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The Molecular Nature and Replication of R Factor 222 in Proteus Mirabilis

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THE MOLECULAR NATURE AND REPLICATION OF R FACTOR 222 IN *PROTEUS MIRABILIS*

by

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INTRODUCTION

The use of antimicrobial agents has had a profound effect on the efficacy of the clinical management of bacterial diseases. Aside from the adverse reactions caused by some antibiotics, the major impediment to antibiotic chemotherapy was presumed to be the occurrence of spontaneous bacterial mutants which were resistant to one or, very rarely, two unrelated antibiotics. Even with the use of combined drug therapy and despite judicious administration of antimicrobial agents, however, a high incidence of bacterial disease has remained in some countries and, within the past decade there has been a significant increase in the number of bacterial isolants which are resistant to multiple drugs. The cause of this ominous public health problem was discovered in 1959 when multiply drug-resistant bacteria were observed to transfer simultaneously the determinants for drug-resistance during mixed cultivation with other bacterial species.

Subsequent studies have shown that the hereditary agents of multiple drug-resistance (R factors) are extrachromosomal genetic elements which can be transferred by cell-to-cell contact among members of diverse bacterial genera. R factors are apparently distributed worldwide and pose a serious threat to efficacious clinical therapy.

The medical importance of R factors has been demonstrated recently by an epidemic of R factor-carrying Shigella in Guatemala; an estimated 112,000 cases of dysentery and 8,300 deaths occurred during a ten month period. In addition to the many known antibiotics to which R factors confer resistance, the rapid emergence of R factors conferring resistance to newly-introduced antibiotics has limited the usefulness of available drugs. To date, varied attempts to limit the formation of

or control the dissemination or consequences of R factors have been unsuccessful. The finding that R factors were present before the commercial introduction of antibiotics has indicated that the development of an effective means for controlling R factors necessitates an understanding of their replicative behavior and the regulation of R factor gene expression.

Although current information on the molecular nature of R factors is substantial, the means by which R factor replication is regulated has not been defined. Such data would provide a molecular basis for future attempts to control R factor dissemination and provide insight into the evolutionary relationships between R factors and other plasmids. Additionally, such a study would provide a better understanding of the basic regulation of deoxyribonucleic acid synthesis.

REVIEW OF LITERATURE

Discovery of Transmissible Drug Resistance

Despite good sanitation practices and the employment of combined antibiotic therapy, bacillary dysentery was rampant in Japan during the 1950's. The observation that administration of a single antibiotic to a patient whose feces contained drug-sensitive organisms resulted in the excretion of organisms resistant to multiple antibiotics was not readily explainable by the mechanism of chromosomal mutation to drug-resistance. Although as early as 1955 patients were observed to harbor simultaneously populations of drug-sensitive and multiply-resistant Shigella and Escherichia coli, the transmissible nature of multiple-drug resistance was first demonstrated in mixed cultivation experiments by Ochiai et al., and Akiba and associates in 1959 and 1960 (51,79). This novel genetic determinant of drug resistance was termed a resistance (R) factor by Mitsuhashi (51).

General Properties of R Factors

Host Range

Many pathogenic and non-pathogenic gram negative bacilli of widely diverse deoxyribonucleic acid (DNA) base compositions have been shown to harbor R factors. Although the members of the family Enterobacteriaceae are often considered to be the "natural hosts", R factors have been transferred to Vibrio, Pasteurella, and Aeromonas (79,82,83)

Recently, transfer of resistance has been observed between Pseudomonas and enteric bacteria (58), and Pseudomonas and members of the Rhizobiaceae family (14). However, the transmissible elements from Pseudomonas appear to be different from the 2 types of known R factors on the basis of male-specific phage susceptibility, host compatibility, and mechanism of transfer.

Physiology of R Factor Transfer

In the early studies on the mechanism of R factor transfer, unsuccessful attempts were made to transfer resistance with the cell-free filtrates of resistant cultures. Together with the observed high frequency (often as high as 10^{-2} per donor cell in one hr) of R factor transmission, these results opposed any hypothesis that R factor transfer occurred by transduction or transformation (30,79). Further studies showed that transfer of drug resistance occurred irrespective of the presence of the fertility factor F and transfer of host chromosome, and that resistance was expressed phenotypically in the recipient cell within minutes after R factor transfer. That R factor transfer required cell-to-cell contact was indicated by the inhibition of transfer observed after blender agitation of a mating mixture and after treatment of donor cells with periodate (79). Evidence for the extrachromosomal nature of the resistance determinants was provided by the observations

that R factors can be cured by acridine dyes and that the R factor is transferred as a single genetic unit by phage P1 kc (79,81).

The process of R factor transfer during cell-to-cell contact appears to be similar to that proposed for F factor transfer. The protein mating tube (pilus) which originates from the donor cell membrane and through which the R factor appears to be transferred to the recipient cell has been identified (5). Cells harboring R factors have been found to contain 1-4 mating or sex pili. These pili can be readily distinguished from common pili on the basis of size, antigenicity, and adsorption of specific phage (49). The ability of periodate to inhibit transfer has been inferred to indicate that a polysaccharide "mating substance" is involved in the process of pair formation between mating cells (79). The frequencies of R factor transfer vary from 10^{-2} to 10^{-7} per donor cell per hour depending upon the particular donor and recipient cells involved and on the physiological state of the donor cells. In contrast to the typical derepressed state of fertility of F factor-harboring cells, the fertility of R factor-harboring cells is repressed. The low frequency of R factor transfer is apparently due to repression of pili synthesis (49,30).

R factors can also be transferred <u>in vitro</u> via transduction. This process however, is not considered a significant mechanism for the dissemination of R factors

in the natural environment. The transmission of R factors via transformation has not been reported.

Comparative Biochemistry of R Factor-mediated Resistance

Although R factors were first recognized as genetic determinants for transferable drug-resistance, they were subsequently shown to also carry the genetic information for self-replication and transfer (49). Some R factors have also been found to code for bacteriocins and to carry the genetic determinants for resistance to bacteriophages, ultraviolet (UV) irradiation, bacteriocins, and certain inorganic divalent cations (49,50,71). R factor-controlled phage- and UV-resistance appears to be mediated by a nuclease and a DNA repair enzyme, respectively (71). The mechanisms of bacteriocin or cation resistance have not been defined.

The levels of drug-resistance imparted by R factors vary dramatically; the level depends not only upon the particular R factor and specific antibiotic involved, but also on the host cell type and growth conditions (71,79). Most R factor-mediated drug-resistance has been found to be due to enzymatic inactivation by periplasmic enzymes. The modes of known enzymatic inactivation include: kanamycin, neomycin, and streptomycin, phosphorylation; bluensomycin, spectinomycin, and streptomycin, adenylation; penicillins including carbenicillin and cephalo-

sporins, hydrolysis by beta-lactamases (58,66,71,81). The biochemical mechanisms of R factor-mediated resistance to gentamycin, viomycin, nalidixic acid, the sulfonamides, the tetracyclines, and the nitrofurans have not been elucidated (71).

Classification

R factors have been classified into 2 groups; fi^+ (fertility inhibition positive) and fi^-. When harbored by either F^+ or Hfr host cells, fi^+ R factors inhibit the normally derepressed F fertility function; fi^- R factors do not inhibit the fertility of F^+ or Hfr cells (79). Fi^+ R factors have been found to code for sex pili, termed F-like, which resemble those produced by F^+ or Hfr cells. In contrast to the derepressed state of F-pili synthesis, the phenotypic expression of R-specific sex pili is normally repressed which results in a low relative frequency of R factor transfer. When both elements are harbored in the same cell, the fi^+ R factor-coded repressor of pili synthesis has been shown to repress F-pili synthesis. Fi^- R factors code for sex pili, termed I-like, which resemble Col Ib sex pili. Although pili synthesis by fi^- R factors, like fi^+ R factors, is naturally repressed, the fi^- R factor-coded repressor does not affect F-pili synthesis or F-fertility. Thus, R factors are readily identifiable on the basis of the type of pili produced since I-like and F-like

pili have been shown to be distinct in antigenicity, in size, and in susceptibility to male-specific phage (13,49,81).

Ecology

Infectious drug resistance has been reported in more than 15 countries; its geographical distribution is apparently world-wide (2,78). R factor-harboring (R^+) bacteria, of many different genera, have been isolated from humans and various domestic as well as aquatic animals (78,79,83).

Numerous epidemiological surveys have demonstrated a positive correlation between the incidence of transferable drug-resistance and the extensive use of antibiotics, both in the treatment of human disease and as prophylactics and growth stimulants in animal feeds (46,52,78). Because of the medical implications of transferable resistance, R factor epidemiology has been studied extensively in nosocomial environments. In a survey of 3,964 Shigella strains isolated in Japan in 1966, 56.6% were observed to carry R factors. A less extensive analysis of other enteric bacteria showed that R factors were harbored by 45% of the E. coli, 42.8% of the Klebsiella, and 11.0% of the Proteus cultures examined. In contrast, only 14 of 4,397 Shigella strains examined in Japan in 1956 were observed to possess transferable drug-resistance (52). The results of a similar survey in the United States on the incidence of R factors in clinical isolants indicated that a high proportion of the multiply

drug-resistant E. coli and Klebsiella strains carry R factors as opposed to a low incidence of R factors in multiply drug-resistant Aerobacter isolants (1). Ninety percent of the causative enterobacteria associated with urinary tract infections in Japan were reported to harbor R factors (82), whereas only about 70% of the causative bacterial strains of similar infections in the United States have been observed to carry transferable drug-resistance (71). Smith (71) also observed that greater than 50% of the drug-resistant Salmonella strains examined harbored R factors.

The results of studies on the incidence of R factor-harboring bacteria in the intestines of normal healthy humans are conflicting, although a recent report cites that about 30% of the healthy individuals examined carried R^+ bacteria (53). R factors have been isolated from the causative Shigella or Salmonella of several epidemics (79). For example, during the first 10 months of an epidemic in Guatemala, an estimated 112,000 cases of bacillary dysentery and 8,200 deaths occurred as a result of an R factor-carrying Shigella (25).

Fi^+ or fi^- R factors have been found to be genetically stable in both E. coli and Shigella. Although fi^- R factors have been observed to exist stably in S. typhimurium, fi^+ R factors spontaneously segregate most drug-resistance determinants at high frequencies (83). About 70% of the

R factors found in E. coli are fi^+, while fi^- R factors comprise the largest group in Salmonella isolants (49). These observations are consistent with the finding that few, if any, fi^- R factors confer resistance to chloramphenicol (79). Of the R^+ Salmonella and E. coli isolants, about 25% have been found to harbor simultaneously an fi^+ and an fi^- R factor (termed hetero-R state) (52,59). The stability and incidence of R factor types in other organisms has not been defined.

The recent isolation of R factors from "preantibiotic" communities and from a culture lyophilized in 1946 indicates that these extrachromosomal elements may have preceded the widespread contamination of the environment with antibiotics (see ref. 16). However, the origin and evolution of R factors remains obscure.

Clinical Management of Infectious Drug Resistance

Cognizant of the threat posed by R factors to efficacious clinical therapy, many investigators have sought a means of controlling the dissemination or minimizing the consequences of R factors. These experimental pursuits include: (a) development of antibiotics resistant to enzymatic attack; (b) direct inhibition of R factor transfer; (c) eradication of R factors by curing; (d) elimination of conditions favorable to R factor emergence; and (e) molecular investigations to determine R factor origin and the regula-

tion of R factor gene expression and replication (6,12). In general, antibiotic derivatives which are less susceptible to enzymatic attack have been found to be less active antimicrobial agents and more toxic than the parental antibiotic. Most compounds which have been observed to inhibit R factor transfer are too toxic for clinical usage, and the physiological bases for transfer inhibition have not been well characterized. However, recent reports of the inhibition of R factor transfer by clinically attainable levels of commonly used antibiotics render this approach promising (7,32). The effectiveness of curing agents, which increase the spontaneous rate of plasmid loss, is limited by their toxicity, by the emergence of R factors insensitive to curing, and by the low frequency of curing (6). Even with a more prudent administration of antibiotics, the complete elimination of R factors would appear to be impossible if these genetic elements originated before the extensive use of commercial antibiotics (16). Molecular studies have provided some insight into the origin and regulation of R factor functions but have not yet disclosed any procedures for controlling infectious drug resistance. At the present time, no effective clinical means of R factor control has been elucidated.

Genetics and Molecular Nature of R Factors

Autonomous State

The non-chromosomal nature of R factors was suspected

from the observation that resistance transfer to drug-sensitive cells occurred independently of host genome transfer (51,79). Furthermore, the observed rapid transfer of R factors from a few R^+ cells to many R^- bacteria indicated that R factor replication occurred more than once per host chromosome duplication (79). Consistent with a hypothesis of autonomous R factor existence, transfer of all resistance markers was found to occur in less than one minute and phenotypic expression of all R factor-mediated resistances could be detected before cell division. Since F^+ cells but not Hfr hosts could be cured of the F factor, the demonstration of R factor curing lent additional evidence for the autonomous state of R factors (51,79). Subsequent studies showed that entire R factors were transduced by phage P1 kc (79). Direct evidence for the autonomous existence of R Factors was obtained by the physical isolation of covalently closed circular R factor molecules (18,54).

Both fi^+ and fi^- R factors have been reported to promote transfer of the bacterial host chromosome, albeit at low frequencies. In general, the observed frequencies of R factor-mediated chromosome transfer are comparable to or slightly lower than those obtained with F^+ cells, and there is apparently no fixed origin or sequence of transfer (49). Recently, Pearce and Meynell (57) reported the isolation of a mutant R factor, derepressed in pili synthesis, which

mediated the oriented transfer of the host genome. To date, however, no cell harboring a stably integrated R factor has been isolated. Although the autonomous state and transmissible nature of R factors have been adequately established, direct evidence for R factor integration is lacking (49).

Chemical Composition

The transmissible and hereditary nature of R factors suggested that these elements were composed of nucleic acids. In addition to the observed inactivation of R factors by incorporated ^{32}P, the observations that R factor stability was decreased by acridine dyes or UV-irradiation lent support to this suggestion (79,80). In 1966, Falkow et al. (19) and Rownd and associates (64) confirmed the autonomous existence of R factors by demonstrating the presence of small satellite DNA bands (about 7% of the total extracted DNA) in CsCl gradients of DNA isolated from R^+ P. mirabilis. The loss or acquistion of an R factor was observed to correspond with the disappearance or presence of the satellite bands, respectively. The 0.015 g/cm^3 increase in R factor buoyant density observed in CsCl after thermal denaturation indicated that R factor DNA was double-stranded. The duplex state of R factor DNA was confirmed by the absorbance increase observed during thermal denaturation and by DNA-DNA hybridization studies of R factor DNA preparations (19,28).

In E. coli, most R factors have been found to comprise a single DNA species which has a density corresponding to a

base composition similar to that of the host genome DNA [50% guanine plus cytosine (GC)] (10,20,55). However, when harbored by P. mirabilis, fi^+ R factors have been observed to exist as 2 satellite DNA species (50 and 58% GC) (10,18,54, 60). Rownd and others (60,61) have shown by density-label and radio-label studies that both of these satellite bands are double-stranded DNA. To date, however, no systematic chemical characterization of R factor DNA has been reported.

Regulation of Pili Synthesis

Although R factors and F factors were known to require cell-to-cell contact for transfer, the mechanism of transfer remained undefined until 1964 when Brinton and collaborators (see ref. 5) demonstrated the presence of novel tubular mating appendages on the surface of F^+ cells. These sex pili, which were shown to be synonymous with the f antigen, were found to be the specific receptor sites for male-specific bacteriophages and to be essential for F factor transfer (5,13). However, in contrast to the typical high frequency (100% transfer after 1 hr) of F factor transfer observed after mating, R factor transfer was observed to occur normally at a lower frequency (10^{-4} to 10^{-6} transcipients in 1 hr). On the other hand, recipient cells which had recently received R factors, termed high frequency resistance transfer (HFRT) cells, were observed to transfer R factors at a high frequency ($\sim$100%

in 1 hr) for several generations before transfer decreased to the typical low frequency (79). From the positive correlation observed between the proportion of bacteria in an HFRT culture which either could be infected by male-specific phage or bore F-like sex pili, and the frequency of R factor transfer, Datta and others (13) concluded that R-specific pili were necessary for R factor transfer and that pili synthesis was regulated negatively by a repressor. The isolation and characterization of fi^+ R factor mutants de-repressed (drd) in pili synthesis, which were observed to transfer R factors at a high frequency and which did not affect F factor fertility function when harbored by F^+ cells, confirmed the above interpretations and indicated that the repressor of fi^+ R-specific pili synthesis can also repress F-specific pili synthesis. The observed decrease in the fertility of cells harboring the drd R factor after super-infection with a wild type, repressed fi^+ R factor indicated that the drd mutants were altered in the i gene product, and were not O^C mutants (13,48,49).

Fi^- R factors, which produce sex pili that are distinctly different from F-like pili but are normally repressed in transfer function, have been found to code for a repressor of pili synthesis which, as expected, does not affect F factor fertility function (13,48,49).

Superinfection Immunity

The phenomenon, termed "superinfection immunity" by

Watanabe (79), in which the frequency of conjugal transfer of a plasmid is reduced by the presence of an identical or closely related hereditary element in the recipient population has been shown to involve 2 quite different processes; entry exclusion and mutual exclusion (20,56).

The term entry exclusion denotes the inhibition of R factor transfer observed between cells carrying closely related or identical extrachromosomal elements. Conjugal R factor transfer from fi^+ R^+ donor cells to fi^+ R factor harboring recipient cells was observed to be significantly reduced (10 to 1,000 fold) when compared to the transfer frequency obtained with R^- recipient cells (82). However, the observation that the frequency of R factor transfer by transduction was identical regardless of the presence or absence of R factors in the recipient cells indicated that transfer was inhibited by the presence of a physical barrier on the surface of R^+ recipient cells (56,81). Consistent with this interpretation, R^+ cells which had apparently lost phenotypic expression of this barrier (R^- phenocopies) were obtained prior to mating by treatment of R^+ recipient cells with periodate, by growth of these cells to the late stationary phase, or by starvation of R^+ recipient cells. R^- phenocopy cells were able to act as fully competent recipients which indicated that entry exclusion results from a genetically-determined barrier at the cell surface (49). Entry exclusion was also observed to occur between cells

harboring fi^- R factors, but was not found to occur between cells harboring opposite types of R factors (i.e. an fi^+ and an fi^-).

Mutual exclusion or plasmid incompatibility refer to the inability of a superinfecting plasmid to establish and maintain itself in a host which already harbors a closely related genetic element (49,56,81). Thus, when an fi^+ R factor was transduced to fi^+ R^+ cells or transferred to fi^+ R^- phenocopies, the entering plasmid could not stably coexist with the isogenic or closely related plasmid because of mutual exclusion. Either the resident or incoming plasmid was observed to be segregated during growth and only cells carrying either but not both plasmids were obtained (49,56). DNA restriction is not involved in mutual exclusion, per se, since isogenic plasmids in identical hosts were observed to exhibit this phenomenon. The maintenance site model, which has been proposed to explain mutual exclusion, assumes that incompatible plasmids have similar attachment-maintenance sites on the host cell membrane which are limiting in number (56). Compatible plasmids are proposed to have different attachment sites and thus may coexist stably. Alternative explanations for the mechanism of mutual exclusion include a proposed common plasmid-coded repressor for replication, and competition between incompatible plasmids for a limited amount of a

cytoplasmic substance required for replication (56).

In general, superinfection immunity has been observed only between elements of the same pilus type (2 fi^+ or 2 fi^- R factors). An fi^+ R factor can coexist stably with an fi^- R factor, and either type can be comaintained with the F factor (50,79). Recently, however, a few members of each class of R factors, which do not exhibit superinfection immunity to closely related elements, have been described (14,49,81).

Restriction and Modification

Both fi^+ and fi^- R factors have been observed to cause restriction and modification of a variety of bacteriophages including λ, P1, P2, P22, T1, T3, T7, and W-31 (50,81). R factor-mediated phage-restriction, which is reflected in the efficiencies of plaque formation, lysogenization, and transduction, has been demonstrated in some cases to be due to an endonuclease that is distant from endonuclease I of *E. coli* (81). The phage-specific structural modification(s) caused by R factors have not been defined. Recently, Hattman et al. (31) reported that certain R factors induced an increased methylation of cytosine in the DNA of phage P22.

Bannister and Glover (4) have proposed that R factor-mediated restriction and modification in governed by a minimum of 3 genes: one gene product is specific for restriction; another gene product is essential for modification; and the third gene product is necessary for both restriction and

modification. Mutant R factors which are either restriction-less (r^-m^+) or modification-less (r^+m^-), or which have apparently lost the ability to modify or restrict (r^-m^-) phage DNA have been identified. Complementation between restriction-modification mutants of an fi^+ and fi^- R factor, or of an R factor and an E. coli host has not been observed; tests for complementation between closely-related R factors is precluded by superinfection immunity.

Genetic Segregation and Recombination

The spontaneous segregation of a portion or of an entire R factor during normal cell growth was recognized in the course of initial investigations of infectious drug resistance. The frequency of spontaneous segregation has been found to be dependent upon the particular R factor and the specific host involved. All spontaneous R factor segregants, which were detected by the loss of one or more of the resistance determinants, were observed to have retained conjugal transmissibility. These observations plus the fact that the majority of transductant colonies obtained by transduction of R factors with phage P1 kc were observed to harbor entire R factors, suggested to Watanabe and associates (51,79) that R factors are normally composite structures which consist of a resistance transfer factor (RTF) linked to the determinants for drug resistance (R-genes). In contrast to the results obtained by P1-promoted transduction of R factors, most of the P22-mediated transductants were observed to contain non-

transferable drug-resistance determinants, some of which were demonstrated subsequently to be integrated into the host genome (51,79). The differences observed between the transductant R factors, obtained by P1-or P22-mediated transduction, were interpreted by Watanabe (81) to be due to the smaller size of phage P22.

Anderson and coworkers (2,3) have presented evidence based on the segregation of R factors in Salmonella that R factors are recombinational assemblages of transfer factors and drug-resistance genes. According to this model, transfer factors and drug-resistance genes exist normally as separate, independent entities but can be found as a composite unit under certain conditions. Preliminary studies on one of Anderson's segregated R factors indicate that for at least some fi^- R factors, the RTF and R-genes can exist independently in the same host cell or as independent replicons in different host cells (70). Recent studies of R factor segregation in Proteus mirabilis indicate that in this host some fi^+ R factors exist as composite molecules which can dissociate under certain conditions into independent RTF and R-genes molecules (10,20,60). In contrast to the results of of Anderson and colleagues (3), only the RTF molecules of these fi^+ R factors have been observed to segregate and act as independent replicons (10). The failure to isolate cells harboring only the non-transmissible R-genes molecule may indicate the necessity of a product coded for by the RTF

molecules for the stability of the R-genes molecules.

Although current evidence seems to support the view that R factors are recombinational assemblages of RTF and R-genes molecules, the molecular state of a specific R factor can vary in different hosts and under different conditions of host cell growth. The mechanism(s) involved in R factor dissociation or segregation has not been defined. Thus, it is not known whether both processes are the result of the same mechanism or of entirely different mechanisms.

Recombination between fi^+ and fi^- R factors or between 2 fi^- R factors following superinfection by conjugation has not been demonstrated. However, recombination between fi^+ R factors has been obtained following conjugal superinfection. Furthermore, the recombination could be effected in recombination-deficient E. coli host cells, which indicated that some fi^+ R factors coded for the production of a recombination enzyme(s) (49,82). By the physical measurement of segregant and recombinant molecules observed in studies of fi^+ R factor 222, Nisioka et al. (55) demonstrated that segregation results from a physical deletion of part of the R factor molecule and that recombinant molecules, obtained by recombination of the 2 different R factor 222 segregant molecules, were identical in size to the wild-type R factor.

When R factors were transduced with phage P22 to plasmid-carrying Salmonella hosts, recombination was observed between fi^+ and fi^- R factors, and between R factors and F factors

or colicin factors. Similarly, recombination between R factors and various phage genomes has been detected during transduction studies. These later recombinational assemblages of resistance-determinants and various other elements appear to indicate a high degree of promiscuity among extrachromosomal elements (50).

Genetic Structure

On the basis of the data obtained from studies of the spontaneous segregants of various R factors Watanabe and colleagues (79) formulated a genetic map and structural model of R factors. The circularity of this model is consistent with the circular R factor molecules isolated from R^+ cells (18,54). In this genetic model the RTF, which controls autonomous replication, pilus formation, conjugal transfer, and various functions other than drug resistance, is postulated to be linked to the determinants of drug resistance. These workers envisioned that the tetracyline resistance determinant is closely linked to one end of the RTF and the chloramphenicol, kanamycin-neomycin, streptomycin, and sulfonamide resistance loci are linked, either in that order or in reverse order, to the opposite end of the RTF. All determinants of resistance were hypothesized to be independent loci except the kanamycin-neomycin determinant, which was never observed to segregate. Segregation patterns for resistance to Hg^{++}, gentamycin, streptomycin, and

sulfonamides, indicated that these functions were closely linked (82). Mitsuhashi and associates (52) have reached similar conclusions about the genetic structure of R factors, and have presented evidence for the close association of the aminobenzyl penicillin resistance determinant with both the tetracycline resistance determinant and the RTF.

The inability to assay genetic linkage in R factors by available techniques prevents any articulate analysis of their gene sequence and any final interpretation of the specific molecular state of R factors observed in various hosts.

Molecular Characterization

Utilizing the CsCl density gradient centrifugation technique, Falkow et al. (19) and Rownd et al. (64) independently identified the genetic material of R factors as double-stranded DNA. When DNA from P. mirabilis harboring any one of several different fi^+ R factors was examined, these investigators observed two R factor satellite DNA bands at buoyant densities of 1.710-1.712 g/cm^3 (19). After examination of the spontaneous segregants of several fi^+ R factors, Falkow and collaborators (19) noted that regardless of the number of remaining resistance determinants, R factor segregants which had lost chloramphenicol resistance never exhibited a satellite band at a density of

1.716 g/cm^3. Since at that time fi$^-$ R factors had not been observed to mediate chloramphenicol resistance, these workers concluded that there was no gross difference in the composition of fi$^+$ and fi$^-$ R factor DNA in CsCl gradients and that the fi$^+$ determinant might be a necessary concomitant for acquistion of the chloramphenicol resistance locus. Segregant R factors which retained only transferable tetracycline resistance were found to exhibit a single satellite DNA band during CsCl gradient analysis, with a buoyant density of 1.708-1.709 g/cm^3. These data were interpreted to mean that the RTF portion of the R factor has a base composition similar to that of the F factor (50% GC) whereas the drug resistance determinants were associated with material of a higher and more heterogeneous GC content (19).

In these initial studies of the molecular nature of R factors, Rownd and associates (64) observed that fi$^+$ R factor NR1 (also termed R factor 222) DNA comprised approximately 1% of the total DNA extracted from R$^+$ E. coli and exhibited densities similar to those observed with DNA from R$^+$ P. mirabilis. However, in DNA extracted from R$^+$ P. mirabilis this R factor comprised 10-15% of the total DNA. Subsequent studies demonstrated that in P. mirabilis harboring R factor NR1 both the amount of R factor DNA and the proportion of each R factor DNA band varied with the growth

phase of the host cell and were affected by the presence of antibiotics in the growth medium (18,61). Both R factor satellite bands observed in DNA from R^+ P. mirabilis were shown to contain covalently closed, circular molecules, which indicated that the DNA species were not fragments generated by the extraction procedure (10,18,54). R factor NR1 has been shown to exist as a single molecular DNA species in E. coli (20), and the 2 R factor satellite bands initially observed in DNA from R^+ E. coli by Rownd et al. (64) were apparently caused by fragmentation of the R factor molecule during isolation.

Nisioka et al. (54) identified 3 different closed, circular R factor molecular species in DNA isolated from P. mirabilis harboring R factor 222/R3 (a segregant of R factor 222 which lost the tetracycline resistance determinant). When DNA from P. mirabilis carrying this fi^+ R factor was examined by CsCl isopycnic centrifugation, 3 R factor satellite DNA bands at densities of 1.708, 1.711, and 1.717 g/cm^3 were observed. By electron microscopic examination, DNA from each satellite band was found to contain nicked circular and covalently closed circular (CCC) molecules. The R factor molecular species banding at 1.717, 1.708, and 1.711 g/cm^3 were observed to have contour lengths which corresponded to molecular weights of 12×10^6, 54×10^6, and 68×10^6 daltons, respectively. Since the largest R factor molecule had an

observed density and length similar to that expected for a composite structure composed of the two smaller molecular species, these investigators suggested that such a recombinational event might reflect the evolutionary development of R factors. In a subsequent report Falkow et al. (20) reported molecular weights of 10 x 10^6, 50 x 10^6, and 60 x 10^6 daltons, estimated from DNA renaturation kinetics, for the 3 molecular DNA species of R factor 222, although the 2 larger molecules appeared as a single satellite DNA band at a density of 1.710 g/cm^3. The finding by these investigators that in E. coli R factor 222 comprised only a single molecular DNA species with a molecular weight of 60 x 10^6 daltons led to the suggestion that the largest R factor molecular species observed in P. mirabilis was the composite R factor.

Studies on the molecular characteristics of other fi^+ R factors have corroborated and extended the results obtained with R factor 222. With R factors R5 and R6, which mediate resistance to 5 and 6 drugs, respectively, 3 R factor molecular DNA species at densities of 1.709, 1.711, and 1.718 g/cm^3, with estimated molecular weights of 55 x 10^6, 65 x 10^6, and 9.5 x 10^6 daltons were observed when DNA from R^+ P. mirabilis was examined. In E. coli these R factors were found to exist predominantly as a single molecular DNA species with a density and length comparable to the largest R factor molecular species isolated from R^+ P. mirabilis (9,10,69).

As mentioned previously, fi^+ R factors were found to com-

prise only 1-2 composite R factor molecules per E. coli host chromosome equivalent throughout the cell growth cycle (55). In P. mirabilis, however, multiple copies of the 3 fi^+ R factor species per host chromosome equivalent were observed. To establish the identity of the 2 smaller R factor DNA species in R^+ P. mirabilis, Cohen and Miller (10) mated R^+ P. mirabilis harboring either R factor R1 or R6 with R^- E. coli and isolated recipient cells which carried only the molecules with a molecular weight of 55×10^6 daltons. As suggested by Rownd et al. (65), this molecular species was observed to fulfill all the functional characteristics of the RTF replicon. These results supported the hypothesis that in P. mirabilis the composite R factor dissociates into RTF and R-genes units which replicate independently. To date, the isolation of host cells which contain only the autonomous R-genes of any fi^+ R factor (ρ= 1.718 g/cm^3) has not been reported. However, the observation of Rownd et al. (65) that the amount of the 1.718 g/cm^3 component of R factor DNA is linearly related to the level of chloramphenicol transacetylase activity, is consistent with the proposal that the molecules which band at this density carry the drug-resistance determinants.

The physicochemical properties of fi^- R factors have been less intensively studied. With few exceptions, fi^- R factors have been observed to comprise only a single satellite DNA band (ρ=1.709-1.710 g/cm^3), when harbored by

either E. coli or P. mirabilis (19,54,55). Each of the fi$^-$ R factors N15, N-3, R-144, and R64 drd 11, which confer resistance to 2,3,1, and 2 drugs, respectively, has been observed to comprise a single class of molecules of the following respective molecular weights; 40 x 10^6 daltons, 46 x 10^6 daltons, 42 x 10^6 daltons, and 76 x 10^6 daltons (28,55,76). Only one fi$^-$ R factor, R28K, which confers resistance to ampicillin and is comprised of duplex DNA of a single density (ρ=1.710 g/cm^3), has been observed to contain 2 DNA species (44 x 10^6 and 28 x 10^6 daltons) of undetermined physicogenic relationship (38). Two fi$^-$ R factors, R6K and Rtsl, have been shown to be composed of DNA with a density (1.704-1.705 g/cm^3) corresponding to a base composition of 45% GC (38,74). Albeit possibly of evolutionary significance, the basis for the difference in density among fi$^-$ R factors remains obscure. Also, in contrast to fi$^+$ R factors, multiple copies of fi$^-$ R factors per host genome have been observed in E. coli (38). Smith et al. (70) have reported recently that certain fi$^-$ R factors segregate into RTF and R-genes units, each of which can be independently and stably maintained. In their preliminary report, the RTF and R-genes of R factor ΔS were estimated to have molecular weights of approximately 60 x 10^6 and 6 x 10^6 daltons, respectively.

Although satellite DNA bands at densities of 1.705, 1.711, and 1.718 g/cm^3 have been observed in DNA isolated from multiply drug-resistant Pseudomonas aeruginosa,

this satellite material was not correlated with drug resistance, per se (23). The molecular properties of a transmissible factor, RP1 in P. aeruginosa that mediated resistance to 4 antibiotics have been reported recently (27). Factor RP1 was composed of CCC molecules, with a molecular weight of approximately 40 x 10^6 daltons and a density of 1.719-1.720 g/cm^3. Multiple copies of RP1 per host genome in P. aeruginosa, E. coli, or P. mirabilis were observed. Since RP1 was observed to coexist stably in the same cell with either fi^+ or fi^- R factors, the molecular relationships between this element and the classical R factors are obscure.

Regulation of Replication

From the current molecular evidence, it would appear that R factors can vary in molecular properties in different hosts and under different conditions of growth. To date, studies of the mechanisms which regulate R factor segregation, dissociation, and replication have been confined primarily to the study of a single fi^+ R factor, NR1 or 222.

In P. mirabilis the composite molecule of R factor 222 (ρ=1.711 g/cm^3) has been observed to dissociate spontaneously into the RTF (ρ=1.709 g/cm^3) and R-genes (ρ= 1.718 g/cm^3) components. Rownd et al. (61) observed that in P. mirabilis R factor NR1 continued to replicate for several hours after the host cells entered the stationary

phase of growth. The increase in R factor DNA content during the stationary phase was shown, by both density-label and radio-label incorporation, to be the result of nascent R factor DNA synthesis. From the results of ^{14}N-^{15}N density-label studies, Rownd and coworkers (60) proposed that copies of the R factor are chosen randomly for replication, that R factor DNA synthesis occurs throughout the cell division cycle, and that the amount of R factor DNA per host chromosome is regulated by a fixed level of synthesis of initiator molecules required for R factor replication.

When P. mirabilis harboring R factor NR1 was grown in drug-free medium, only a single R factor satellite DNA band (ρ=1.712 g/cm^3) was observed. In contrast, after shifting R^+ cells to medium containing any one of the drugs, except tetracyline, to which the R factor confers resistance, additional R factor bands were observed at densities of about 1.715 and 1.718 g/cm^3. After prolonged growth in the presence of drugs, all R factor DNA was manifested as a single satellite DNA band at a density of 1.718-1.719 g/cm^3. On the basis of these results, Rownd et al. (62) hypothesized that in the absence of drugs R factor NR1 dissociates into the RTF and R-genes components which replicate under relaxed (more than once per cell division) and stringent (once per cell division) control, respectively. Thus, the single copy of the R-genes would not be detected readily during growth of R^+ P. mirabilis in drug-free medium. The antibiotics, except tetracycline, to which the R factor confers

resistance are envisioned to induce the reassociation of the RTF and R-genes and to select for cells harboring composite R factor molecules. During prolonged growth in the presence of drugs, the R-genes replicons are hypothesized to reassociate sequentially with one RTF to give large polygenic molecules of increasing density, and eventually all R factor DNA would increase to a density of 1.718 g/cm^3. Since the presence of drugs in the medium would necessitate increased cellular resistance, these workers argued that the relaxed replication of the composite molecule carrying more than one set of R-genes would easily allow for a large increase in gene number and, consequently, in the concentration of drug-inactivating enzymes. Since tetracycline failed to effect the density transition from 1.712 to 1.718 g/cm^3, Rownd et al. (63) proposed that the tetracycline resistance locus must be located on the RTF portion of the R factor. However, this hypothetical model is not consistent with many of the results of molecular studies reported by other investigators for the 222 or related R1 and R6 fi^+ R factors (10-12,54,69). The proposal of polygenic molecules is incongruent with the results obtained from electron microscopic measurement and sucrose gradient centrifugation of R factor DNA (10,21,54,62). Furthermore, the mechanism proposed does not explain how the positively controlled and stringently regulated R-genes

molecules could replicate in the presence of drugs at a rate, not only faster than in drug-free media, but also rapid enough to produce the immense number of R-genes molecules required to recombine with one RTF to give polygenic composite structures of density 1.718-1.719 g/cm^3. Thus, the predictions of the model of Rownd et al. (62,63) do not satisfactorily correlate with the observations of other investigators on the molecular nature of R factors.

In agreement with genetic evidence, fi$^+$ R factors have been found to exist predominantly in E. coli as a single molecular DNA species which comprises 1-2 R factor copies per host genome (9,20,55). On the basis of the isolation from R$^+$ E. coli of a few circular molecules with a molecular weight of about 10 x 10^6 daltons, Cohen and Miller (9) have suggested that replication and dissociation of fi$^+$ R factors is stringently regulated in E. coli, whereas both of these processes are under relaxed control in Proteus.

The similar densities of R factor and E. coli genome DNA have hindered studies of the regulation of R factor replication in this host. However, Vapnek et al. (76) have demonstrated that only a single DNA strand is passed to R$^-$ cells during R factor transfer, and Falkow and others (21) have concluded, from a study of the R factor replicative intermediate molecules in E. coli that R factor replication in this host is consistent with the rolling circle model for DNA replication.

The replicative behavior of fi^- R factors has not been examined as thoroughly as that of fi^+ R factors. Most fi^- R factors exist apparently as multiple copies per host genome in both E. coli and P. mirabilis. Although fi^- R factor R28K was found to exist as 2 different molecular DNA species per cell, the proportion and amount of each DNA species remained constant throughout the cell growth cycle, which indicates a relaxed but consistent regulation of replication (38). In contrast, the number of copies/host genome of fi^- R factor R6K in E. coli varied from 13 in the exponential phase to 38 in the stationary phase of host cell growth (38) and resembled the replicative behavior of fi^+ R factors in Proteus (62). Preliminary studies of fi^- R factor ΔS indicate that the RTF and R-genes of this R factor exist as 1 and 10 copies/genome, respectively, when harbored by E. coli (7). It seems apparent that no conclusive interpretation of fi^- R factor replication can be drawn from the limited evidence available.

Phylogenetic Relationships to Other Plasmids

The known transmissible, extrachromosomal genetic elements include R factors, F factors, Col factors, the P-lac factor, the K-88 antigen (K-88) factor, the hemolysin (Hly) factor, and the entertoxin (Ent) factor (50). With the exception of F factors, these elements have been characterized and classified on the basis of a specific

determinant(s). However, some R factors have been found to code for the production of a specific colicin, and some Col factors, identified on the basis of transmissible colicin production, have been observed to carry drug-resistance determinants. In addition, recombination between R factors and F factors, or R factors and Col factors has been observed when superinfection was conducted by transduction (49). Thus, the potentially large variation in the number of accessory genetic determinants which can be harbored by any of these elements has confused the taxonomy of transferable genetic elements.

When the transmissible plasmids are compared on the basis of pili type and superinfection immunity, R factors appear to be closely related to certain plasmids. For example, fi^+ R factors and F-like Col factors have been observed to code for the production of F-pili and to resist superinfection by other F-like elements (except by F or Col V factors). Similarly, I-like or fi^- R factors have been found to produce pili resembling those specified by the Col 1b factor and have not been observed to coexist stably in the same cell with other I-like elements, which include many of the Col factors (79). F factors or the Col V factors have been observed to coexist in the same cell with fi^+ R factors or any I-like element, although both F and Col V factors have been shown to express immunity to a superinfecting F or Col V factor (49,50). In contrast to the typical repressed

state of all R factors and most Col factors, the F factor and Col V factors have been observed to be derepressed in fertility function (49). Thus, there appears to be at least 3 different compatibility groups among transmissible elements. However, the observation that fi^+ but not fi^- R factors can complement transfer-deficient F factors would appear to indicate a close relationship between F and fi^+ R factors.

The K88, Hly, and Ent factors have been shown to be F-like elements similar to the F and Col V factors. Although F, R, and Col factors have been observed to promote chromosome transfer, only F and some Col factors have been demonstrated conclusively to exist in the integrated state (50).

Physicochemical studies of F, P-lac, and Col factors revealed that these elements are composed of DNA with an average base composition of approximately 50% GC (20). Likewise, the transfer portion of most R factors are composed of about 50% GC (19,64). Though these observations indicate a possible common origin for all transfer factors, the chemical and evolutionary basis for the observed differences in density (1.710 g/cm^3 vs. 1.705 g/cm^3) between certain R factors is not apparent. Limited evidence indicates that Col elements have about the same molecular weight (40×10^6 to 70×10^6 daltons for trans-

missible Col factors) as most R factors and F factors examined to date (28).

Possibly, the best single measure of the relatedness between R factors and other transmissible plasmids has been obtained from DNA-DNA renaturation studies. The DNA of fi^+ R factor R1 was observed to be 75% homologous to both the RTF from R1 and to R factor 222 DNA, about 50% similar to Ent factor DNA, and approximately 40% homologous to F factor DNA. In contrast, only about 15% relative binding was observed between R1 DNA and the DNA from 3 different I-like factors (28). These results suggest either an uncommon origin or a different evolutionary development for the F-like and I-like transfer factors. Cohen et al. (12) have shown that the homologous nucleotide sequences between F and fi^+ R factor DNA are located on one-half of the R factor molecule. These data indicate not only that the determinants of transfer of fi^+ R factors and the F factor are similar, but also that these genes have been contiguously conserved on the R factor molecule.

From one point of view, transferable plasmids appear to to bridge the gap in genetic development between bacteria and bacteriophages. The relationships among R factors, other plasmids, and bacteriophages, however, remain speculative. Possibly of greater significanece is the undefined association between transferable elements and male-specific phages.

Currently, however, even the similarities and differences in the genetic constitution of plasmids within a single group (I-like or F-like) have not been clearly defined.

Summary

R factors are infectious extrachromosomal genetic elements which confer upon the bacterial host resistance to one or many antimicrobial agents. These autonomous units of inheritance are conjugally transmissible among a variety of pathogenic and non-pathogenic bacteria. Although R factors are apparently distributed world-wide, their origin and evolutionary development remains obscure.

R factor-harboring bacteria pose a serious threat to the effective management of clinical disease, as exemplified by several documented epidemics and in the increased incidence of drug-resistant, R factor-harboring clinical isolants from nosocomial environments. To date, attempts to eliminate R factors have not been successful and despite varied efforts, no effective clinical means of controlling R factors has been elucidated. Limited evidence from molecular studies indicates that R factors are composed of double-stranded DNA which has been observed to vary in molecular properties in different hosts and under various host cell growth conditions. To date, the chemical nature of R factor DNA has not been systematically examined nor have the specific regulatory requirements for R factor replication or molecular dissociation been defined. An understanding of the chemical properties and replication of these elements would pro-

vide a basis at the molecular level for future efforts to eliminate or control infectious drug-resistance in bacteria.

SCOPE OF THE PROBLEM

Resolution of the replicative requirements and the specific chemical nature of R factors might offer a selective approach at the molecular level to the elimination of these transmissible elements from the bacterial host cell. Aside from the public health concern of R factors, R^+ bacterial hosts which harbor multiple R factor replicons per host genome provide an excellent biological system for studying the basic processes involved in the regulation of DNA replication.

The objective of this investigation was to study the physical and chemical properties of R factor 222 in P. mirabilis and the manner in which the replication of R factor 222 is regulated in this host. Specifically, the following aspects were examined.

1. Characterization of the effect of various cultural conditions and inhibitors of protein and DNA synthesis on R factor 222 replication.
2. Density analysis of native and denatured R factor 222 DNA in neutral and alkaline CsCl gradients.
3. Chemical hydrolysis of R factor 222 DNA and evaluation of its specific chemical composition.

MATERIALS AND METHODS

Media

All cultures were grown in Bacto-Penassay broth (PAB, Antibiotic Medium #3; Difco) with or without additives as indicated. For several shift experiments minimal medium supplemented with 0.2% glucose was prepared as described by Davis and Mingioli (15). Penassay agar (PAA), employed in the antibiotic disc assays and for culture maintenance, consisted of PAB plus 1.5% Bacto-Agar (Difco). For evaluating R factor transfer or enumerating total viable count, MacConkey agar (MAC, Difco) fortified with 1.5 g each of Bacto-Beef Extract and Bacto-Yeast Extract (Difco) per liter was utilized. KCN broth (Difco) was employed as an enrichment medium in R factor transfer studies.

Bacterial Strains and R Factor

The *Proteus mirabilis* strains used in this study were kindly supplied by H. J. Welshimer (Department of Microbiology, Medical College of Virginia, Richmond, Va.). Biochemical characteristics of these strains are described in the Results section. The strain designated Pm-5 was selected as a suitable R factor recipient on the basis of its antibiotic sensitivity pattern and ability to stably maintain R factor 222.

Escherichia coli K-12 substrain CSH-2 and an isogenic strain harboring the R factor 222 were provided by T. Watanabe (Department of Microbiology, Keio University School of Medicine, Tokyo Japan). CSH-2,a methionine-and proline-requiring auxotroph of

E. coli K-12, was mated with Shigella flexneri 2b carrying R factor 222 to obtain a CSH-2 harboring R factor 222 (84).

R factor222 was originally discovered by Nakaya et al. in a strain of Shigella flexneri isolated from a dysentery patient. This R factor is of the fi^+ type and specifies resistance to chloramphenicol, streptomycin, tetracycline, sulfadiazine, and spectinomycin (37).

From a culture of E. coli CSH-2 carrying the R factor 222, a mutant derepressed in fertility function was selected by the procedure of Meynell and Datta (48). P. mirabilis harboring R factor 222 was isolated from an 18 hr mating of R^+CSH-2 (222) and R^- P. mirabilis as described in Results.

All E. coli strains were maintained on PAA slants at 4 C; PAA slants containing 20 µg each of chloramphenicol and streptomycin per ml were used to maintain the P. mirabilis (222) strain. Fresh stock slants were prepared every four months.

Cultural Conditions, Media Shifts, and Growth Measurements

Inocula for R factor transfer experiments were grown in PAB at 35 C for 24 hr in 10 ml stationary cultures. Mating mixtures were prepared by adding 1 ml of the donor and 1 ml of the recipient culture to 8 ml of fresh PAB. Selection of R factor-harboring recipient cells (transcipients) is described in Results.

For regulation studies R factor replication was examined under various conditions which inhibit host-cell protein or DNA synthesis. For these experiments inocula were grown for 18 hr at 35 C in 10 ml

stationary cultures of PAB containing 25 µg chloramphenicol per ml. Liter cultures of prewarmed PAB with or without additives were inoculated with approximately 10^8 cells and incubated at 35 C on a gyratory shaker (New Brunswick) at 125 rev per min.

Shift experiments were carried out by mixing 500 ml of prewarmed PAB containing the indicated additive with one liter of a 12 hr (exponential phase) or 24 hr (stationary phase) culture followed by continued incubation for the indicated interval. In shifts to minimal medium, 12 or 24 hr PAB-cultured cells collected by centrifugation at 8,000 x g for 10 min at 30 C were suspended in 500 ml prewarmed minimal medium and incubated at 35 C for one generation without shaking. Shifts to low temperature were conducted by placing 24 hr cultures at 5 C for 2, 10, or 15 days.

Growth of P. mirabilis (222) in PAB and in PAB containing 25 µg chloramphenicol per ml was monitored by viable counts and by turbidity at 650 nm with a Klett-Summerson Colorimeter. Total viable counts and total resistant viable cells were enumerated by plating on MAC and MAC containing 25 µg chloramphenicol per ml, respectively.

Antibiotics and Other Inhibitors

Chloramphenicol (Chloromycetin Kapseals, Parke-Davis & Co.; CAM) was prepared by dissolving 250 mg capsules in 10 ml sterile 0.1 N HCl and diluting with saline to the desired concentration. The resulting suspension was shaken and quickly pipetted to obtain accurate quantities. A fresh CAM solution was prepared for

each experiment.

Streptomycin sulfate (E. R. Squibb & Sons: STR) and tetracycline hydrochloride (Tetrachel, Rachelle Laboratories, Inc.; TET), both supplied as dry powders, were dissolved in sterile 0.9% NaCl and stored at a concentration of 250 mg/ml.

Kanamycin sulfate (Kantrex, Bristol Laboratories; KAN) and neomycin sulfate (E. R. Squibb & Sons; NEO) were obtained in solution and diluted with sterile 0.9% NaCl to yield a stock solution containing 75 mg/ml.

Puromycin dihydrochloride (Nutritional Biochemicals; PUR) was obtained, used, and stored in the dry state.

Nalidixic acid tablets (NegGram, 500 mg, Winthrop Laboratories; NAL) were each suspended in 10 ml sterile 0.9% NaCl. The mixture was shaken, quickly pipetted, and discarded after each experiment.

Phenethyl alcohol (Eastman Organic Chemicals; PEA) was added directly to cultures to a final concentration of 0.25%.

Disc Assay for Antibiotic Resistance

The disc diffusion technique was employed to assay the antibiotic sensitivity pattern of cultures. Seeded agar was prepared by adding 0.1 ml of a 10^{-2} dilution of an 18 hr PAB culture to 10 ml melted PAA tempered at 48 C. The inoculated medium was mixed and poured into sterile 100 X 15 mm petri plates. Discs (Difco Laboratories) containing low and high concentrations of nine chemotherapeutic agents (NAL, CAM, COL, KAN, NEO, STR, TET, SUL, and ampicillin) were used in each assay. Plates were incubated for 18 hr at 35 C. The diameter of the zone of inhibition around each

disc recorded in mm, for any given culture, was generally reproducible to within ($\pm$) 1 mm. Due to occasional overgrowth around the SUL discs, zones were measured after 12-14 hr incubation.

Biological Assay for Chloramphenicol

To assess the extent of R factor-mediated inactivation of CAM during growth of P. mirabilis (222) in PAB plus CAM, a quantitative disc assay was employed. Biologically active CAM remaining in the culture medium was measured by adding 0.1 ml of the culture filtrate, prepared by passage through a 0.45 µm filter (Millipore Corp., HA type, 25 mm), to a sterile 12 mm absorbent disc. Air-dried discs were placed aseptically on PAA plates seeded with approximately 10^5 cells of E. coli CSH-2 (minimal inhibitory concentration, less than 2 µg CAM/ml). and the diameter of the zone of inhibition around each disc was measured after 18 hr incubation at 35 C. The concentration of CAM in the culture medium after various intervals of incubation was determined by reference to a standard curve (See Appendix Figure 1) obtained by assaying known quantities of CAM (1-50 µg/disc) in an identical manner.

Extraction of Deoxyribonucleic Acid

Cells were collected by centrifugation at 8,000 x g at 4 C, washed twice in saline-EDTA (0.15 M NaCl, 0.1 M ethylenediaminetetraacetate: pH 8.0), and stored at -20 C. DNA from 2-3 g frozen cells homogenized in 25 ml saline - EDTA was isolated according to the procedure of Marmur (42). To ensure extensive

cell lysis, 4 mg lysozyme/ml (Calbiochem) was added to the initial cell suspension and the mixture incubated for 80 min at 37 C. The material obtained from the first ethanol precipitation was similarly treated with 100 µg ribonuclease/ml (Calbiochem) for 60 min at 37 C to minimize ribonucleic acid (RNA) contamination. DNA from the final isopropanol precipitation step was dissolved in either 0.1 X or 0.01 X SSC before adjusting the final salt concentration to 1 X SSC (0.15 M NaCl containing 0.015 M sodium citrate, pH 7.0). Concentrations of DNA ranging from 500 to 1000 µg/ml were stored over a drop of chloroform at 4 C. DNA was quantitated spectrophotometrically with a Zeiss PMQ II at 260 nm based on an $E_{1\ cm}^{0.1\%}$ of 24.

Cesium Chloride Isopycnic Centrifugation

To determine the buoyant density and base composition of the DNA preparations, the CsCl density gradient centrifugation technique of Meselson et al. (47) was employed with the use of a Beckman Model E analytical ultracentrifuge. The centrifuge cell consisted of a 12 mm, 4° sector Kel-F centerpiece, a plain quartz lower window, and a 1° negative wedge upper quartz window. Samples for centrifugation were prepared by adding 0.23 ml of a 0.1 X SSC solution containing 0.5 µg of reference DNA and 1-2 µg of sample DNA to 0.84 ml of stock CsCl (Matheson, Coleman, and Bell; optically pure) consisting of 13 g CsCl/7.0 ml 0.02 M Tris [tris-(hydroxymethyl-) aminomethane; Calbiochem] buffer at pH 8.5. The mixture was adjusted, if necessary, to $[\eta]^{25\ C}$ of 1.4010 ± 0.0001 with the aid of an Abbe 3L refractometer (Bausch and Lomb) prior to

centrifugation at 44,000 rev/min for 20-24 hr at 25 C in an analytical D rotor. Streptomyces venezuelae mycelial DNA (ρ = 1.7300 g/cm^3) obtained from S. G. Bradley (Department of Microbiology, Medical College of Virginia, Richmond, Va.) served as the density reference.

In all studies where the proportion and amount of the R factor DNA components were determined, the preparation for centrifugation contained 3.5 µg of sample DNA and 0.35 µg of reference DNA. Ultraviolet (UV) absorption pictures taken throughout the run and at various exposure times (10-40 sec) after 24 hr were developed in D-11 developer (Eastman Kodak). To provide reproducible and correlative results, care was taken to ensure that the linear density range of the Kodak commercial film used for UV absorption photography was not exceeded; i.e. a maximal change in absorbance at 550 nm of < 1.4 between the lightest and darkest sections of each film.

Microdensitometry and Density Profile Analysis

UV absorption films were traced with a recording microdensitometer supplied by E. Berry (Department of Biophysics, Medical College of Virginia, Richmond, Va.) at a scan speed of 3.9 mm/min and chart speed of 2 inches (5 cm) per min (35).

The equations of Schildkraut et al. (67) were combined and reduced to simplify the determination of buoyant density and base composition. The following expressions resulted;

$$(1)\quad \rho = \rho_o + 0.0092\ {}^{db}/_{b'}\left({}^{db}/_{b'}+2A\right) - {}^{cb}/_{b'}\left({}^{cb}/_{b'} + 2A\right)$$

$$(2)\quad \text{Mole \%}_{GC} = \rho_o/0.098 - 16.94 + 0.0939\ {}^{db}/_{b'}\left({}^{db}/_{b'} + 2A\right) - {}^{cb}/_{b'} + 2A)$$

where ρ = density of the sample DNA in g/cm^3

ρ_o = density of the reference DNA in g/cm^3

A and b were known to be 5.7 cm and 1.6 cm, respectively, for the Model E and the remaining values were obtained from the tracing as described previously (67). Thus, the ratios $^{db}/_{b'}$ and $^{cb}/_{b'}$ which express the actual position of the sample and reference DNA bands in the centrifuge cell were calculated from the tracings and incorporated into either (1) or (2) to obtain the buoyant density or guanine plus cytosine content of the DNA samples, respectively.

For density profile analysis the relative areas of the R factor DNA bands and the P. mirabilis chromosomal DNA band were measured by planimetry (Keuffel & Esser model #4236) of the manually resolved, essentially Gaussian distributions of the tracings. The percentage of the total area comprised by each peak was calculated, i.e. the percent of the 50% GC R factor component, the percent of the 58% GC R factor component, and the percent of the host genome relative to total DNA extracted. The number of copies of each R factor component per P. mirabilis genome equivalent was calculated by using the values 2.8×10^9 daltons, 50×10^6 daltons, and 10×10^6 daltons for the molecular weights of the Proteus genome (64), the 50% GC, and the 58% GC R factor components (20), respectively. Since the relative areas obtained by planimetry represent the relative quantity of each DNA species, the number of copies of each R factor component/genome equivalent was calculated using the following formulas, in which the values

280 and 56 represent the ratio of molecular weights of the *Proteus* genome and the 58% GC R factor component, and of the *Proteus* genome and the 50% GC R factor component, respectively:

$$\frac{\text{58 GC Copies}}{\text{Genome Equivalent}} = (280)\ \frac{\text{Percentage 58\% GC DNA}}{\text{Percentage Proteus DNA}}$$

$$\frac{\text{50\% GC Copies}}{\text{Genome Equivalent}} = (56)\ \frac{\text{Percentage 50\% GC DNA}}{\text{Percentage Proteus DNA}}$$

Thermal Denaturation of Deoxyribonucleic Acid

The temperature at which one-half of the double-stranded DNA in a solution is denatured or Tm (midpoint of thermal denaturation) has been shown to be linearly related to the DNA base composition (43). The change in absorbance at 260 nm (A_{260}) with increasing temperature (1 C per 3-4 min) was measured in a Gilford Model 2400 or Zeiss PMQ II spectrophotometer against a blank solution containing the same salt concentration as the DNA solutions (0.1 X SSC). Each sample DNA preparation had an original A_{260} of 0.4-0.6. Both the midpoint of the hyperchromic shift and the percent increase in absorbance after complete denaturation were obtained from the recording. Base compositions were calculated from the equations of Mandel et al. (41):

$$\text{Mole \% G+C} = (\text{Tm } 0.1 \text{ X SSC}/50.2) - 0.990$$

$$\text{G+C}_x = \text{G+C}_{std} + 0.0199\ (\text{Tm}_x - \text{Tm}_{std})$$

The latter equation was employed when a reference DNA of known G+C content was analyzed along with sample DNA preparations. Percent increase in absorbance was expressed relative to the absorbance prior to heating.

Determination of Base Composition by Depurination

Treatment of DNA at a pH of 1.6 has been found to cause the selective cleavage of the β-glycosidic linkages between the purine bases and their deoxyribose residues resulting in apurinic acid. This technique was employed, as originally described (73), to obtain DNA free of purines so that the pyrimidines could be investigated by hydrolysis and chromatography. Five ml of a DNA preparation (1 mg/ml) in 1 X SSC was adjusted to pH 1.6 with 1N HCl and dialyzed against 1 liter of HCl (pH 1.6) for 24 hr, against a liter of fresh 1 X SSC (pH 7.0), and finally against a liter of distilled water for 1 hr. The dialysate was evaporated to dryness, hydrolyzed, and chromatographed.

Application of the above technique to the determination of base composition as described previously (33) was also employed. A 0.5 ml solution of DNA (100-800 µg/ml) was dialyzed against 1 X SSC for 12 hr followed by adjustment of the pH to 1.58 with 1N HCl and dialysis against 5 ml SSC (pH 1.58) for 24 hr with gentle shaking at 37 C. The absorbance of the dialysates was determined

in a recording Beckman DB spectrophotometer against a blank SSC solution of pH 1.58. The % GC of DNA preparations were calculated by the following equations of Huang and Rosenberg (33).

$$R = \frac{(1-X_G)\,a + X_G\,(c)}{(1-X_G)\,b + X_G\,(d)}$$

where $R = A_{265}/A_{280}$ ratio of the sample dialysate.

X_G= mole fraction of guanine relative to adenine

a = molar absorptivity of adenine at 265 nm

b = molar absorptivity of adenine at 280 nm

c = molar absorptivity of guanine at 265 nm

d = molar absorptivity of guanine at 280 nm

Using the extinction coefficients for adenine and guanine at these wavelengths and under these conditions, the % GC (X_G) equals:

$$X_G = \frac{13.1-5.0\,R}{5.8+1.9\,R}$$

Nucleic Acid Hydrolysis

DNA preparations for chemical hydrolysis were sometimes treated with 0.5 mg pancreatic ribonuclease/ml (Calbiochem; self-digested for 30 min at 80C) for 8 hr at 37 C and precipitated twice with isopropanol before final dissolution in 0.1 X SSC. The stock preparations were stored at a final concentration of 1 X SSC over a drop of chloroform at 4 C. A portion of each DNA preparation was precipitated with isopropanol, washed twice in 95% ethanol, and dried under vacuum over $CaSO_4$ dessicant. For comparative purposes,

small quantities of each preparation were precipitated immediately prior to hydrolysis, washed as described above, and dried under a gentle stream of air for 20 min. Salmon sperm DNA (Calbiochem; lyophilized), dissolved in SSC and treated identical to sample preparations, served as the hydrolysis control.

The perchloric acid hydrolysis method of Marshak and Vogel (44) was employed following the procedures outlined by Wyatt (86). Forty µl of 70% $HClO_4$ (Allied Chemical Co.) was added to 3 mg of dried DNA in 100 X 13 mm screw cap tubes and hydrolysis was carried out at 100 C for 60 min. The diluted hydrolysate (10-15 µl) was spotted for chromatography.

Formic acid hydrolyses (86,87) of 1-3 mg samples were conducted in 6 X 50 mm sealed glass tubes. After adding 0.5 ml of 90% formic acid (Fisher) the tubes were closed about 20 mm above the surface of the liquid, and heated for 30 min at 175 C. Dried hydrolysates, evaporated under vacuum at 60 C, were dissolved in 25-50 µl 1 N HCl of which 10-15 µl was spotted for chromatography.

Hydrolysis with 0.5 ml 6 N HCl was conducted in sealed tubes by following the formic acid hydrolysis procedure. After incubation at 125 C for 2 hr, the hydrolysate was dried in a rotary evaporator at 55 C, dissolved in 25 µl distilled water, and spotted on chromatograms.

To assess the extent of degradation of free bases by different hydrolytic conditions, standard bases and standard base mixtures were hydrolyzed by the same procedures and analyzed chromatographically.

Enzymatic Digestion of Deoxyribonucleic Acid

To check for the presence of abnormal bases in DNA preparations without the use of organic or inorganic acid hydrolysis, sequential digestion of each sample with deoxyribonuclease I followed by snake venom phosphodiesterase was employed (24). DNA samples dissolved in 20 mM Tris-HCl, pH 7.4, containing 10 mM $MgSO_4$ were treated with 0.10 mg deoxyribonuclease I (Calbiochem) per ml at 37 C for 3 hr. These partially digested preparations were further hydrolyzed with 10 µl of venom phosphodiesterase/ml (Calbiochem, 5 mg/ml) after the pH was adjusted by adding 100 µl 1 M glycine-NaOH, pH 9.2, per ml of the partially degraded sample. After 1.5 hr at 37 C, the reaction was stopped by adjustment of the pH to 4.0. The resulting 5'- mononucleotides were then subjected to acid hydrolysis and separated by chromatography.

Paper Chromatography

Chromatography was carried out on Whatman #1 paper by the descending method. Isopropanol-hydrochloric acid-water (65 : 16.6 : 18.4, v/v)(86) was the primary solvent system for separation of bases. This solvent required about 24 hr to run 40 cm at 25 C. A system composed of ethyl acetate-formic acid-water (70 : 20 : 10, v/v) (22) was employed for analysis of eluted spots or as the second solvent system in bidirectional chromatography of hydrolysates. This solvent ran 40 cm in 16-18 hr at 25 C.

All chromatograms were spotted, with 5-15 µl of sample hydrolysates or standard solutions. Both individual base standards and base mixtures were included to establish R_f values.

Carbohydrate standard solutions (glucose, ribose, and deoxyribose) were also run for comparative purposes.

Elution and Spectral Analysis of Bases

Chromatographed purines, pyrimidines, nucleosides, and nucleotides were detected with a short wave UV lamp (257 nm, Mineralight). Carbohydrates were located by development with $AgNO_3$ under alkaline conditions (75). Preliminary identification of spots was based upon both comparison with R_f values of the standards and with reported R_f values (22).

Base spots located with UV light were circled lightly with a lead pencil, cut into strips, and placed in small tubes containing 1.5 ml 0.01 N HCl for 12 hr at 25 C. Spectral analysis of the eluted spots was conducted in a recording Beckman DB spectrophotometer. One ml eluates were scanned from 320 to 200 nm against both a blank solution of 0.01 N HCl and an eluted blank spot from a similar area of the chromatogram. Spectra were read at pH 2.0, 7.0, and pH$\sim$13 by adjustment of both sample and blank solutions with 1N NaOH.

In addition to R_f values, identification of bases was accomplished by comparisons of observed λ max, λ min, and ratios of absorbance at different wavelengths ($^{250}/_{260}$, $^{280}/_{260}$) to reported values. Base quantitation was carried out by utilizing the extinction coefficients at 3 different wavelengths and averaging the calculated quantities. All spectral constants were obtained from a recent compilation unless otherwise noted (17).

RESULTS

Selection of *Proteus mirabilis* host.

Antibiotic sensitivity assays of 5 different clinical isolants, preliminarily identified as *P. mirabilis*, were conducted in order to select a drug-sensitive strain suitable for use in these studies. All cultures were indole negative and produced urease and hydrogen sulfide. Three of the *P. mirabilis* strains were resistant to one or more of the antibiotics to which R factor 222 confers resistance and these strains were discarded. Biochemical properties of the two drug-sensitive isolants were characteristic of *P. mirabilis* strains. Each strain exhibited swarming on MAC, produced gas from glucose, and gave a positive methyl red test, utilized citrate as the sole carbon and energy source, and grew in KCN broth. The cultures did not produce acetylmethylcarbinol or lysine decarboxylase, utilize malonate, or ferment lactose, sucrose, or dulcitol. The antibiotic sensitivities of these strains, Pm-3 and Pm-5, are given in Table 1.

Transfer of R factor 222 from *E. coli* to *P. mirabilis*

The ability of *Proteus* to grow in KCN broth and the inability of this species to utilize lactose were the basis of the following enrichment and selection procedures designed to separate recipient *P. mirabilis* from the donor R^+ *E. coli* cells. Donor [CSH-2 (222)] and recipient (Pm-3 or Pm-5) cells were prepared for mating by growing each culture in PAB at 35 C for 24 hr. The

Table 1

Antibiotic sensitivity patterns of donor and recipient cultures from R factor transfer studies.

	Diameter of Zones of Inhibition (mm)[2]																	
	AMP[1]		CAM		COL		KAN		NEO		STR		TET		SUL		NAL	
Culture	2	10	5	30	2	10	5	30	5	30	2	10	5	30	50	300	5	30
Pm-3	23	32	21	32	R	R	16	24	16	22	12	22	R	10	S	S	16	30
Pm-5	24	32	17	29	R	R	23	26	17	23	15	24	R	15	S	S	16	28
CSH-2	18	30	22	33	14	19	21	28	18	25	15	23	26	S	S	S	20	32
CSH-2 NAL-R	20	30	24	32	15	20	16	26	15	24	14	21	29	S	S	S	R	R
R^+ CSH-2(222)	20	30	R	R	14	19	23	30	18	27	R	18	R	12	R	R	19	29
R^+ CSH-2 NAL-R(222)	17	32	R	R	16	22	15	27	15	25	R	R	R	11	R	R	R	R
R^+ Pm-5(222)	25	33	R	9	R	R	23	25	17	23	R	15	R	R	R	R	15	25
R^+ Pm-5(KAN,NEO,STR,TET)	22	30	16	30	R	R	12	20	12	20	R	15	R	R	S	S	15	27

[1] Concentrations in µg/disc listed below each antibiotic.
[2] R = resistant (no zone); S = sensitive (zone either very large, or for sulfadiazine, zone size not meaningful).

mating mixture consisted of 1 ml each of the donor and a recipient culture plus 10 ml of fresh PAB. After incubation for 15-18 hr at 35 C, the Proteus recipients were enriched by incubating 0.2 ml of the mating mixture in 10 ml KCN broth for 48 hr. The enrichment culture was plated on plain MAC and on MAC containing 25 µg colymycin/ml plus one of the antibiotics, with the exception of sulfonamides (SUL), to which R factor 222 confers resistance. Final concentrations of these drugs were: 25 µg CAM/ml, 20 µg STR/ml, and 20 µg TET/ml. The lactose-fermenting E. coli cells appeared as red colonies on MAC whereas P. mirabilis colonies were whitish and opaque. Colymycin was employed to inhibit the growth of the donor E. coli cells while CAM, STR, and TET were added to select for P. mirabilis recipients which had received the R factor (transcipients). To isolate a Proteus harboring the R factor 222, Pm-5 and CSH-2 (222) were mated, and a number of potential transcipient colonies were picked from the selective media and restreaked on identical media. An isolated colony was picked from each of the restreaked plates after 24 hr incubation and used to inoculate 10 ml PAB. After incubation for 24 hr, PAB cultures were used to inoculate a stock PAA slant, to assay for antibiotic sensitivities, to inoculate a urea agar slant, and to check for culture purity by streaking on MAC. Isolants which degraded urea and formed whitish, opaque colonies on MAC, and which had acquired resistance to CAM, STR, TET, and SUL were, thus, Pm-5 (222).

The results of a typical mating between Pm-5 and CSH-2 (222)

are shown in Table 2. The selective medium containing TET was not effective in preventing the growth of R^- P. mirabilis. However, over 50% of the P. mirabilis colonies isolated from plates containing CAM or STR exhibited newly acquired resistance. When single colonies from the selective antibiotic plates were restreaked on identical media, two colony types were observed. The majority of plates contained smooth-edged colonies which had acquired resistance to CAM, STR, TET, and sulfonamides, and these cells were assumed to carry R factor 222. A few plates contained entirely rough-edged colonies which possessed relatively high levels of resistance to STR and TET and lower levels of resistance to KAN and NEO. The antibiotic sensitivity patterns of these two types of transcipients are given in Table 1. Further analysis of these R factor-harboring P. mirabilis are described in the following sections. When Pm-3 was similarly mated several times with CSH-2 (222), no transcipients were obtained. Since this Proteus strain was either a poor recipient or could not stably maintain the R factor, no further studies were conducted with this culture.

Genetic Studies on R Factor 222

On the basis of resistance to CAM, STR, TET, and SUL, P. mirabilis transcipients which formed smooth-edged colonies appeared to harbor R factor 222. To determine the transmissibility of the acquired drug resistances matings between these transcipients and E. coli CSH-2 NAL-R (chromosomally resistant to > 1000 µg nalidixic acid/ml) were conducted. Procedures for

Table 2

Effect of various antibiotics in the selection medium on the enumeration of P. mirabilis transcipients.

SELECTIVE MEDIA	COLONY TYPES	
	CSH-2(222)	Pm-5
	Viable Counts (per ml)[1]	
Plain MAC	19×10^6	35×10^7
MAC + 25 µg COL/ml + 25 µg CAM/ml	N.G.[2]	20×10^4
MAC + 25 µg COL/ml + 25 µg STR/ml	N.G.	5×10^2
MAC + 25 µg COL/ml + 25 µg TET/ml	N.G.	TNTC[3]

[1] Counts obtained on selective media after 24 hr incubation at 35 C.
[2] No growth (no red colonies detected).
[3] Too numerous to count (R^- P. mirabilis were not inhibited by this concentration of TET).

effecting and detecting R factor transfer were as described previously except that KCN enrichment was deleted and E. coli CSH-2 NAL-R transcipients were selected on medium containing CAM, STR, or TET plus 1000 µg NAL/ml in place of COL. These Pm-5 transcipients were observed to transfer resistance to CAM, STR, TET, and SUL as a unit (i.e. the entire 222 R factor) to CSH-2 NAL-R. Antibiotic sensitivity patterns of R^- and R^+ CSH-2 NAL-R cultures are given in Table 1. The aberrant transcipient, Pm-5 (KAN, NEO, STR, TET), did not transfer any resistance-determinants to CSH-2 NAL-R under identical mating conditions. Thus, it would appear that these transcipients lacked the RTF portion of R factor 222. The non-transmissible aberrant "R factor" was apparently derived from 222, although it has not been observed previously to confer resistance to KAN or NEO. In numerous antibiotic sensitivity assays the aberrant transcipient population was always observed to express resistance at relatively high levels to STR and TET, and at lower levels to KAN and NEO, but neither CAM nor SUL resistance was detected.

Since R factor 222 has been reported to segregate either the TET resistance-determinant or the CAM, STR, and SUL resistance genes, the nature of the (KAN, NEO, STR, TET) factor was obscure. Several matings between Pm-5 and E. coli CSH-2 (222) were conducted to establish the typical segregant types of R factor 222 detectable under these conditions. From three different matings four types of P. mirabilis transcipients with the following patterns of resistance were obtained: (CAM, STR, TET, SUL), the

typical resistances conferred by R factor 222; the aberrant resistance pattern (KAN, NEO, STR, TET); and the 2 patterns of segregation reported previously, (TET), and (CAM, STR, SUL). Transcipients containing the 222 or the aberrant resistance patterns accounted for greater than 90% of all transcipients observed. In each case, the Pm-5 (222) but not the aberrant transcipient cells were found to transfer the acquired resistance to E. coli CSH-2 NAL-R. The transferability of resistances from the other two types of transcipients was not assessed.

To determine if the KAN, NEO, STR, and TET resistance genes in the aberrant transcipient were autonomous or integrated, an attempt was made to mobilize these genes with a transmissible R factor which confers only resistance to ampicillin (AMP). For this experiment, Pm-5 carrying the (KAN, NEO, STR, TET) factor was mated simultaneously with both R^{+} CSH-2 NAL-R harboring the AMP R factor and R^{-} CSH-2 NAL-R. If the AMP factor could mobilize the aberrant R-genes, CSH-2 NAL-R transcipients possessing resistance to AMP, KAN, NEO, STR, and TET should be detected. Although several triple matings were conducted, no such transcipients were isolated.

A P. mirabilis transcipient harboring a transferable R factor 222, isolated from these experiments, was employed in all subsequent studies of the molecular nature and replication of R factor 222.

Molecular Characterization of R factor 222 in P. mirabilis.

To determine the molecular nature of R factor 222 in Proteus, DNA isolated from R^- and R^+ Pm-5 cells was subjected to CsCl isopycnic centrifugation. Microdensitometer tracings of UV-absorption films of R^- and R^+ P. mirabilis DNA preparations after CsCl centrifugation are shown in Fig. 1. DNA from R^- P. mirabilis exhibited a single unimodal, symmetrical band at a density of 1.698 g/cm^3 which corresponded to 38% GC (Fig. 1A). Irrespective of the cultural conditions or physiological state of the cells, no satellite DNA was detected in any DNA preparation from R^- P. mirabilis. In contrast, DNA from P. mirabilis harboring R factor 222 showed 2 satellite R factor DNA bands at densities of 1.710 and 1.718 g/cm^3 in addition to the P. mirabilis genome band (Fig. 1B). The densities of the R factor bands, corresponding to base compositions of 50 and 58% GC, respectively, were never observed to vary but the relative amounts of R factor DNA in each band varied considerably in cells grown under different cultural conditions (Fig. 2). When DNA preparations from Pm-5 (KAN, NEO, STR, TET) cells grown under a variety of conditions were analyzed in a similar manner, no satellite DNA bands were detected.

The effects of variation of host physiological state or of cultural conditions which selectively interfere with protein or DNA synthesis on the replication of R factor 222 was examined by quantitating the percentage of each R factor satellite DNA band relative to the total DNA and the number of copies of each R factor component per host genome equivalent.

Figure 1. Density profiles of R^+ and R^- P. mirabilis. A, DNA isolated from R^- P. mirabilis grown for 48 hr at 35 C in PAB; B, DNA isolated from P. mirabilis harboring R factor 222 after growth in PAB for 48 hr at 35 C.

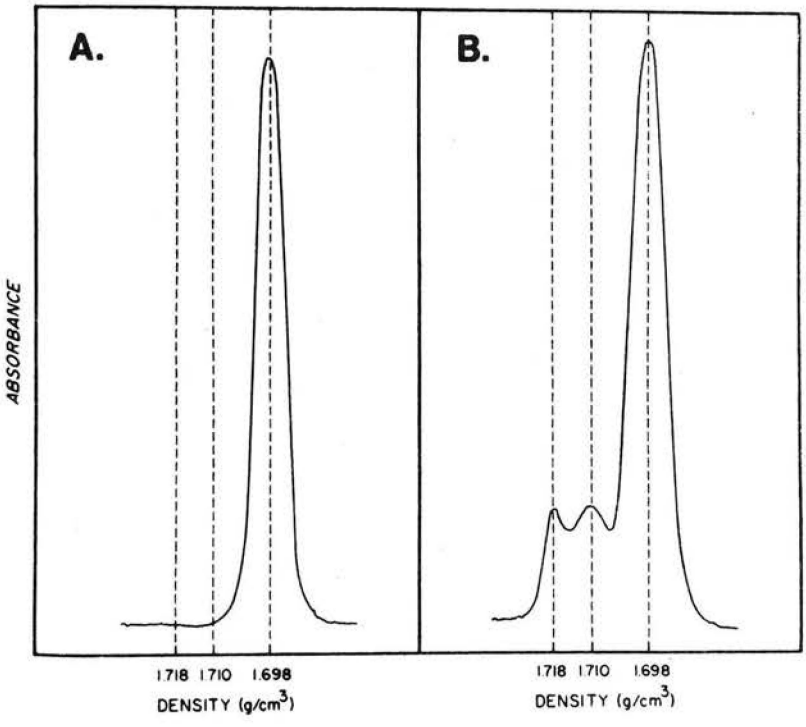
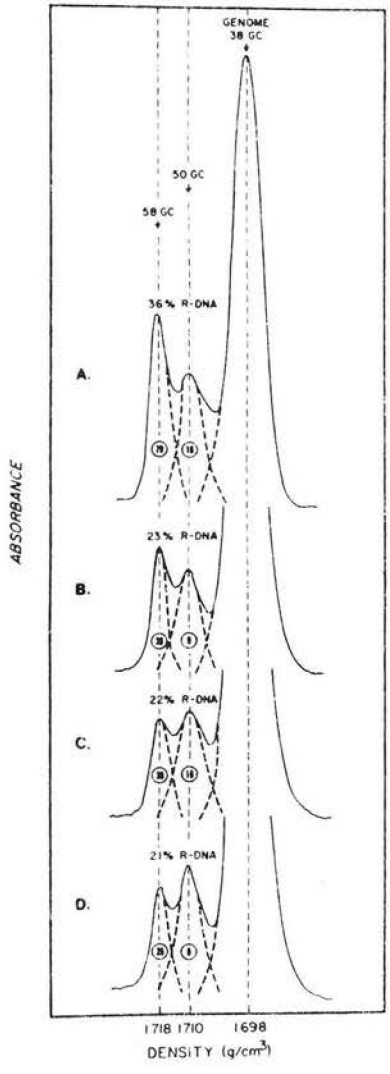

A.
B.
ABSORBANCE
1.718 1.710 1.698
DENSITY (g/cm3)
1.718 1.710 1.698
DENSITY (g/cm3)

Figure 2. Density profiles of R^+ P. mirabilis DNA containing various amounts and proportions of the R factor components. Percent R factor DNA (% R-DNA), given above each tracing, is expressed as a percentage of the total DNA extracted. Numbers under the 58% GC and 50% GC peaks represent the copies per P. mirabilis genome calculated as described in Materials and Methods.


GENOME
38 GC
50 GC
58 GC
36% R-DNA
A.
23% R-DNA
B.
22% R-DNA
C.
21% R-DNA
D.
ABSORBANCE
1718 1710 1698
DENSITY (g/cm³)

A series of ultraviolet absorption film tracings illustrating the variation both in percentage of total R factor DNA and in quantities of each R factor band is shown in Fig. 2. In Fig. 2A, R factor DNA comprised 36% of the total extracted DNA and was composed of 79 copies of the 58% GC and 16 copies of the 50% GC R factor components per P. mirabilis genome equivalent. Variation in the percentage of total R factor DNA was not necessarily associated with a change in the composition of the R factor DNA, as indicated by a comparison of Fig. 2A and 2B. However, the relative amounts of each R factor component can vary as shown by comparisons of Fig. 2B, 2C, and 2D. All subsequent studies on the replicative behavior of R factor 222 in P. mirabilis were analyzed by these parameters. Although the values from a single experiment are given, the results of duplicate experiments were similar.

Effect of Physiological Age of R^+ P. mirabilis on R Factor Replication

The variation in R factor 222 DNA content in R^+ P. mirabilis during growth in PAB is shown in Table 3. After growth of the R^+ P. mirabilis for 12 hr, R factor DNA comprised 16% of the total extracted DNA; the 50% GC and 58% GC components were, proportionately, 65% and 35% of the total R factor DNA, respectively. This corresponded to seven copies of the 50% GC and 19 copies of the 58% GC components per host genome. Total R factor DNA content reached a maximum of 22% after 24 hr and remained constant between 24 and 72 hr. Similarly copies of the 50% GC component remained relatively constant after 24 hr, whereas 58% GC copies decreased from a maximum of 36 at 24 hr to a constant level of about 30 after 36 hr of incubation.

Table 3

Variation in R factor DNA content in R^+ P. mirabilis during growth[1].

Incubation Time (hr)	%R-DNA[2]	50% GC		58% GC	
		%[3]	Copies[4]	%[3]	Copies[4]
12	16	65	7	35	19
24	22	55	9	45	36
36	21	62	9	38	28
48	22	62	10	38	30
72	22	64	10	36	28

1 R^+ P. mirabilis grown in PAB at 35 C.
2 Percentage of total DNA extracted.
3 Relative percentage of total R factor DNA.
4 Copies per P. mirabilis genome equivalent based on molecular weights given in Materials and Methods.

Fig. 3 illustrates the relationship between R factor DNA composition and the physiological age of the host. The ratio of 58% GC:50% GC copies increased from 2.7 during exponential growth (12 hr) to a maximum of 4.0 after 24 hr, and then declined to a relatively stable value of about 3.0. The generation time under these conditions was 45 min. A 20 to 25% decrease in viability was noted during the first 12 hr in the stationary phase, or about the time when the maximal 58% GC:50% GC ratio and percentage of R factor DNA were observed. Although not shown, turbidimetric measurements of growth paralleled the viability curve shown in Fig. 3, and viable counts obtained when 25 µg CAM/ml was added to the enumeration medium were 25-30% lower between 9 and 36 hr of incubation, indicating the presence of a significant number of viable sensitive cells during this growth interval.

Thus, R factor replication continued in the stationary phase of growth in PAB and R factor DNA content reached a maximum of 22% after 24 hr incubation.

The Influence of Chloramphenicol on R Factor Replication During the Growth Cycle of R^+ P. mirabilis.

When the replication of R factor 222 was studied in P. mirabilis grown in PAB containing 25 µg CAM/ml, the results shown in Table 4 were obtained. During exponential growth in this medium, the percentage of R factor DNA (17) was similar to that observed after 12 hr in drug-free PAB. However, the proportion

Figure 3. Relationship between the physiological age of R^+ P. mirabilis and replication of the R factor components. Cultures were grown with shaking in PAB at 35 C for the indicated time of incubation. Viable cells (●) were enumerated by plating on MAC. Percent R factor DNA (■) is expressed as a percentage of the total DNA extracted. Ratios (◐) of copies per P. mirabilis genome equivalent of the 58% GC to the 50% GC components were calculated using the molecular weights given in Materials and Methods.

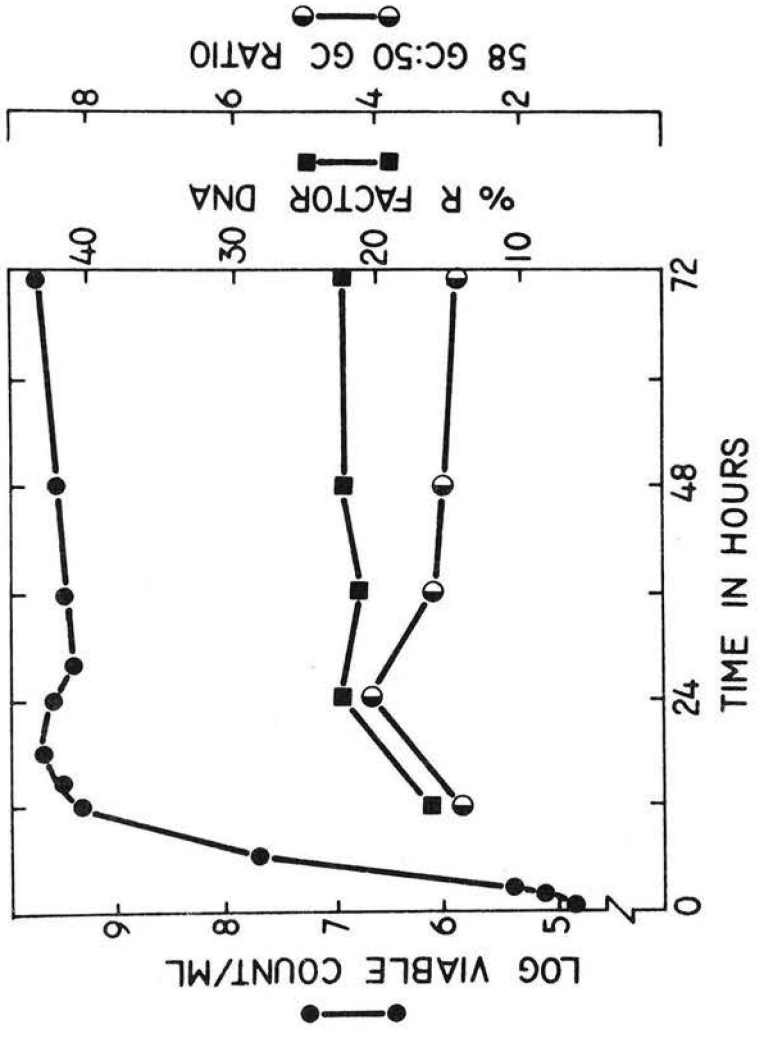

LOG VIABLE COUNT/ML
% R FACTOR DNA
58 GC:50 GC RATIO
TIME IN HOURS
0
24
48
72
5
6
7
8
9
10
20
30
40
2
4
6
8

Table 4

Variation in R factor DNA content in R^+ P. mirabilis during growth in the presence of chloramphenicol[1].

Incubation Time (hr)	%R-DNA[2]	50% GC		58% GC	
		%[3]	Copies[4]	%[3]	Copies[4]
12	17	50	6	50	29
24	31	53	13	47	59
36	36	50	16	50	79
48	26	39	8	61	60
72	27	37	8	63	65

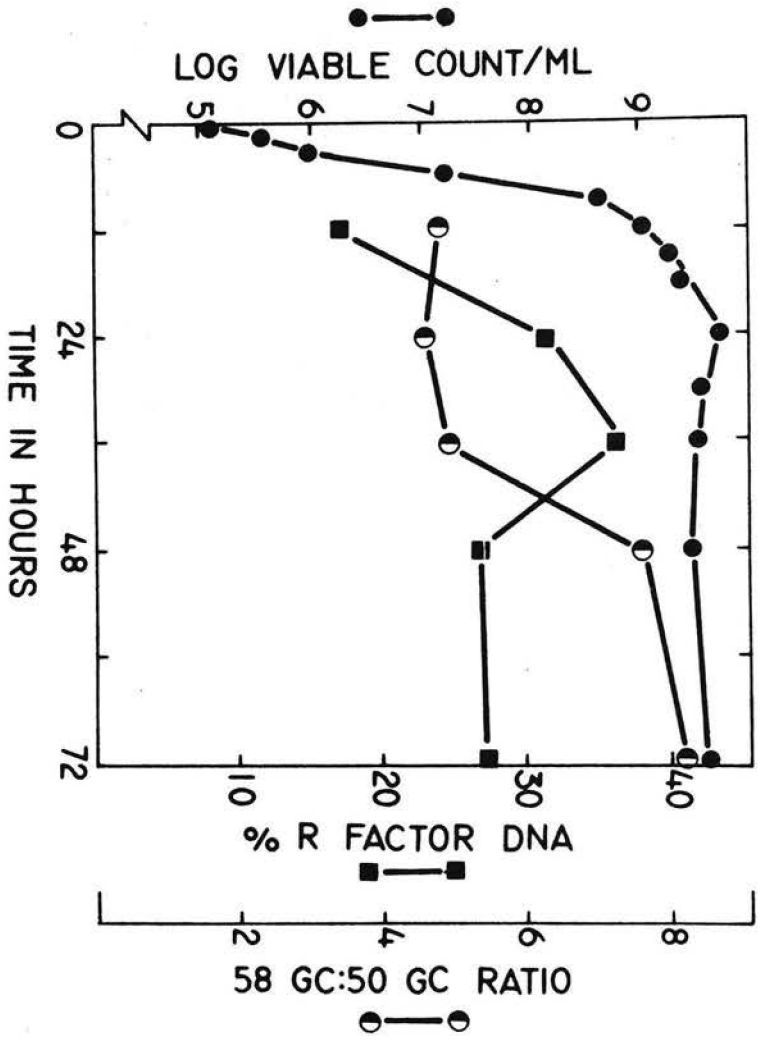
[1] R^+ P. mirabilis grown in PAB containing 25 µg CAM/ml at 35 C.

[2,3,4] See Table 3.

of the total R factor DNA represented by each R factor component (R-component) was observed to be 50%. During the stationary phase, R factor DNA content continued to increase to a maximal value of 36% after 36 hr incubation. Each R-component comprised 50% of the total R factor DNA which corresponded to 16 copies and 79 copies of the 50 and 58% GC replicons per genome, respectively, and represented an increase of 64% in the maximal percentage of R factor DNA over that observed in drug-free medium. Between 26 and 72 hr of incubation the number of 50% GC components was halved, whereas 58% GC copies were reduced only slightly and percent R factor DNA leveled at a value of 26-27. In contrast to values of 60-65% observed in drug-free PAB, the relative proportion of the total R factor DNA composed of 50% GC components in medium containing CAM was always about 50% or less.

Variation in R factor DNA composition as a function of growth of R^+ P. mirabilis in PAB containing 25 µg CAM/ml is depicted in Fig. 4. The generation time was 50 min in this medium. The 58% GC:50% GC ratio was relatively stable (about 5.0) during the first 36 hr of incubation. However, it increased to 7.5 and 8.1 after 48 and 72 hr, respectively, suggesting a more relaxed regulation of 58% GC replication. As noted in drug-free PAB, a 20-25% decrease in viable cells was observed in the PAB plus CAM culture, and was likewise associated with the observed maximal percentage of R factor DNA (36%). Bioassays for CAM activity remaining in the growth medium showed that less than 5 µg CAM per ml remained after 24 hr incubation whereas no CAM was detectable after 36 hr. As observed during growth in drug-free medium,

Figure 4. Effect of chloramphenicol in the growth medium on replication of the R factor components. Cultures were grown with shaking in PAB containing 25 µg CAM/ml at 35 C for the indicated time of incubation. Viable cells (●), percent R factor DNA (■), and ratios (◑) of copies per P. mirabilis genome equivalent of the 58% GC to the 50% GC components were determined as described in Fig. 3.


LOG VIABLE COUNT/ML
5
6
7
8
9
TIME IN HOURS
0
24
48
72
10
20
30
40
% R FACTOR DNA
2
4
6
8
58 GC:50 GC RATIO

turbidimetric measurements of growth in CAM followed the viability curve shown in Fig. 4, and 25-30% of the population between 9 and 36 hr of incubation failed to grow on enumeration medium containing 25 µg CAM/ml.

The observed doubling in the maximal number of copies of each R-component per genome equivalent in R^+ P. mirabilis when grown in the presence of low CAM concentrations suggested that CAM may act by interfering with the production of an inhibitor involved in the regulation of replication, thus allowing increased R factor replication. The increased 58% GC:50% GC ratio in CAM-grown cells during both the exponential and stationary phases of growth indicated that, under these conditions, regulation of 58% GC replication was more relaxed.

In a separate experiment, R^+ P. mirabilis cells were grown in PAB containing a higher CAM concentration (100 µg/ml). Under these conditions few 50% GC copies could be detected after 24 hr of incubation even though R factor DNA comprised 25-30% of the total extracted DNA. After 36 hr of incubation R factor DNA comprised 40-45% of the total extracted DNA; the 50% GC copies had reached a level of 10, whereas 58% GC copies had increased to 150 per genome equivalent. Hence, 58% replication was again observed to be more relaxed in regulation. The small quantity of the 50% GC component after 24 hr incubation indicated that protein synthesis may be required for the replication of this replicon.

Relationship Between the Physiological State of R^+ P. mirabilis and R Factor Replication After Shifting to Medium Containing Chloramphenicol.

To define more clearly the requirements for R factor replication, R^+ cells grown in PAB in the presence or absence of CAM for 12 and 24 hr were shifted to media containing high CAM concentrations for one generation. The differential effects on R factor replication observed when cells grown in low CAM concentrations were shifted to PAB containing high CAM concentrations are summarized in Table 5. After one generation in 30 µg CAM/ml, the percentage of R factor DNA increased from 17 to 25 in exponential phase cells, which represented primarily a doubling in the number of 58% GC copies per genome. The increased number of 58% GC replicons in conjunction with the constancy in 50% GC copies was reflected in the 58% GC:50% GC ratio which increased from 4.8 to 8.6. Under similar conditions stationary phase cells decreased in total R factor DNA content from 31% to 24% after one generation in 100 µg CAM/ml, due entirely to a reduction in 50% GC copies/genome from 13 to 7. In both instances, the increased ratio of 58% GC:50% GC copies indicated more relaxed 58% GC replication. Thus, it appeared that low CAM concentrations reduced the synthesis of some inhibitor but still allowed R factor replication. In high CAM concentrations, where the rate of protein synthesis was abruptly reduced during the first generation, 50% GC replication was almost completely inhibited while 58% GC

Table 5

R factor replication in R^+ P. mirabilis after shifting cells from low to high concentrations of chloramphenicol[1].

	Before Shift	One Generation Postshift
EXPONENTIAL PHASE CELLS[2]		
Percent R-DNA[4]	17	25
50% GC Copies[5]	6	7
58% GC Copies[5]	29	60
Ratio 58% GC:50% GC	4.8	8.6
STATIONARY PHASE CELLS[3]		
Percent R-DNA	31	24
50% GC Copies	13	7
58% GC Copies	59	57
Ratio 58% GC:50% GC	4.5	8.2

[1] Preshift cells were grown in PAB plus 25 µg CAM/ml at 35 C. Postshift cells were incubated for one generation.
[2] Postshift CAM concentration was 30 µg/ml.
[3] Postshift CAM concentration was 100 µg/ml
[4,5] See Table 3.

replication was unaffected. This suggested that protein synthesis was required for 50% GC replication.

If R factor replication is regulated in a negative manner, the inhibitor must attain effective concentrations during the early stationary phase in cells grown in drug-free medium, where maximal R factor DNA as well as 58% GC copies were observed. Thus, one would predict that exponential phase, PAB-grown cells would not contain effective levels of inhibitor and that increased R factor replication would occur upon a similar shift to high CAM concentrations unless positive control (presumably initiator synthesis) was required. On the other hand, in identically cultured stationary-phase cells in which effective inhibitor and maximal R factor DNA levels had been obtained, both R factor replicons would be inhibited upon shifting to CAM. The results of such an experiment are shown in Table 6. In the exponential phase cells, R factor DNA content doubled as a result of rapid 58% GC replication; 50% GC replication did not increase. No increased R factor replication occurred after the shift of stationary-phase cells grown in PAB to CAM, and the 58% GC:50% GC ratio remained constant as compared to the rise in ratio to 9.6 observed for the exponential-phase cells. Thus, initiator synthesis appears to be necessary for 50% GC but not 58% GC replication. These results concur with the hypothesis that stationary-phase cells contain inhibitor at levels sufficient to prevent rapid replication of the 58% GC component, which implies that replication of each R factor component, at best, parallels

Table 6

Effect of physiological state of R^+ P. mirabilis on R factor replication after shifting cells to medium containing chloramphenicol[1].

	Before Shift	One Generation Postshift
EXPONENTIAL PHASE CELLS[2]		
Percent R-DNA[4]	16	30
50% GC Copies[5]	7	8
58% GC Copies[5]	19	77
Ratio 58% GC:50% GC	2.7	9.6
STATIONARY PHASE CELLS[3]		
Percent R-DNA	22	20
50% GC Copies	9	8
58% GC Copies	36	29
Ratio 58% GC:50% GC	4.0	3.6

[1] Preshift cells were grown in PAB at 35 C. Postshift cells were incubated for one generation.
[2] Postshift CAM concentration was 25 µg/ml.
[3,4,5] See Table 5.

genome replication during the first generation under these conditions.

Effect of Physiological State of R^+ P. mirabilis on R factor Replication After Shifting to Minimal Medium.

Previous results indicated that 50% GC replication requires positive control and that replication of both R factor replicons is regulated in a negative manner. However, the possibility that these observed effects were due only to some type of inductive phenomenon specific for CAM and not to reduction of protein synthesis had to be eliminated. For this purpose, shifts to minimal medium, where the rate of protein synthesis abruptly decreased to the lower rate characteristic for the slower growth rate in minimal medium, were conducted. The results obtained after shifts of exponential- and stationary-phase cells grown in the presence or absence of CAM to minimal medium for one generation are compared to data for preshift cells in Table 7. The observed changes in R factor DNA content after shifts to minimal medium are practically identical to those which occurred in shifts to CAM. Exponential-phase cells cultured in PAB or PAB plus CAM both exhibited increased R factor DNA due entirely to increased 58% GC replication after one generation in minimal medium. Although cells from both growth media contained about equal amounts of R factor DNA before the shifts, a greater increase in percentage of R factor DNA was noted in the CAM-grown cells after one generation in minimal medium. The differential effect on R factor replication observed after

Table 7

Effect of physiological state of R^+ P. mirabilis grown in the presence and absence of chloramphenicol on R factor replication after shifting cells to minimal medium.

PHYSIOLOGICAL STATE OF CELLS	% R-DNA[1]	COPIES PER GENOME[2] 50% GC	58% GC	RATIO 58% GC:50% GC
		CELLS GROWN IN PAB		
Exponential				
Preshift cells[3]	16	7	19	2.7
One generation postshift	22	6	52	8.7
Stationary				
Preshift cells	22	9	36	4.0
One generation postshift	23	8	42	5.3
		CELLS GROWN IN PAB + CAM		
Exponential				
Preshift cells	17	6	29	4.8
One generation postshift	27	7	69	9.9
Stationary				
Preshift cells	31	13	59	4.5
One generation postshift	28	7	71	10.1

[1,2] See Table 3.
[3] Values at time of shift.

shifts to minimal medium of cells grown in CAM or of exponential-phase PAB-grown cells, which presumably have less than an effective inhibitor concentration, was also reflected by the increased 58% GC:50% GC ratios. No increase in the replication of either R factor component was observed after shifts of stationary-phase, PAB-grown cells. In contrast, when cells of similar physiological age grown in CAM were shifted to minimal medium, 50% GC replication appeared to be virtually inhibited but 58% GC copies/genome increased from 59 to 71. Thus, although total R factor DNA content decreased only slightly from 31 to 28% in these cells, the 58% GC:50% GC ratio increased from 4.5 to 10.1. These data support the hypothesis that 50% GC but not 58% GC replication requires protein synthesis. Furthermore, these results indicate that the level of inhibitor which affects the replication of both R factor components is higher in stationary-phase cells than in exponential-phase cells, and is also higher in more rapidly growing cells of the same chronological age, i.e. grown in PAB in contrast to PAB plus CAM.

R Factor Replication in R^+ *P. mirabilis* After Inhibition of Protein Synthesis With Puromycin.

The change in R factor DNA content after R^+ cells were shifted to bacteriostatic concentrations of puromycin (PUR) is shown in Table 8. As noted in shifts to minimal medium and 100 µg CAM/ml, increased 58% GC replication occurred in exponentially growing cells from both culture media after 3 hr in puromycin. In each case,

Table 8

Effect of physiological state and growth in chloramphenicol of R^+ P. mirabilis on R factor replication after shifting cells to bacteriostatic concentrations of puromycin.

PHYSIOLOGICAL STATE OF CELLS	% R-DNA[1]	COPIES PER GENOME[2] 50% GC	58% GC	RATIO 58% GC:50% GC
	CELLS GROWN IN PAB			
Exponential				
Preshift cells[3]	16	7	19	2.7
Shifted to PUR[4]	27	7	67	9.6
Stationary				
Preshift cells	22	9	36	4.0
Shifted to PUR	23	9	38	4.2
	CELLS GROWN IN PAB + CAM			
Exponential				
Preshift cells	17	6	29	4.8
Shifted to PUR	25	6	60	10.0
Stationary				
Preshift cells	31	13	59	4.5
Shifted to PUR	49	19	172	9.1

[1,2,3] See Table 7.
[4] Final PUR concentration was 5 µg/ml. Postshift cells were incubated for 3 hr at 35 C.

total R factor DNA and the ratio of 58% GC:50% GC copies increased while 50% GC replication remained constant. No detectable change in R factor DNA occurred in stationary-phase, PAB-grown cells as evidenced by the constancy in all parameters examined. In stationary-phase, CAM-grown cells R factor DNA increased to 49% of the total extracted DNA which corresponded to 19 copies of the 50% GC and 172 copies of the 58% GC replicons per Proteus genome. The doubling of the 58% GC:50% GC ratio under these physiological conditions again indicated more relaxed 58% GC replication. The slight increase in the number of 50% GC copies observed after shifting stationary-phase cells grown in PAB plus CAM to puromycin might reflect the presence of preformed initiator or preferential lysis of cells containing large numbers of 58% GC copies.

Thus, the similarity of effects observed after shifts to CAM, minimal medium, or puromycin suggests that the means by which protein synthesis is inhibited is irrelevant to the subsequent effects on R factor replication as long as the rate of protein synthesis is abruptly reduced.

The Effect of Shifts to Medium Containing Streptomycin or Tetracycline on R Factor Replication in R^+ P. mirabilis.

Increased R factor DNA concomitant with augmented 58% GC replication has been reported previously by Rownd and others (37,60-63,65) to occur in R^+ P. mirabilis cultures when grown in

the presence of any of the antibiotics, except tetracycline (TET), to which the R factor confers resistance. The data presented in the previous sections indicate that this effect is a result of decreased inhibitor synthesis and the protein synthesis requirement for 50% GC replication. Although the results obtained in the minimal medium and PUR shifts are incongruent with a theory of selective induction of R factor replication by the antibiotics to which the R factor confers resistance, shifts to STR and to TET were conducted (Table 9). After shifts of stationary phase, PAB-grown cells, in which maximal R-DNA and effective inhibitor levels should have been attained, to 100 µg of either STR or TET/ml, R factor replication remained constant or decreased slightly. In contrast, cells cultured in PAB plus CAM before the shift exhibited increased 58% GC but not 50% GC replication after 3 hr in TET. When stationary-phase cells grown in PAB plus CAM were shifted to STR for 3 hr, 50% GC replication appeared to be completely inhibited while the continued 58% GC replication resulted in an increase in the 58% GC:50% GC ratio to 9.8. These data are consistent with the predictions of the regulatory systems suggested from the previous shift experiments.

Selective Replication of the 58% GC R Factor Component During Incubation of R^+ P. mirabilis at 5 C.

Additional evidence that 50% GC but not 58% GC replication requires protein synthesis (i.e. initiator), and that effective inhibitor levels are attained in PAB-cultured but not in PAB

Table 9

R factor replication in R^+ *P. mirabilis* after shifting cells to medium containing streptomycin or tetracycline.

SHIFT ADDITIVE	% R-DNA[1]	COPIES PER GENOME[2] 50% GC	58% GC	RATIO 58% GC:50% GC
		CELLS GROWN IN PAB		
Preshift cells[3]	22	9	36	4.0
Shifted to TET[4]	20	9	28	3.1
Shifted to STR[4]	14	6	18	3.0
		CELLS GROWN IN PAB + CAM		
Preshift cells	31	13	59	4.5
Shifted to TET	30	9	76	8.4
Shifted to STR	24	6	59	9.8

[1,2,3] See Table 7.

[4] Cultures grown for 24 hr were shifted to medium containing STR or TET at a final concentration of 100 µg/ml and incubated for 3 hr at 35 C.

plus CAM-grown cells after 24 hr was obtained in shifts to 5 C (Fig. 5). The results obtained when cells grown in drug-free PAB for 24 hr were shifted to 5 C for 2,10, or 15 days are shown in Fig. 5A. In these cells, R factor DNA increased gradually from 22% to 29% during 15 days at 5 C. The increase in R factor DNA content was a result of 58% GC replication exclusively; 58% GC copies/genome increased from 36 to 75. The viable population of R^+ cells did not change during the 15 day incubation period. A more rapid increase in 58% GC replication was observed after shifts of CAM-grown cells (Fig. 5B). In these cells R factor DNA increased from 31 to 43% of the total extracted DNA after 10 days at 5 C. This represented 14 copies of the 50% GC and 139 copies of the 58% GC components per host genome equivalent or a 58% GC: 50% GC ratio of 9.9. Replication of the 50% GC component was not observed. After 15 days at 5 C, the viable count of these R^+ cells had decreased by approximately 90%, and the remaining cells contained less total R factor DNA with an increased 58% GC:50% GC ratio.

Differential R Factor Replication After Shifting R^+ P. mirabilis in Various Physiological States to Medium Containing Nalidixic Acid or Phenethyl Alcohol.

When exponential-phase cells grown in PAB with or without CAM were shifted to PAB containing inhibitory concentrations of either NAL or PEA, R factor DNA increased (Table 10). The increase was due primarily to 58% GC replication which was reflected in the increased 58% GC:50% GC ratios. In stationary-

Figure 5. Differential replication of the R factor components in R^+ P. mirabilis at 5 C. Cultures grown for 24 hr at 35 C in PAB (A) or PAB plus 25 µg CAM/ml (B) were shifted to 5 C. DNA isolated from cells after holding for 2, 10, and 15 days at 5 C is compared to 24 hr cultures (0 days at 5 C). Percent R factor DNA (●), viable cells (O), and copies per P. mirabilis genome equivalent of the 50% GC (□) and 58% GC (■) components were determined as described in Fig. 3.

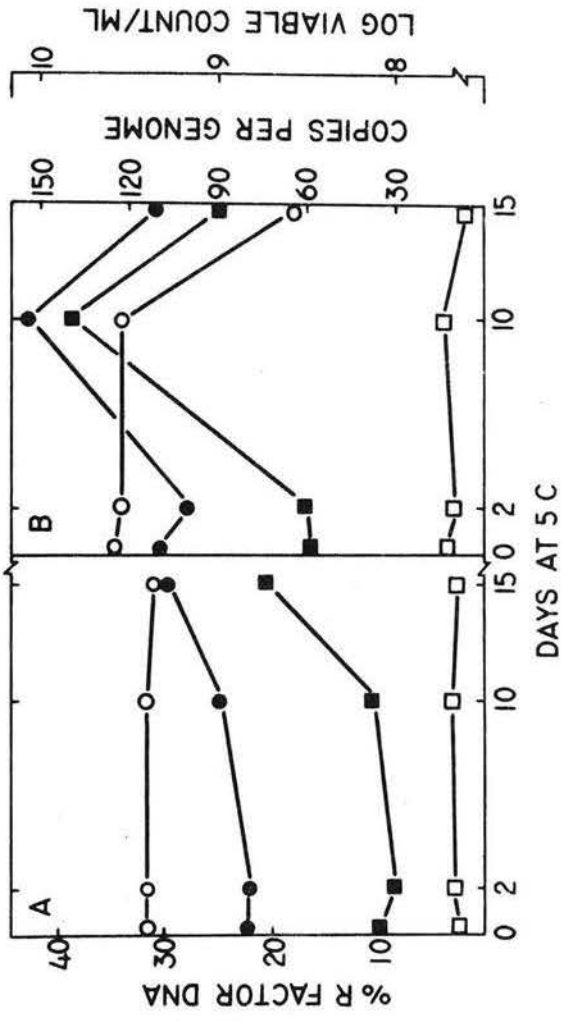

% R FACTOR DNA
40
30
20
10
A
0 2
10
15 0 2
10
15
B
DAYS AT 5 C
COPIES PER GENOME
150
120
90
60
30
LOG VIABLE COUNT/ML
10
9
8

Table 10

Differential replication of the R factor components in R^+ P. mirabilis after shifting cells to medium containing phenethyl alcohol or nalidixic acid.

CULTURAL CONDITIONS	% R-DNA[1]	COPIES PER GENOME[2] 50% GC	58% GC	RATIO 58% GC:50% GC
	EXPONENTIAL PHASE CELLS			
PAB				
Preshift cells[3]	16	7	19	2.7
Shifted to PEA[4]	32	10	81	8.1
Shifted to NAL[5]	23	7	50	7.1
PAB + CAM				
Preshift cells	17	6	29	4.8
Shifted to PEA	25	6	63	10.5
Shifted to NAL	24	6	57	9.5
	STATIONARY PHASE CELLS			
PAB				
Preshift cells	22	9	36	4.0
Shifted to PEA	24	9	42	4.7
Shifted to NAL	19	7	32	4.6
PAB + CAM				
Preshift cells	31	13	59	4.5
Shifted to PEA	39	14	111	7.9
Shifted to NAL	25	7	59	8.4

[1,2,3] See Table 7.

[4] Final PEA concentration was 0.25 %. Postshift cells were incubated for 3 hr at 35 C.

[5] Final NAL concentration was 500 µg/ml. Postshift cells were incubated for 3 hr at 35 C.

phase PAB-grown cells, replication of neither R-component was observed to increase after 3 hr in NAL or PEA, as would be expected if both replicons were naturally repressed. After shifting to PEA, CAM-grown stationary-phase cells increased in R factor DNA content, as a result of an increase in the number of 58% GC copies from 59 to 111. When similarly cultured cells were shifted to NAL, 50% GC copies were reduced in half, whereas 58% GC copies remained constant, resulting in decreased total R factor DNA and an increased ratio of 58% GC:50% GC copies. As observed in previous shifts, the uncontrolled replication of the 58% GC R-component was lethal to greater than 90% of the PEA-inhibited cells.

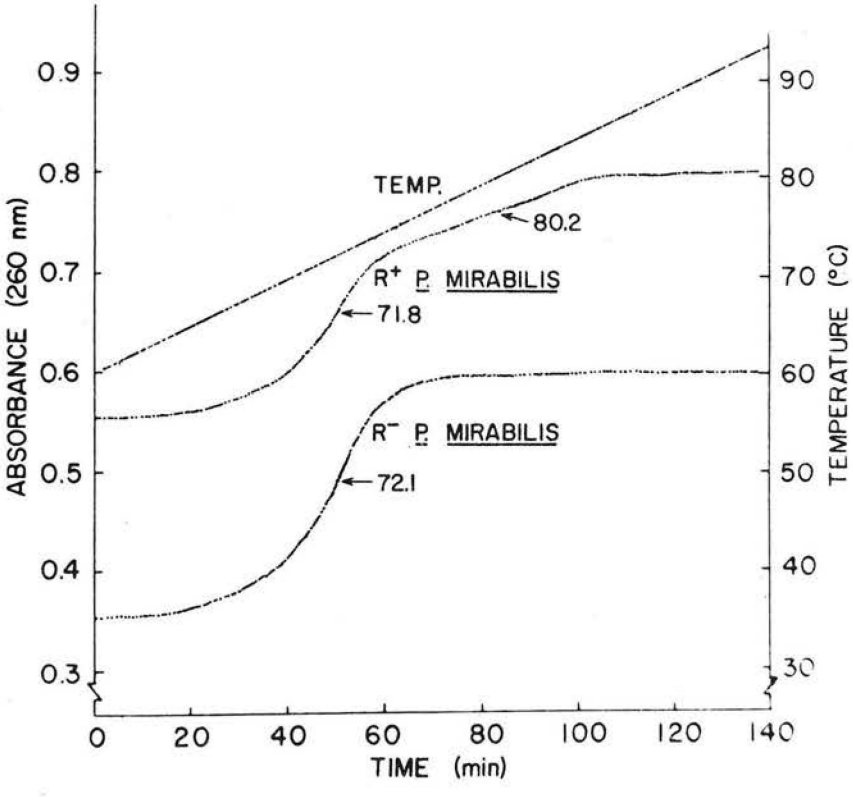
Since NAL and PEA would not be expected to affect protein synthesis under these conditions, absence of 50% GC replication is apparently due to some other affect. Since PEA has been shown to interfere with chromosomal DNA attachment and initiation in E. coli, the above data might reflect the necessity of a membrane maintenance or replication site for the 50% GC replicons.

Thermal Denaturation of R Factor 222 DNA.

Falkow (19) and Rownd (64), in 1966, independently reported that R factors are composed of typical double-stranded DNA on the basis of the observed shift in buoyant density in CsCl after denaturation. To date, however, the chemical nature of R factor DNA has not been investigated systematically. The studies presented in this and the remaining sections represent an attempt to define more clearly the physical and chemical properties of R factor DNA.

The typical hyperchromic shifts observed when DNA from R^- and R^+ P. mirabilis was thermally denatured are illustrated in Fig. 6. DNA from R^- P. mirabilis showed a single sigmoid absorbance increase of 38%, the midpoint of which corresponded to 72.1 C (39% GC). When DNA from R^+ P. mirabilis (which contained 39% total R factor DNA of which 62% was comprised of the 58% GC R factor molecular species) was similarly treated a more complex thermal transition curve was observed. The P. mirabilis chromosomal DNA in this preparation denatured at a temperature corresponding to 71.8 C (39% GC) and showed an apparent hyperchromic shift of 40%. The temperature at the midpoint of the hyperchromic transition representing the R factor DNA appeared to be 80.2 C which corresponded to an average base composition of 56% GC. However, the increased absorbance due to the R factor DNA was only about 20%. Similar low and skewed hyperchromic shifts for R factor DNA were observed when other DNA preparations from R^+ P. mirabilis were examined. These results indicated that the R factor DNA in these preparations was either partially single-stranded or of atypical composition. However, no DNA bands corresponding to single-stranded R factor DNA were observed in these DNA samples after CsCl isopycnic centrifugation and the marked heterogeneity of the thermal transition curve did not correspond to the homogeneous appearance of the R factor DNA in CsCl gradients.

Figure 6. Thermal denaturation profiles of R^+ and R^- P. mirabilis DNA. The linear increase in temperature (TEMP.) is shown in the top line. The procedures employed in this study are described in Materials and Methods.


ABSORBANCE (260 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
TEMP.
80.2
R+ P. MIRABILIS
71.8
R− P. MIRABILIS
72.1
90
80
70
60
50
40
30
TEMPERATURE (°C)
0
20
40
60
80
100
120
140
TIME (min)

Physicochemical Studies on R Factor 222 DNA

DNA preparations from R^- and R^+ P. mirabilis were exposed to various denaturing conditions prior to CsCl density gradient centrifugation to ascertain the effects of these conditions on the physical state of R factor DNA. In one series of experiments 2.0 ml of a DNA solution (120 µg/ml) in 0.1 X SSC was cooled to 0 C in an ice-H_2O-acetone bath, the pH was adjusted to 3.5, 10.5, or 12,5 with 1N HCl or 1N NaOH and then held at 0 C for 15-30 min. Samples were neutralized, rapidly mixed with neutral CsCl, and centrifuged at 44,000 rev/min as previously described. For thermally denatured samples, DNA solutions in 0.1 X SSC were heated at 100 C for 20-30 min, mixed quickly with reference DNA and neutral CsCl, and centrifuged. In alkaline CsCl isopycnic centrifugation studies, DNA samples were adjusted to pH 12.5 before addition to alkaline CsCl (13g CsCl/7.0 ml 0.04 M K_2HPO_4, pH 12.5) (77).

The results obtained from neutral CsCl gradient analysis of DNA from R^- and R^+ P. mirabilis after complete denaturation by heat or alkaline treatments and from alkaline CsCl gradient analysis are compared to the densities of native, double-stranded Proteus and R factor DNA in Table 11. After denaturation, P. mirabilis DNA showed a typical increase in buoyant density in neutral CsCl of 0.016 g/cm^3. The observed shifts in density after denaturation of both R factor components were less than expected; the 50% GC component increased by only 0.010 g/cm^3 in neutral CsCl. Although the change in

Table 11

Shifts in buoyant density of P. mirabilis and R factor DNA after denaturation.

	Neutral CsCl			Alkaline CsCl	
DNA Species	Native	Denatured	Δρ	Denatured	Δρ
P. mirabilis	1.698[1]	1.714	.016	1.760	.062
R factor 222					
50% GC	1.710	1.722	.012	1.772	.062
58% GC	1.718	1.728	.010	1.779	.061

[1] Density in g/cm^3.

buoyant density associated with denaturation in neutral CsCl has been shown to decrease slightly with increasing GC content, the density shifts observed for both R-components were considerably lower than the minimum expected and again indicated an atypical chemical composition. In alkaline CsCl, where the guanine and thymine residues are fully titrated, P. mirabilis DNA and both R factor DNA components were observed to undergo the typical 0.061-0.062 g/cm^3 increase in density when compared to the respective densities of the native DNA species (Table 11). Thus, one might predict that any abnormality in the R factor DNA would not be directly associated with guanine or thymine.

To determine if the decreased density shifts observed in neutral CsCl for both R factor components after denaturation were associated with a modification that is sensitive to mild acid or alkaline treatment, DNA samples from R^+ P. mirabilis were treated at pH 3.5 or 10.5 prior to centrifugation. However, no change in density of the R factor DNA bands was observed in neutral CsCl gradients.

Chemical Analysis of R Factor 222 DNA

The abnormal buoyant density shifts in CsCl, and the relatively low and heterogeneous hyperchromic shift observed upon denaturation of R factor 222 DNA strongly indicated an unusual composition. The base composition of DNA from R^- and R^+ P. mirabilis was determined by paper chromatographic analysis of formic and perchloric acid hydrolysates of DNA samples.

The results obtained are illustrated diagramatically in Fig. 7. The relative positions of the nucleic acid base standards in each of the solvent systems employed are given at the top and bottom of the diagram. Although not shown, R^- P. mirabilis DNA contained only the four typical DNA bases. The hydrolysates of DNA from P. mirabilis (222) contained an additional UV-absorbing spot designated "X". As shown in the top section of Fig. 7, "X" had a slightly higher R_f value than 5-methyl cytosine and migrated parallel to 5-hydroxymethyl uracil in the HCl-isopropanol solvent. Although the observed quantity of "X" was variable, a comparison of samples hydrolyzed for various time intervals indicated that spot "X" was not partially hydrolyzed material. When the cytosine spot from these chromatograms was eluted, concentrated, and re-chromatographed in the same solvent system, three spots were observed [Pm-5 (222) cytosine]. Most of the material rechromatographed to its original position, but spots corresponding in R_f to the 5-methyl cytosine standard and "X" in the first chromatogram were observed. When the material in spot "X" was similarly rechromatographed in HCl:isopropanol, the majority of UV-absorbing material corresponded in R_f values to "X" and the uracil standard. A small spot was also detected near the solvent front. In the formic acid:ethyl acetate solvent system, the eluted cytosine yielded spots with R_f values corresponding to cytosine and 5-hydroxymethyl uracil, plus unidentified material near the solvent front. When material "X" was chromatographed in this solvent system, the majority of material moved to a position

Figure 7. Chromatographic properties of unknown base in hydrolyzed DNA from R^+ P. mirabilis. Spots obtained after chromatography of base standards, P. mirabilis 222 DNA hydrolysates, or the eluted cytosine or unknown base from the R^+ P. mirabilis DNA hydrolysate are shown in both solvent systems employed. Adenine, A; guanine, G; cytosine, C; thymine, T; uracil, U; unknown base, X; 5-methylcytosine, MC; 5-hydroxymethyl cytosine, HMC; 5-hydroxymethyl uracil, HMU.

ISOPROPANOL-HCL
BASE STANDARDS
G
A
C
HMC
MC
HMU
U
T
P. MIRABILIS (222)
G
A
C
X
T
PM-5(222)CYTOSINE
C
MC
X
UNKNOWN BASE
X
FORMIC ACID-ETAC
PM-5(222) CYTOSINE
C
X
UNKNOWN BASE
X
BASE STANDARDS
G
HMC
C
A;MC
HMU
U
T

parallel to the 5-hydroxymethyl uracil standard, although a small spot was observed near the solvent front. From these results it would appear that the hydrolysate of R^+ P. mirabilis DNA contained a compound which chromatographed with cytosine in the HCl: isopropanol solvent and which could give rise to 5-hydroxymethyl uracil, uracil, and 5-methyl cytosine. A compound which has been found in bacteriophage DNA and which possesses these characteristics is 5-hydroxymethyl cytosine.

Although the DNA employed in this study was isolated from the same batch of R^+ P. mirabilis cells, some hydrolysates did not give rise to the spot designated "X". Attempts to define the optimal hydrolysis conditions for the recovery of material "X" were unsuccessful. Chromatograms of hydrolyzed R^+ P. mirabilis DNA, that contained only the 4 typical DNA base spots, were used to calculate the base composition of these DNA samples. Table 12 shows the chemically determined base composition of P. mirabilis and R factor 222 DNA. DNA from R^- P. mirabilis was composed of 40.3% GC, as compared to calculated values of approximately 39% GC from thermal denaturation and buoyant density studies. R^+ P. mirabilis DNA contained an average base composition of 46.7% GC. On the basis of the amount of each R factor component in the DNA prior to hydrolysis, (given in Table 12), the predicted base composition of the R factor DNA was calculated to be 55.6% GC. For these calculations, values for the quantity of 50 and 58% GC R factor components was determined by CsCl gradient analysis. The observed base composition of the R factor DNA represents the excess proportionate quantities of each base in

Table 12

Base composition of P. mirabilis and R factor 222 DNA.

DNA	Mole Percent				Pur/Pyr	% GC
	A	G	C	T		
R^- P. mirabilis	29.5	20.2	20.1	30.2	0.99	40.3
R^+ P. mirabilis[1]	26.8	23.4	23.3[3]	26.5	1.01	46.7
R factor 222 calculated[2]	22.2	27.8	27.8	22.2	1.00	55.6
observed	22.7	28.2	28.1[3]	21.0	1.04	56.3

[1] Composed of 60% P. mirabilis DNA and 40% R factor DNA. The 58% GC and 50% GC R factor components comprised 28% and 12%, respectively, of the total DNA.

[2] Based on R factor DNA composition of 30% of 50% GC and 70% of 58% GC components.

[3] Total material eluted and quantitated as cytosine.

R^+ P. mirabilis DNA, normalized to 100 moles. The extinction coefficient of cytosine was used to quantitate the material in the spot corresponding in R_f to the cytosine standard. The observed value for the average base composition of the R factor DNA is in close agreement with the expected or calculated value. The spectral properties at acid and alkaline pH of all eluted spots corresponding in R_f to the typical DNA bases, with the exception of the cytosine spot from R^+ P. mirabilis DNA hydrolysates, were similar to published values. The material which corresponded in R_f to cytosine in hydrolysates of R^+ P. mirabilis DNA yielded spectral ratios significantly different from those observed for the cytosine standard.

The spectral characteristics of compound "X" are compared with those of nucleic acid bases which have similar spectral properties in Table 13. In agreement with the chromatographic properties of "X", its spectral characteristics resemble most closely those of 5-hydroxymethyl uracil and 5-hydroxymethyl deoxyuridine. Although the material in spot "X" and in the cytosine spot from R^+ P. mirabilis should have been hydrolyzed to free bases, both of these spots gave positive reactions when chromatograms were developed to detect carbohydrates.

To date, no satisfactory quantitation of the unknown base has been made. Preliminary attempts, however, indicate that the unusual base, tentatively identified as 5-hydroxymethyl cytosine, may replace as much as 35-40% of the cytosine in R factor DNA. In

Table 13

Spectral characteristics of the unknown base and related compounds.

COMPOUND	ACID[1] λ_{max} (nm)	ACID[1] λ_{min} (nm)	ACID[1] 250/260	ACID[1] 280/260	ALKALI[2] λ_{max} (nm)	ALKALI[2] λ_{min} (nm)	ALKALI[2] 250/260	ALKALI[2] 280/260
Unknown Base X	261	231	0.75	0.30	261	240	0.92	0.42
5-hydroxymethyl uracil	261	231	0.76	0.35	285	245	0.66	1.75
5-hydroxymethyl cytosine	279	241	1.97	0.45	283	254	0.83	2.75
5-hydroxymethyl deoxyuridine	264	233	0.68	0.52	264	243	0.78	0.44

[1] pH 2.0.
[2] pH 13.0.

the HCl:isopropanol solvent system 5-hydroxymethyl cytosine chromatographs with cytosine and has been shown to break down under these conditions to 5-hydroxymethyl uracil, 5-methyl cytosine, and uracil.

DISCUSSION

R factor 222 has been reported previously to segregate either the TET resistance determinant or the determinants for resistance to CAM,STR, and SUL (19,55,79). Results reported herein indicate that a third type of segregant can occur in P. mirabilis. This segregant has lost resistance to CAM and SUL, retained resistance to STR and TET, and gained resistance to KAN and NEO.

During transfer of R factor 222 from E. coli to P. mirabilis, recipients harboring the aberrant R factor segregant were observed about one-fourth as frequently as those carrying R factor 222 and greater than 10 times more frequently than other R factor 222 segregant types. The high frequency of isolation of recipient cells harboring the aberrant R factor from plates containing only COL and STR argues against the possibility that chromosomal mutants were selected. Since one would not expect to find a large number of the recipient cells that had stably integrated the same six resistance genes, it would appear that the resistance determinants of the aberrant segregant are in the autonomous state. However, this R factor segregant was not transferable and could not be visualized in CsCl gradient studies.

Although isolation of such an R factor segregant would very likely have been precluded by the conditions for segregant isolation employed in previous studies (19,55,79), the possibility remains that the aberrant R factor segregant was a product of the conditions employed in this study.

As reported previously (18,19,61,64), during CsCl isopycnic centrifugation of DNA from R^+ P. mirabilis the 222 R factor appeared as 2 satellite DNA bands at densities corresponding to 50 and 58% GC(Fig. 1). Although the amount and proportion of each R factor DNA species varied with the physiological age of the host cell and under different growth conditions, the densities of the R factor DNA species remained constant (Fig. 2).

The results of previous investigations indicated that R factor 222 spontaneously dissociates in P. mirabilis into RTF (50% GC) and R-genes (58% GC) molecules which exist as multiple copies per cell, whereas this R factor was found to exist in E. coli as a single molecular DNA species and to comprise only 1-2 copies per cell (9,10,20,55). To determine the mechanisms responsible for the paradoxical replicative behavior of R factor 222 in different hosts, the effects of various growth conditions on R factor replication in P. mirabilis were examined.

The results of these studies on the differential effects of various inhibitors and cultural conditions on the replication of the R factor components have led to the following working hypothesis of the regulation of R factor 222 replication in P. mirabilis. In this host R factor 222 exists as three independently replicating molecular species: the composite R factor; the RTF or transfer portion; and the R-genes or drug-resistance determinants. Replication of these three replicons is regulated differentially by both positive and negative effectors. An inhibitor, coded for by the RTF replicon, acts in a negative manner to inhibit the replication of both the

RTF and the R-genes replicons. In addition, the RTF molecules are under the positive control of a self-coded initiator. A maintenance site on the host cell membrane is essential for RTF replication whereas replication of the R-genes replicon is strictly cytoplasmic. According to this hypothesis, the composite R factor molecule would be under the same regulatory control and would synthesize the same regulatory products as the RTF replicon. Segregation or dissociation of the composite R factor molecule into two independently replicating molecules and their subsequent replication is most simply explained by the rolling circle model of DNA replication formulated by Gilbert and Dressler (26) and more recently adapted as a mechanism for plasmid replication by Novick (56). According to this proposed replication model, synthesis of the R factor molecule proceeds from the RTF or 50% GC portion in one of 2 ways: (1) complete replication to yield another composite molecule as occurs principally in E. coli (10,20,55); or (2) scission at the RTF terminus to produce 50% GC (RTF) and 58% GC (R-genes) replicons, both of which are capable of either further replication or supercoil formation (10,11,54,69). Thus, both the composite molecule and RTF require initiator synthesis and membrane attachment for replication, and are negatively regulated by an inhibitor. In contrast, the R-genes replicon is subject only to negative control of replication, does not require a membrane maintenance site, and would be predicted to undergo a malignant

or uncontrolled type of replication in the absence of the regulatory inhibitor of replication. To avoid confusion with the previously described R factor-coded transcriptional repressor for pili synthesis (48,49), the negative regulatory product is referred to as an inhibitor rather than a repressor.

As described in the literature review, Rownd and associates (37,60-63,65) have presented a different interpretation of the events observed during the replication of R factor 222 in P. mirabilis. These workers have proposed that R factor 222 replication in P. mirabilis is controlled only in a positive manner by initiator synthesis, and that the observed variation in the amount of each R factor satellite DNA species is entirely a result of some undefined mechanism of association-disassociation between the RTF and R-genes. According to this proposal, R factor 222 normally dissociates into 2 molecular species; the R-genes replicon is under stringent control and exists as 1 copy/cell, whereas RTF replication is relaxed and allows for multiple copies of this replicon/cell. When R^+ cells are grown in the presence of any of the antibiotics to which the R factor confers resistance, except TET, the R-genes molecule is induced to associate with an RTF replicon, the product of which replicates under the relaxed RTF regulatory system in such a way as to give a large molecule consisting of one RTF and many R-genes molecules. These large molecules gradually increase in density from 1.712 to 1.718 g/cm^3. In a more recent account of this model, Rownd and Mickel (63) have argued that the TET resistance determinant is

linked convalently to the RTF unit since TET was not effective in inducing the reassociation of the R-genes with the RTF.

The experimental data which corroborate the interpretations summarized in the working hypothesis resulting from this study are presented and contrasted with the predictions of the model of Rownd et al. (37,60-63,65) in the following paragraphs.

Several lines of evidence support the concept of negative control for R factor replication. A comparison of the amount of total R factor DNA and the number of copies of each R factor component at the time when maximal amounts of R factor DNA were observed in R^+ P. mirabilis grown in drug-free PAB (Table 3) to values observed in cells grown in PAB containing 25 µg CAM/ml (Table 4) strongly suggests that a specific cytoplasmic inhibitor is involved in the regulation of R factor replication. Total R factor DNA increased by 64% in the CAM-grown cells and both R-components increased significantly in copies per genome. This indicated that the inhibitor must act on both the 50 and 58% GC replicons. The inhibitor is most easily visualized as being analogous to the repressor in the model for transcriptional control proposed by Jacob and Monod (36) except that this cytoplasmic inhibitor acts negatively to inhibit DNA replication. During growth in CAM (Fig. 4), where the host generation time was 50 min, maximal R factor DNA was not only increased but also was observed later in the stationary phase than in PAB-cultured cells (Fig. 3). These data were interpreted to mean that in drug-free PAB, where the cells are dividing with a generation time of 45 min, the

inhibitor attains significant levels between 12 and 24 hr or incubation and normally represses R factor replication during this interval. However, in the presence of CAM inhibitor synthesis is reduced during the exponential phase and the copies/genome of both R factor replicons are increased between 12 and 36 hr of incubation. Thus, only after a delay which corresponds to the inactivation of CAM are effective inhibitor concentrations attained. This interpretation is consistent with the observed effects of the physiological state of the R^+ Proteus cells on the replication of the R factor components after shifting to minimal medium (Table 7), or to medium containing high CAM concentrations (Tables 5 and 6), STR (Table 9), TET (Table 9), or PUR (Table 8, where protein synthesis was reduced or inhibited. Increased R factor replication was never observed in any of these shifts involving stationary-phase cells grown in drug-free PAB, where effective levels of inhibitor would be predicted to exist.

When 24 hr cultures grown in PAB or PAB plus CAM were incubated for various intervals at 5 C, where the rate of protein synthesis would be drastically reduced, only 58% GC replication was observed (Fig. 5). In the PAB-grown cells little replication occurred before 10 days at 5 C, whereas the number of 58% GC copies in the CAM-grown cells increased rapidly after 2 days incubation at 5 C. These results concur with the interpretation that stationary-phase, PAB-grown cells contain inhibitor at concentrations which are effective in preventing rapid R factor replication. However, as will be discussed later, only 58% GC replication

occurred at 5 C presumably because this replicon does not require positive control. On the other hand, if R factor replication is regulated only by positive control and the R-genes replicon is regulated stringently under drug-free conditions, as proposed by Rownd and colleagues (37,60-63,65), one would not expect an increase in 58% GC replicons/genome after PAB-grown R^+ cells are shifted to 5 C. Similarly, if protein synthesis does occur minimally after R^+ cells grown in CAM are shifted to 5 C, one would expect both the RTF and the associated RTF-R-genes composite molecules to increase slightly in copies/genome. However, 50% GC replication was not observed in either case, and 58% GC copies/genome increased gradually after shifting PAB-grown cells to 5 C. Thus, the results obtained after shifts to 5 C do not conform to the predictions of a theory based solely on positive control.

The increased 58% GC:50% GC ratio observed in R^+ P. mirabilis grown in CAM (Table 4) reflects the proportionate decrease in 50% GC R factor DNA and indicates that CAM differentially affects the replication of the different R factor replicons. When R^+ cells were cultured in 100 µg CAM/ml, both cell growth rate and 50% GC replication but not 58% GC replication were reduced relative to the values observed for growth in drug-free PAB. After 36 hr incubation the 58% GC replicons comprised 150 copies/genome or 75% of the total R factor DNA. Thus, although low CAM concentrations caused an

increase in both R factor components per genome, high CAM concentrations significantly reduced replication of the 50% GC replicon and promoted an increased 58% GC replication. Combined with the observations from the various shift experiments that protein systhesis is required for RTF replication, it appears that growth in CAM affects R factor replication in 2 ways: reduced synthesis of an initiator necessary for 50% GC replication; and reduced synthesis of an inhibitor that acts on both the 50% GC and 58% GC replicons. The positive regulatory molecule is assumed similar to that described by Jacob et al. (35) and functions to initiate DNA replication. Similar and predictable replicative patterns of the R factor components were observed after shifts to all conditions that reduced or inhibited protein synthesis. These data are inconsistent with the proposal of Rownd et al. (37,60-63,65) that variation in the proportion of the R factor components is due to an inductive type of phenomenon specific for the drugs, except TET, to which the R factor confers resistance. Based on their proposal, it would be difficult to visualize how puromycin, a highly specific inhibitor of protein synthesis (72) and a drug to which the R factor does not confer resistance, can produce effects comparable to those observed with CAM or STR. Similarly, their model does not explain the differential replication of the R factor components observed after shifting to minimal medium or 5 C. Furthermore, the effects of shifts to TET, reported

herein, were comparable to those observed with CAM or STR. Hence, it seems unlikely that the observed effects are related to something other than an alteration of the synthesis of a protein initiator and inhibitor.

As discussed by Jacob and colleagues (35), a maintenance-attachment site on the host membrane is necessary to ensure the segregation of an extrachromosomal element to all daughter cells. Novick (56) has noted that even 60 copies/cell of an element which exists cytoplasmically are not sufficient to avoid hereditary instability. The observed hereditary persistence of both the composite R factor molecule and the RTF replicon is consistent with a membrane maintenance site requirement. When R^+ P. mirabilis were shifted to NAL or PEA (Table 10), both of which affect genome-membrane attachment and initiation of DNA synthesis in E. coli (39), 58% GC replication but not 50% GC replication was increased in all cases except in stationary-phase, PAB-grown cells where the inhibitor effectively repressed rapid 58% GC replication. Since protein synthesis was not affected under these conditions, these data were interpreted to mean that the 50% GC replicons (composite R factor and RTF) require membrane-attachment, and that the 58% GC replicons exist cytoplasmically. Perhaps the viable population (25-30%) which appeared drug-sensitive between 12 and 36 hr of incubation in PAB or PAB plus CAM was the result of unequal segregation of 58% GC replicons to daughter cells. Falkow et al. (21) have

shown recently by membrane-DNA gradient techniques that a large proportion of the composite molecules are membrane-attached during R factor replication in E. coli.

The computations of the number of copies of each R factor component were based on the molecular weights of the R factor 222 components reported by Falkow et al. (20). Accordingly, the R-genes replicon is one-fifth the size of the RTF and one might expect a theoretical 58% GC:50% GC ratio of 5 if both replicons are equally sensitive to the inhibitor and possess identical initiation requirements. Deviations from this predicted ratio might reflect the following: replication of the composite R factor molecule; a DNA extraction procedure more efficient for isolation of one of the R factor DNA species; or a differential regulation of replication of the RTF and R-genes. The composite molecular species has been shown to account for a significant amount of the total R factor DNA only during the early exponential phase of host cell growth (10,20), and similar 58% GC:50% GC ratios and amounts of each R factor component have been observed after extraction of DNA by other methods from comparably grown cells (20). Thus, deviation from the predicted ratio most likely resulted from a differential control of replication. Additionally, the fact that the few composite molecules present would be quantitated as 50% GC (RTF) replicons or the possibility of an inefficient extraction of one of the R factor components would have little, if any, effect on the overall interpretation of these results. If the

molecular weights reported by Nisioka et al. (54) for the 222 R factor components were employed in calculating copies/genome for different replicons, the interpretations of these data would remain unaltered but values for copies/genome of each R-component would be decreased slightly.

In these studies, quantitation of DNA was based on measurement of relative band areas after CsCl centrifugation. When DNA is fragmented during isolation, as occurs with Marmur's procedure (42), this method of quantitation necessitates the assumption that each DNA band is intramolecularly homogeneous with respect to base composition. During these studies, DNA from R^- P. mirabilis always exhibited a unimodal, symmetrical peak of constant bandwidth. An increase in bandwidth, which would reflect heterogeneity in base composition, was not observed for any of the bands of R^+ P. mirabilis DNA examined during these studies. Besides justifying the above assumption, these observations indicated that few of the heterogeneous composite molecules were present.

Rownd and associates (37,60-63,65) have reported that upon transferring R^+ P. mirabilis from drug-free medium, where only the 50% GC component was detectable, to drug-containing medium, intermediate molecular species with densities between 1.712 and 1.718 g/cm^3 were observed. These species eventually shifted to a density of 1.718 g/cm^3 as a result of a transition phenomenon of association-disassociation between the RTF and R-genes. A

reciprocal back transition was observed upon further growth after shifting to drug-free medium. These workers have proposed that, in order to increase the copy number, the stringently controlled R-genes associate with the RTF in the presence of drugs, and the resulting composite structure replicates under relaxed control. However, if RTF replication is only regulated positively by a protein initiator, as they suggest, it seems contradictory that RTF molecules which had associated with the R-genes could replicate at a relatively faster rate in the presence of protein synthesis inhibitors (i.e. CAM, STR, minimal medium, or PUR). Also these investigators theorize that the DNA band which eventually appears at a density corresponding to 58% GC contains large polygenic molecules which are composed of one RTF and many tandemly redundant R-genes. However, in molecular investigations by Rownd (62) and others (12,54) on the physical length of the R factor DNA in each R factor satellite band, no significant population of molecules which had a length greater than that of the composite R factor (i.e. one RTF plus one R-genes) were detected. In fact, Nisioka et al. (54) found only molecules of a length analogous to the R-genes molecule, when DNA which banded at a density corresponding to 58% GC was examined. In the studies reported herein and by others (10,19, 20) only 2 R factor DNA bands were detected in preparations from R^+ P. mirabilis. Although the quantities of DNA in each band varied considerably, the densities of both bands remained

constant. Possibly differences in the Proteus host or in the methods of isolation and analysis of DNA may account for the incongruity between these results and those of Rownd et al. (37,60-63,65).

Thus, in these studies replication of the 58% GC component was not prevented by bacteriostatic concentrations of any inhibitor of protein or DNA synthesis tested, or by shifts to physiological conditions that inhibited protein and host chromosome synthesis. In exponential-phase cells from either PAB or PAB plus CAM and early stationary-phase cells grown in CAM, where R factor replication had not yet been repressed, replication of the 58% GC R-components became malignant when the Proteus cells were subjected to any bacteriostatic condition. Thus, interference with the synthesis of the inhibitor of replication, presumably coded for by the RTF, eliminated all regulatory control over the R-genes.

The proposal that the genetic loci for inhibitor and initiator synthesis are most likely located on the 50% GC portion (RTF and composite R factor replicons) is supported by several observations. Replication of the 50% GC components was observed to parallel host genome replication during exponential growth of R^+ P. mirabilis, even though multiple-copies of the RTF replicon/genome were present. However, copies/genome of this replicon were increased during the early stationary phase of host cell growth and during growth in CAM, which indicated that regulation of the host genome and that of the 50% GC replicons were separable. In conjunction with the

observation that fi^+ R factors replicate in E. coli minicells (12,34,40) which contain little or no host chromosomal DNA, these results suggest that the gene(s) for initiator synthesis are located on the R factor. The fact that either the individual composite R factor or the RTF replicon can persist hereditarily in P. mirabilis host cells indicates that the locus for initiator synthesis is situated on the RTF portion of the R factor. Host cells harboring only the R-genes replicon have not been isolated, which suggests that both the initiator and inhibitor are synthesized by the RTF replicon and that R-genes replication would be lethal to the host cells in the absence of the regulatory inhibitor coded for by the RTF portion.

The rolling circle model of replication (26) affords a simple mechanism by which R factor segregation or dissociation can occur. In theory, replication of a particular replicon, according to this model, could produce not only monomers but also dimers and trimers in either the duplex or single-strand state. This prediction is consistent with the variety of DNA fragments observed in purified R factor DNA by Rownd and coworkers (62). In R^+ P. mirabilis, where 3 different molecular species of R factor DNA corresponding to the composite R factor, the RTF, and the R-genes molecules have been identified, linear, nicked circular, and covalently closed circular molecules of each size class have been isolated (10,11,12,54). In contrast, R factor 222 exists predominantly in the composite state in E. coli.

Linear composite R factor molecules as well as nicked circular molecules have been isolated from R^+ E. coli during R factor replication, whereas the covalently closed circular (supercoiled) molecules appear to be a molecular resting state between replication cycles (21).

The differences between the distinctly dissimilar replicative patterns of R factor 222 observed in E. coli and P. mirabilis might be due only to the presence in Proteus of an endonuclease which effects scission of the R factor molecule at the RTF terminus to yield individual RTF and R-genes molecules. The identification of such a hypothetical endonuclease involved in R factor dissociation in Proteus and its absence in E. coli might provide a basic explanation for the apparent instability and segregation of R factors in other hosts.

In the composite form, as it exists in E. coli, R factor replication appears to be closely associated with host cell division. Although replication in this host may be under both positive and negative control, in the absence of segregation the R factor would appear to be subject only to positive control. Closely related to this observation is the report that fi^+ R factors replicate in P1-infected E. coli after cessation of host genome synthesis (18) which indicates that R factor replication is not intimately linked to host genome synthesis or to cell division.

Besides affording an explanation of the replicative behavior of R factor 222 in different hosts, the working hypothesis presented herein can be used to formulate testable predictions. One such prediction is that the 58% GC replicon cannot stably exist in P. mirabilis cells in the absence of the regulatory inhibitor, coded for by the RTF, since a lethal vegetative type of replication would occur. This might be reflected in the lysis observed in R^+ P. mirabilis during the stationary phase of growth or after shifts to bacteriostatic conditions, and might explain the failure of attempts to isolate cells harboring only the R-genes replicon of R-222.

The relationships among the systems for regulation of replication of different extrachromosomal elements remain undefined. Preliminary evidence indicates that the R-genes portion of at least some fi^- R factors can exist stably in the absence of the RTF replicons (70). The similarity, if any between the immunity repressors of certain lysogenic bacteriophage and the proposed regulatory inhibitor of R factors remains speculative. The paucity of information on the replication of most bacterial plasmids and episomes prevents any overall interpretation of correlation of their replicative behavior. However, the replication of Col E_1 factor, a nontransmissible plasmid which specifies the production of a bacteriocin, has been studied in considerable detail. Somewhat analogous to the R-genes of 222, Col E_1 replication appears uncontrolled in the presence of CAM or during growth under conditions of amino acid starvation (8). It is, however, inhibited by rifampicin, an antibiotic which is known to inhibit RNA synthesis and which has

not been employed in R factor replication studies to date.

At the present time, no systematic investigation of the physiocochemical or chemical properties of R factor DNA has been reported. Although preliminary, the results of these studies on the physical and chemical properties of R factor DNA are strongly suggestive of an atypical chemical composition. During thermal denaturation R factor DNA consistently exhibited a skewed hyperchromic shift of relatively low absorbance increase (Fig. 6) but the midpoint of the hyperchromic transitions for both Proteus genome and R factor DNA corresponded to base compositions that were not unexpected. The presence of single-stranded R factor DNA which would have been reflected in a low absorbance increase during denaturation was not detected in CsCl gradient analysis of the DNA preparations employed in these studies. Despite the fact that the hyperchromic shifts due to denaturation of the genome and R-components were overlapping these results were consistent with the interpretation that R factor DNA has an atypical chemical composition.

The double-stranded nature of R factor DNA was originally concluded from the observed increase in buoyant density in CsCl after denaturation (19,64). When DNA isolated from R^+ P. mirabilis was denatured by heat or alkaline treatment and analyzed subsequently in CsCl gradients, abnormally low shifts in buoyant density were observed for the R factor components (Table 11). In contrast to the typical 0.016 g/cm^3 increase in density observed for the host genome DNA after denaturation, the 50 and 58% GC

R factor components increased in density by only 0.012 and 0.010 g/cm^3, respectively. Albeit the change in density associated with denaturation has been shown to decrease slightly with increasing GC content (77), the low values observed for the denatured R factor components were indicative of an abnormal base composition. The lack of agreement between these data and previously reported values might reflect the fact that only the 50% GC R factor component was examined in the earlier studies and that this component comprised only a small amount of the total extracted DNA (18,64). When similar DNA preparations from R^+ P. mirabilis were examined in alkaline CsCl gradients, where the guanine and thymine residues are fully titrated (77), all bands were observed to increase in density by the expected quantity (0.061-0.062 g/cm^3) (Table 11). This indicated that the altered residues were probably not guanine or thymine. In addition to the above studies, DNA samples were exposed to mild acid or alkaline conditions prior to CsCl gradient analysis, but no alteration in buoyant density was noted for any of the DNA species in these preparations.

On the basis of the evidence obtained from thermal denaturation and CsCl gradient analyses of several DNA preparations containing various quantities and proportions of each R factor component DNA species that R factor DNA was atypical in composition, chemical hydrolyses and chromatographic analyses were conducted. The hydrolysates of DNA from R^- P. mirabilis were observed to contain material corresponding to the 4 typical DNA bases. However, in addition to these 4 bases, a fifth UV-

absorbing spot was detected on the chromatograms of hydrolysates of R^+ P. mirabilis DNA (Fig. 7). This unusual material, designated "X", had a R_f value equal to that of the 5-hydroxymethyl uracil standard. An evaluation of the chromatographic properties of spot "X" and the cytosine spot, from hydrolysates of R^+ P. mirabilis DNA, in 2 different solvent systems indicated that the material corresponding in R_f to cytosine was giving rise to the unknown material "X" as well as to 5-methyl cytosine, uracil, and a small amount of undetermined material at the solvent front. Consistent with these observations, the material with an R_f value corresponding to cytosine was also observed to differ significantly in spectral ratios from both published values and the values observed for cytosine standards. These data indicated that R factor DNA might contain 5-hydroxymethyl cytosine, a base found in the DNA of T-even bacteriophage, which parallels cytosine in chromatographic mobility in the HCl:isopropanol solvent system and is known to give rise to 5-methyl cytosine, 5-hydroxymethyl uracil, and uracil. When the resulting spots from the various chromatograms were eluted and quantitated, DNA from R^- P. mirabilis was observed to have a chemically determined base composition of 40.3% GC (Table 12) which approximates the values of 38-39% GC estimated from Tm and buoyant density measurements. DNA from R^+ P. mirabilis exhibited an average base composition of 46.7% GC or an observed R factor composition of 56.3% GC, which was not exceptional. Thus, although the chromatographic data indicated the presence of an unusual base component, the chemically determined base composition of R factor DNA appeared to be normal.

Perhaps the discrepancy in these calculations lies in the fact that the material corresponding to cytosine had abnormal spectral ratios but nevertheless was quantitated by using the published values for cytosine.

The spectral characteristics of the unknown compound "X" were observed to resemble most closely those of 5-hydroxymethyl uracil and 5-hydroxymethyl deoxyuridine (Table 13) and corroborated interpretations of the chromatographic data. In addition, both the unknown material "X" and the material corresponding to cytosine reacted positively to tests for carbohydrates.

Thus, although preliminary, the available data strongly indicate that R factor DNA contains an unusual base, presumably identified as 5-hydroxymethyl cytosine on the basis of its chromatographic behavior and on the spectral properties of a degradation product. Hydroxymethyl cytosine, normally found in the glucosylated state in T-even bacteriophage DNA, has been observed to affect the buoyant density shifts associated with DNA denaturation (87). The unusual constituent in R factor DNA which may effect the abnormal density shifts observed after denaturation reacted positively to tests for carbohydrate which might be indicative of a secondary modification. Several preliminary attempts at quantitation of material "X" indicate that possibly as much as 35-40% of the cytosine residues in R factor DNA could be replaced by this compound. The potential physiological role of such a nucleotide constituent remains speculative but the positive results of this investigation warrant further efforts to elucidate the nature of this unusual

material.

SUMMARY

The molecular nature and replicative behavior of R factor 222 was examined in Proteus mirabilis. In deoxyribonucleic acid (DNA) from R^+ P. mirabilis, R factor 222 was identified by CsCl density gradient centrifugation as 2 satellite DNA bands at densities corresponding to 50 and 58 moles percent guanine plus cytosine (% GC). Replication of the 50 and 58% GC components of R factor 222 in P. mirabilis was analyzed during growth in the presence and absence of chloramphenicol (CAM) and after shifting exponential- and stationary-phase cells to conditions which inhibit host protein or DNA synthesis. CAM reduced the cellular growth rate but increased the amount of both R factor components relative to host chromosomal DNA. However, the 58% GC component showed a larger proportionate increase. This was inferred to indicate reduced synthesis of an inhibitor that acts on both R factor components and an initiator required for replication of the 50% GC component. Replicative patterns observed after shifting exponential- and stationary-phase cells grown with or without CAM to minimal medium or CAM for one generation, or puromycin for 3 hr, corroborated this interpretation. After shifts of exponential-phase cells from either medium, replication of the 50% GC components paralleled host replication, thus indicating a requirement for protein synthesis. Under these conditions, replication of the 58% GC replicon increased due to reduced inhibitor synthesis. R factor DNA content remained constant after shifting stationary-phase

cells from drug-free medium, whereas increased replication of the 58% GC component occurred after identical shifts of CAM-grown cells of the same chronological age. This indicated that effective concentrations of the regulatory inhibitor were attained in the stationary-phase cells grown in drug-free medium. Similar responses were observed after shifts to 5 C or to medium containing streptomycin or tetracycline. Absence of replication of the 50% GC component after shifting to medium containing nalidixic acid or phenethyl alcohol and the hereditary persistence of this replicon during growth indicated that the 50% GC replicon was attached to the membrane. Thus, in P. mirabilis the three replicons of R factor 222 are regulated as follows: the composite R factor and transfer portion (RTF) replicons, represented by the 50% GC component, require protein synthesis and membrane attachment for replication and are negatively regulated by an inhibitor; the 58% GC or resistance determinants replicon exists cytoplasmically and is subject only to negative replicative control.

The unusually low hyperchromic shift and the abnormal buoyant density shifts in CsCl observed after thermal denaturation of R factor DNA indicated an abnormal chemical composition. DNA from P. mirabilis harboring R factor 222 was examined chromatographically after enzymatic and chemical hydrolyses. Preliminary results indicated the presence of an unusual chemical component in R factor DNA which reacts positively to carbohydrate development and possesses chromatographic and spectrophotometric properties similar to 5-hydroxymethyl cytosine.

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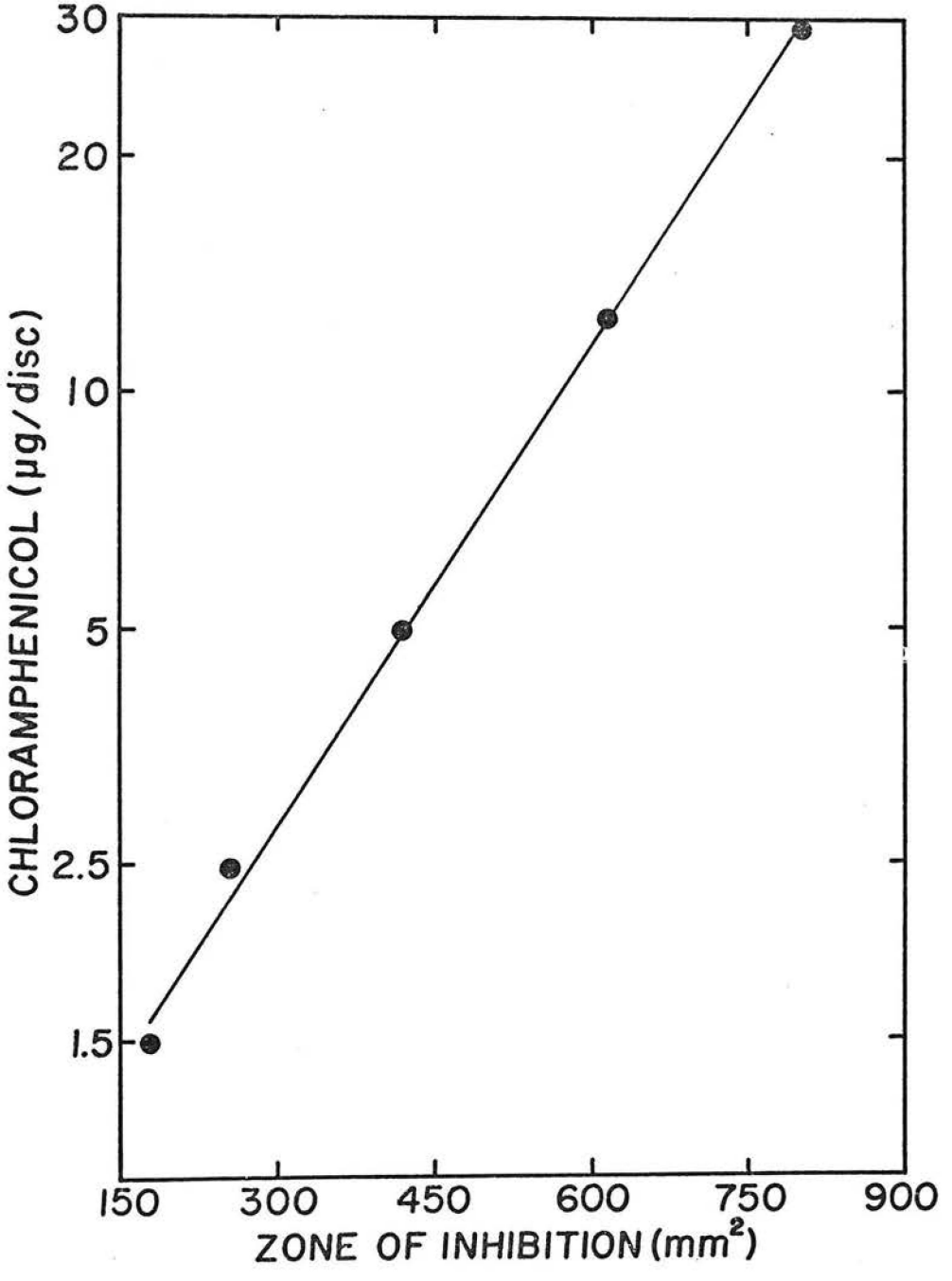
APPENDIX

Appendix Figure 1. Dose response curve for the microbiological assay of chloramphenicol .


30
20
10
5
2.5
1.5
CHLORAMPHENICOL (µg/disc)
150
300
450
600
750
900
ZONE OF INHIBITION (mm²)