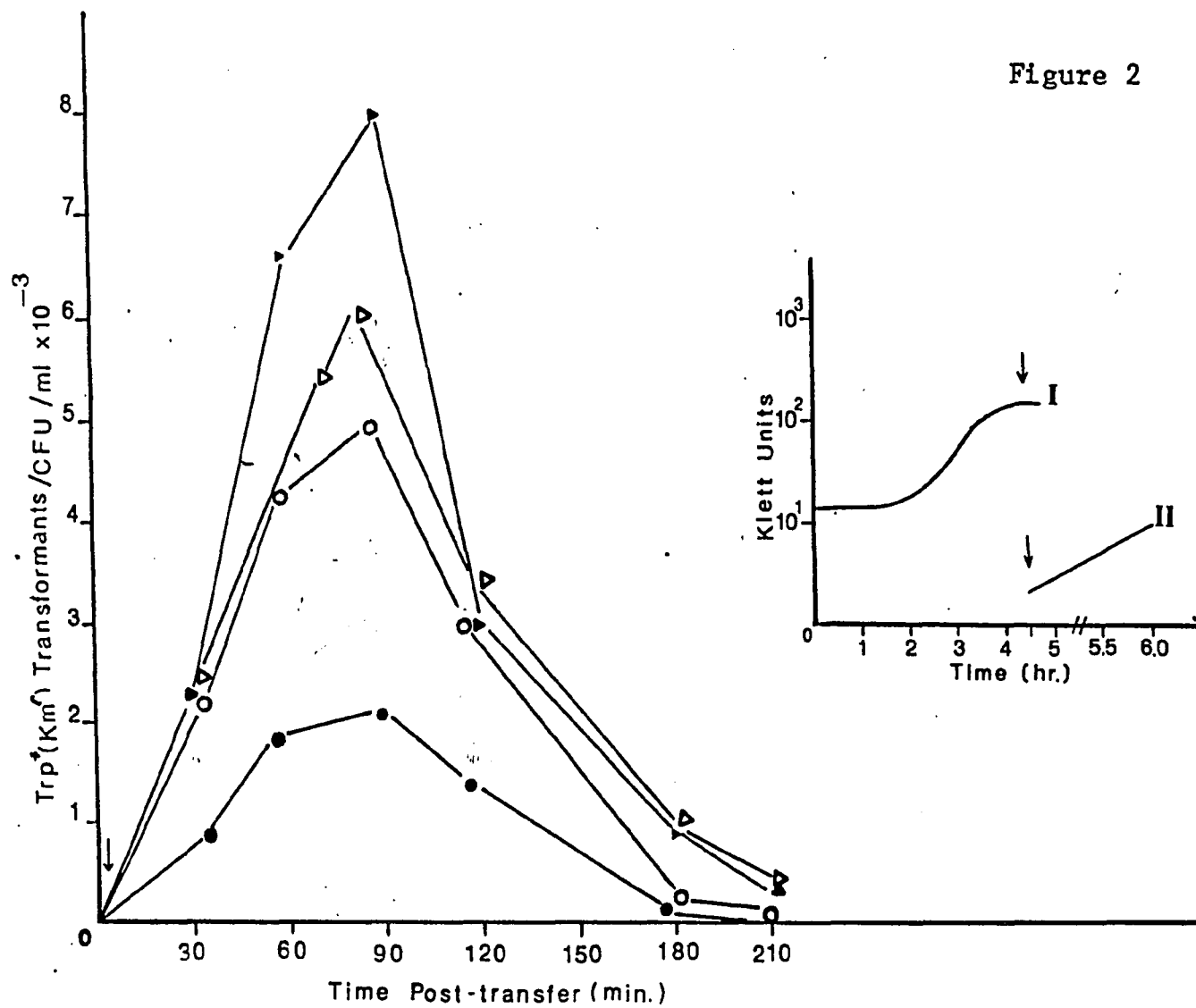


Figure 3

Specific activity of native and treated DNA. DNA preparations from B. subtilis were transformed into auxotrophs at various weight concentrations and protrophic colonies scored after 48 hrs. Transformation was in 1 ml. of competent cells grown 90' in Spizizen II medium. Recipient strain BR 151 (trpC2, metB10, lys-3) was used with native W23 DNA for the trp⁺ (●—●), met⁺ (x—x), lys⁺ (O—O) genes. A sheared sample (10% residual trp⁺) (Δ—Δ) measures trp⁺ activity also. Eco RI digested DNA generates restricted trp⁺ (●---●), met⁺ (x---x), lys⁺ (O---O) colonies. BD29 (argA2, leu-1) is used to measure specific activity of Eco RI treated arg⁺ (□---□) transformation.

Figure 3

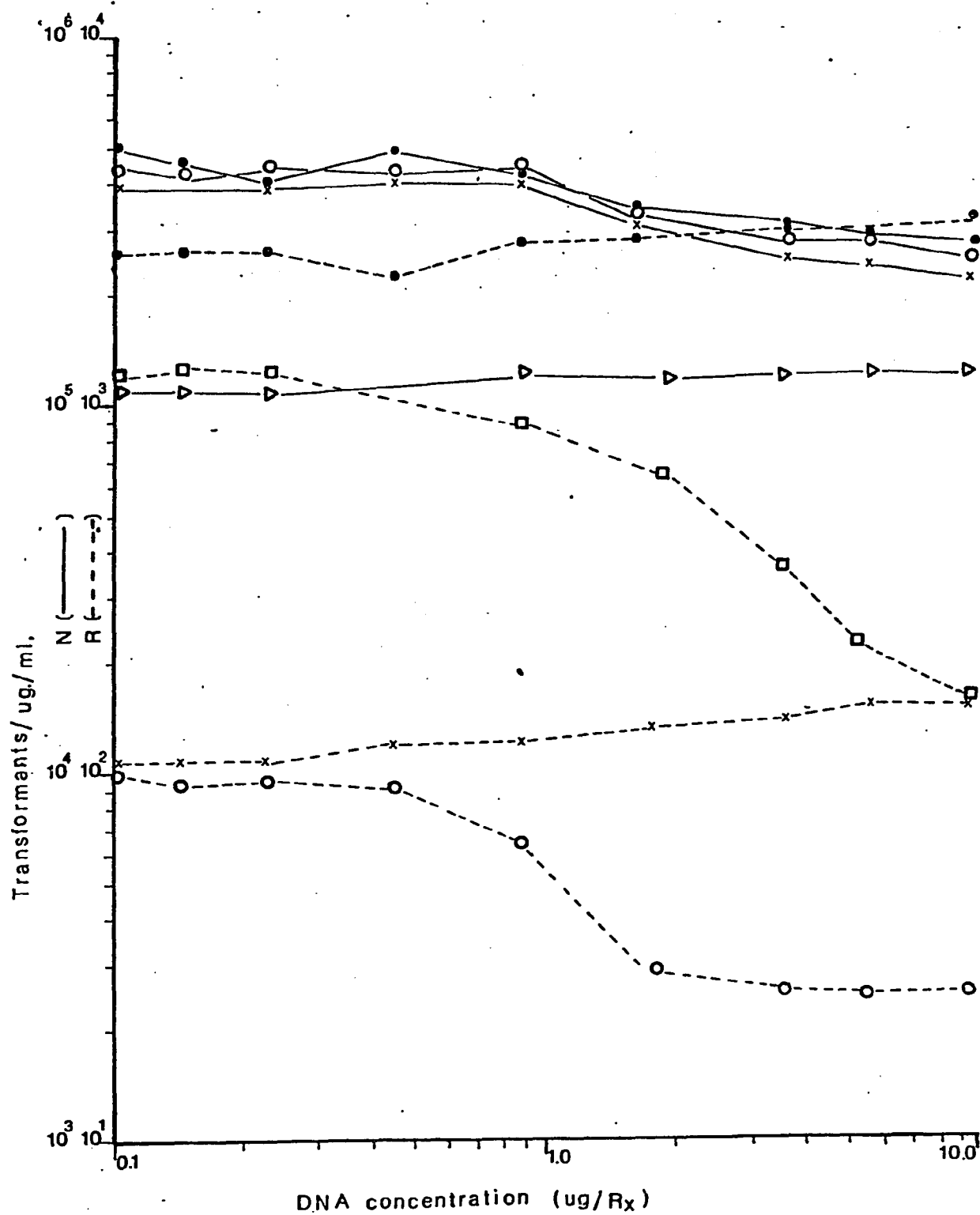


Figure 4

Agarose electrophoresis of various species of DNA. This composite photograph is a 0.75% agarose gel pattern of

a) lambda x EcoRI B) lambda x Hind III c) ϕ 105 x EcoRI

d) native B. subtilis DNA e) B. subtilis chromosomal DNA x EcoRI f) B. subtilis x Hind III g) calf thymus DNA x EcoRI

h) B. subtilis x EcoRI* activity i) B. subtilis sheared to 10% native biological activity j) B. subtilis x DNAase I

k) pUB110 native l) pUB110 x EcoRI.

Figure 4

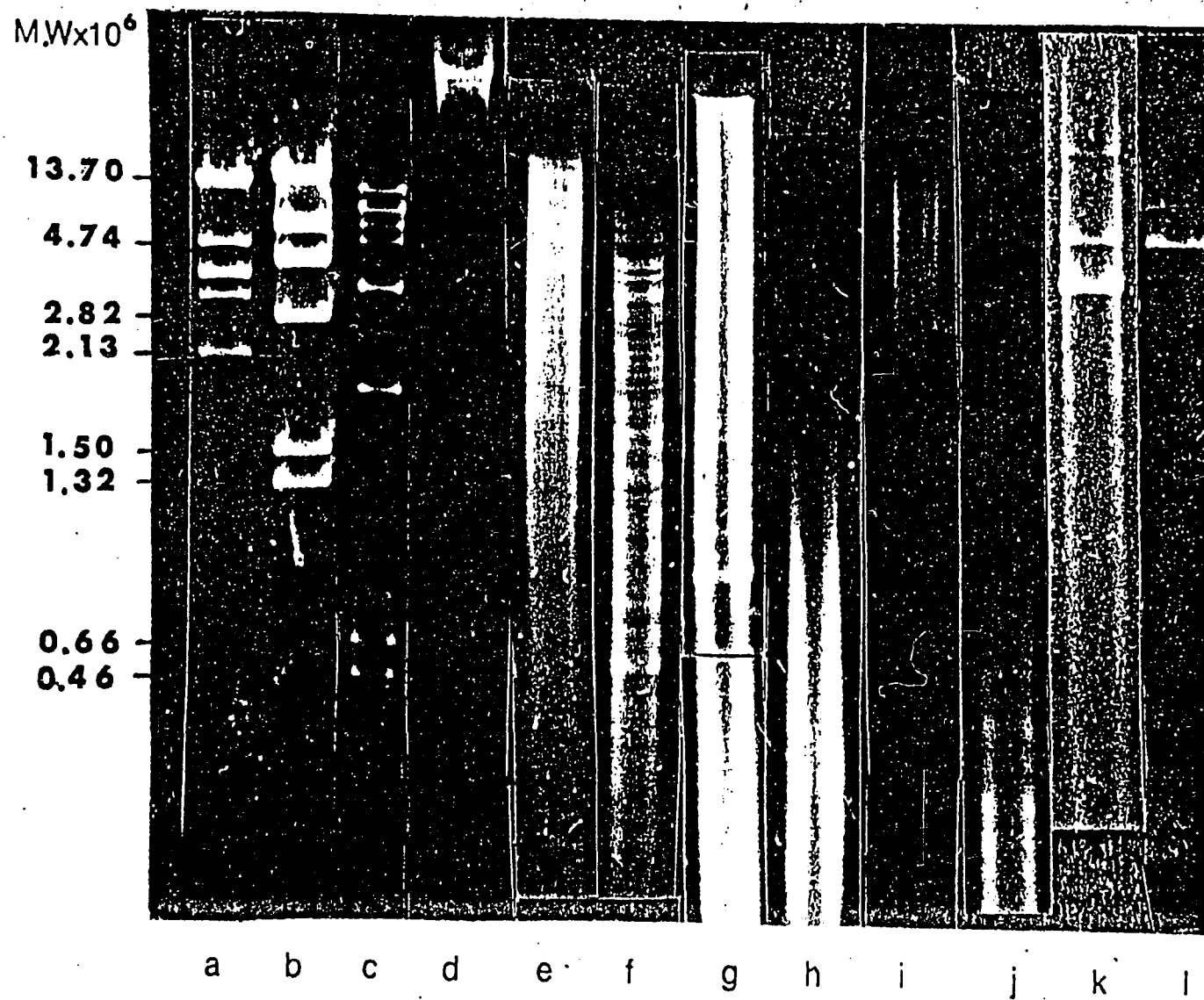


Figure 5

Standard curve of electrophoretic mobility vs. M.W. on 0.75% agarose gel. Bacteriophage lambda is digested to completion with Hind III nuclease and electrophoresed as in Methods. Anode is on right of migration scale. EtBr stained bands are measured and plotted vs. M.W. on log scale (see insert) or converted to negative 2/3 power by procedure of Bearden (1979). Where necessary gels longer than usual (9 cm) were run in order to detect the 0.32 Md. fragment. M.W. is from Schroeder and Blattner (1978). Eco RI digested lambda or ϕ 105 DNA was used to extend the weight range of the analysis.

Figure 5

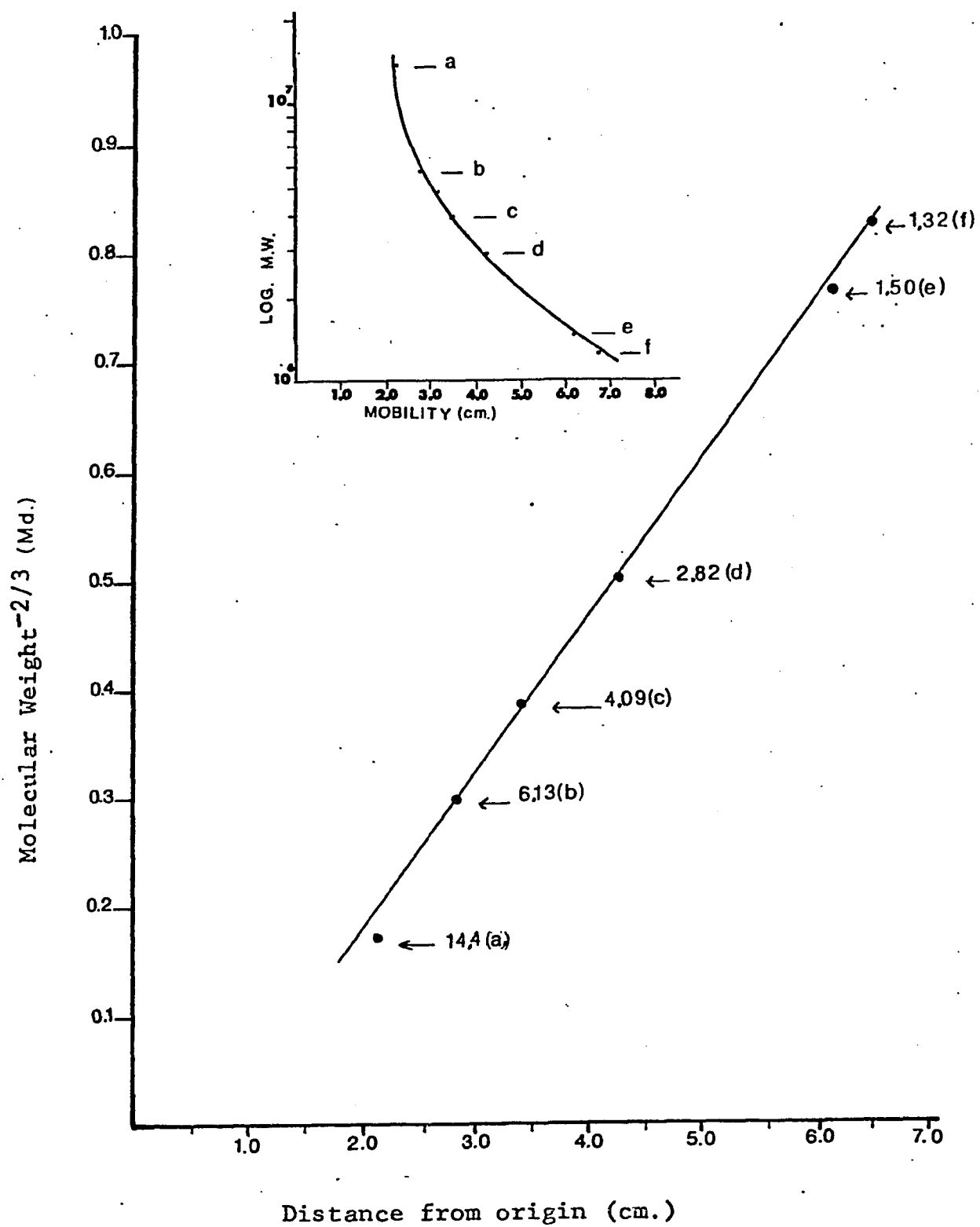


Figure 6

Labeling of protrophic W23 cells with H^3 -thymidine.

Cells were grown overnight in penassay broth and transferred to minimal broth (see Methods). Overnight minimal cultures were centrifuged, washed in dilution saline and brought to a Klett of 15 in fresh minimal media with or without 200 μ g/ml 2'dA, filter sterilized. After 15' growth at 37°C with agitation, 5 μ Ci/ml H^3 -Thymidine was added. TCA insoluble counts were measured from 0.5 ml. aliquots. Klett was recorded for control (Δ — Δ) and 2'dA ($\circ$ — $\circ$) supplemented cultures as was H^3 incorporation ($\blacktriangle$ — $\blacktriangle$) and ($\bullet$ — $\bullet$) respectively. Specific activity of 2'dA DNA was generally greater than 10^5 cpm/ μ g. 2'dA is 2' deoxyadenosine.

Figure 6

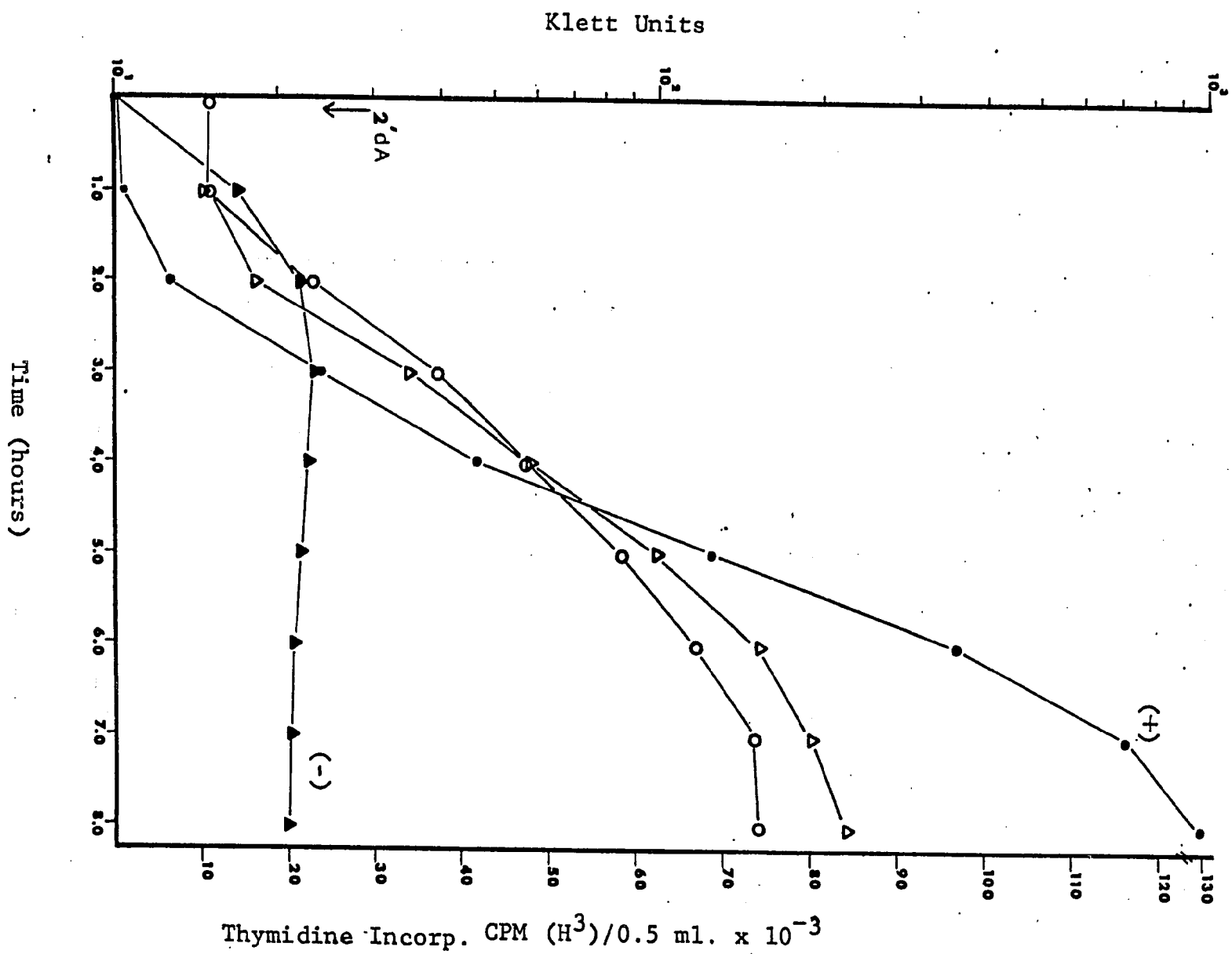


Figure 7

Decay kinetics of transforming activity following Eco RI digestion. Native and digested DNA samples were assayed at 1.0 - 2.0 ug/ml. Percent survival (RTA) is calculated as restricted transformants/ml divided by native values. Approx. 3 enzyme units/ug DNA were employed in the digestion mix and loss of activity with time followed by removing aliquots, terminating with 65°C heat for 10' and sterilization with chloroform. The arrow indicates the point at which an additional 100 units of enzyme activity were added to the system. Aliquots (0.1 ml) of DNA were added to 0.9 ml competent SB25 (trpC2, hisB2) for trp⁺ and his⁺; BD170 (trpC2, thr-5) for thr⁺; BD29 (argA2, leu-1) for arg⁺ and leu⁺; BR151 (trpC2, metB10, lys-3) for lys⁺. Transformed cells were selected after 48 hour incubation on minimal, supplemented media. Typical native transformants/ml were in the uniform range of 5 - 10 x 10⁵/ml.

Figure 7

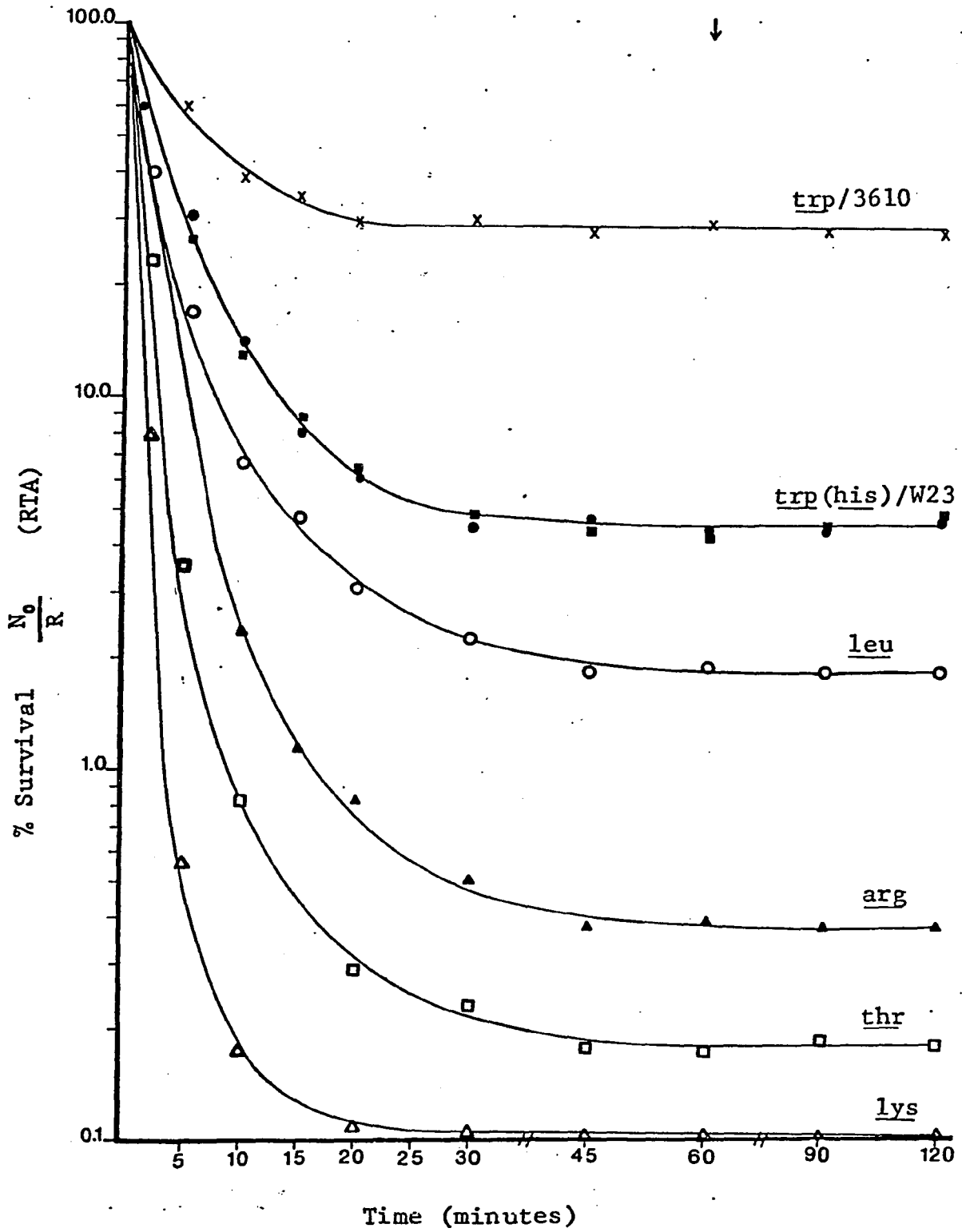


Figure 8

Effect of random scission on biological activity. Samples of B. subtilis DNA were transformed into auxotrophic BR151 cells brought to competency after 90' growth in Spizizen medium II. DNA conc./1.0 ml assay was 2.0 ug. Shearing was at an initial concentration of 20 ug/ml at 4°C in 1X SSC at setting 5 of a Sorvall Omni-mixer in a 2.0 ml cuvette. DNAase I from Worthington (0.02 ug/ml) was added to a similar DNA preparation in dilution saline containing 10mM MgCl₂ at 25°C. BR151 (trpC2, metB10, lys-3) was the recipient for W23 DNA trp⁺ (●—●), met⁺ (□—□), lys⁺ (○—○) transformants; DNAase residual trp⁺ activity is given by (X—X). Native samples were similarly maintained in the respective buffers and transformed for each time point.

Figure 8

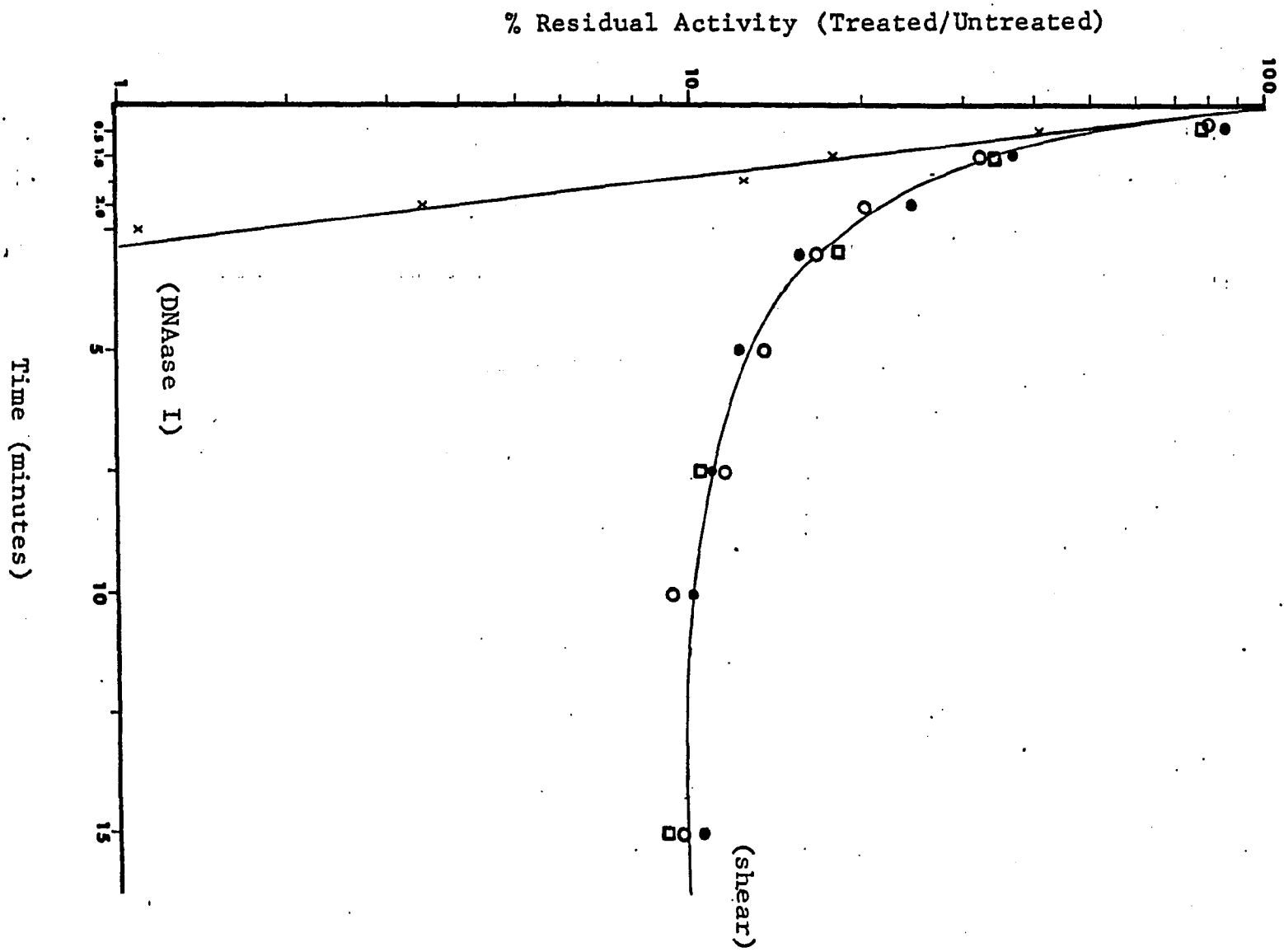


Figure 9

Survival of biological activity in plasmid pUB110 following restriction cleavage. A CsCl - EtBr gradient purified preparation of pUB110 was isolated from strain BD - 366. Initial DNA conc. 25 ug/ml. Treatment was with EcoRI or Hind III nuclease (1.0 unit/ug) in buffer (as per supplier; tbs. 3, 4) at 37°C. Transformation was at 2.5 ug/reaction (1.0 ml) and km^r colonies were scored by overlay with 10 ug/ml kanamycin sulfate following a 90' expression lag. Recipient cells were BD 170 (trpC2, thr-5, km) which generated 8.6×10^5 and 1.2×10^6 trp⁺ or thr⁺ transformants/2ug native chromosomal W23 DNA. Native plasmid yielded 3×10^5 km^r colonies/2.5ug DNA. Time samples (0.1ml) were removed and terminated by heating to 65°C for 10'. The insert displays the loss of pUB110 km^r donating ability vs. EcoRI conc. during a 30' digestion period. Also shown is a schematic of the pUB110 EcoRI recognition palindrome.

Figure 9

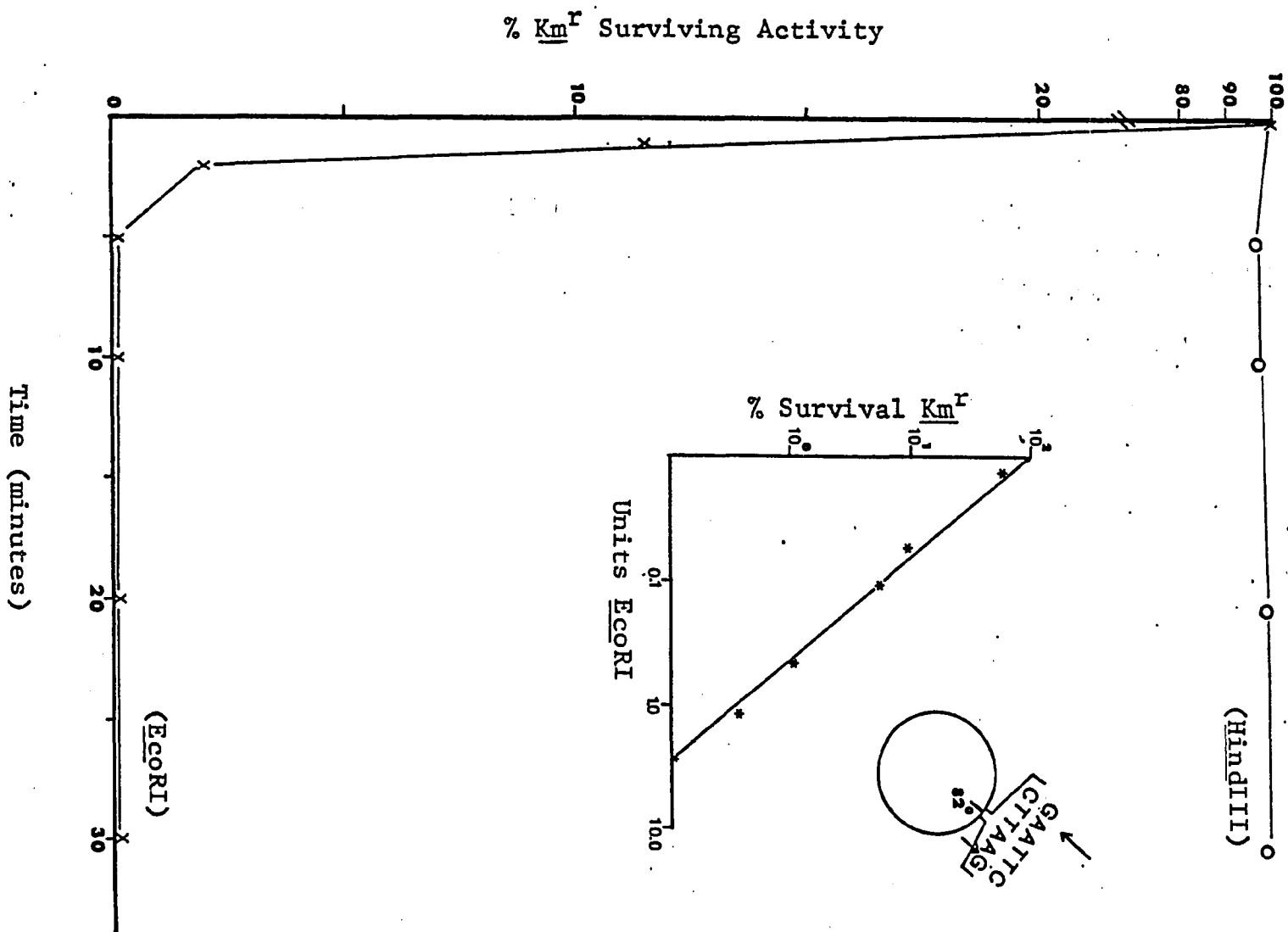


Figure 10

Product distribution following EcoRI cleavage of B. subtilis chromosomal DNA. Tritiated W23 (●—●) or 3610 (X—X) DNA was digested overnight with 2 units/ug DNA EcoRI nuclease at 37°C. A 50 ml aliquot (approx 1.0 ug) was electrophoresed in 0.75% agarose gel at 20 ma. until the bromphenol blue marker reached the 9.0 cm. position. Gel was EtBr stained, photographed and sliced into 1 - 2 mm sections, dissolved in 0.2 ml NH_4OH at 80°C followed by scintillation in Bray's solution. Molecular weight distribution is via direct measurement of EcoRI treated lambda and $\phi 105$ DNA and Hind III treated lambda on the same run. W23 and 3610 specific activity was approximately 4.8×10^5 and 3.9×10^5 cpm/ug respectively. Native undigested DNA migrated less than 0.6 cm from the origin (at position 0).

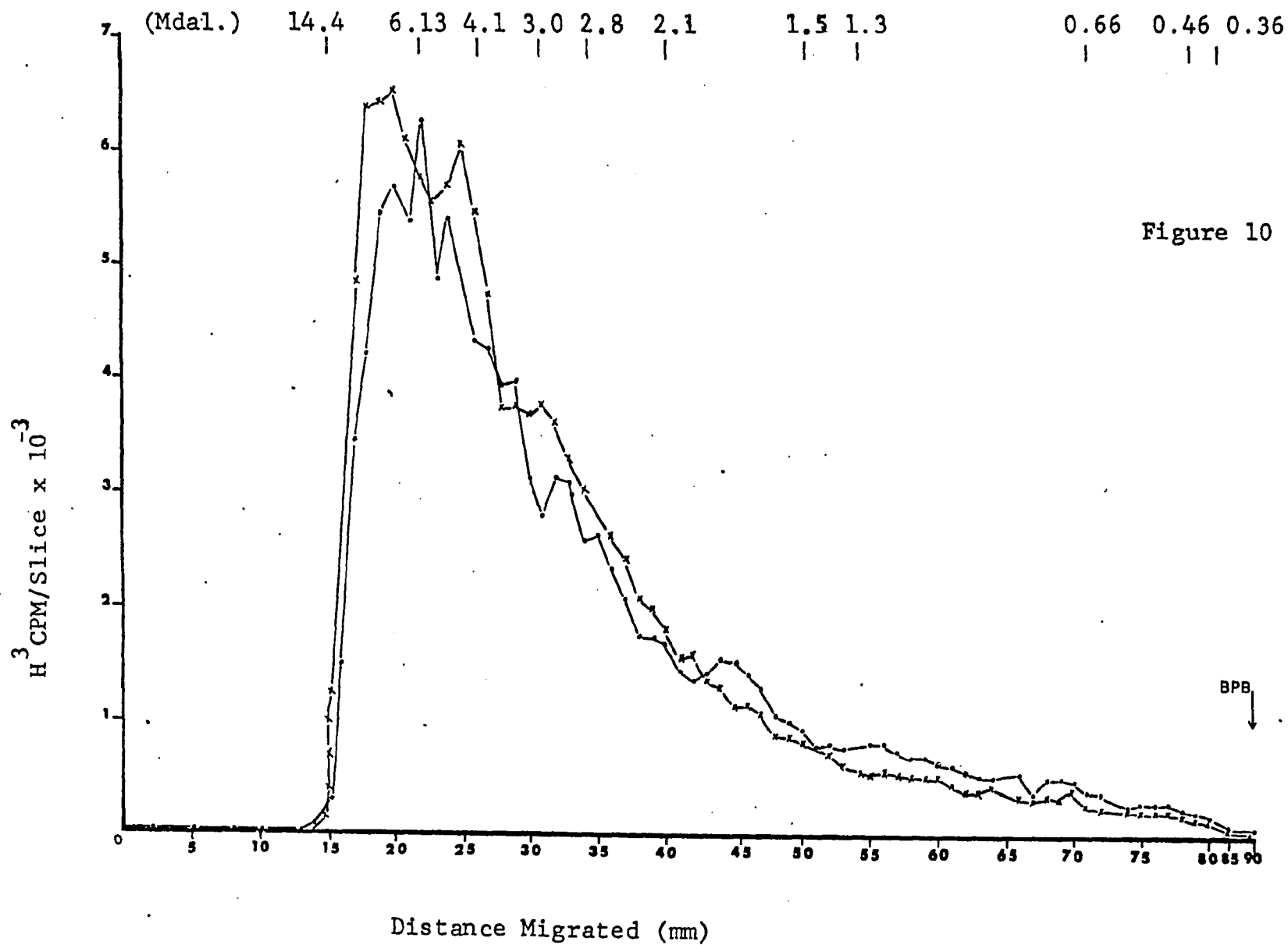


Figure 10

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Figure 11

Product distribution following EcoRI and EcoRI* digestion of B. subtilis DNA. Conditions are as in Fig. 10. W23 DNA was digested with EcoRI (X—X) or Eco RI* (●—●) specific conditions.

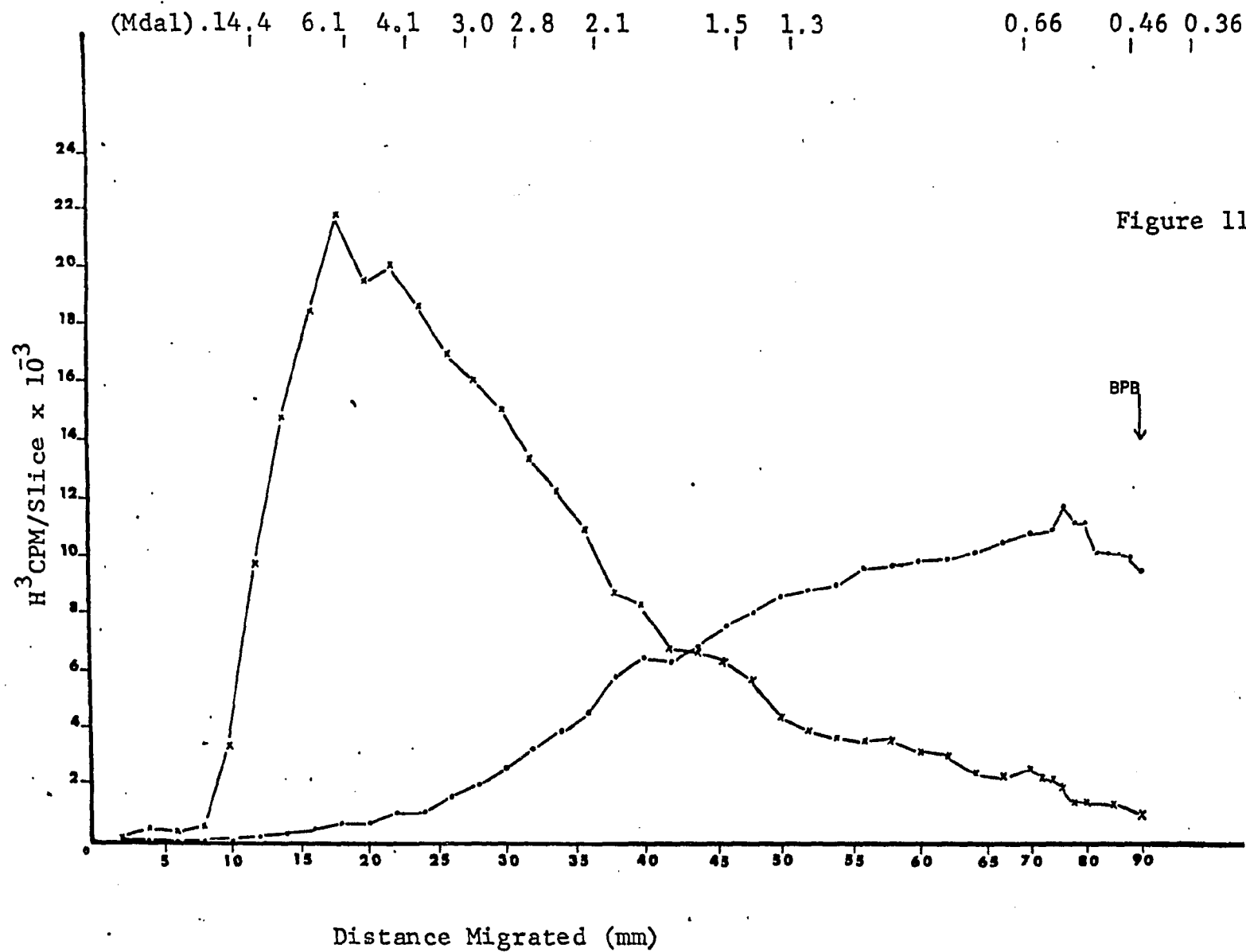


Figure 12

Effect of shearing on W23 chromosomal fragments. Samples of W23, tritium labeled, were sheared mechanically in a small volume Sorvall Omni-mixer vessel for various periods of time on ice, in 1 x SSC. Biological activity by transformation of BR151 (trpC2, metB10, lys-3) was reduced to 10% of native and 1% of native by controlled shearing for 1' (O—O) and 15' (X—X) respectively. Native transformants were trp⁺ 5.2×10^5 , metB10, 5.8×10^5 , lys⁺ 5.6×10^5 . Shearing was with 20 ug/ml unlabeled carrier DNA to which 5 ug/ml tritiated W23 had been added. Transformation analysis was at 2 ug/reaction. Aliquots of 50 ul. were electrophoresed on 0.75% agarose and processed as described in methods. Specific activities are given in Fig. 10.

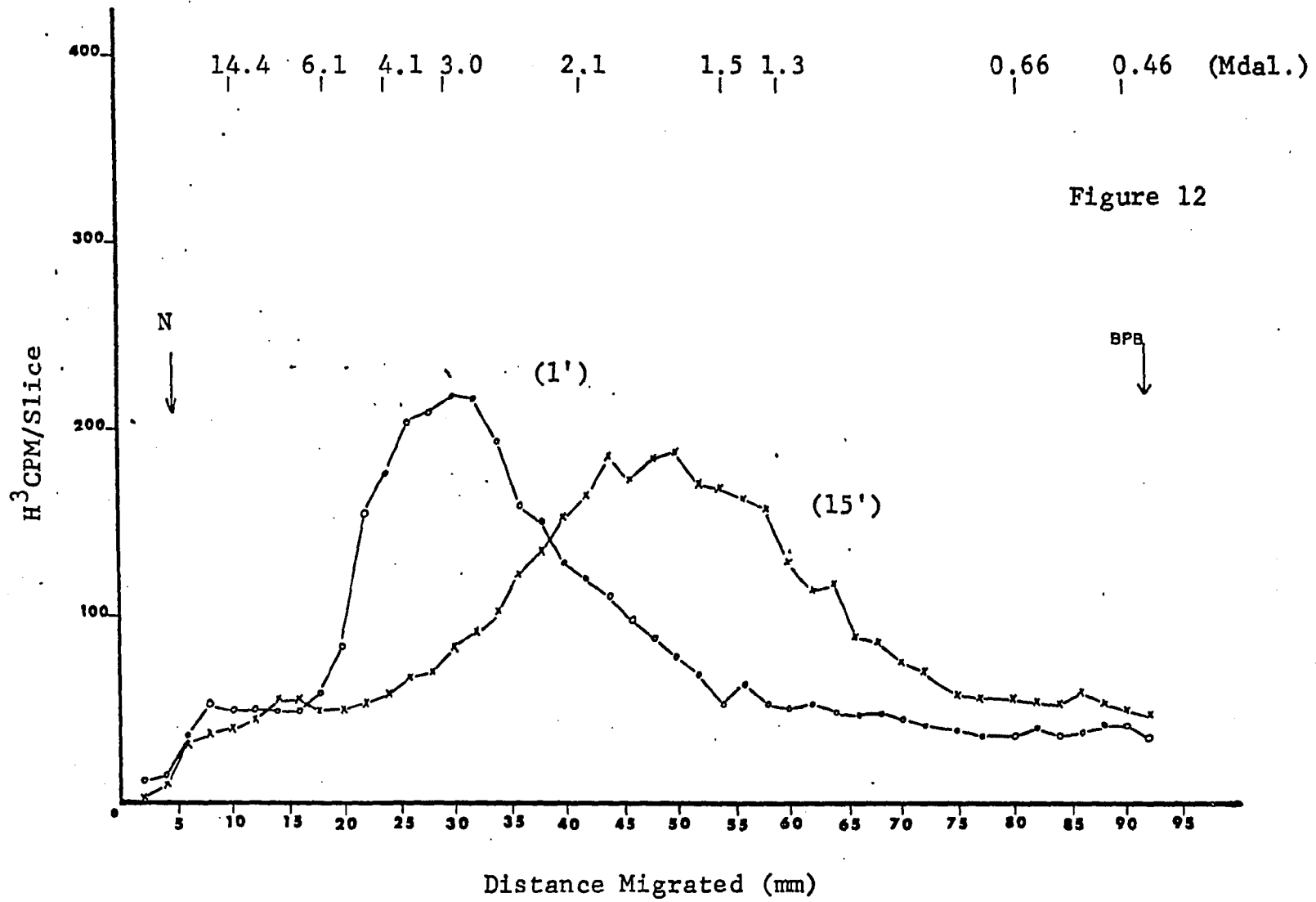


Figure 13

Product distribution following Hind III digestion.

Chromosomal DNA from W23 (X—X) and 3610 (O—O) was cleaved with restriction nuclease Hind III and run on a 0.75% agarose gel as described. Sections 1mm each were processed for tritium distribution.

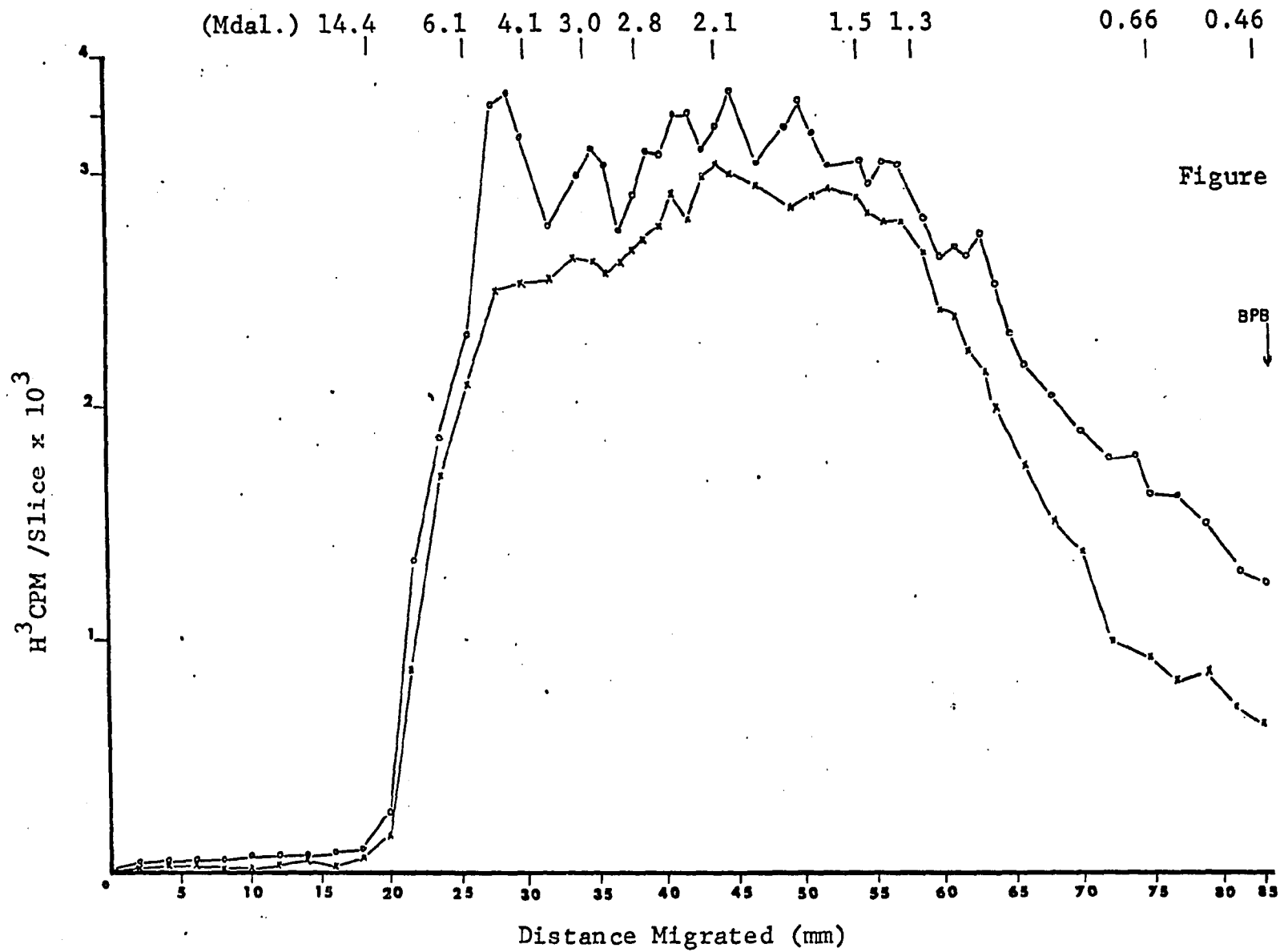


Figure 13

Figure 14

Gel electrophoresis of DNA molecules. A composite photograph of ethidium bromide stained DNA species was generated by gel electrophoresis. Lane a) lambda x EcoRI b) lambda x Hind III c) W23 Nd) W23 x EcoRI e) W23 x Hind III f) W23 EcoRI digest, ligated g) B. subtilis DNA shear 1' (10% biol. activity) h) shear 2' i) shear 5' j) shear 10' k) shear 15' (1% biol. activity) l) Hind III digest-partial digest m) B. pumilis x EcoRI n) pUB110 x EcoRI - ligated o) pRR-6 native p) pRR-6 x EcoRI q) pRR - 6 x HindIII r) pRR-6 x EcoRI/HindIII

Figure 14

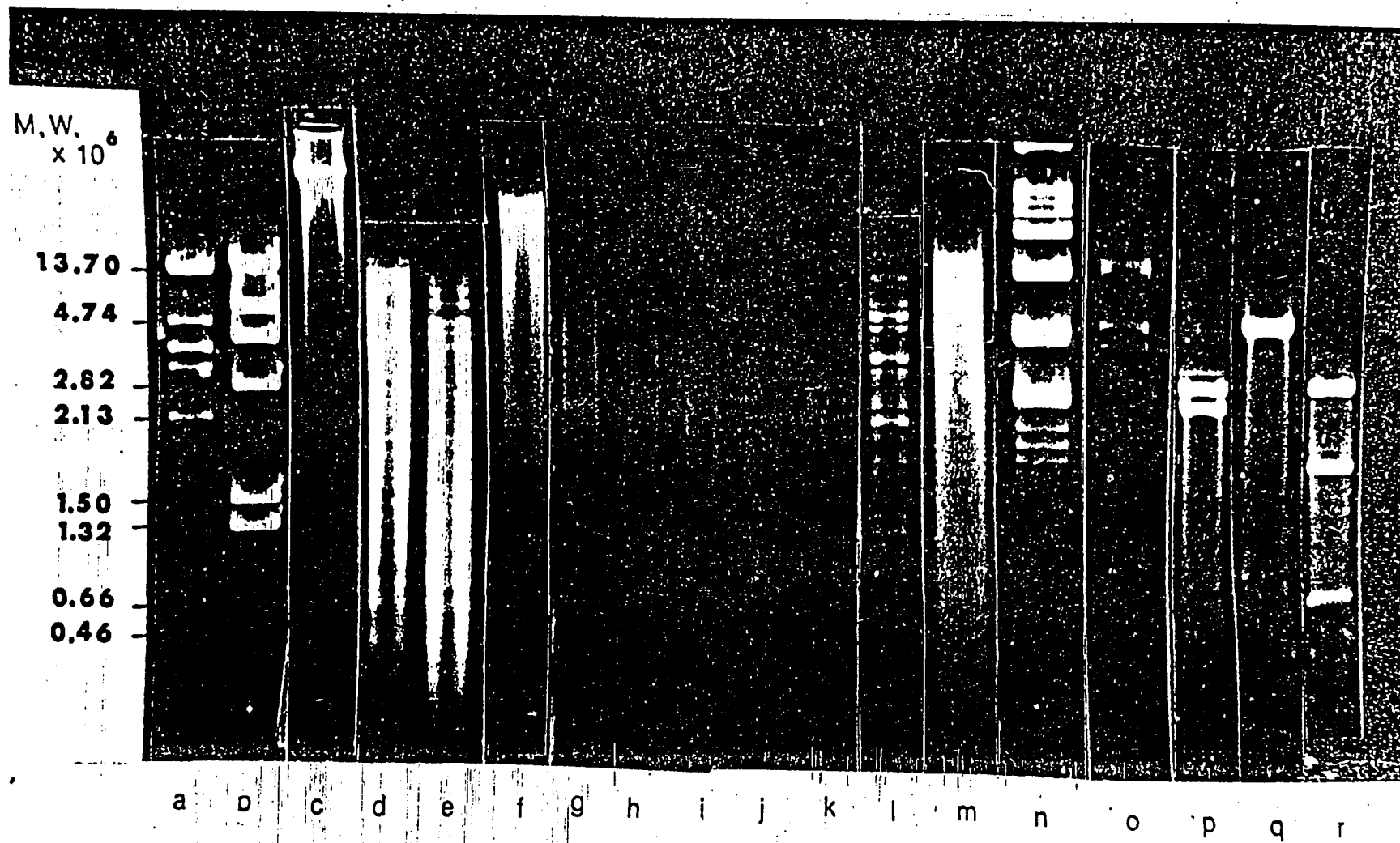


Figure 15

Enzyme dose curve of biological activity following EcoRI digestion of W23 DNA. EcoRI was diluted in 10mM KPO_4 buffer, pH7.0; 7mM 2-mercaptoethanol; 1mMEDTA; 200mM NaCl; 0.1% gelatin, and added to native chromosomal DNA in EcoRI digestion buffer. RTA was calculated after a 1 hour digestion at 37°C. Transformation was at 2 ug/rx. The reactions were terminated by heat to 65°C for 10'. Aliquots of 0.1 ml were removed and added to 0.9ml competent cells, followed by 30 min. incubation prior to selection of prototrophic transformants. Buffer conditions are as in Table 3. The arrow indicates the 50% RTA level.

Figure 15

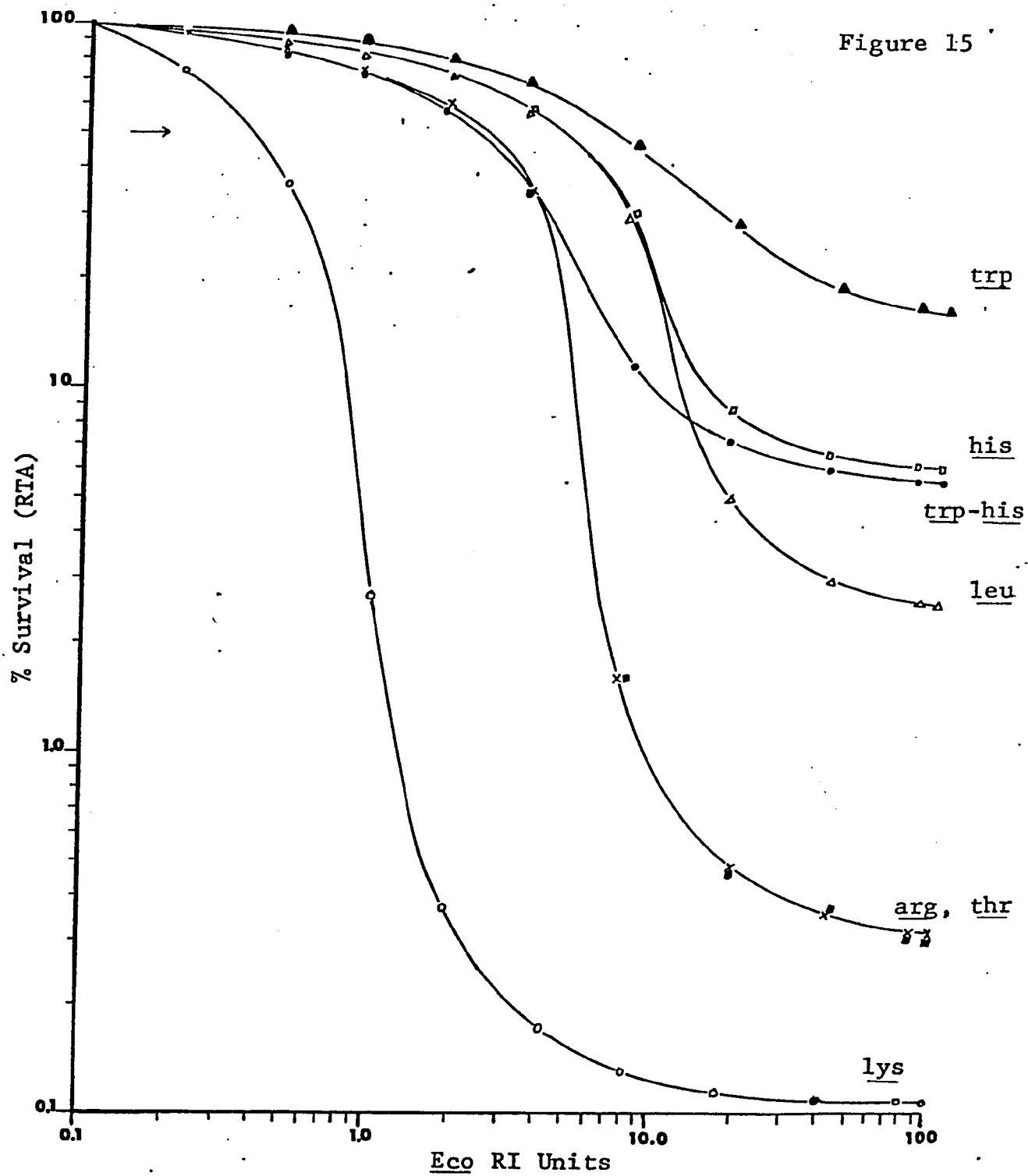


Figure 16

Marker survival with Hind III enzyme dose. DNA from W23 at 20 ug/ml was cleaved as in Fig. 15 with various concentrations of Hind III enzyme. BD 170 (trpC2, thr-5) is used to measure trp⁺ and thr⁺ transformants at a DNA conc. of 2 ug/rx. Buffer conditions are as in Table 4. Native transformation frequency was 7.8×10^5 and 8.4×10^5 /ml for trpC2 and thr-5 prototrophic transformants respectively. There were approximately 3.5×10^8 viable cells in the recipient culture. The arrow indicates the 50% RTA level.

Figure 16

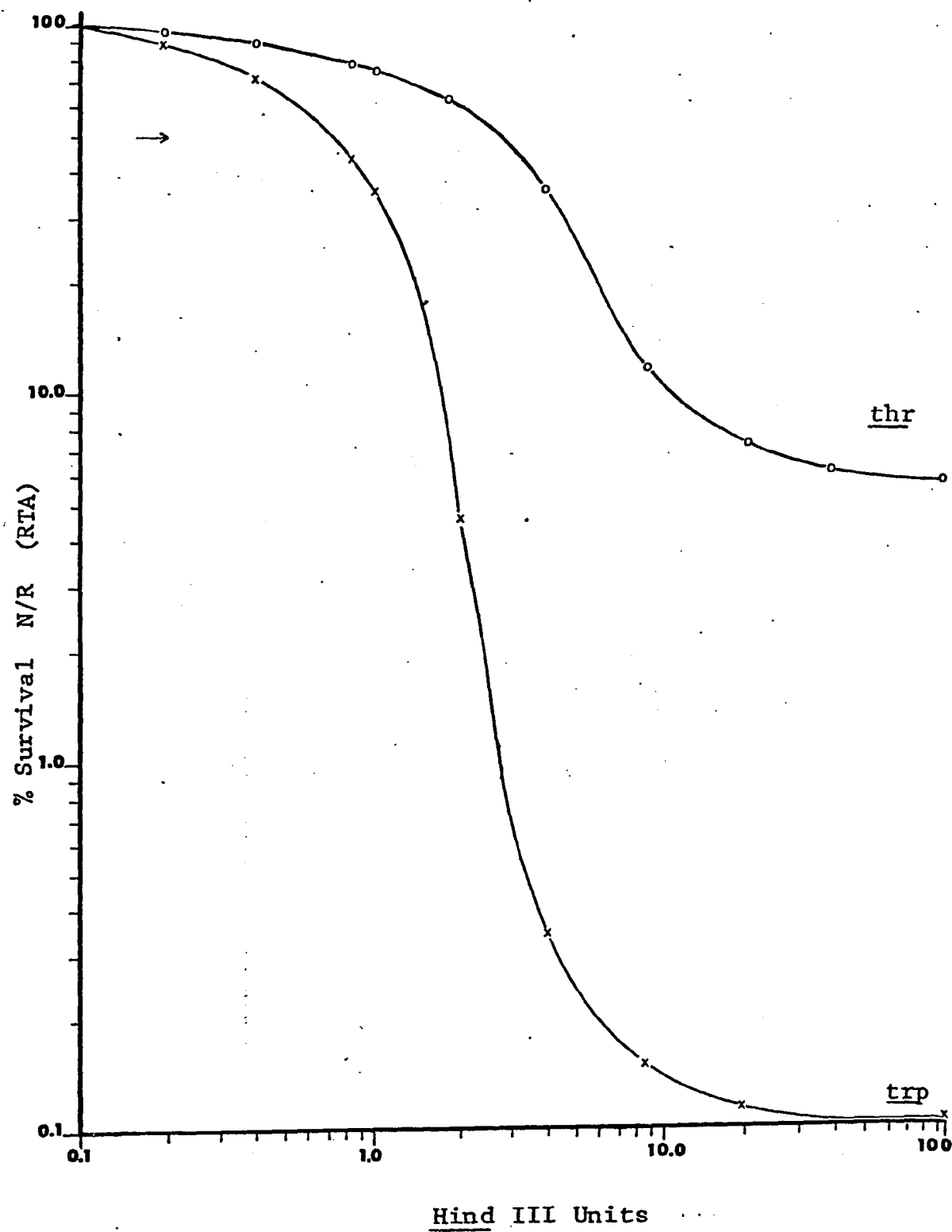


Figure 17

Marker decay kinetics with EcoRI enzyme dose. The data of Fig. 16 were selected and transposed to a linear scale. Activity of W23 trp⁺ and thr⁺ loci was determined in BD170 (trpC2, thr-5) while arg⁺ and leu⁺ were tested in recipient BD29 (argA2, leu-1).

Figure 17

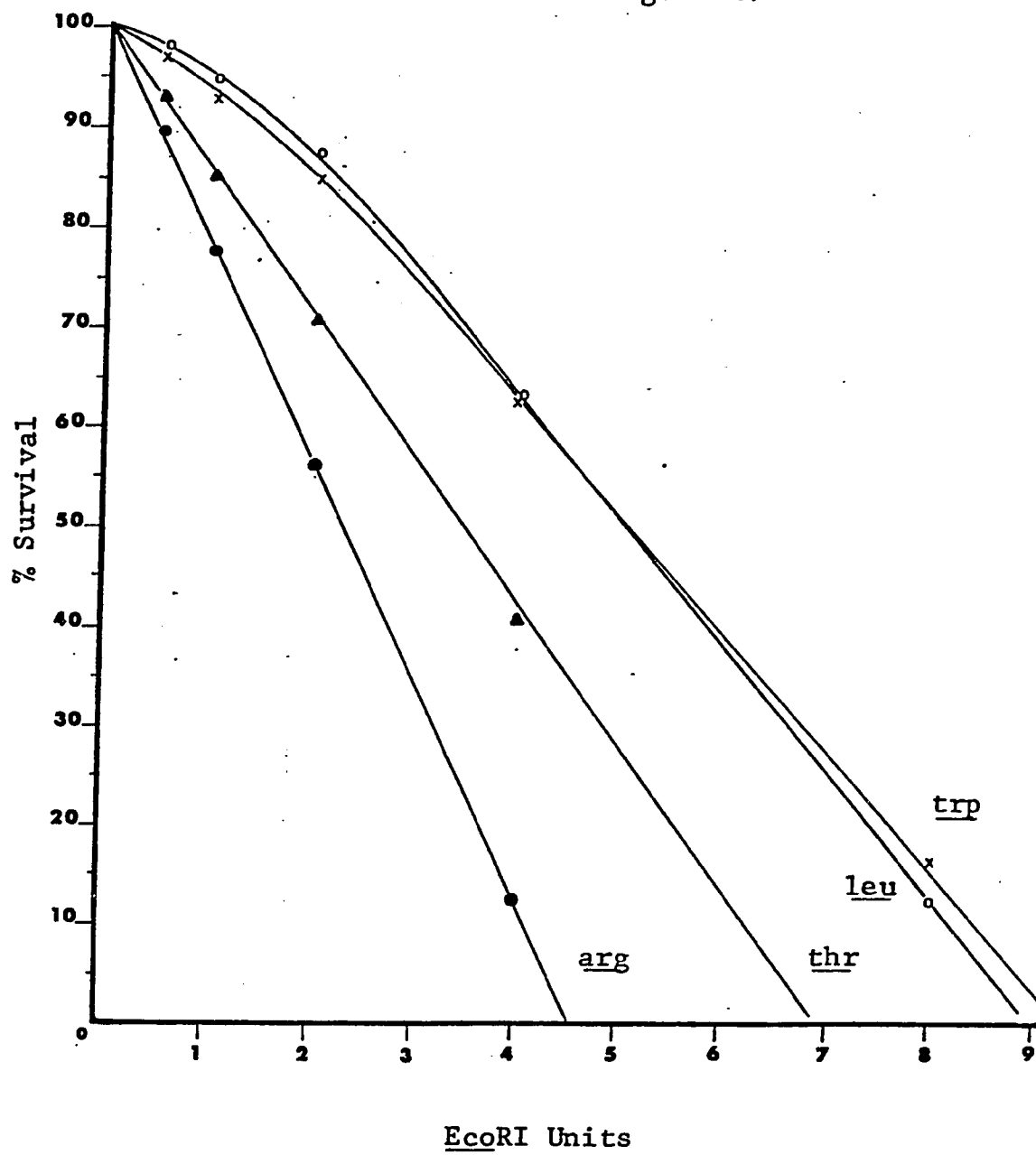


Figure 18

Cotransformation of gene activity versus EcoRI enzyme concentration. The trpC2 - hisB2 linkage group and the argA2 - leu-1 pair were tested for simultaneous cotransformation at 1.0 ug/ml in SB25 (trpC2,hisB2) and BD29 (argA2, leu-1) respectively. Native cotransfer frequencies were 65.8% and 2.0% in SB25 and BD29 respectively. Digestion was for 60' in the presence of various concentrations of nuclease. Cotransfer at each point was normalized to the native, undigested frequency. Cotransfer was calculated as $a^+b^+/0.5(a^+b+ab^+)$. Individual gene survival (RTA) following EcoRI digestion were 10%, 8%, 0.12%, 2.5% for trpC2, hisB2, argA2 and leu-1 respectively. A schematic of restriction cleavage palindromes relative to the prototrophic gene sequences is presented.

Figure 18

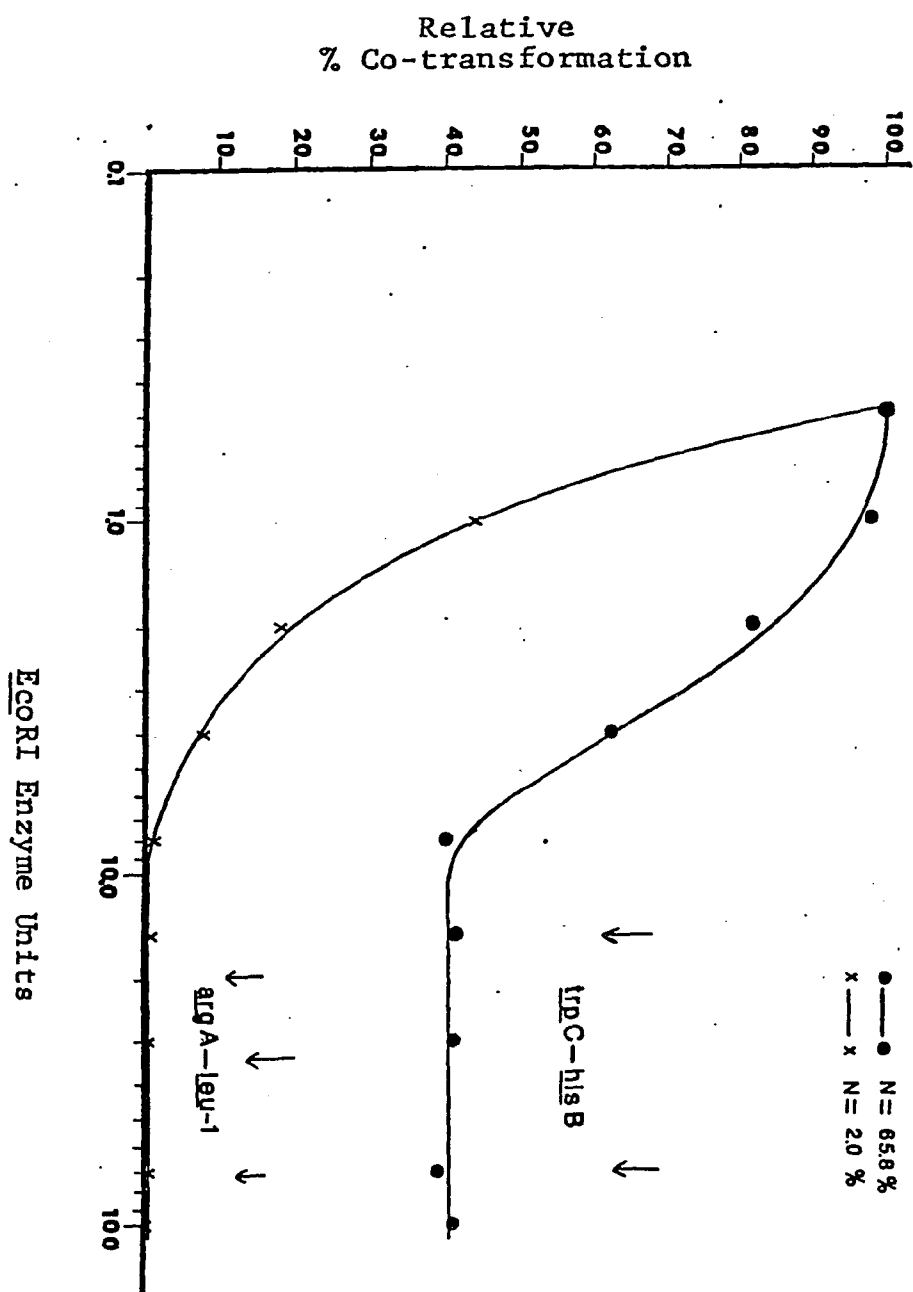


Figure 19

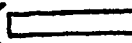
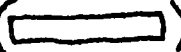
Gel profile of biological activity following restriction. Chromosomal W23 DNA was digested with 150 units of EcoRI or Hind III at a concentration of 50 ug/ml overnight. Aliquots of 50 ul. were loaded onto 0.75% agarose gels and separated until the dye marker reached 10 cm. Gels were sliced perpendicular to the direction of migration and the slices of 3 parallel lanes pooled for each distance increment. DNA was extracted as in Methods and transformed into mutant competent recipients. The total colony forming activities along the gel profile were totaled and activity in a given size range expressed relative to the total recovered restricted biological activity. (A) W23 EcoRI digest trpC2() or hisB2() complementing activity in SB25. (B) W23 EcoRI argA2() , leu-1() activity in BD29. (C) W23 Hind III digested thr-5() and leu-1() activity in BD170 and BD29. Molecular weight along the profile is provided by known restricted standards. Total EcoRI generated trp⁺, his⁺, arg⁺, leu⁺ colonies equal 1.6×10^5 , 1.5×10^5 , 2.7×10^4 , 7.1×10^4 /ml. Total Hind III thr⁺ and leu⁺ 4.8×10^5 , 6.8×10^4 . DNA conc. are approximate and about 1.0 ug/rx.

Figure 19

14.4 4.75 3.02 2.13 1.50 1.32 0.66 0.46 $\times 10^6$

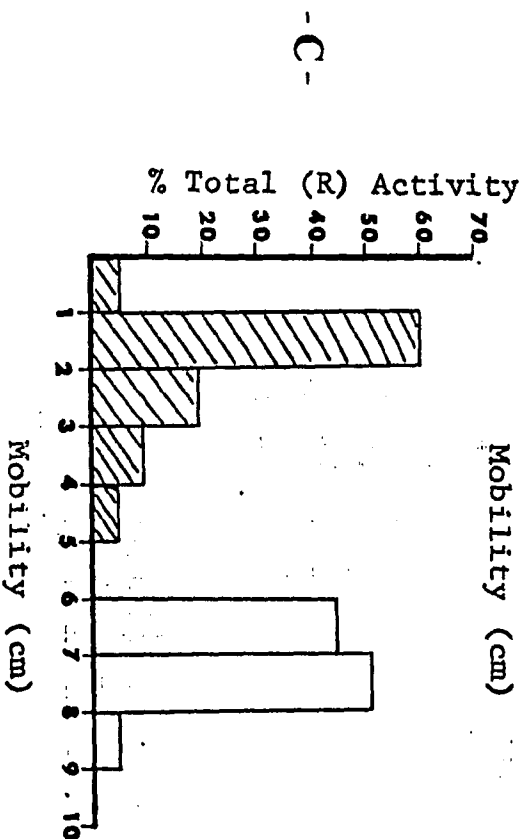
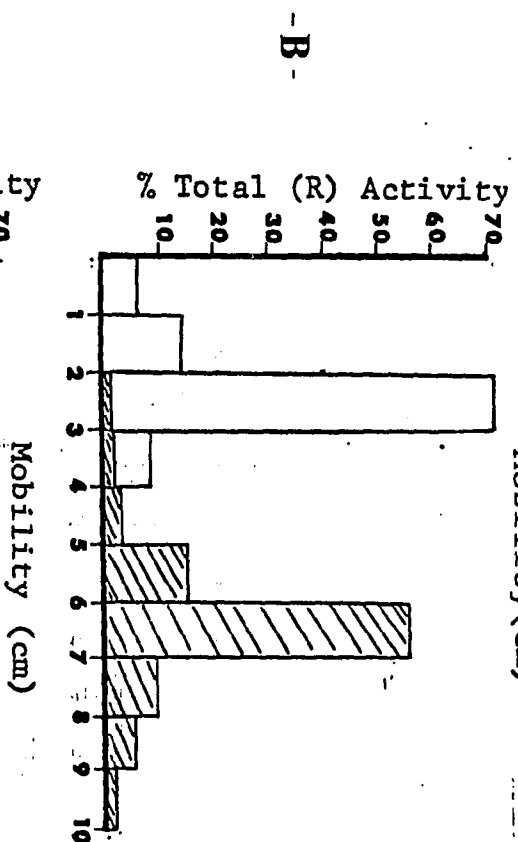
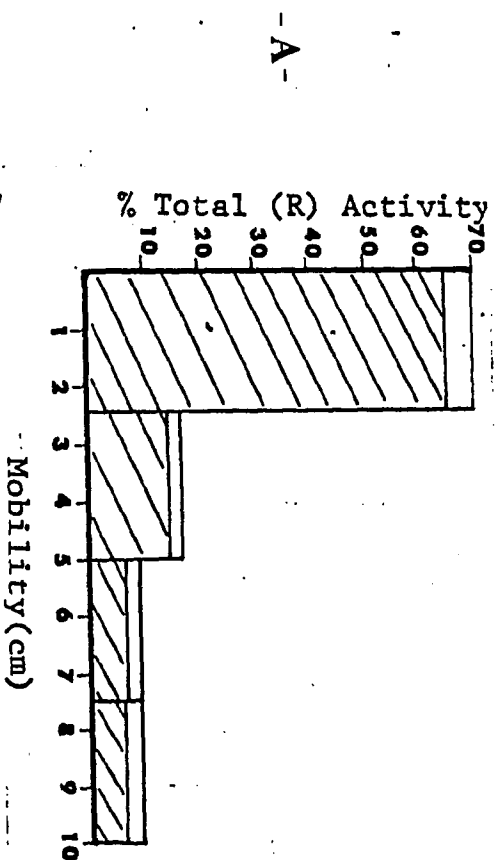


Figure 20

CsCl-EtBr fractionation of restricted and ligated B. subtilis chromosomal DNA. W23 (H^3) DNA (20 ug/ml) was digested overnight with 100 units EcoRI (total volume 2.5 ml) at $37^\circ C$. The enzyme was denatured at $65^\circ C$ for 10 min followed by ligation overnight at 4° in EcoRI buffer (Table 3) supplemented with 10mMDTT, 2mMATP, 50 ug/ml BSA, 2 units T4 DNA ligase (total volume 1 ml). This was sterilized with $CHCl_3$, assayed by transformation and a 0.5 ml aliquot centrifuged as in Methods, fractions collected via pump (5 drop) and 25 ul samples counted on filters. Fractions were collected from bottom to top. Area under curves was calculated by integration with a Ti58 calculator. Biological activity in BR 151 is as in Table 15. Total gradient volume is 8.0 ml.

Figure 20

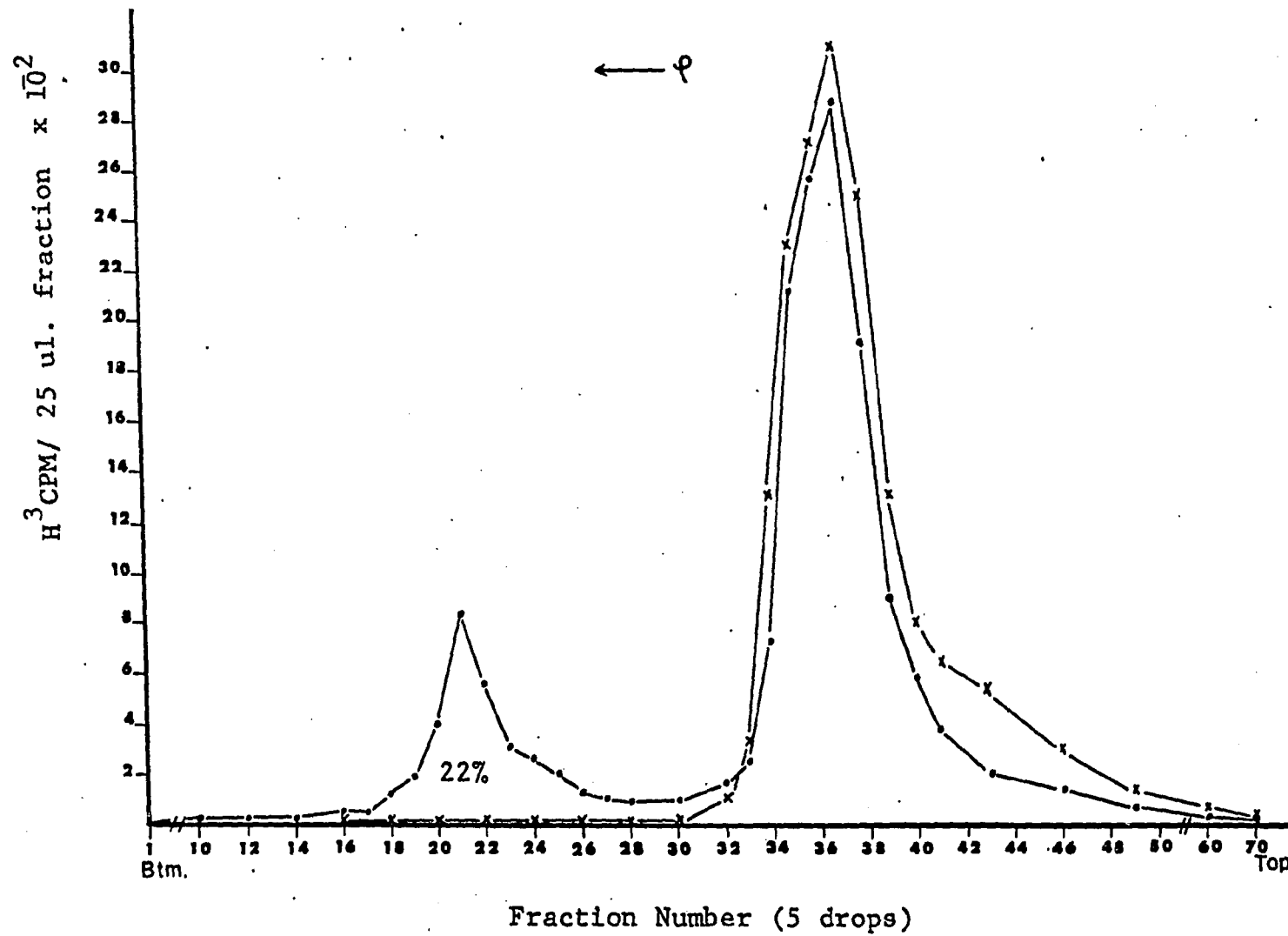


Figure 21

Determination of copy number of pUB110. Uncleared lysates of pUB110 infected BD170 were prepared from tritium labeled B. subtilis cells grown in the presence of 2'dA as in Methods. 0.25 ml. of such a 30 ml. lysate was applied to a dye-density gradient. The radioactivity in the CCC region is expressed as a percent of total by area integration and copy number calculated as: $\text{Copy} = \frac{\text{fraction CCC} \times \text{M.W. host chromosome}}{\text{M.W. plasmid}}$. Centrifugation and filter processing were as in Methods. Plasmid genotype was monitored by spot testing host before and after plasmid infection. Plasmid M.W. (3.0×10^6) was verified by gel electrophoresis. Total gradient volume approx. 8.0 ml. Growth medium was minimal broth supplemented with 200 ug/ml 2'dA, 25 ug/ml tryptophan and 25 ug/ml thr, 10 ug/ml kanamycin. Final klett was 170 units.

Figure 21

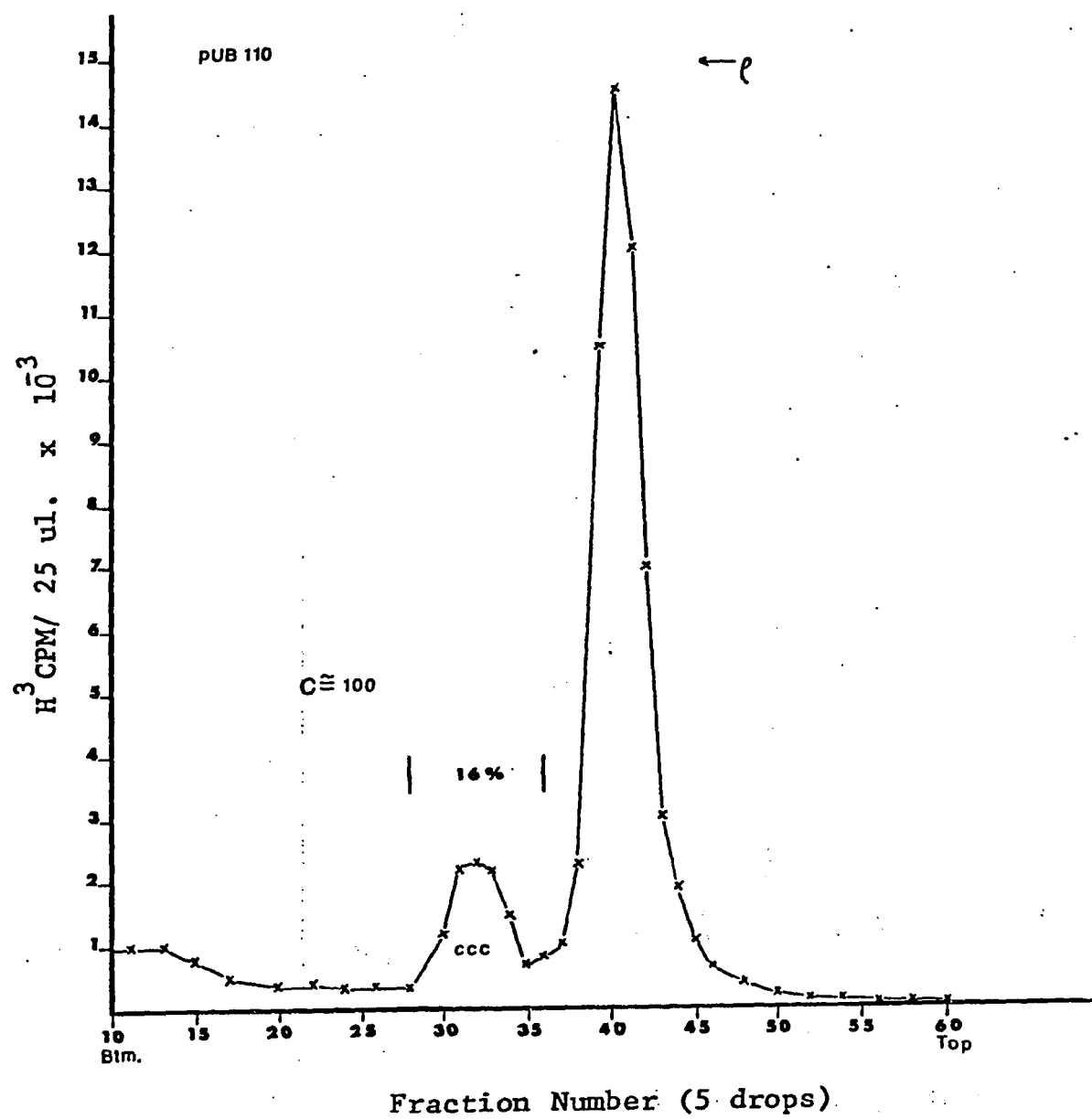


Figure 22

Determination of copy number of plasmid pRR-6. The pUB110-trp⁺(B. pumilus) chimera was passed into BD170 and labeled with H³-(CH₃)-thymidine in the presence of 200 ug/ml 2'dA. An uncleared, total lysate was prepared as in Fig. 21 and dye-gradient analysis performed. Growth was in minimal medium containing 10 ug/ml kanamycin, 25 ug/ml threonine as in Methods. Cell genotypes were monitored by spot testing on selective media. Final klett was 150 units.

Figure 22

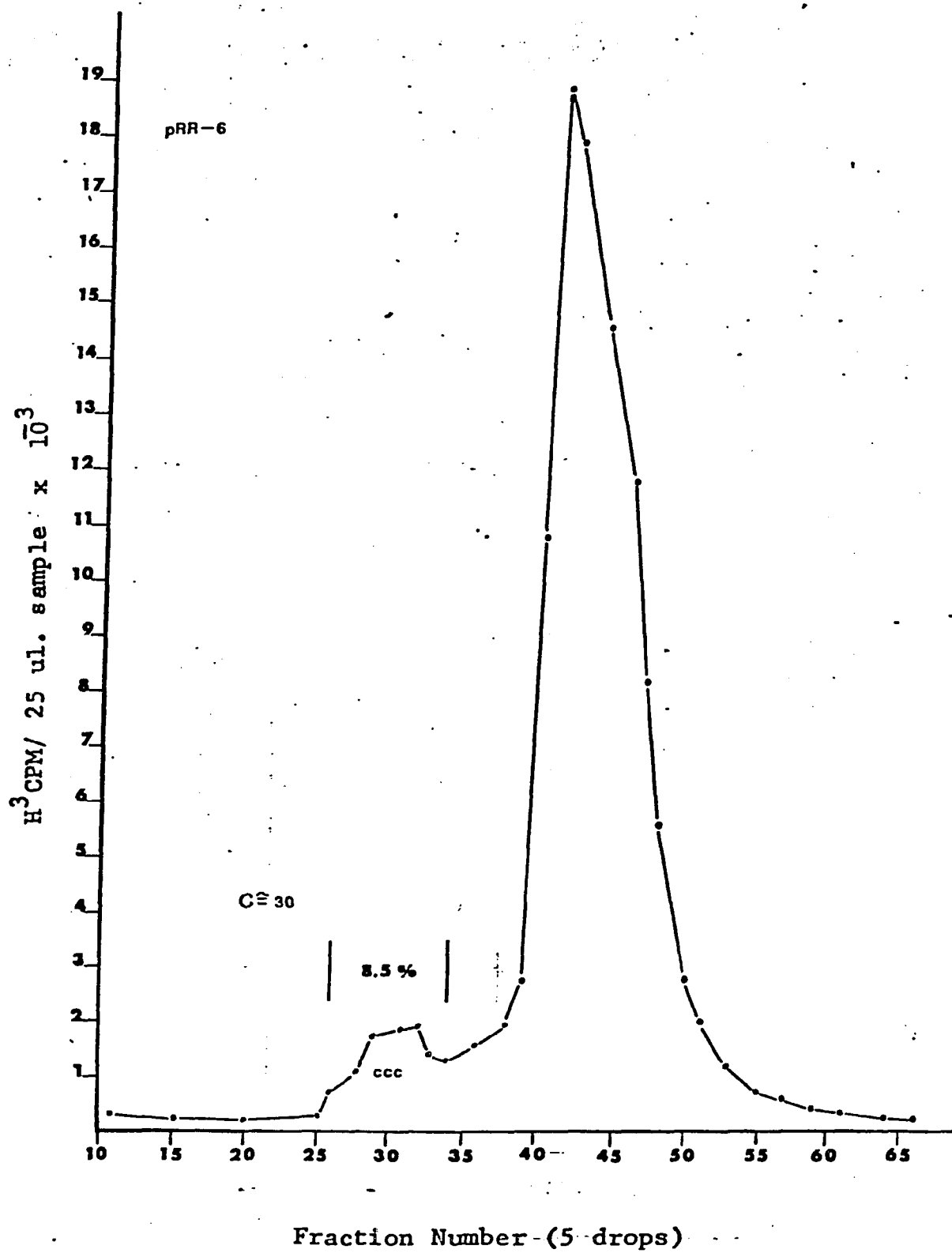


Figure 23

Restriction cleavage of pRR-6 clone. The plasmid was digested with restriction nuclease and analyzed on a 0.7% agarose gel. Lane a) λ x HindIII b) pRR-6 x BglIII/EcoRI c) EcoRI/HindIII d) BclI e) HpaII f) HaeIII g) ϕ 105 x EcoRI. See Table 20 for fragment sizes.

Figure 23

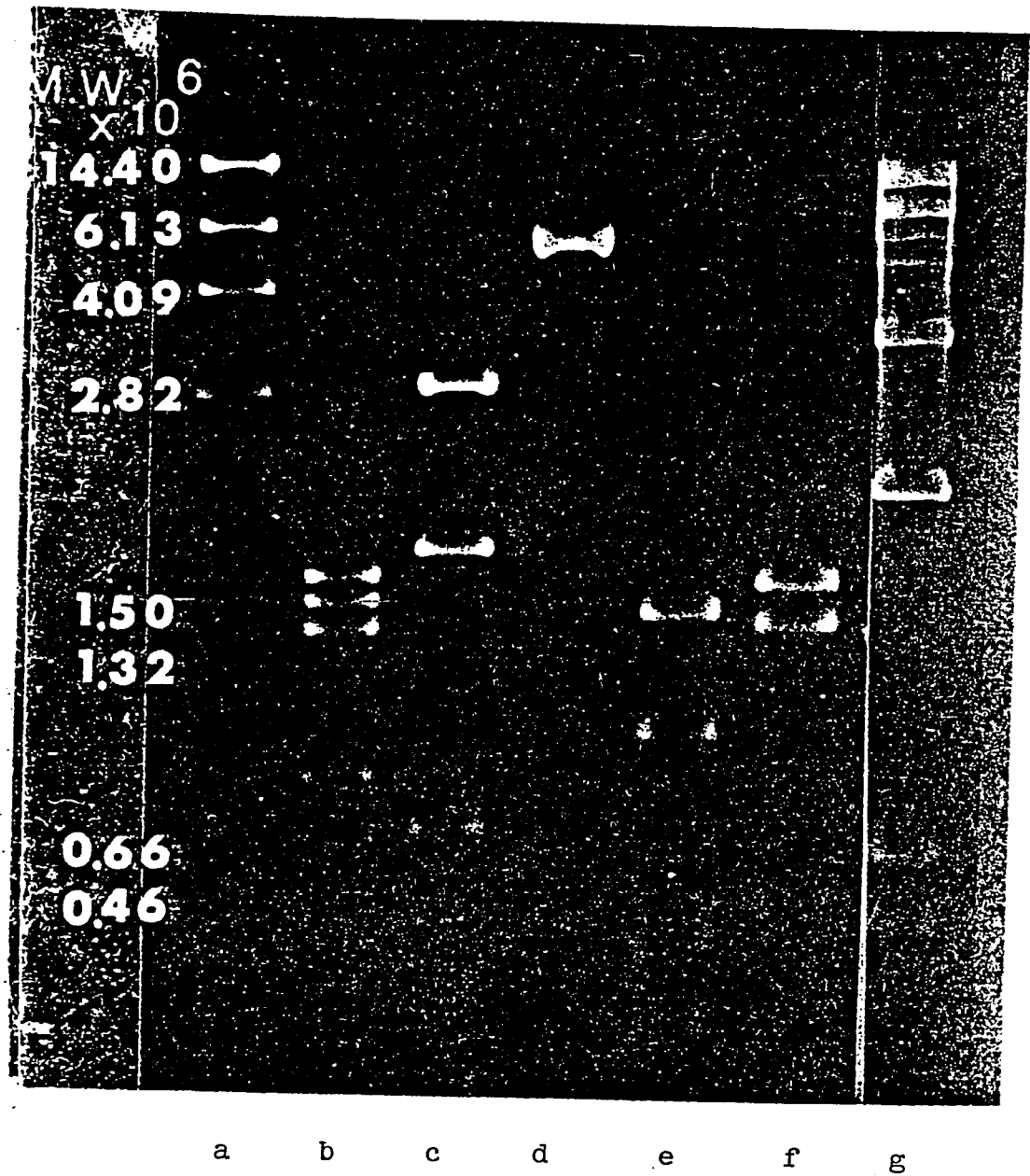
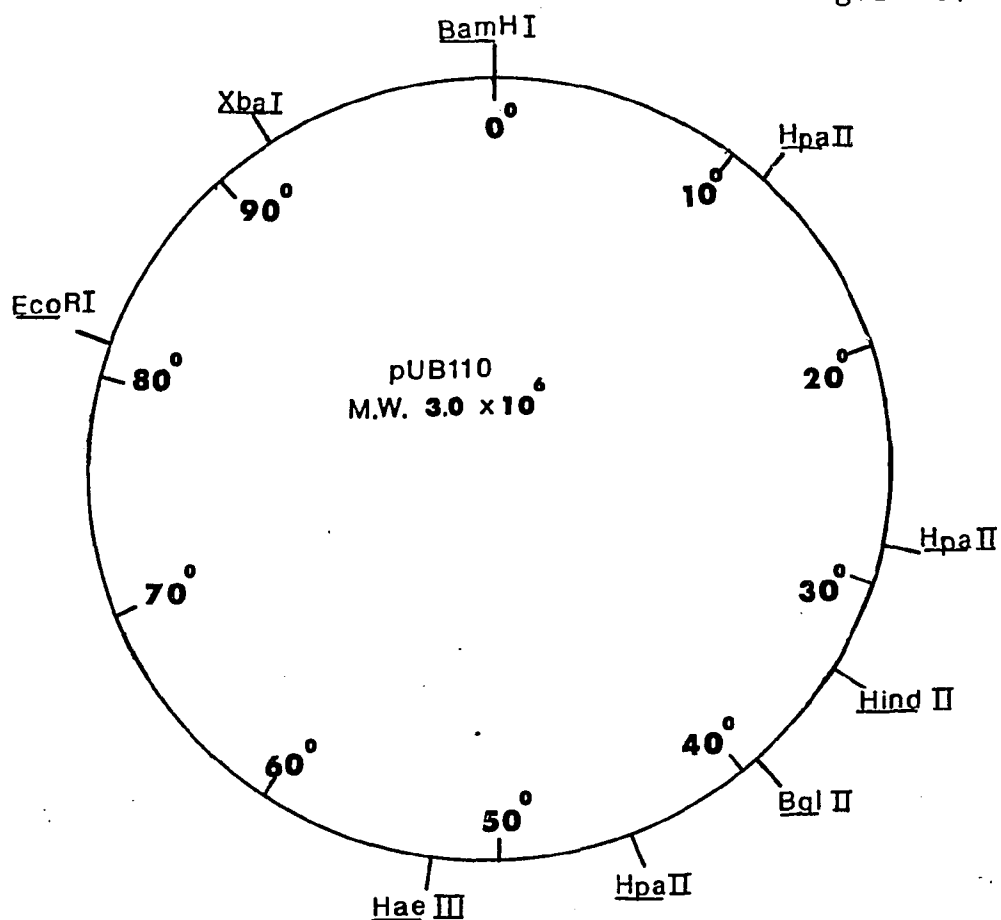


Figure 24



Enzyme	A	B	Fragments (Mdal.)			E
			C	D		
<u>EcoRI</u>	3.00	-	-	-	-	-
<u>HindIII</u>	(3.00)	(no recognition sites)				
<u>EcoRI/BamHI</u>	2.45	0.52	-	-	-	-
<u>EcoRI/HpaII</u>	1.10	0.71	0.50	0.45	(?)	-
<u>BglIII</u>	3.00	-	-	-	-	-
<u>EcoRI/BglIII</u>	1.55	1.40	-	-	-	-

Data of Gryczan, T., Contente, S., Dubnau, D.
J. Bact. 134:p. 318-329, 1978.

Figure 25

Restriction fragment map of pRR-6. The trp⁺ Eco RI fragment of B. pumilis origin is pictured as an insert in the km^r pUB110 plasmid vector. Restriction sites are located, along with their molecular size (arrows), within the insert (—) bounded by vector sequences (----). Site position was by comparison of pRR-6 cleavage (Table 20) with scheme of vector pUB110 as determined by Gryczan et al (1977) (Fig. 24) and by cleavage of gel purified fragments. Cistron size is not drawn to relative scale (Table 16), nor is mutant allele position clear. However, approximate position of cleavage on the genetic coding span is close to that predicted by protein size. Cistron content is based on mutant complementing capacity (Table 19). Coding capacity of trp (E,D,C,F,B,A) operon relative to total protein M.W is 0.27, 0.18; 0.09, 0.1, 0.27, 0.08 respectively while capacity of insert, based on protein M.W relative to trp E (84,000 d.), is 1.0, 0.68, 0.32, 0.36 for E, D, C, F respectively.

Figure 25

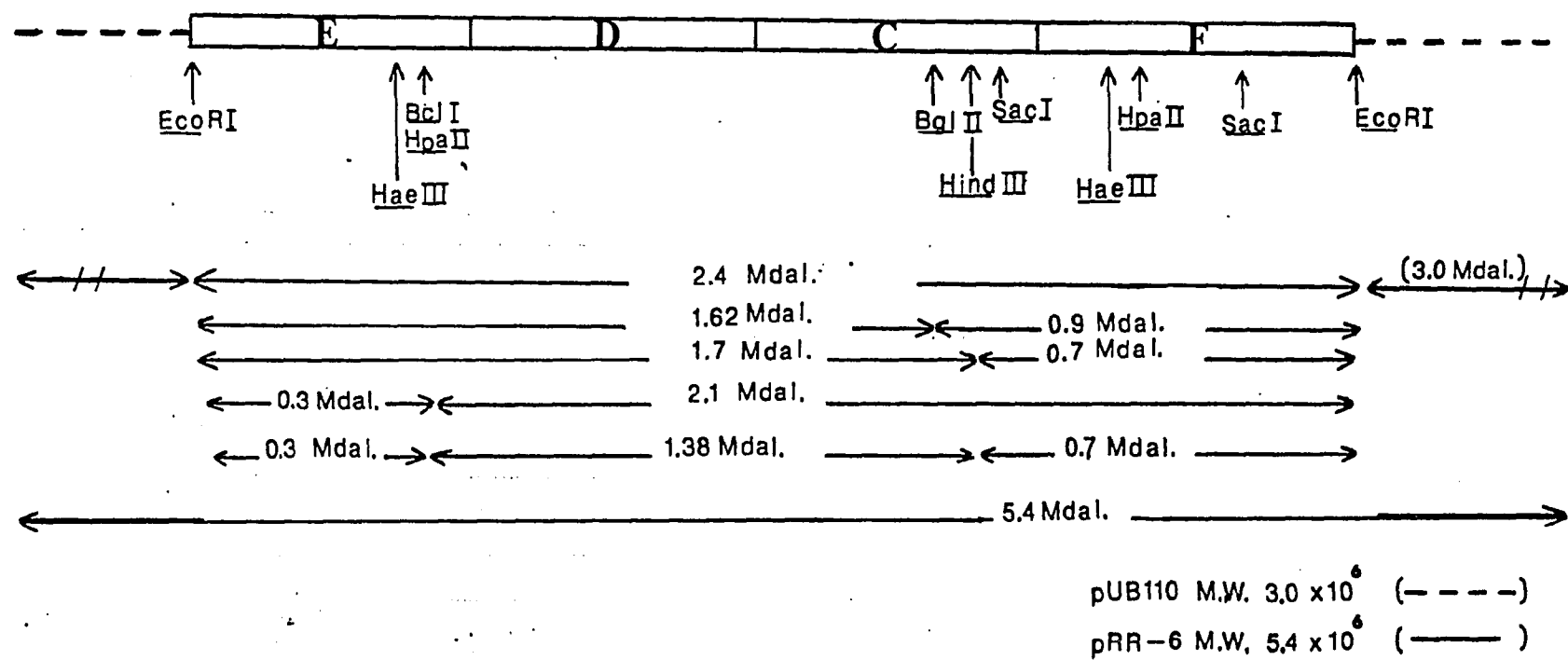


Figure 26

Nick translation kinetics of plasmid pUB110. Density purified sample of plasmid (1.0ug) was labeled with H^3 -(CH₃) thymidine-5'-triphosphate (78.1Ci/mM) according to Rigby et al (1977) and as described in Methods with 200 pmoles labeled dTTP and 300 pmoles each unlabeled dGTP, dCTP, dATP. At various times after addition of 0, 0.1 or 0.5 ng DNAase I and 2.0 units of Pol I, aliquots (2.0 ul) were terminated by addition to 0.2 ml acid-phosphate (see Methods) stop solution followed by counting acid insoluble counts on filters. Total reaction volume was 100 ul. Reaction temp. was 10°C.

Figure 26

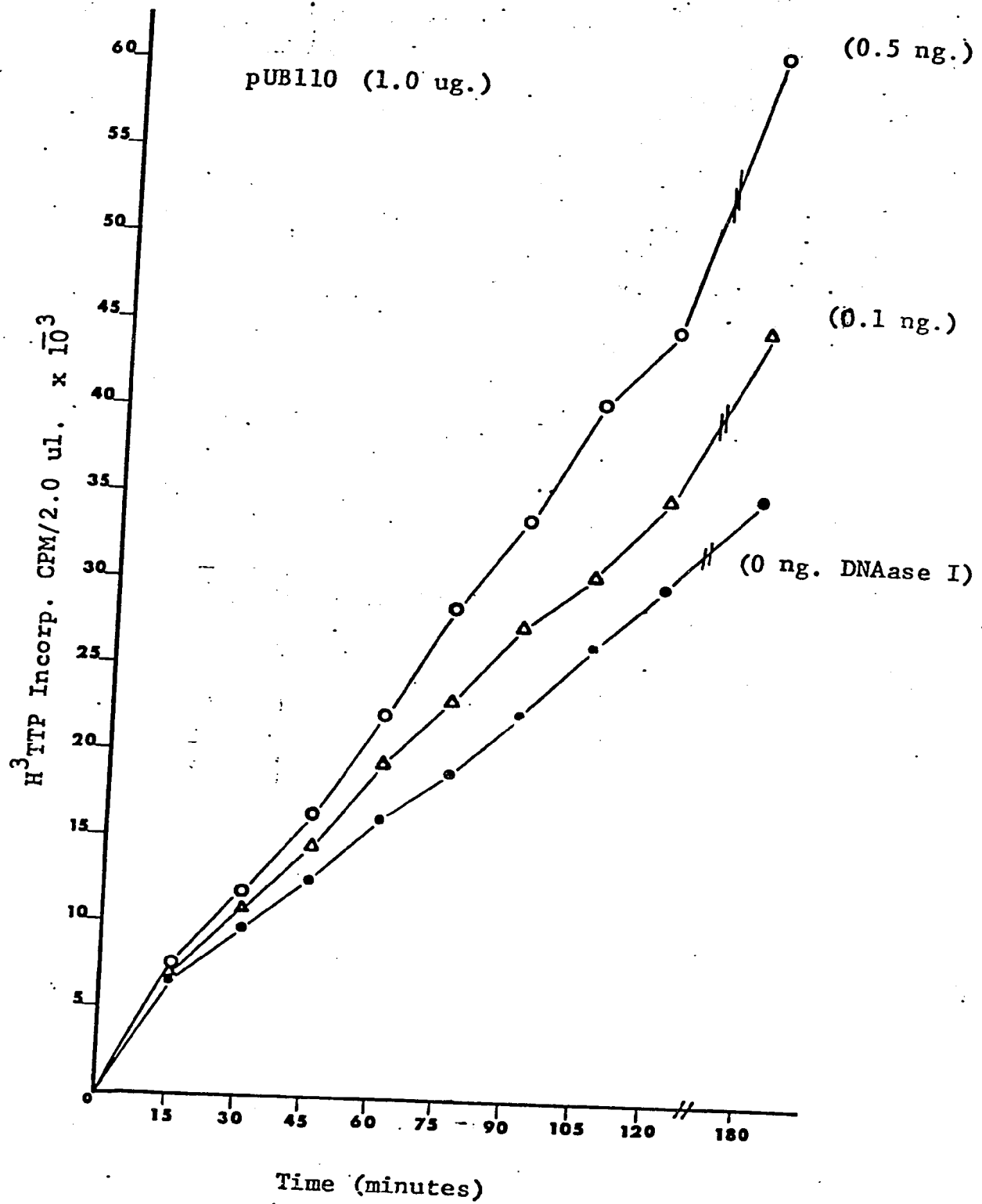


Figure 27

Nick translation kinetics of B. subtilis chromosomal DNA. Conditions were as in Fig. 26. In addition, similar reactions, 100 ul total volume, were carried out in presence of 100 ng and 500 ng DNAase I. Counting protocol was as previously described and in Methods.

Figure 27

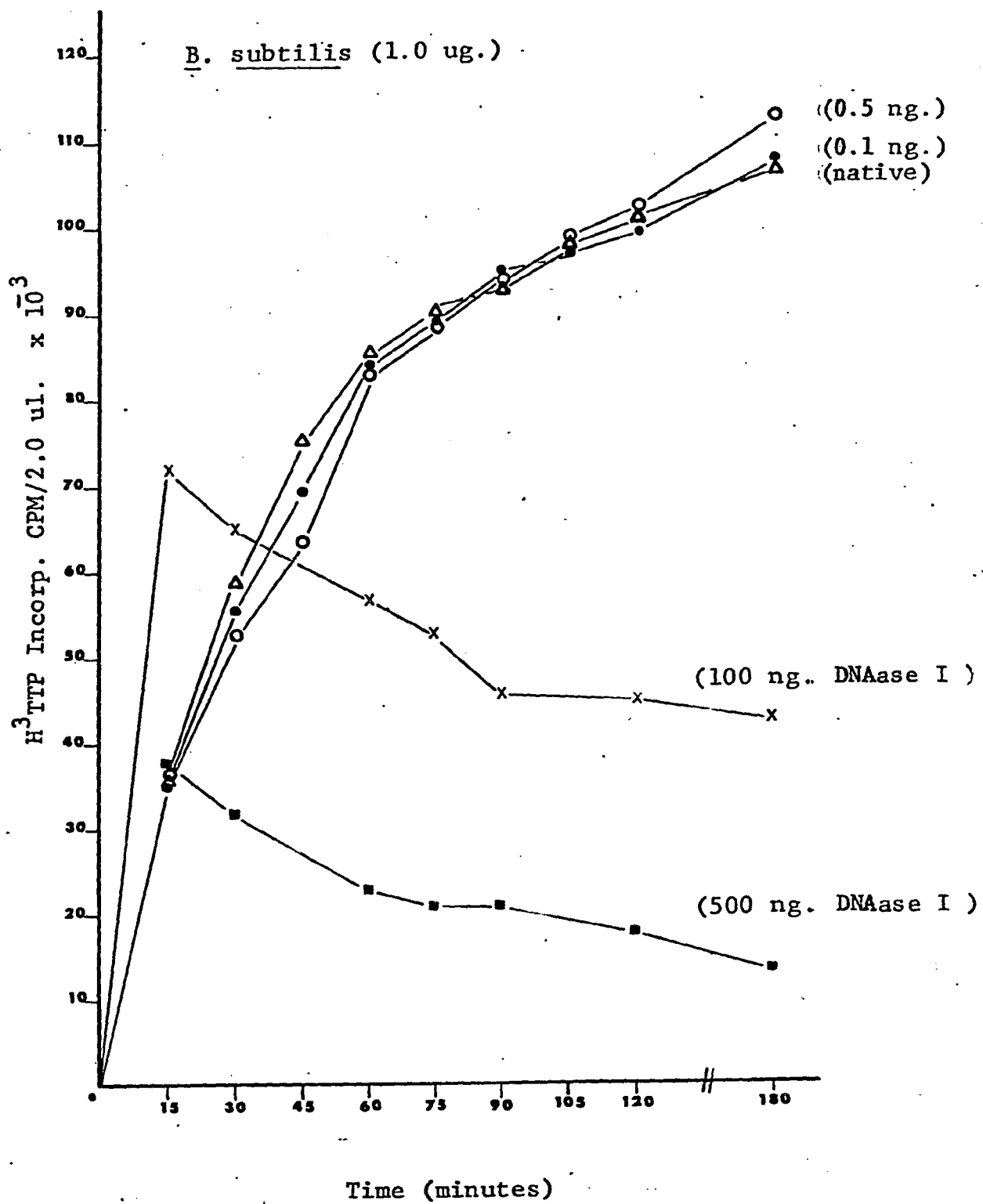


Figure 28

Sephadex G-50 exclusion chromatography. The product of a typical nick translation reaction, such as Fig. 26, was loaded onto a 5.0 ml pre-swollen column and eluted with buffer (Methods). Prior to loading, the reaction was phenol and ether partitioned and marked with 1 ul. of 0.001% BPB tracking dye. Radioactivity was followed by counting 1.0 - 5.0 ul aliquots of each fraction (2.0 drops) or monitored by means of a mounted survey meter (P^{32}). Curve area was calculated as described. Specific activity assuming complete column recovery is generally 8×10^5 - 1×10^6 cpm/ug with (H^3) label and greater than 10^7 when (P^{32}) was utilized.

Figure 28

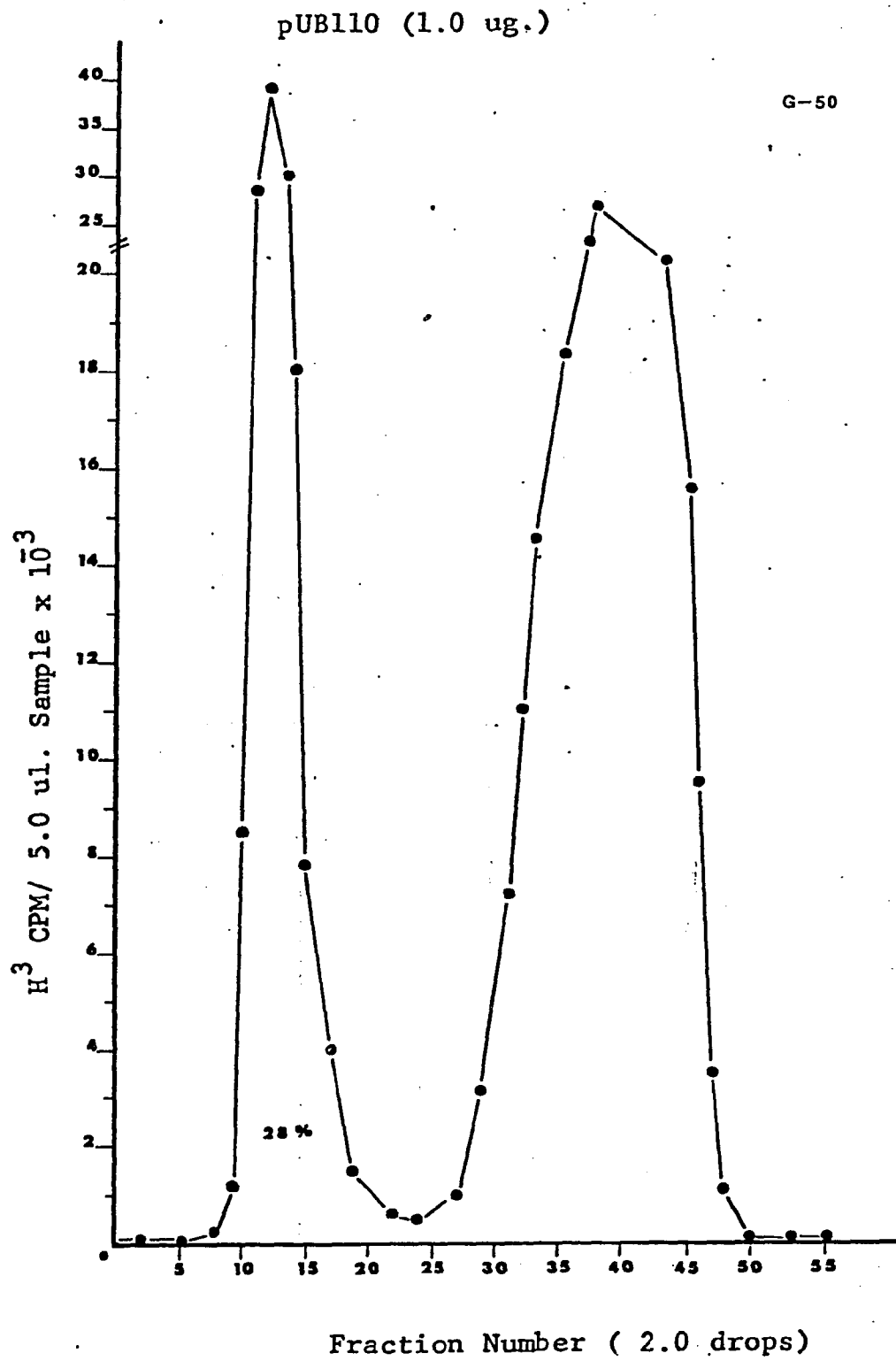


Figure 29

Southern blot hybridization of *B. pumilus* genome DNA. P^{32} nick translated pRR-6 or specific gel purified subfragments were used as probe. Lane a) molecular weight standards b) pRR-6 x EcoRI c) pRR-6 x EcoRI/HindIII d) pUB110 vector x EcoRI. Hybridization lanes are e) EcoRI digested *B. pumilus* DNA x 2.4 Md. EcoRI pRR-6 trp probe f) HindIII *B. pumilus* DNA x 2.4 Md. pRR-6 probe g) EcoRI/HindIII *B. pumilus* DNA x 2.4 Md. probe h) HindIII *B. pumilus* DNA x 1.7 Md. pRR-6 Eco/Hind fragment i) HindIII *B. pumilus* DNA x 0.7 Md. Eco/Hind pRR-6 fragment j) EcoRI/HindIII cleaved *B. pumilus* DNA x 1.7 Md. pRR-6 probe k) Eco/Hind digested DNA x 0.7 Md. probe l) Bgl II digested DNA x total EcoRI 2.4 Md. probe m) Bgl II/Hind III DNA x EcoRI 2.4 Md. probe n) EglIII/EcoRI DNA x 2.4 Md. probe o) BamHI DNA x 2.4 Md. probe p) pRR-6 digested with EcoRI/HindIII x total pRR-6 probe q) Eco/Hind pRR-6 x 1.7 Md E/H probe r) Eco/Hind pRR-6 x 0.7 Md probe s) *B. pumilus* digested with EcoRI

Figure 29

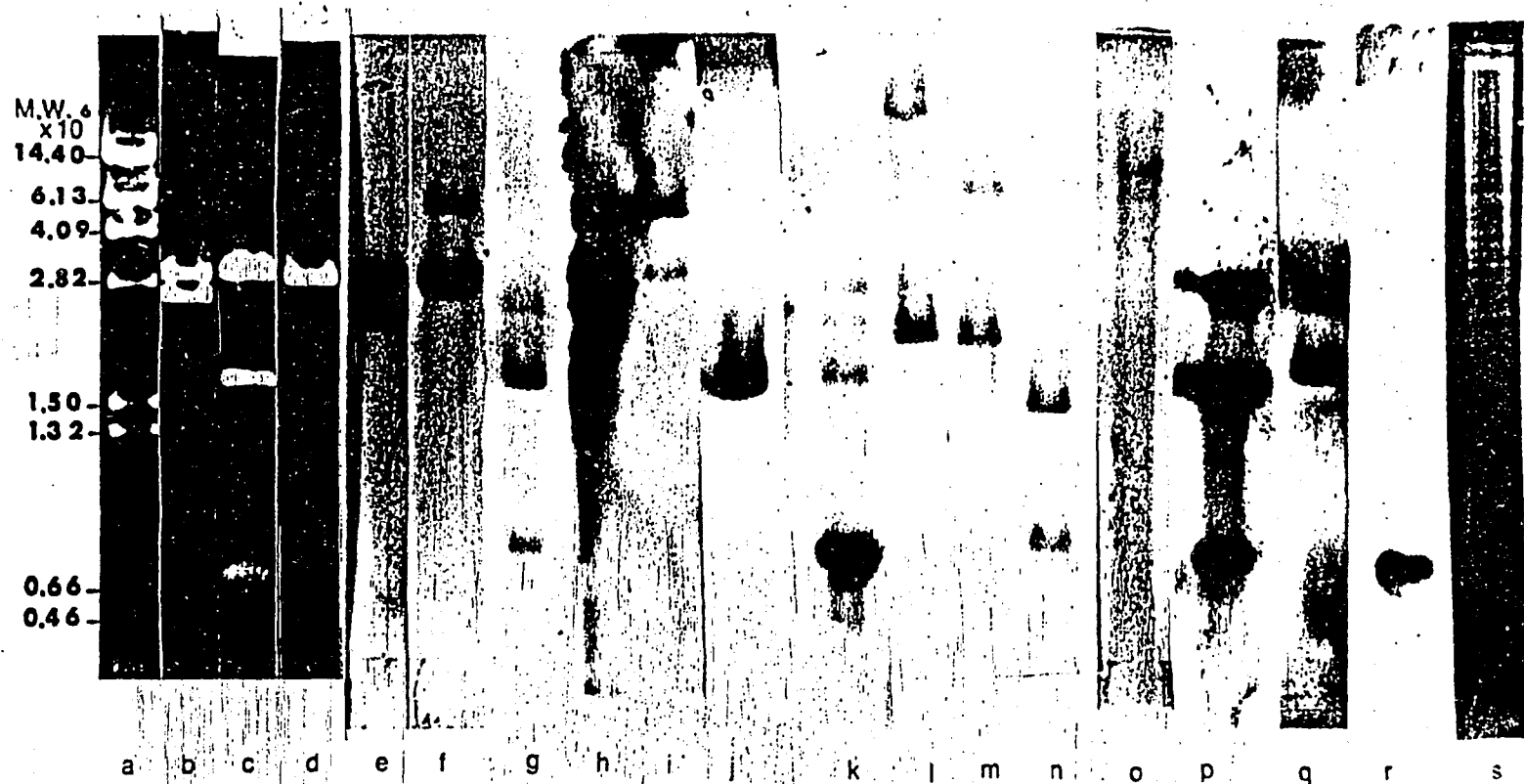


Figure 30

Restriction site organization of the B. pumilus chromosomal trp⁺ region. Orientations (I and II) are presented for the tryptophan operon cluster of the B. pumilus genome. The sequences, based on biological activity, which appear in the chimera pRR-6 are given by (▬) while chromosomal sequences are (~~~~). Relative coding size of the operon members is given in detail in Fig. 25. EcoRI and HindIII sites are positioned by means of the data of Tbls. 20, 21; Figs. 25, 29. Sequences beyond the flanking Hind III sites are not known. Assignment of genetic capacity is schematic.

Figure 30

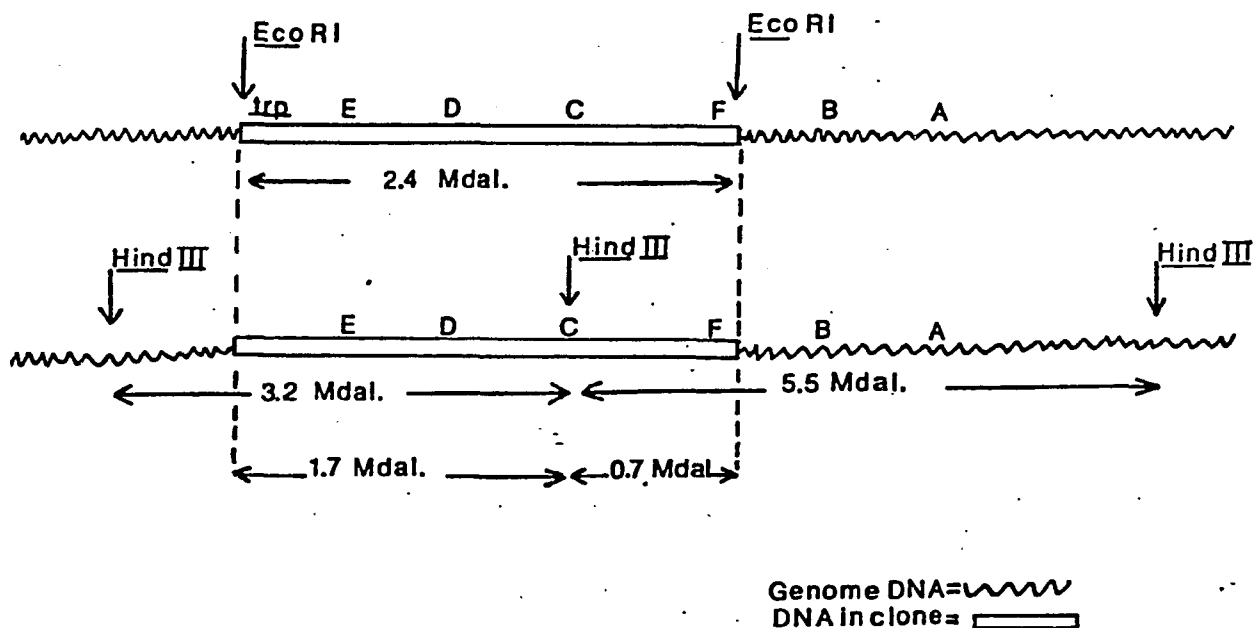
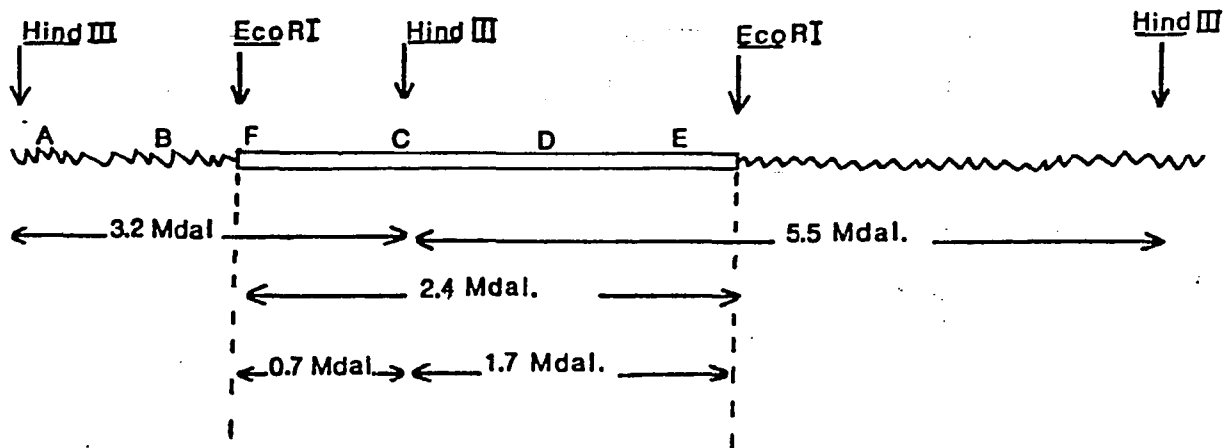
Restriction Site Organization of the *B. pumilus* *trp* RegionOrientation I:Orientation II:

Figure 31

Southern blot hybridization of Bacillus DNA to trp specific cloned probe. Lane a) molecular weight standard b) pRR-6 x EcoRI c) pRR-6 x EcoRI/Hind III d) pUB110 x EcoRI. Hybridization lane 3) EcoRI B. pumilus DNA f) Hind III B. pumilus DNA g) B. licheniformis DNA Hind III digest h) BR 151 Hind III digest i) BR 151 DNA Eco/Hind III digest j) W23 DNA EcoRI digest k) W23 DNA x Hind III l) W23 DNA x Eco RI/Hind III digest m) 3610 DNA x EcoRI digest n) 3610 DNA x Hind III digest o) 3610 DNA x Eco/Hind digest p) 168 DNA x EcoRI digest q) B. subtilis W23 DNA EcoRI digestion pattern. Probe in all above lanes was isolated P³² labeled 2.4 Md. pRR-6 trp specific cloned fragment.

Figure 31

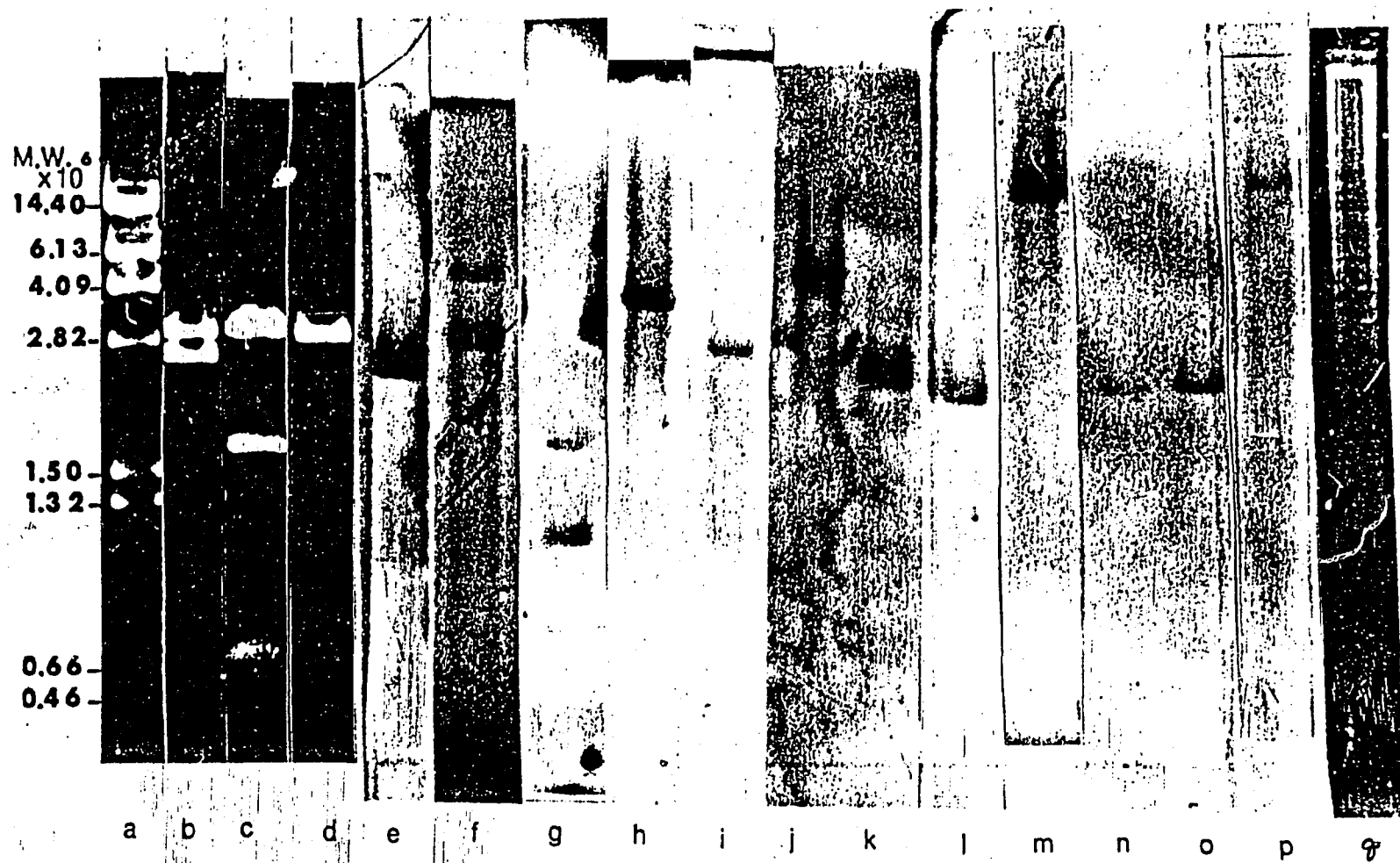
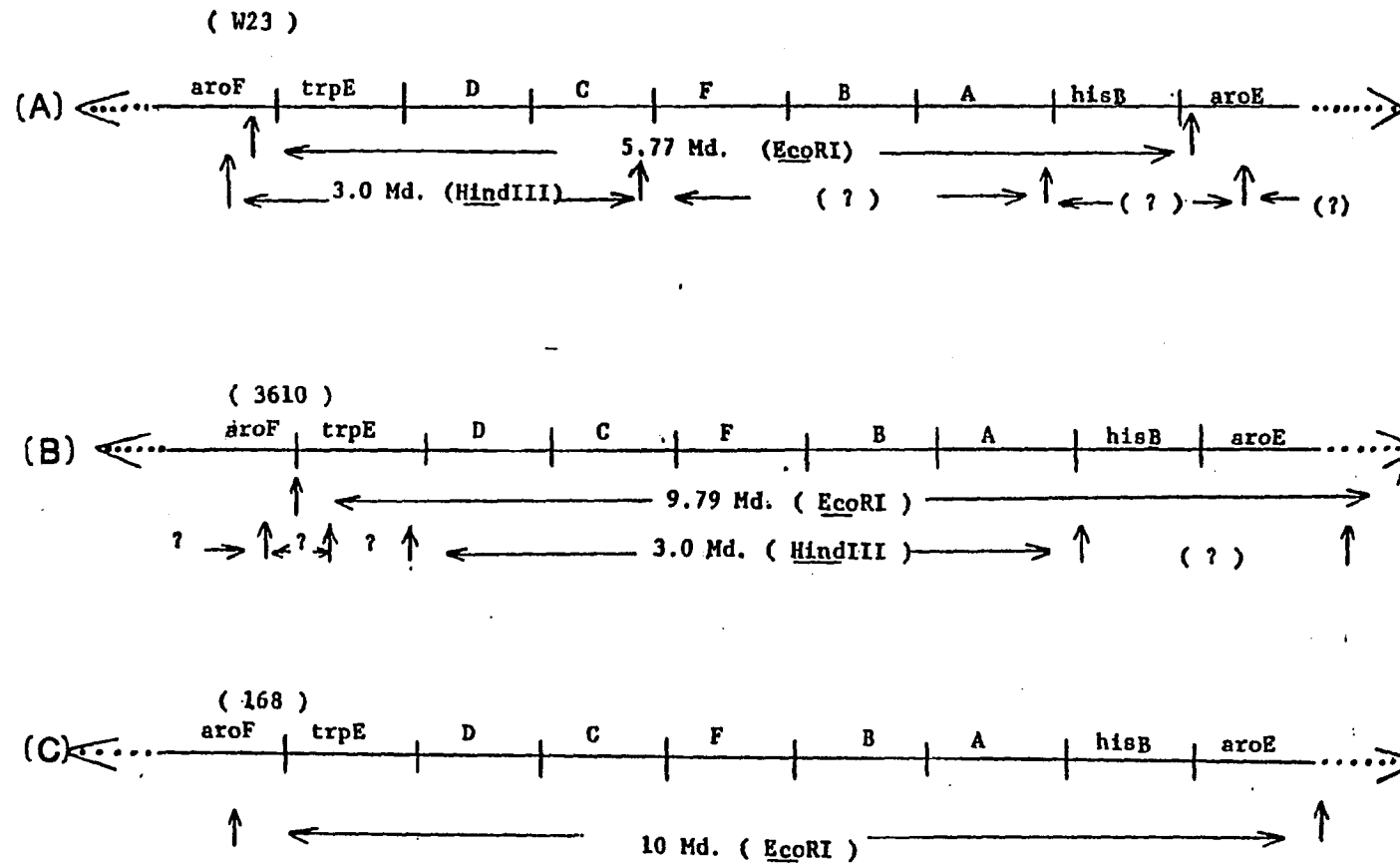


Figure 32

Restriction site organization of the B. subtilis W23, 3610 and 168 genomes. EcoRI and Hind III cleavage sites are schematically positioned and inferred relative to the tryptophan region based on hybridization data of Fig. 31 and Tbls.3, 4, 6, 7, 11, 12, 16, 21. Figure 32a is a composite schematic based on RTA calculations and hybridization blot data. Figure 32b presents Southern blot trp specific fragment patterns inferred relative to the aromatic cluster for B. subtilis strands and B. pumilus genomic DNA.

Figure 32a



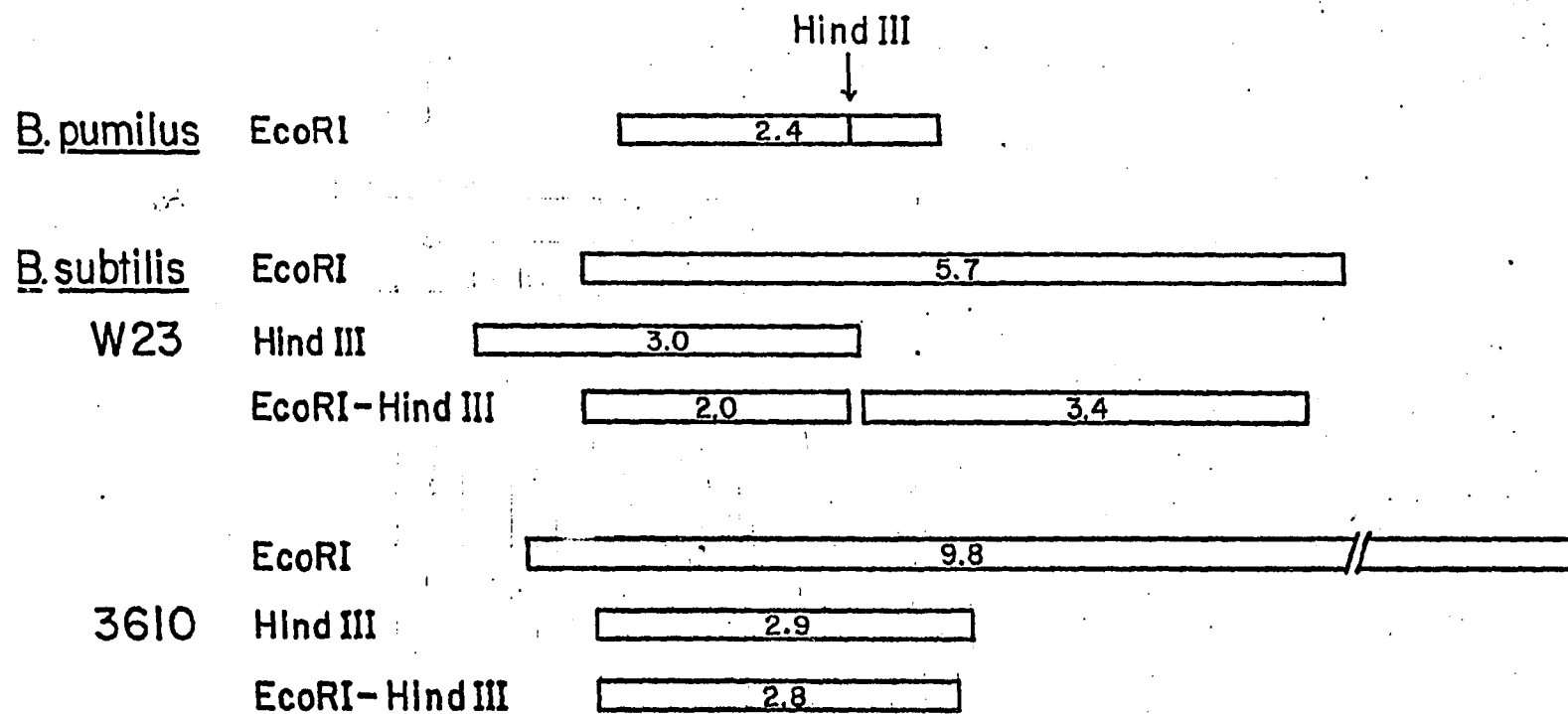
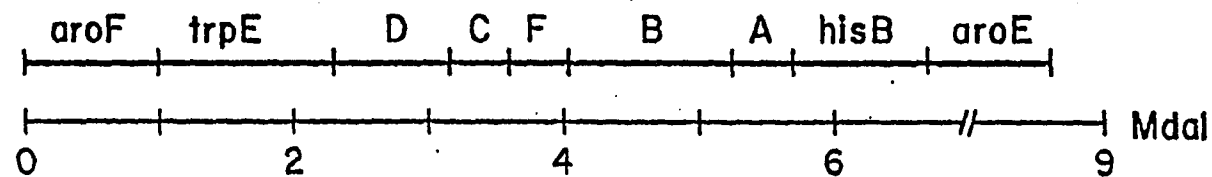


Figure 32b

Figure A-1

Methyl (H^3) labeling of various DNA's with EcoRI methylase. Samples of DNA were labeled with S-(Methyl- H^3)-adenosyl-L-Methionine (specific activity 5532 cpm/pM) as described in Methods for plasmid pBR322, plasmid pUB110, phage lambda and phage T7. Samples of EcoRI digested lambda DNA were also included. To measure acid insoluble H^3 incorp. into B. subtilis chromosome DNA, label of specific activity 30,094 cpm/pM was employed. E. coli DNA PolI labeling of the EcoRI cohesive ends, as a comparison, failed in our hands. Total reaction volume 100 - 200 ul. Initial DNA conc. W23 (○—○) 56 ug/ml; 3610 (Δ—Δ) 53 ug/ml; lambda (●—●) 100 ug/ml; pBR322 (■—■) 20 ug/ml; pUB110 (▲—▲) 6.0 ug/ml; T7/RI-λ (X—X). Calculations are based on H^3 incorporation (pM 6meA/pM genome) and presented as H^3 receptive palindormes/genome assuming $2CH_3$ /EcoRI site. Also shown is the 6-methyl-adenine moiety and its hydrogen bonding positions (←—→). (?) refers to a peculiarity of the pUB110 replicative cycle which involves concatenation. Observed incorporation most probably reflects an average site(EcoRI) number in the native population.

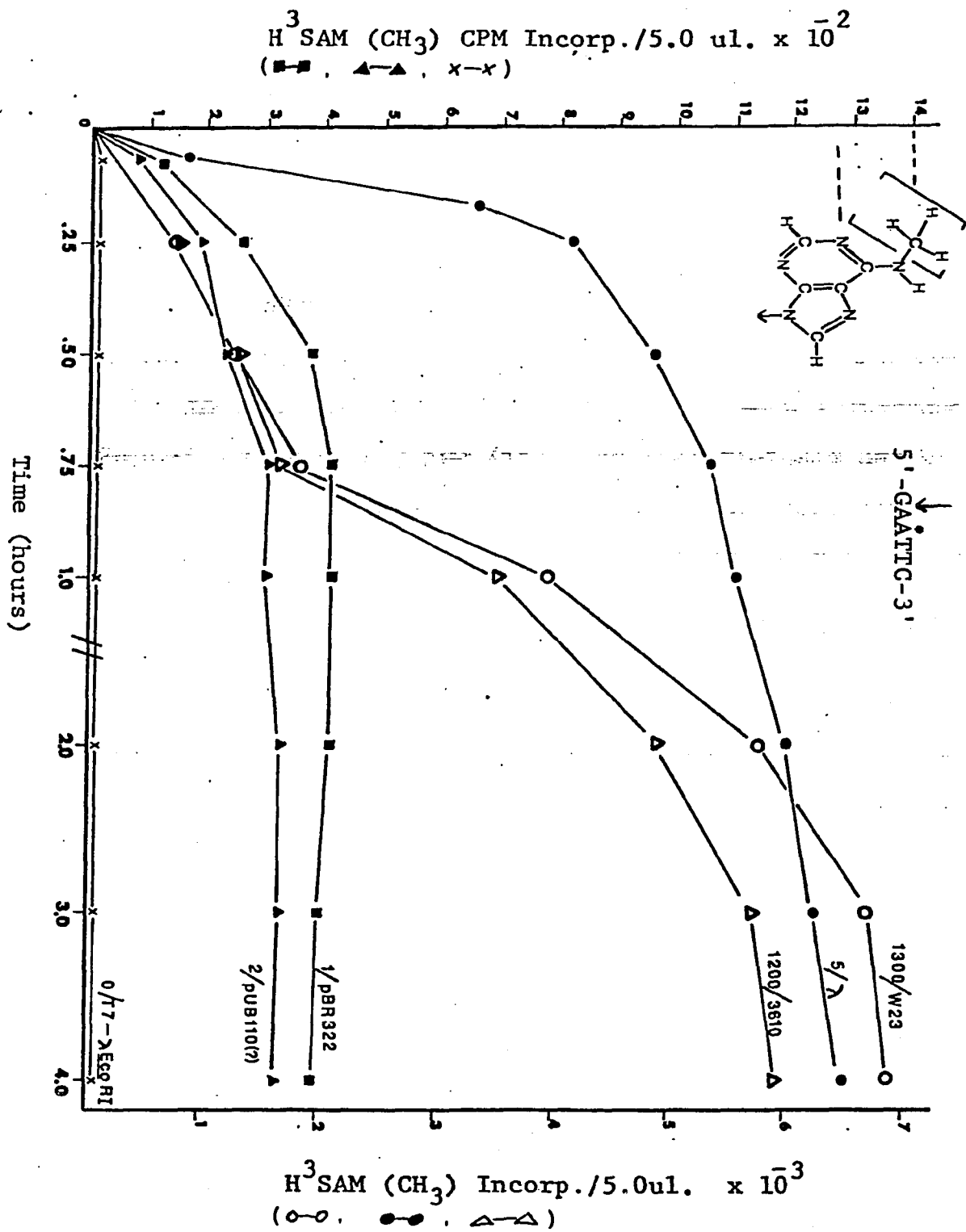


Figure A-1