PHYSIOLOGY OF ESCHERICHIA COLI K-12

DURING CONJUGATION

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CONTENTS

SUMMARY

The effects of conjugation on recipient cell physiology and metabolism have been examined in this thesis.

Previous reports have shown that recipient (F^-) cells of Escherichia coli K-12 were sensitive to an excess of donor (Hfr) cells. This phenomenon of lethal zygosis was observed as a decrease in the number of viable F^- cells and, as it represented a major change in the physiology of recipient cells, was further characterized in this study.

Parameters found to affect lethal zygosis included the ratio of donor to recipient cells, the cultural conditions of both parental cell types prior to mixing, and the conditions employed throughout the experiment. Results also confirmed that lethal zygosis was mediated by events associated with conjugation.

The phenomenon, which was normally observed in liquid media, also occurred on solid media and use of these two methods allowed an examination of strains of different mating type. Whereas most Hfr strains showed killing activity, no F^+ and only one F-prime donor examined produced lethal zygosis to the extent observed with killing Hfr strains. In general all F^- strains were sensitive, including recombination-deficient mutants (recA, recB, and recC).

An examination of cell functions over a range of donor:recipient ratios showed that conjugation was accompanied by the following changes in recipient cell physiology; (i) inhibition of deoxyribonucleic acid synthesis, (ii) inhibition of β-galactosidase induction, (iii) altered transport and accumulation of galactosides, and (iv) leakage of β-galactosidase into the supernatant fluid. These results are discussed

in terms of possible conjugation induced changes that at high Hfr:F^- ratios lead to lethal zygosis.

In order to assist further characterization of primary metabolic events accompanying conjugation, the selection of lethal zygosis resistant F^- mutants was attempted. One class of survivors (P190 series of strains) from solid media plate tests was found to be insensitive to lethal zygosis, but had become sensitive to male-specific bacteriophages and possessed F^+-like donor ability. The acridine orange sensitivity of these characteristics suggested that the P190 series possessed an extrachromosomal sex factor. From studies with these strains, and with known F^+ and Hfr donors, it was inferred that there exists an immunity to lethal zygosis (Ilz^+) associated with the F factor. Donor strains with deletions or point mutations in their F factor were examined in order to locate $\underline{ilz}^+$ on the F factor genetic map.

In a further attempt to obtain lethal zygosis resistant strains, a number of colicin resistant, colicin tolerant, and bacteriophage resistant mutants were screened. All were found to be sensitive to lethal zygosis, with the exception of one group of mutants which were resistant to Kox bacteriophage and tolerant to colicins K and L. The latter mutants were also conjugation-defective in crosses with either Hfr or F-prime donors. Examination of the cell envelope of one of these strains revealed alterations in a major cell wall protein. The relationship between resistance to colicins, bacteriophage, and lethal zygosis is discussed.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

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CHAPTER 1

INTRODUCTION

Bacterial conjugation may be defined as a process in which transfer of genetic material is accomplished by direct contact between the members of a sexually differentiated pair of bacterial cells. Such exchange by conjugation has been reported to occur within the following bacterial genera; Escherichia (Lederberg and Tatum, 1946a,b), Salmonella (Zinder, 1960), Serratia (Belser and Bunting, 1956), Pseudomonas (Holloway, 1955), Pasteurella (Lawton, Morris and Burrows, 1968), Rhizobium (Heumann, 1968), Enterobacter (de Graaf, Tieze, Bonga and Stouthamer, 1968) and Vibrio (Bhaskaran, 1958). In addition, intergeneric conjugation has been described between strains of a number of genera (for reviews see Baron, Gemski, Johnson and Wohlhieter, 1968; Jones and Sneath, 1970).

In this introduction, there has been no attempt to review the conjugation literature in exhaustive detail, but rather to provide a survey pertinent to the topic of the thesis.

THE CONJUGATION SYSTEM IN ESCHERICHIA COLI K-12

Conjugation in bacteria was first described by Lederberg and Tatum (1946a,b) who observed the formation of prototrophic recombinants when different multiple auxotrophic derivatives of E. coli K-12 were mixed. This process was later shown to require intact bacteria in direct contact since mixtures of supernatant fluids or filtrates, from either parent, with cells of the other strain failed to yield recombinants (Tatum and Lederberg, 1947). Furthermore, a similar result was obtained when fluid

cultures of each parental type were separated by a sintered glass filter which allowed only the fluid media to interchange (Davis, 1950).

Hayes (1952) provided evidence that parental cells do not play identical roles in conjugation, and further studies (Lederberg, Cavalli and Lederberg, 1952; Cavalli, Lederberg and Lederberg, 1953; Hayes, 1953a,b) clarified the heterothallic nature of conjugation. These investigations revealed that the fertility observed depended upon the presence of a transmissible agent, the fertility factor F (Lederberg et al., 1952). Cells which harboured F were called F^+ donors (or males) and were capable of transferring chromosomal markers, at a low frequency (10^{-4} to 10^{-6}), to F^- recipient strains (or females) which lacked this agent. $F^+ \times F^+$ matings were found to be less fertile, whereas crosses between F^- strains were infertile. When F^+ donor cells were mixed with an excess of F^- cells, the recipients were converted to donors at a rate faster than the bacteria could multiply. This suggested that the sex factor F was capable of epidemic spread among the recipient population.

Further understanding of the F factor and its role in conjugation was provided by the isolation and characterization of donor strains that yielded a high frequency of recombinants (Hfr) when mixed with F^- cells (Cavalli-Sforza, 1950; Hayes, 1953b). These strains were isolated from F^+ donors but differed from them in that only a small proportion of the recombinants from Hfr x F^- crosses inherited the donor state. All recombinants appeared to receive a unique segment of the donor chromosome (Hayes, 1953b). In a series of classical experiments (Wollman and

Jacob, 1955, 1958; Wollman, Jacob and Hayes, 1956), the chromosome transfer from Hfr donor to F^- recipient was shown to have a specific orientation such that a leading locus or origin always entered the female cell first. This origin was followed by various markers which could be arranged in a continuous gradient with respect to their frequency of transfer. Studies with Hfr strains lysogenic for various inducible prophages showed that the frequency of induction of a phage, on transfer to a nonlysogenic recipient (zygotic induction), became smaller the more distally located the prophage was from the origin of transfer (Jacob and Wollman, 1958b). This suggested that the gradient of markers observed was due to random breakage of the chromosome during transfer.

Analysis of chromosome transfer in a number of Hfr strains isolated from a single F^+ donor (Jacob and Wollman, 1958a) led to the hypothesis that the bacterial chromosome is circular and that Hfr strains arise among an F^+ population by insertion of the sex factor F into the chromosome at a number of specific sites (Jacob and Wollman, 1961). Rupture of the circle at the point of F attachment generates a linear structure with unique direction and orientation of transfer. Since the only recombinants inheriting donor properties in a Hfr x F^- cross were those receiving terminally transferred markers (Wollman et al., 1956), it was postulated that the F factor was linked to the distal extremity of the Hfr chromosome.

Interrupted matings with a number of Hfr donors have now shown that the minimum time for transfer of the entire chromosome, at 37C, is approx-

imately 90 min (Taylor and Thoman, 1964). By combining data from such studies with that from other methods of genetic analysis, it has been possible to construct a detailed circular linkage map of E. coli K-12 (Taylor and Trotter, 1972).

In addition to the F^+ and Hfr donors, a third type, the F-prime (F') donor, has also been described (Jacob and Adelberg, 1959; Adelberg and Burns, 1960; Hirota and Sneath, 1961; Pittard, Loutit and Adelberg, 1963). The distinguishing feature of these strains, which arose from Hfr donors, was their ability to transfer not only a fragment of the chromosome (merogenote), but also donor ability, at a high frequency. Subsequent studies have indicated that the sex factor in these strains had become detached from the original Hfr chromosome by reciprocal genetic exchange. This led to the formation of a circular F' element which carried a segment of the chromosomal material (Broda, Beckwith and Scaife, 1964; Scaife, 1966; Berg and Curtiss, 1967; Freifelder, 1968; Low, 1968).

The isolation and characterization of these F-prime strains provided considerable support for the model of genetic interaction between episomes and the bacterial chromosome, proposed by Campbell (1962). Discussion of the nature of sex factor insertion and detachment, and the mechanism of chromosome mobilization by secondary F' donors, has been included in a number of reviews (Adelberg and Pittard, 1965; Hayes, 1966, 1968; Scaife, 1967; Novick, 1969; Willetts and Broda, 1969; Adelberg and Bergquist, 1972; Low, 1972).

In addition to the transfer mediated by the sex factor F, conjugation associated with other transmissible plasmids has been described in a number of strains of the Enterobacteriaceae. Donor strains carrying transferrable colicinogenic (Col) factors and those carrying infectious drug resistance (R) factors are also capable of transferring chromosomal markers at low frequencies (Clowes, 1961; Ozeki and Howarth, 1961; Sugino and Hirota, 1962). The sex factor activities of these, and other plasmids, as well as the complex interrelationships that exist between transmissible and non-transmissible plasmids, have been excellently reviewed by a number of authors (Falkow, Johnson and Baron, 1967; Meynell, Meynell and Datta, 1968; Novick, 1969; Watanabe, 1969; Reeves, 1972).

NATURE AND FUNCTION OF THE SEX FACTOR F

The sex factor F has been shown to be a small deoxyribonucleic acid (DNA) molecule which can be isolated as a covalently closed circular (CCC) duplex (Freifelder, 1968), a form in which it is thought to occur for at least some of its intracellular existence (Clowes, 1972). From hybridization studies between an Flac element and the DNA of its host (Falkow and Citarella, 1965) it was concluded that F, which was equivalent to about 2% of the E. coli chromosome, had approximately 40% nucleotide sequence homology with that structure. The F sex factor was also found to be composed of two distinct molecular regions; one-tenth of the F DNA had a base composition of 44% G + C, whereas the remainder had a G + C

content of 50%, as did the host chromosome.

The molecular weight of the F factor has been estimated by a number of techniques, including neutral sucrose sedimentation (Freifelder and Freifelder, 1968; Bazaral and Helinski, 1970) and X-ray inactivation (Freifelder, 1968); however, the values obtained ranged from 35 to 75 megadaltons. More recently, measurements of F DNA contour length, by electron microscopy, have led to a value of 61 to 64 megadaltons (Kline and Helinski, 1971; Clowes, 1972; Palchaudhuri, Mazaitis, Maas and Kleinschmidt, 1972). From a similar measurement, the F DNA has been estimated to be equivalent to 94,500 base pairs (94.5 kilobase), a number sufficient for approximately 90 genes (Sharp, Hsu, Ohtsubo and Davidson, 1972).

In addition to the common property of fertility, the presence of the sex factor F in F^+, F-prime, and Hfr donors imposes a number of properties on the cells of these strains, which readily distinguish them from a recipient (F^-) cell. Some of these properties are described in the following sections.

Surface properties of male cells

It has been shown that F^+ cells have a greater affinity for acidic dyes, and agglutinate more readily in buffers at higher pH, than do F^- cells (Maccacaro, 1955; Maccacaro and Comolli, 1956). Electrophoresis confirmed that donor cells are less electronegatively charged than recipient cells (Turri and Maccacaro, 1960), a difference that has been

employed in the separation of these two cell types by ion-exchange chromatography (Zsigray, Fulk and Lawton, 1970).

The existence of a surface antigen, f^+, on cells harbouring the F factor was demonstrated by Ørskov and Ørskov (1960), and subsequent studies (Ishibashi, 1967; Knolle and Ørskov, 1967; Lawn, Meynell, Meynell and Datta, 1967) showed this antigen to correspond to the F pilus, a filamentous structure, the synthesis of which is under the control of F (Brinton, Gemski and Carnahan, 1964). These appendages can be readily distinguished from the common pili (fimbriae) and flagella of the cell, since ribonucleic acid (RNA) donor-specific phages attach along their sides (Crawford and Gesteland, 1964) and DNA donor-specific phages adsorb specifically to the F pilus tip (Caro and Schnös, 1966).

A considerable body of evidence has now accumulated in support of F pilus involvement in, at least, the early stages of conjugation (for discussion see: Brinton, 1965, 1971; Curtiss, 1969; Valentine, Silverman, Ippen and Mobach, 1969). The evidence is largely derived from studies employing donor cells that lack F pili, a state that can be brought about by any of the following: blending (Novotny, Carnahan and Brinton, 1969; Novotny, Raizen, Knight and Brinton, 1969), starvation of donor cultures (Curtiss, Caro, Allison and Stallions, 1969), aerated overnight growth of donor cells (Lederberg et al., 1952; Brinton, 1965), and mutation (Ohtsubo, Nishimura and Hirota, 1970; Achtman, Willetts and Clark, 1971). In all cases, loss of pili resulted in decreased ability to form specific pairs. This ability was also reduced when the

pilus tip was blocked by treatment of the donor cell, prior to mixing, with either DNA donor-specific phage (Ippen and Valentine, 1967; Novotny, Knight and Brinton, 1968) or with 10^{-3}M Zn^{2+} (Ou and Anderson, 1972).

Brief exposure of male cells to periodate also markedly reduced their ability to pair with female cells (Sneath and Lederberg, 1961). The periodate-sensitive site may well be associated with the F pilus, as such treatment has also been shown to inhibit the adsorption of RNA and DNA male-specific phages (Dettori, Maccacaro and Piccinin, 1961; Raizen, cited by Ou and Anderson, 1972).

F pili are 85 to 95A wide (Brinton, 1965, 1971; Lawn, 1966) and are thought to reach about 1.1μ in length before separating from the cell (Brinton, 1971), although greater lengths have also been observed (Lawn, 1966; Curtiss et al., 1969). The mean number of pili per donor cell increases from 1.4 to 2.7 as the growth conditions become more anaerobic (Curtiss et al., 1969). A physical and chemical analysis of these multifunctional appendages has shown that they are assemblies of a phospho-glyco-protein unit, F pilin, of molecular weight 11,800 (Brinton, 1971).

It should be noted that the majority of sex factors can now be placed into one of two groups depending upon whether they produce F-like or I-like pili (Meynell et al., 1968). F, ColV, ColB, Hly, Ent, K88, and fi^+ R factors produce F-like pili, whereas I-like pili are determined by

the ColI plasmid, the sex factor of ColE1a, and a number of fi⁻ R factors (Meynell and Datta, 1969). There is also evidence for a further pilus type that is specified by the N group of plasmids (Datta and Hedges, 1971).

While the role of F pili in specific pair formation is now generally accepted (see above), there is some controversy as to F pilus involvement in the passage of genetic material from donor to recipient.

Electron microscopy of conjugating bacteria (Anderson, Wollman and Jacob, 1957) revealed that donor and recipient cells could be joined by a cellular bridge. This observation led to the suggestion that transfer of DNA occurred via areas of localized fusion, formed as a result of close wall to wall contact between the mating pair. This "conjugation bridge" model of pair formation and transfer was in accord with the earlier observation made by Davis (1950); however, the discovery of a relationship between F pili, the F factor, and male-specific phages led Brinton (1965) to propose the "F pili conduction model". In this model the F pilus serves a dual role: as the site of attachment of male to female, and as the tube through which the DNA is transferred (the F pilus was thought to have an axial hole 25A in diameter). Brinton (1971) has recently restated the case for the pilus in transfer, and has proposed four different models in which the F pilus is represented as two parallel protein rods, each composed of an assembly of F pilin monomers.

Curtiss (1969) has examined the evidence for and against pilus conduction and has proposed a conjugation model in which the F pilus initially connects the male and female cells, then retracts into the donor

cell so bringing the mating pair into close wall to wall contact to allow transfer by the classical "bridge" model. Independently, Marvin and Hohn (1969) specified a similar "F pili retraction model" for RNA phage infection and DNA conjugational transfer. They envisaged a pilin depolymerizing mechanism activated by recipient cell contact. Results from studies of Pseudomonas aeruginosa pili, after RNA phage adsorption, have been cited as evidence in support of the retraction model (Bradley, 1972a,b). Ou and Anderson (1970) examined conjugating pairs by micro-manipulation and concluded that F pili were involved in drawing mating pairs into "close contact". The latter situation was found to be twice as fertile as that in which pairs were "loosely associated", presumably by F pili alone.

F^- cells, unlike donor cells, showed increased ability to form mating pairs when incubated in the presence of 10^{-3}M Zn^{2+}, prior to mating (Ou, 1973). Furthermore, this increased recombination frequency was also observed when Hfr donors, sheared free of their pili, were employed. This result suggests the existence of a donor site, other than F pili, which is involved in pair formation by direct wall to wall contact.

Surface exclusion

Exponential cultures of donor cells, carrying an F factor, are poor recipients in matings with other donors harbouring the same plasmid (Lederberg et al., 1952; Cavalli et al., 1953). This phenomenon of exclusion (Meynell, 1969; Novick, 1969) is manifested against the transfer of both F and chromosomal material, and it has been suggested that a sex

factor-specific barrier to the physical transfer of DNA is involved (Novick, 1969; Falkow, Tomkins, Silver, Guerry and LeBlanc, 1971). This hypothesis is supported by evidence showing reduced recovery of F factor DNA from excluding minicells, compared to that from F^- derived minicells (Sheehy, Orr and Curtiss, 1972); however, the exact nature of the barrier to transfer is not understood. Coulter counter measurements of mating pairs formed with surface exclusion proficient (Sex^+) or deficient (Sex^-) Flac mutants as recipients, have indicated that exclusion may operate, in part at least, to reduce pair formation (Achtman et al., 1971).

Donor cells lose the property of exclusion and gain recipient ability (F^- phenocopies) either when grown into late stationary phase (Lederberg et al., 1952) or when starved for a required amino acid (Curtiss et al., 1969). Although these physiological conditions also bring about the loss of F pili (see previous section), it does appear that the presence of an F pilus is not essential for the expression of exclusion (Curtiss et al., 1969). This conclusion is supported by the inability of donors to mate with F^+ or F' derived minicells, which rarely possess pili (Cohen, Fisher, Curtiss and Adler, 1968), and by the fact that many transfer-defective (Tra^-) mutants lack pili but still exhibit exclusion (Achtman et al., 1971). Curtiss (1969) has suggested the necessity for a donor component responsible for exclusion, to be located at or near the surface of the donor cell; however, no donor-specific surface structure, other than F pili, has been detected (Brinton, 1971).

Although loss of pili by a donor cell does not eliminate exclusion,

Meynell and Ewins (1973) have found that the exclusion by an F^+ cell (as a recipient) did not operate when the Hfr donor cell also carried an F-like R factor and produced mixed pili; however, these F^+ cells could exclude if the donor Hfr carried an I-like R factor and produced discreet F-like and I-like pili. These results suggested that there is an exclusion specificity associated with the type of piliation produced.

Incompatibility

Bacteria harbouring one plasmid cannot normally be stably superinfected by another isogenic or closely related plasmid. This phenomenon of plasmid incompatibility (Novick, 1969) allows a resident plasmid to impose an obstacle, in addition to exclusion, to the establishment of a second plasmid within its host. Therefore, even if exclusion is removed by treatments described above, Flac cannot be stably maintained in an Hfr cell (Scaife and Gross, 1962; Dubnau and Maas, 1968), although inherited functions can be observed for a number of generations. However, if Flac is transferred to a cell harbouring an Fgal plasmid, the segregants carry either Flac or Fgal but not both (de Haan and Stouthamer, 1963). There are exceptions to this incompatibility, for donor strains with two homologous integrated sex factors have been isolated (Clark, 1963).

While the molecular basis for plasmid incompatibility is not understood, it has been postulated that replication attachment sites on the cytoplasmic membrane (maintenance sites) are saturated by one plasmid in a cell, so blocking the replication of a competing plasmid (Jacob, Brenner

and Cuzin, 1963). Compatible plasmids are presumed to have distinct membrane attachment sites. This model, as well as one involving negative control of plasmid replication (Pritchard, Barth and Collins, 1969), has been discussed by Novick (1969) and Clowes (1972).

Fertility inhibition

Unlike the fertility factor F, the majority of F-like plasmids do not express donor ability (Meynell et al., 1968), and a repressor-operator type mechanism has been implicated in the regulation of transfer functions. Derepressed fertile mutants of F-like R factors have been isolated (Meynell and Cooke, 1969).

The F sex factor appears to be defective in producing a repressor, although some plasmids are able to repress F transfer and have been termed fi^+ (fertility inhibition), whereas those plasmids lacking this property are fi^- (Watanabe, Nishida, Ogata, Arai and Sato, 1964). In a recent model for fertility repression, it was proposed that the transfer inhibitor is formed by the interaction of a non-specific component, the fin^+ product (formerly fi^+ product; encoded on a repressed F-like plasmid), with a plasmid specific component, the P product (coded for by traP on the F factor) (Finnegan and Willetts, 1971, 1972; Willetts and Finnegan, 1972). It was further suggested that this inhibitor interacts with a specific site on the F factor (traO) so preventing the synthesis of the traJ product (see below) which is, in turn, required for the synthesis of other tra gene products.

Female-specific phage restriction

A number of bacteriophage are known to have lowered efficiency of plating, and reduced plaque size, on E. coli K-12 strains carrying the sex factor F. Included among these female-specific or F restricted bacteriophages are ØI (Dettori et al., 1961), ØII (Wollman, 1947), T3 (Schell, Glover, Stacey, Broda and Symonds, 1963), T7 (Dettori et al., 1961; Mäkelä, Mäkelä and Soikkeli, 1964), H (Molnar and Lawton, 1969), and W-31 (Watanabe and Okada, 1964).

This phenomenon does not appear to be an example of the classical restriction system (Arber and Linn, 1969). It has been shown that ØII is capable of adsorbing to, and killing, donor cells (Cuzin, 1965); macromolecular syntheses in the donor cell are inhibited a few minutes after infection (Linial and Malamy, 1970). Further studies indicated that the presence of an F factor in a cell prevented the synthesis of two of the three classes of protein required for T7 development, even though all known T7 messenger RNA was present (Morrison and Malamy, 1971). These authors therefore suggested that two genes (pifA and pifB) on the F factor, code for the production of inhibitors of T7 translation control mechanisms. Support for this model was provided by the isolation of F factor mutants ($PifA^-$) which allowed only one additional T7 protein to be synthesized and gave partial restriction. A second class of mutants ($PifA^- PifB^-$) allowed all three T7 proteins to be synthesized and had lost all ability to inhibit T7 development.

It should be noted that the efficiency of plating of a number of

female-specific phages depends upon the host strain carrying the F factor (Linial and Malamy, 1970) and on the E. coli strain employed for propagation (Monner and Boman, 1970; Williams and Meynell, 1971).

In addition to the properties discussed above, the F sex factor specifies a replication and segregation system and may well encode for its own integration and recombination systems. These functions are discussed in a number of recent reviews (Novick, 1969; Helinski and Clewell, 1971; Clowes, 1972; Achtman, 1973a).

Genetics of sex factor F

Complementation analysis and deletion mapping of conditional and absolute mutations have permitted the construction of a genetic map of sex factor F (Willetts, 1972a).

By performing complementation tests between transfer-defective (tra) mutants of an F-prime plasmid and a compatible R factor, Ohtsubo et al. (1970) were able to identify seven cistrons involved in genetic transfer. Similar analyses utilized transient heterozygotes carrying two different Tra$^-$ Flac mutants which had been introduced into the same cell by conjugation (Achtman et al., 1971) or by P1 transduction (Willetts and Achtman, 1972). These latter studies identified a further five cistrons traA, traB, traH, traJ, and traK in addition to traI, traD, traG, traF, traC, and traE which correspond, respectively, to the complementation groups A, B, C, D, E, and F of Ohtsubo et al. (1970); traL (Willetts, 1973) is equivalent to group H (Ohtsubo et al., 1970). These cistrons have been mapped by complementation analysis with a series of Fgal deletions

(Ohtsubo, 1970) and with a series of Hfr deletions (Ippen-Ihler, Achtman and Willetts, 1972; Willetts, 1973), and the following order has been determined traJ, A, L, E, K, B, C, F, H, G, D, I.

In addition to these twelve cistons required for F transfer, the following have been located on a Flac map: $\emptyset II^r$ (Ippen-Ihler et al., 1972), a locus for inhibition of female-specific phage development which may be equivalent to pifA and pifB (Morrison and Malamy, 1971); ori, the origin of transfer (Willetts, 1972b); inc, a locus determining incompatibility of an integrated F factor towards a superinfecting F-prime (Willetts, 1972a). The approximate location of traP, the cistron coding for the P product involved in transfer inhibition, has been reported (Finnegan and Willetts, 1972).

Characterization of transfer-defective (tra) mutants has shown that at least ten of the twelve cistrons identified are involved in F pilus formation; mutations in traH, F, C, B, K, E, L, A, J, and some in traG completely lack F pili, whereas traD mutants possess pili but are unable to be infected by the RNA phages such as f2, MS2, and R17 which can adsorb to them (Walker and Pittard, 1969, 1972; Ohtsubo et al., 1970; Achtman et al., 1971, 1972). It is possible that traG mutations are in two different cistrons, as a second group of traG mutants possessing pili has been isolated (Achtman et al., 1972).

F-like R factors are able to complement mutations in most tra cistrons (Willetts, 1971; Alfaro and Willetts, 1972). Exceptions include

mutations in traI, traA, and traJ, and it has been suggested that the products of these cistrons are plasmid specific. The traI product has been implicated in the initiation of transfer replication, whereas traJ has been postulated to be a positive control gene that regulates all tra cistrons (Finnegan and Willetts, 1971, 1972; Willetts, 1971; Willetts and Finnegan, 1972; Achtman, 1973a,b), since traJ mutations lack F pili, plasmid specificity (traI), pilus specificity (traA), and surface exclusion (traS). The existence of the traS cistron is based on recipient abilities of the Hfr deletion strains employed by Ippen-Ihler et al. (1972). Deletions ending in traI and some in traD retain surface exclusion, whereas others in traD and all those extending into traG and further have lost exclusion (Willetts, 1972a and personal communication).

Electron microscope heteroduplex studies with Fgal deleted plasmids have allowed physical map distances for a number of tra cistrons to be determined (Sharp et al., 1972). The total F map is 94.5 kb (a unit of distance is one kilobase equal to 1,000 base pairs) and at least part of the tra region is located between 50 kb (traE) and 94.5 kb (traI) (from Achtman, 1973a).

PHYSIOLOGY OF CONJUGATION

Studies on the physiology of conjugation have largely been concerned with two broad questions. Firstly, there was a need to define optimum conditions for conjugation such that mating pair formation and all subsequent steps leading to recombinant formation could be maximized.

Experiments to determine the effects of varying nutritional additives, population density, temperature, pH, and prior growth conditions of cells on chromosome transfer and recombinant formation, are included in this area.

The second major group of physiological studies have been concerned with the requirements for cellular activities, such as energy metabolism and macromolecular syntheses, during conjugation. These experiments were designed, in the main, to provide an understanding of the conjugation process at each of its various stages. The following stages of conjugation have been described and discussed by Clark and Adelberg (1962) and by Curtiss (1969); (i) formation of specific pairs between donor and recipient cells, (ii) conversion of specific pairs to effective pairs by the formation of a conjugation bridge or tube, (iii) chromosome or sex factor mobilization - including conversion of a circular chromosome to one that can be transferred linearly, (iv) chromosome transfer, and (v) recombinant formation. Physiological studies have also provided evidence for the active role of both donor and recipient cells in transfer (Curtiss and Charamella, 1966; Freifelder, 1967; Curtiss, Charamella, Stallions and Mays, 1968) for it had previously been suggested (Fisher, 1957b) that F^- cells were passive partners at this stage.

Requirements for energy metabolism during conjugation

It was shown by Fisher (1957a) that conjugation was an energy

dependent process requiring aerobic metabolism of a carbohydrate via the tricarboxylic acid (Krebs) cycle, and in a further series of experiments the stages of the conjugation process that required such oxidative energy were examined (Fisher, 1957b). It was found that mating mixtures held under anaerobic conditions for varying periods, all formed recombinants at the same rate once oxygenation was commenced, and it was concluded that even the earliest step in conjugation, mating pair formation, was endergonic. Fisher (1961) later showed that Hfr and F^- cells mixed in the presence of the metabolic inhibitor, 2:4 dinitrophenol (DNP), went on to form recombinants when they were diluted at various times to prevent further collision. This suggested pair formation had occurred in the presence of DNP. Curtiss and Stallions (1967) resolved this question by employing the de Haan and Gross (1962) method for separating the stage of pair formation from that of chromosome transfer; specific pairs were able to form in the absence of energy metabolism in either one or both of the mating parents.

The stage of conjugation that has been shown to be endergonic is that of chromosome transfer. In one experiment, Fisher (1957b) withdrew samples of a mating mixture at various intervals after mixing and added them to media containing DNP. He found that this metabolic inhibitor prevented the formation of new recombinants, and concluded that energy was required throughout the period of transfer. The effect of temperature on the kinetics of chromosome transfer has been examined (Hayes, 1957), and the results indicated that such a limitation slowed the rate

of chromosome transfer. Wood (1968) has confirmed and extended these observations using a wider range of temperatures.

The recombinant frequencies observed with cells starved in unsupplemented buffer prior to mixing, led Fisher (1957b) to suggest that the Hfr donor was active during chromosome transfer, whereas the recipient was passive. However, it is now known that starvation of donor cells leads to loss of F pili (Curtiss et al., 1969), and, as pointed out by Curtiss (1969), only limited conclusions can be drawn from Fisher's results. It was shown by Curtiss et al. (1968) that starved F^- cultures yield higher recombinant numbers than unstarved cultures, when mated in the presence of an energy source. These authors also demonstrated the need for active energy metabolism in both parents; maximum frequencies of recombinant formation and zygotic induction were achieved only when both parents were capable of utilizing supplied carbohydrates. It is of practical interest that conjugation can occur, at normal frequencies, in an anaerobic environment, provided a rich mating media is used (Stallions and Curtiss, 1972).

Effects of environmental conditions on conjugation

In addition to the effects of temperature on conjugation (Hayes, 1957; Woods, 1968), the influence of a number of other environmental factors have been examined.

The rate of recombinant formation in buffer + glucose + sodium aspartate was found to double when the pH was reduced from 7.2 to 6.2 (Hayes, 1957), although the effect was not observed when mating was

performed in broth. An optimum pH of 6.1 for synthetic mating media was observed by both Fisher (1957b) and Brinton (1965). A higher rate of recombinant formation, like that found with buffer at pH6.2, was also observed when undiluted suspensions of parental cells were employed in the mating mixture (Hayes, 1957), and it was concluded that the lower pH brought about a surface effect that allowed increased collisions to occur. Brinton (1965) found that stable mating pairs formed immediately after mixing parental cells, and that the number of pairs increased rapidly in accordance with simple collision theory. He drew the conclusion, based on the collision rate constant, that F pili were capable of making effective contacts at multiple sites on the recipient cell surface.

The effect of nutritional supplements on recombinant formation was examined by Gross (1963a) who found reduced recombinant yields when parental cells, grown and mated in rich media, were plated on synthetic media. The effect could be overcome by the inclusion of small amounts of broth or casein hydrolysate in the synthetic medium.

Effects of parental cell growth conditions and growth phase

The physiological condition of a parental cell greatly influences its ability to engage in conjugation. We have seen that the properties of fertility, surface exclusion, and piliation were altered when donor cells were starved for an amino acid prior to mating (Fisher, 1957b, 1966; Krisch and Kvetkas, 1966; Curtiss et al., 1968, 1969) or when donor cultures were vigorously aerated into late stationary phase

(Lederberg et al., 1952; Cavalli et al., 1953; Brinton, 1965). The latter treatment might also be considered as a starvation process.

The effect of these conditions on donor cell physiology has been examined, in detail, by Curtiss et al. (1969). These authors found that donor cells grown under anaerobic conditions (N_2), or without aeration, had higher mean numbers of F pili per cell and longer pili, than did cells grown with vigorous aeration; such cells were more able to form mating pairs and initiated chromosome transfer earlier than the cells grown aerobically. Starvation of donor cells resulted in loss of F pili, loss of ability to form mating pairs, and an increase in recipient ability; restoration of normal growth conditions reversed these effects.

Amino acid-starved recipient cells have also been shown to have altered mating ability, although results from different laboratories are contradictory (Krisch and Kvetkas, 1966; Curtiss et al., 1968; Wlodarczyk and Kunicki-Goldfinger, 1969). Curtiss et al. (1968) found that starvation of F^- cells for an amino acid prior to mating led to a more rapid appearance of donor markers in the recipient, and suggested that a specific stage in the chromosome replication cycle was required before the F^- cell could participate in chromosome transfer. A similar conclusion was also reached from data obtained with synchronized F^- cultures; maximum recipient ability correlated with the stage in the cell division cycle during which initiation of a new round of replication occurred (Špelina and Stárka, 1968; Mycielski, Kociszewska-Kiljańczyk

and Kunicki-Goldfinger, 1969; Kociszewska-Kiljańczyk, Mycielski and Kunicki-Goldfinger, 1970). In contrast to the results with F^- cells, studies indicate that the capacity of an Hfr donor cell to transfer the chromosome is independent of the chromosome replication cycle or the cell division cycle (Blinkova, Bresler and Lanzov, 1965; Špelina and Stárka, 1968; Mycielski et al., 1969).

It is apparent from the discussion above that the phase of growth does influence donor ability, at least at the stage of pair formation. With F^- cells the effect of growth phase on recipient ability is not at all clear. Claims have been made that either logarithmic-phase (Fisher, 1965; Kunicki-Goldfinger and Rumińska, 1967) or stationary-phase cultures (Curtiss et al., 1968) made better recipients. These contradictory results could be accounted for by the different mating methods and growth conditions employed.

Requirements for macromolecular syntheses

As pointed out previously, studies of the requirements for macromolecular syntheses during conjugation have largely been undertaken to define the nature and mechanism of processes that are associated with this remarkable sexual act. Many of the early investigations were performed prior to the establishment of optimal mating conditions and, in addition, the precise mode of action of a number of the metabolic inhibitors used was not understood. Consequently, in many cases, unphysiological conditions were employed and ambigous data have resulted.

There are, of course, complexities in selectively examining processes in cells of one parental type (e.g., recipient) while the members of an opposite type (e.g., donor) continue normal mating activities. It is only with the recent advances in techniques, and with the employment of suitable genetic blocks, that unequivocal evidence has emerged regarding the nature of conjugational metabolism and physiology.

In the preceding section some of the evidence for the role of protein synthesis was examined and it can be concluded that the Hfr cell, at least, needs to be able to synthesis proteins prior to, and perhaps during, the early stages of conjugation. In addition to these studies based on amino acid starvation, there are studies that have made use of inhibitors of protein synthesis. Fisher (1957a) found that the addition of chloramphenicol (10μg/ml) to the mating mixture had no effect on recombinant yields, and he concluded that protein synthesis was not required for conjugation. In contrast, Friszke (1970) observed an 85% decrease in recombinant frequency when chloramphenicol was added within the first 30 min after mixing Hfr and F^- cells. The latter author also examined the effect of chloramphenicol treatment on donor and recipient cells prior to mixing and, as expected, found lowered Hfr donor ability, but the recipient ability of F^- cells displayed a cyclical response with time of treatment. This was suggested to be associated with the phase of the replication cycle at which protein synthesis was inhibited, a conclusion also reached from amino acid starvation and synchronization

studies (see above).

Although no data are available, Curtiss, Mays, and Stallions (1967) have claimed that Hfr cells are required to be able to undergo protein and DNA syntheses, within the first 5 min of mating, to allow initiation of transfer. Inhibition of protein, DNA, and RNA syntheses did not alter the rate of chromosome transfer. Apart from this preliminary report little else is known of the need for RNA synthesis during conjugation, although rifampicin, an inhibitor of transcription, has been shown to influence F gene expression and donor ability (Riva, Fietta, Silvestri and Romero, 1972).

Whereas there has been little focus on the requirements for RNA and protein syntheses, the role of DNA synthesis, during conjugation, has been the subject of intensive investigation over the past decade. In the replicon model (Jacob et al., 1963), replication of each independent unit, which includes the bacterial chromosome and the sex factor F, is controlled by an initiator which interacts specifically with a particular site, the replicator. This interaction results in initiation, at the replicator, of a new round of replication. In the integrated state (Hfr) the replication system of F does not function, as it does in the autonomous state, and F is replicated passively by the chromosome machinery.

This replicon hypothesis was extended (Jacob et al., 1963) to provide the following model (Jacob-Brenner-Cuzin) for genetic transfer during

conjugation. Upon effective contact with an F^- cell, an F specific initiator is synthesized and allows the F replicator to assume control over chromosome replication, which is initiated by a single strand break near the origin of transfer. The replication machinery is so orientated as to drive one newly formed daughter chromosome into the recipient via the conjugation bridge. F factor control of chromosome replication ("integrative suppression") has been described recently (Nishimura, Caro, Berg and Hirota, 1971).

Bouck and Adelberg (1963) proposed an alternative model for transfer in which completion of a full cycle of replication is followed by transfer of one of the daughter chromosomes before ring closure can take place. In other words, the free end, provided on completion of replication, becomes the origin of transfer. The Jacob-Brenner-Cuzin model therefore requires F directed DNA synthesis during transfer, whereas the Bouck-Adelberg model requires DNA replication only prior to transfer. These predictions have provided the basis for the immense amount of work on DNA synthesis during conjugation, that has been reviewed recently by a number of authors (Kunicki-Goldfinger, 1968; Curtiss, 1969; Brinton, 1971).

Unfortunately, many conflicting observations and conclusions exist in the literature and, as yet, one cannot unequivocally state whether DNA transfer can or cannot proceed in the absence of DNA replication. However, experiments utilizing nalidixic acid (Hollom and Pritchard, 1965; Barbour, 1967; Bresler, Lanzov and Lukjaniec-Blinkova, 1968; Cohen

et al., 1968; Fisher and Fisher, 1968; Bouck and Adelberg, 1970; Hane, 1971), edeine (Piekarowicz, Wlodarczyk and Kunicki-Goldfinger, 1968), or thymine starvation (Pritchard, 1965; Curtiss et al., 1968) to prevent DNA synthesis and studies employing isotopic labelling techniques to measure DNA synthesis (Ptashne, 1965; Gross and Caro, 1966) have provided evidence compatible with the Jacob-Brenner-Cuzin model (Jacob et al., 1963).

Results from experiments with DNA replication (dna) mutants indicate that vegetative chromosome replication in either donor or recipient cell is not required for genetic transfer, although DNA synthesis associated with conjugation, and thought to be transfer mediated, has been observed (Bresler et al., 1968; Marinus and Adelberg, 1970; Moody and Lukin, 1970; Stallions and Curtiss, 1971). This mating-stimulated DNA synthesis, which is independent of the dnaB gene product, has been further analyzed by Vapnek and Rupp (1970, 1971) in a series of elegant studies and, together with earlier data, their conclusions form the basis for the following mechanism of transfer replication.

After an effective mating pair is formed a unique single strand of DNA is transferred to the recipient with a 5' - 3' orientation (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968), probably by a mechanism similar to the rolling circle model (Gilbert and Dressler, 1968; Ohki and Tomizawa, 1968). In the case of F^+ donors it is known that the denser of the two F DNA strands (in CsCl-poly U,G) is transferred to the

recipient where a complementary strand is synthesized to reform the CCC F sex factor DNA molecule; the lighter strand remains in the donor where a heavy complement is synthesized (Vapnek and Rupp, 1970, 1971). Mating-dependent DNA synthesis has also been observed in the recipient during a Hfr x F^- cross, the extent of which approximated the amount of DNA transferred (Wilkins, Hollom and Rupp, 1971).

EFFECTS OF CONJUGATION ON RECIPIENT CELL PHYSIOLOGY

In contrast to the attention given to the question of the physiological requirements for conjugation, as outlined in the previous sections, there has been little interest shown in the physiological changes induced in a cell as a result of mating, other than DNA transfer replication.

One example of such an alteration in recipient cell physiology was first described by Alfoldi, Jacob and Wollman (1957) and Alfoldi, Jacob, Wollman and Mazé (1958). These authors carried out a series of crosses involving different Hfr strains, all colicinogenic for E1. When each was mixed with a non-colicinogenic F^- strain (Hfr $\underline{col}^+$ x $F^-\underline{col}^-$), it was found that the extent of transfer of the E1 character varied with the Hfr strain used. In reciprocal crosses between various Hfr $\underline{col}^-$ strains and an $F^-\underline{col}^+$ recipient (Hfr $\underline{col}^-$ x $F^-\underline{col}^+$) a large percentage of the F^- cells were non-viable after mixing with an excess of the Hfr cells. This latter phenomenon was named "lethal zygosis" and the extent to which it occurred depended upon the Hfr strain employed; those Hfr strains

providing high $\underline{col}^+$ transfer were also able to produce high levels of lethal zygosis in the reciprocal cross. This apparent correlation led these authors to suggest that the ColE1 factor was located at a chromosomal site in the Hfr but was released from this on transfer to an F^- cell to form an autonomous element. In the reciprocal cross, the penetration of the $\underline{col}^-$ character into the $F^-\underline{col}^+$ cell was thought to release the recipient from immunity to the colicin it produced, thereby leading to the killing observed; a situation analogous to an inverse zygotic induction (Jacob and Wollman, 1961).

Detailed examination of the transfer of the colicinogenic (Col) factors E2, I, and V (Nagel de Zwaig, Anton and Puig, 1962) and of ColE1 (Clowes, 1963; Nagel de Zwaig and Puig, 1964) provided evidence which led to the now accepted conclusion that these Col factors are maintained and inherited as extrachromosomal elements. These studies also showed that the lethal zygosis observed, when an excess of non-colicinogenic Hfr cells were mixed with colicinogenic recipients, occurred when both mating types were non-colicinogenic. The extent of lethal zygosis varied according to the types of Hfr and F^- strains used and with the conditions employed (Clowes, 1963).

Gross (1963b) analyzed the effect of the Hfr:F^- ratio on mating and found a progressive fall in the number of recombinants obtained as the Hfr concentration increased above one cell per five F^- cells. In a further experiment, samples from a mating mixture (Hfr:F^- ratio of 4:1)

were collected and diluted at various times after mixing. The results led Gross (1963b) to conclude that additional contacts between F^- and Hfr cells, that would occur with time, reduced rather than increased recombinant yields. Irreversible damage to the F^- cell was thought to occur with an excess of Hfr cells within 20 min after mixing. From their observations both Clowes (1963) and Gross (1963b) suggested that lethal zygosis resulted from multiple mating between one F^- cell and several Hfr cells.

There have been comments on lethal zygosis in a number of reviews and reports (Gross, 1964; Reeves, 1965, 1972; Nomura, 1967; Kunicki-Goldfinger, 1968; Stallions and Curtiss, 1971) but no further detailed studies have been reported on this physiological change associated with conjugation. Clowes (personal communication) obtained some evidence for leakage of ^{14}C-labelled material from prelabelled F^- cells during lethal zygosis, but the results did not correlate well with the loss of viability observed. Tentative experiments also indicated that Hfr cells possessed a cell wall enzyme (a glucuronidase) which might mediate the Hfr-F^- interaction (Clowes, Silver and Moody, cited by Clowes, 1965 and personal communication). Under conditions of multiple mating such activity would result in extensive F^- cell wall damage and lysis would ensue.

In addition to the loss of F^- viability described above, other conjugation induced physiological changes have been reported. It was

found that induction of the enzyme β-galactosidase, in F^- cells, was reduced when those cells were mixed with a three-fold excess of Hfr cells (Riley, Pardee, Jacob and Monod, 1960). This change was accompanied by an inhibition of division of exconjugant cells. Anderson (1958) also observed altered cell division associated with conjugation. Using the techniques of micromanipulation and microscopy he found many of the descendants of a Hfr x F^- cross, to be unable to divide or to increase in size, whereas others went on to form filaments. Ou and Anderson (1970), who were able to distinguish between "loose", pili associated, pairs and "close", wall to wall, pairs, found a higher proportion of non-viable cells with the latter form of contact.

Some of these physiological changes associated with conjugation were cited by Reeves (1965, 1972) in support of a hypothesis to explain the relationship between a colicin and its target cell. Reeves suggested that the possession of a receptor which allows the adsorption of a colicin molecule must, in some way, be advantageous for that cell. The presence of a fertility system would provide such an evolutionary advantage and it was proposed, on that basis, that the colicin itself was a modified fertility recognition site which was released from one cell and interacted at a complementary fertility site on a sensitive cell (the colicin receptor). Such an interaction would lead to the perturbation of the metabolic responses which would normally accompany the conjugation event. On this hypothesis, multiple mating could lead to an

amplification of these conjugation induced responses and result in death of the cell. Nomura (1967) has also drawn the analogy between F and Col factors in their ability to bring about changes in the bacterial regulatory mechanisms as a result of surface contact.

OBJECT OF THE STUDY

The primary objectives of this study were as follows:

1. To characterize the phenomenon of lethal zygosis, a major physiological change associated with conjugation.
2. To examine the physiology of conjugating F^- cells at various Hfr:F^- ratios, in order to define alterations in macromolecular syntheses, and other metabolic activities, that are conjugation induced.

CHAPTER 2

MATERIALS AND METHODS

MEDIA

Nutrient broth (Difco-0003) was prepared double strength plus 0.5% (w/v) sodium chloride; nutrient agar was blood-agar base (Difco-0045) prepared as directed, without the addition of blood. Minimal liquid medium was that described by Davis and Mingioli (1950). Minimal agar was prepared by the addition of 2% (w/v) agar (Difco-0140) to minimal liquid medium. Glucose (0.5%, w/v) was generally employed as a carbon source in minimal agar and minimal liquid. Growth supplements and other carbon sources were used at the following final concentrations except where noted: purines, pyrimidines, and amino acids, 20μg/ml; vitamins, 1μg/ml; glycerol, 0.4% (v/v); arabinose, galactose, and melibiose, 0.5% (w/v); lactose, maltose, mannitol, and xylose, 1.0% (w/v).

Tryptone broth was prepared as 0.8% (w/v) tryptone (Difco-0123) with addition of 0.5% (w/v) sodium chloride. Tryptone yeast extract broth was prepared as 1.0% (w/v) tryptone with the addition of 0.5% (w/v) sodium chloride and 0.25% (w/v) yeast extract (Difco-0127). Casein hydrolysate (acid) (Oxoid-L41) was used as a supplement in some experiments.

Fermentation characteristics of strains or recombinants were determined by plating cultures on either eosin-methylene blue (EMB) agar (Difco-0511) or tetrazolium agar [prepared as described by Meynell and Meynell (1970)] with sugars added at the levels indicated above.

Preconditioning of media was by growth of the appropriate transfer defective (Tra^-) donor strain to an optical density of approximately 0.3 (see below), followed by centrifugation to remove the majority of cells and filtration of the supernatant fluid (Sartorius membrane filter SM11306; pore size, 0.45μm). The filtrate, which was used on the day of preparation, was prewarmed to 37C immediately before use.

BACTERIAL STRAINS

The strains of E. coli K-12 used, together with their source or derivation, are listed in Table 2-1, with the following exceptions: the Hfr strains derived from χ209 (Curtiss and Stallions, 1969) with the origins OR80 (χ900), OR64 (χ884), OR76 (χ896), OR7 (χ437), OR66 (χ886), OR56 (χ876), OR75 (χ895), OR77 (χ897), OR63 (χ883), and OR49 (χ869), kindly provided by R. Curtiss III; the P190 series (Table 6-1) derived from solid media matings between the strains AB1133 and AB259; the Hfr deletion strains (Fig. 6-4) of the KI series (Ippen-Ihler et al., 1972) (which have a phenotype similar to KI529 or KI534 except that they are Lac^-), kindly provided by N. Willetts; the colicin resistant and tolerant mutants derived from AB1133 (Tables 7-1 and 7-2), kindly provided by J.K. Davies and those strains described by Reeves (1966, 1972) including P114, P115, P116, P117, P118, P119, P120, P125, P134 and P138; the bacteriophage resistant mutants derived from P400 (Table 7-3), kindly provided by R. Hancock. The origins and directions of transfer for the Hfr strains used are shown in Fig. 2-1. All strains were stored as freeze

TABLE 2-1

Bacterial strains[i]

Strain no.	Mating type	Genotype	Source[ii] derivation
K-12	F^+	prototroph (λ)	Wild type
P201	F^+	thr leu thi lac mal gal ara xyl mtl str ton (λ)	W677 str[iii]
P107	F^-	thr leu lac gal ara mal xyl mtl (λ)	Cavalli 42[iii]
P703	HfrR1	met (λ)	
P300	HfrR2	thr leu thi lac mal gal ara xyl mtl ton (λ)	
P301	HfrR3	as P300	
P702	HfrR4	met (λ)	
P816	HfrR5	thi mtl lacY gal mal xyl λ^r	
RC748	F^-	met pro tsx str (λ)	a. $W1:M^-P^-$[iii]
P513	F^-	met tsx str (λ)	RC748
P882	F^-	met proA tsx str (λ)	AB1133 $\xrightarrow{P1kc}$ P513
P978	F^-	met proA tsx str thyR[iv] (λ) .	P882
P322	F^-	met proA tsx str lacY (λ)	P882
RC759	HfrP4X(J2)	met (λ)	a.
RC749	HfrP10(J4)	thr leu thi mal lac gal ton (λ)	a.
RC12	F^-	thr leu thi lac mal gal ara xyl mtl str ton azi (λ)	a. W677 str azi[iii]
AB1133	F^-	thi argE his proA thr leu mtl xyl ara galK lacY str supE λ^-	b.

TABLE 2-1 Continued

P122	F^-	as AB1133 but (λ)	AB1133
AB1157	F^-	as AB1133 but *tsx sup-37*	c.
AB1884	F^-	as AB1157 but *uvrC4*	c.
AB1885	F^-	as AB1157 but *uvrB5*	c.
AB1886	F^-	as AB1157 but *uvrA6*	c.
JC2917	F^-	as AB1157 but *recA12*	d.
JC2921	F^-	as AB1157 but *recA1*	d.
JC2926	F^-	as AB1157 but *recA13*	d.
JC2929	F^-	as AB1157 but *recA65*	d.
JC5489	F^-	as AB1157 but *recC22*	d.
JC5743	F^-	as AB1157 but *recB21*	d.
AB313	Hfr	*thi thr leu lacZ str* (λ)	e. CGSC313
AB312	Hfr	*thi thr leu lacZ str* λ^-	e. CGSC312
AB259	HfrH	*thi rel* λ^-	e. CGSC259
P980	HfrH	*thi rel tra* λ^-	AB259
P969	HfrH	*thi rel* (λ)	AB259
P332	HfrH	as AB259 but *tsx*	AB259
P333	HfrH	as P980 but *tsx*	P980
BW40	F^-	as AB1157 but *uvrB tdk*	f.
PA309	F^-	*thr leu thi his arg lacY str trp* (λ)	g.
P948	F^-	as PA309 but *tdk* trp^+	BW40 $\xrightarrow{P1kc}$ PA309
AB261	HfrP4X	*metB* (λ)	e. CGSC261
P729	HfrP4X	*metB tra* (λ)	AB261
P726	HfrP4X	*metB tsx* (λ)	AB261
P727	HfrP4X	*metB tsx tdk* (λ)	P726
P766	HfrP4X	*metB tsx tdk tra* (λ)	P727
Ra-2	Hfr	*sfa-4* (*sfa-5*) *supE* λ^r *mal*	e. CGSC4241
B7	Hfr	*metB* λ^r λ^- *rel*	e. CGSC4237
P722	HfrC	*met* (λ)	h.
P721	HfrC	*met tra* (λ)	h.

TABLE 2-1 Continued

KL20	F^+	thi rel λ^-	i.
KL16	Hfr	as KL20	e. CGSC4245
KL84	Hfr	as KL20	e. CGSC4238 (may have point of origin as P4X)
KL96	Hfr	as KL20	e. CGSC4243
KL99	Hfr	as KL20 but lac	i.
KL174	Hfr	as KL20 but his	i.
P315	Hfr	as KL174 but str	KL174
KL175	F^+	as KL20 but his	i.
P314	F^+	as KL175 but str	KL175
KL182	F^-	as KL20	i.
KL184	F^-	as KL182 but str	i.
P348	F^+	as AB1133	KL20 x AB1133
P356	F^+	as P882	KL20 x P882
χ57	HfrH	thi λ^-	j.
χ15	F^+	prototroph λ^-	j.
χ493	HfrOR11	prototroph λ^-	j.
χ503	HfrOR21	prototroph λ^-	j.
χ289	F^-	prototroph λ^-	j.
χ545	F^-	proB-lac^{del} str λ^-	j.
χ818	F'ORF-207	Flac/proB-lac^{del} λ^-	j.
χ573	F'ORF-4	Flac proC purE/lac-purE^{del} λ^- ser/gly	j.
χ517	F'ORF-1	Flac proC purE/lac-purE^{del} λ^-	j.
χ594	F'ORF-1	as χ517	j.
χ985	F^-	thr pyrA lacY proC tsx purE λ^- pdxC pyrC trp his str ilv met	j.

TABLE 2-1 Continued

χ42	F^+	his (λ)	j.
χ209	F^+	prototroph λ^-	j.
χ1088	F^-	thr leu pro lac tsx trp his lys str xyl ilv	j.
P347	F^+	as AB1133	χ209 x AB1133
P357	F^+	as P882	χ209 x P882
P350	donor	as P107	$P324^{(v)}$ x P107
P353	donor	as P882	P324 x P882
P601	F'	Fgal/met (λ)	
P995	F'	Flac/thi lac^{del}	k. RV/Flac(iii)
CA241	HfrH	thi lac^{del} λ^-	l.
P311	HfrH	thi lac^{del} (λ)	CA241
P310	HfrH	thi lac^{del} tra (λ)	P311
102	F^-	thi thr leu lac λ^- str	m.
342	F^-	as 102 but (P1)	m.
JC3272	F^-	his trp lys tsx lac mal gal str λ^r λ^{imm}	n.
JC6583	F'JCFL0	Flac/trp lac	n.
P373	F'JCFL0	Flac/as JC3272	JC6583xJC3272
JC6608	F'JCFL1	Flac traA1/trp lac supD	n.
P371	F'JCFL1	Flac traA1/as JC3272	JC6608xJC3272
M156	F'JCFL90	Flac traJ90/trp lac supD	n.
P372	F'JCFL90	Flac traJ90/as JC3272	M156xJC3272
ED2177	F'EDFL33	Flac $tra^+(Sex^-)$/as JC3272	n.
JC6536	F'JCFL4	Flac tra-4/trp lac supD	n.
P370	F'JCFL4	Flac tra-4/as JC3272	JC6536xJC3272
E267	F^-	his trp proA lac gal tsx str	n.
ED381	F^-	lac^{del} gal tsx	n.

TABLE 2-1 Continued

KI534	Hfr	tsx	n.
KI529	Hfr	bio uvrB tsx	n.
D11	F^-	trp pro his str tsx (λ)	o.
D21	F^-	as D11 but ampA1	o.
D31	F^-	as D21 + unmapped (Amp^r-III)	o.
D31m4	F^-	as D31 + unmapped (ϕW^r)	o.
ASH10	F^-	thi met thy leu lac str (λ)	p.
ASH102	F^-	as ASH10 but CetB	p.
A-9	F^-	as AB1133 but tolA-9	q.
P-305	F^-	as AB1133 but tolP-305	q.
P-507	F^-	as AB1133 but tolP-507	q.
P400	F^-	as AB1133 but non-9 his^+	r.

(i) The abbreviations and nomenclature are essentially that of Demerec, Adelberg, Clark, and Hartman (1966) and Taylor and Trotter (1972). The terminology for episomal mutations has been described by Achtman et al. (1971).

(ii) Strains were kindly provided by:

a, R. Clowes; b, A.L. Taylor; c, P. Howard-Flanders; d, A.J. Clark; e, B.Bachmann, Coli Genetic Stock Center (CGSC) numbers shown ;. f, B. Wilkins; g, R. Nagel de Zwaig; h, E. Meynell; i, K. Brooks Low; j, R. Curtiss III; k, M. Malamy; l, J. Scaife; m, W. Arber; n, N. Willetts; o, D. Monner; p, I.B. Holland; q, B. Rolfe; r, R. Hancock.

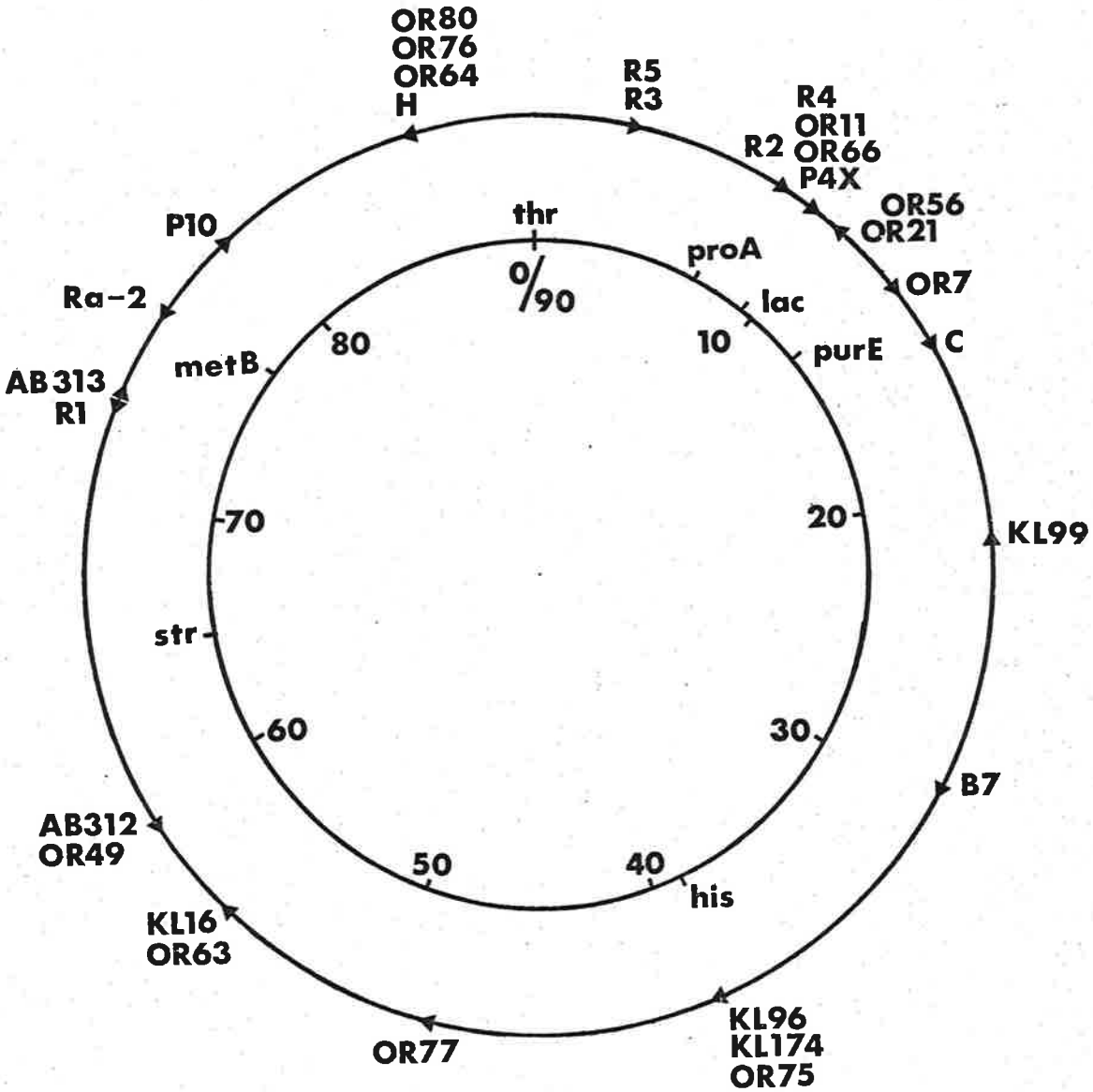
(iii) Synonym

(iv) The symbol thyR is employed for a low level thymine requiring strain when it is not known whether the locus affected is drm or dra (Bachmann, 1972).

(v) P324 is described in Table 6-1.

FIG. 2-1

Chromosome map of *E. coli* K-12. Arrows indicate origins and direction of transfer of Hfr donors used [from Taylor and Trotter (1967), Curtiss and Stallions (1969), Stallions and Curtiss (1971), Low (1972), and from Coli Genetic Stock Center information supplied by B. Bachmann].


OR80
OR76
OR64
H
R5
R3
R4
OR11
OR66
P4X
R2
OR56
OR21
OR7
C
KL99
B7
KL96
KL174
OR75
OR77
KL16
OR63
AB312
OR49
AB313
R1
Ra-2
P10
thr
0/90
proA
lac
purE
10
20
30
40
his
50
60
str
70
metB
80

dried cultures and working stocks were maintained on nutrient agar slopes at 4C.

RC748 (Clowes, 1963), a W1 recombinant isolated by Gross (1963b), was recovered as Met$^-$, having apparently lost proline auxotrophy (P513), and a proA marker was introduced from AB1133 by P1kc transduction to give P882. Strain P978 is a low level thymine (1μg/ml)-requiring mutant of strain P882, selected, after the introduction of a thyA mutation by the method of Stacey and Simson (1965). Strain P322 was selected as a lactose-negative colony on tetrazolium agar after P1kc transduction of a LacY mutation from strain PA309 to strain P882. The absence of the lactose permease was confirmed by the inability to utilize the α-galactoside melibiose at 42C (Prestige and Pardee, 1965). The strain produced normal levels of β-galactosidase on induction.

The transfer-defective (Tra$^-$) mutants, P310, P729, P766, and P980 were selected essentially as described by Cooke, Meynell and Lawn (1970). Approximately 10^9 male-specific (MS2) phage were spotted onto a culture of the parental Hfr strain which had been spread on a nutrient agar plate. After overnight incubation, colonies were selected from the zones of inhibition, purified and tested for resistance to MS2 phage. The presence of the F factor in these Tra$^-$ mutants was determined by ability to give recombinants for proximal markers at low frequency (10^{-5} to 10^{-7} per cell) and for continued resistance to bacteriophage T3 (Schell et al., 1963). These mutants were also resistant to the male-specific phage fd, and lacked F pili (by electron-microscopy).

The strains P370, P371, and P372, carrying Flac episomes with

amber-suppressible tra mutations, were selected by transfer of the episome indicated from an Su^+ host into the F^-/Su^- strain JC3272.

λ lysogens of various strains were obtained by plating approximately 10^2 λ phage with 0.2 ml of a bacterial culture in a 3 ml overlay of "soft agar" (equal parts nutrient broth and nutrient agar held at 44C). After overnight incubation, suspected lysogens were picked from the centre of turbid plaques and purified. Colonies resistant to λ phage but sensitive to λ vir were assumed to be λ lysogens.

Streptomycin-resistant mutants were selected by the liquid enrichment method described by Meynell and Meynell (1970). Tsx mutants were selected directly against phage T6 and tested for resistance to colicin K.

Strain P727 was derived as follows. A trp marker from strain PA309 was transferred by P1kc transduction to a T6-resistant mutant (P726) of strain AB261. The tdk-1 allele (Hiraga, Igarashi and Yura, 1967; Igarashi, Hiraga and Yura, 1967) was cotransduced with trp^+ from strain BW40 into this trp mutant. Selection of Tdk^- cells was based on their inability to utilize thymidine in the presence of 5-fluorouracil (Hiraga et al., 1967). The absence of deoxythymidine kinase (EC2.7.1.21) was confirmed by the inability of Tdk^- cells to incorporate ^{3}H-thymidine to levels greater than 0.1% of the incorporation by Tdk^+ strains. Strain P948, derived from PA309, was also selected following transfer of the tdk mutation.

BACTERIOPHAGE STRAINS

Male-specific phages used were MS2 (Davis, Strauss and Sinsheimer, 1961), kindly supplied by E. Meynell and fd (Marvin and Hoffmann-Berling, 1963), kindly supplied by R. Hull. The female-specific phages used were T7 (Dettori et al., 1961; Mäkelä et al., 1964), ØII-T (Morrison and Malamy, 1971), W-31 (Watanabe and Okada, 1964), and T3 (Schell et al., 1963) kindly supplied by R. Hancock, and ØII, ØW, and Ø3 (Monner, Jonsson and Boman, 1971), kindly supplied by D. Monner. T6 (Demerec and Fano, 1945) was a strain held in this laboratory. These bacteriophages were either used as supplied or propagated by the agar plate method (Adams, 1959). It should be noted that both ØII and ØII-T were samples of the ØII described by Cuzin (1965), whereas ØW was a sample of the original ØII described by Wollman (1947).

λ phage was obtained by ultraviolet (UV) induction of wild type E. coli K12, and λ vir (Jacob and Wollman, 1954) was kindly provided by W. Woods.

PREPARATION OF BACTERIAL CULTURES

All cultures were incubated at 37C, the temperature used in all experiments. Nonaerated overnight (16 to 20 hr) cultures were obtained by subculturing, from a slope or single colony, to 10ml of media (nutrient broth unless otherwise indicated). These generally had an optical density (OD) at 650nm between 0.5 and 0.8. Overnight (20 hr) stationary-phase cultures were inoculated in a similar way (10ml

volumes in 100ml flasks or bottles) and aerated by agitation on a reciprocating shaker (120 strokes/min; 32mm amplitude). These had an OD_{650} between 2.0 and 3.5 depending upon the media employed. Early stationary-phase cultures were prepared by dilution (5- to 10-fold) of a nonaerated overnight culture, followed by incubation with aeration for 4 to 5 hr and then had an OD_{650} of 1.5 to 2.5 depending on the medium employed.

Logarithmic cultures were prepared by diluting an overnight nonaerated culture 50- to 100-fold in fresh media (50ml in 100ml bottles) and then aerating with a reciprocating shaker as above. Early logarithmic cultures were collected at 2.5 to 3 hours ($OD_{650} \sim 0.3$ and a viable count of about 2×10^8 cells/ml) and late logarithmic cultures were taken at OD_{650} between 1.0 and 1.5 after 4 to 5 hr of incubation. All cultures, where required, were diluted immediately prior to use with fresh prewarmed media to an OD_{650} of 0.2 to 0.3 and then had a viable count of about 2×10^8 cells/ml.

BACTERIOPHAGE METHODS

Sensitivity to bacteriophages was determined by cross-streaking, by spot tests, or by plaque assay. Spot tests were performed as follows. A 0.2 ml amount of a nonaerated overnight culture of the bacterial strain under test was added to 3ml of soft agar and poured onto a nutrient agar plate. After the agar had set, 0.01ml volumes of

appropriate dilutions of the phage to be tested were spotted on and the plates incubated overnight at 37C. Efficiency of plating (EOP) was determined by mixing 0.2ml of a nonaerated overnight (or logarithmic-phase) culture of the bacterial strain to be tested with 0.1ml of an appropriate phage dilution in 3ml of soft agar and pouring onto a nutrient agar plate. Plaques were examined and counted after incubation at 37C overnight.

MATING PROCEDURES

Liquid media matings, for transfer of F and examination of donor or recipient ability, were carried out by mixing equal volumes (1ml) of logarithmic-phase cultures of donor and recipient strains. After 60 min incubation with gentle agitation, samples of the mating mixture were mechanically agitated, with a "Whirlimixer" (Fisons Scientific Apparatus Ltd.), suitably diluted, and plated on selective media.

Surface exclusion was measured by using the test strain as recipient and selecting for $His^{+}Str^{r}$ recombinants in crosses with the Hfr strain KL96, which transfers $\underline{his}^{+}$ proximally. Equal volumes (1ml) of logarithmic-phase cultures of KL96 and either logarithmic-phase or diluted stationary-phase cultures of the recipient were mixed, incubated for 60 min, and samples were plated as described above.

The frequency of mating pair formation was estimated essentially as described by de Haan and Gross (1962). Equal volumes (1ml) of logarithmic-phase cultures, of the strains under test, were mixed and incubated with gentle agitation. After 5 min for pair formation, 0.05ml of the mating

culture was carefully withdrawn and added to 25ml of prewarmed minimal liquid medium supplemented with glucose and nutrient broth (1% v/v). Incubation was continued with gentle agitation and samples were removed, vigorously agitated, and plated on selective media.

In all matings, samples were diluted in minimal liquid medium supplemented with glucose and either 0.04% (w/v) casein hydrolysate or 1% (v/v) nutrient broth. The inclusion of supplements in the dilution medium allowed traces of nutrient broth or amino acids to be plated with the samples on glucose minimal agar and so limited metabolic imbalance which could lead to reduced recombinant yields (Gross, 1963a).

STANDARD CONDITIONS FOR LETHAL ZYGOSIS

The following conditions were used unless otherwise stated. A stationary-phase culture of the F^- strain (or other streptomycin-resistant strain under test) was diluted with fresh media to about 2×10^8 cells/ml. A 0.25ml amount of this was mixed with an early logarithmic-phase culture of a streptomycin-sensitive strain, and media where required, to give the desired ratio (usually 20:1) in a final volume of 5ml. The mixed culture, in a 100ml flask or bottle, was gently aerated with a reciprocating water bath shaker (60 strokes/min: 20mm amplitude). The F^- strain viable count at commencement was about 1×10^7 cells/ml, apart from the exceptions noted.

At intervals, 0.1ml samples were diluted in supplemented minimal liquid and vigorously agitated with a "Whirlimixer" to separate mating

pairs. Viable counts were estimated by adding 0.1ml volumes of suitably diluted samples to a 3ml overlay of soft agar and pouring onto nutrient agar plates. The plates were incubated overnight at 37C. In the case of survivor viable counts, streptomycin (100µg/ml) was added to the overlay to prevent growth of streptomycin-sensitive strains, or in some cases contraselection against the Hfr strain was made on selective minimal agar without streptomycin. Survivors and total cell counts are expressed as a percentage of the input number of the respective cell type. Viable counts were similar whether dilutions were performed in nutrient broth or the dilution media described above.

Recombination frequency was estimated by plating, immediately following agitation, 0.1ml volumes of the diluted samples on selective media and incubating for 24 to 36 hr at 37C. Since the F^- strain was present at approximately the same initial titre in all experiments described, recombinants are expressed as a percentage of input F^- cells.

LETHAL ZYGOSIS ON SOLID MEDIA

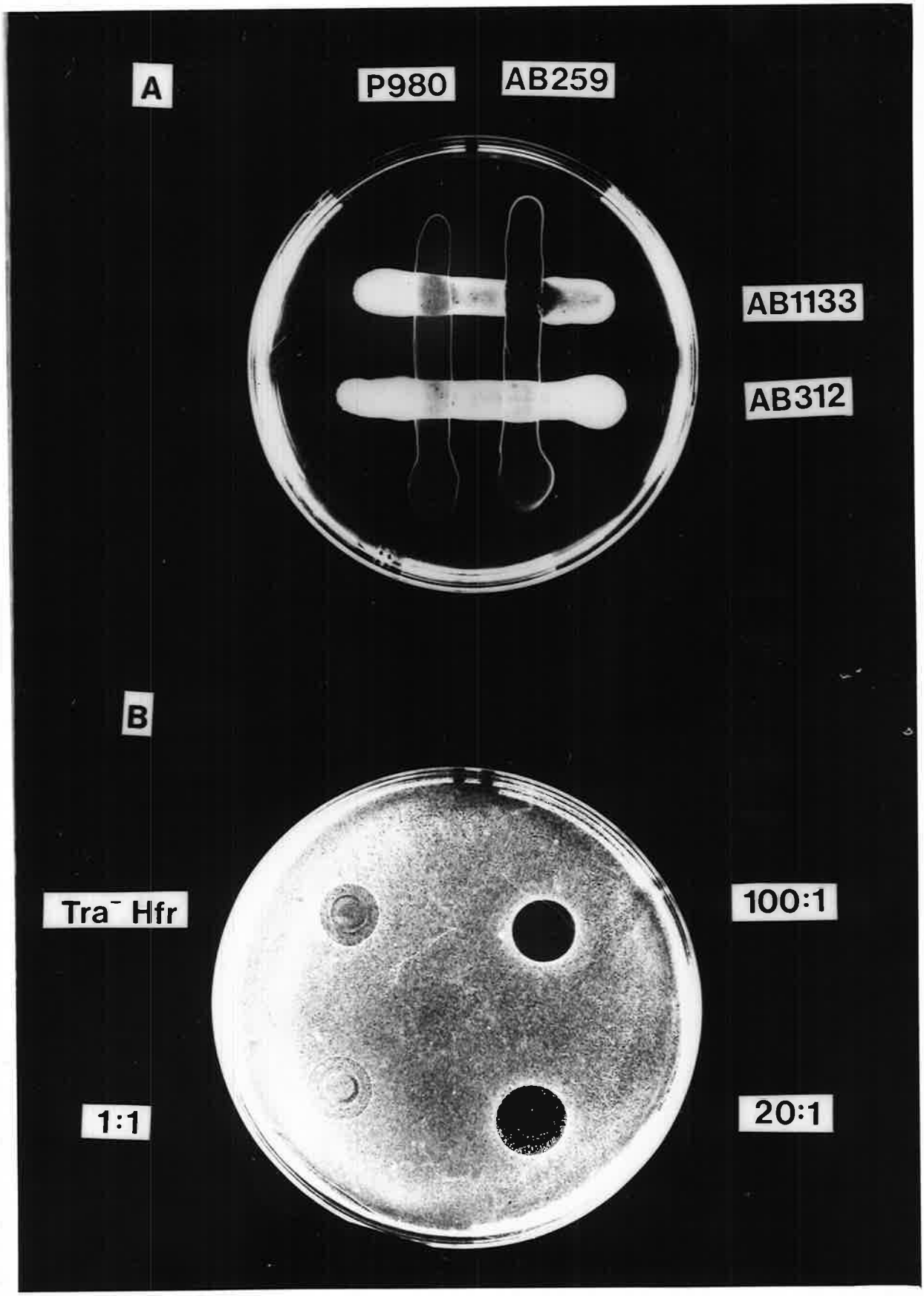
The cross-streak method (Fig. 2-2A) was as follows: volumes (0.1ml) of overnight nonaerated cultures of streptomycin-sensitive strains were streaked on nutrient agar plates and allowed to dry. A 1:100 dilution of a culture of a streptomycin-resistant strain was then streaked across this at right angles and allowed to dry. The plate was incubated at 37C for 2.5 to 3 hr and then sprayed (De Vilbiss atomizer - 15) with streptomycin at 10^4 units/ml to prevent further growth of the

FIG. 2-2

Lethal zygosis on solid media with AB259, a streptomycin-sensitive HfrH strain, and the streptomycin-resistant F^- strain AB1133.

A. Cross-streak method (see text). Controls included were the Tra^- mutant P980 which did not produce inhibition, and the Hfr strain AB312, which, like other donors, was insensitive to the phenomenon.

B. Spot test. A 1-100 dilution (about 5×10^6 cells/ml) of an nonaerated overnight culture of AB1133 was spread onto the surface of a nutrient agar plate and allowed to dry. A small volume (0.02ml) of a nonaerated overnight (about 5×10^8 cells/ml) culture of the Hfr strain AB259 (or the Tra^- Hfr strain P980) was spotted directly onto this lawn, to give a Hfr:F^- ratio of approximately 100:1. The Hfr culture was also diluted 1 in 5 and 1 in 100, then spotted, to give ratios of 20:1 and 1:1, respectively.


A
P980
AB259
AB1133
AB312
B
Tra⁻ Hfr
100:1
1:1
20:1

streptomycin-sensitive strain under test. After a further 18hr of incubation, a zone of inhibition was observed if the streptomycin-sensitive strain could kill the other by lethal zygosis. Levels of inhibition were distinguished as: ++, complete inhibition; +, inhibition with slight growth; -, no inhibition.

An alternative method for demonstrating lethal zygosis on solid media (Fig. 2-2B) was to spot cultures of streptomycin-sensitive strains directly onto a diluted culture of a streptomycin-resistant strain that had been spread on a nutrient agar plate and to incubate and streptomycin treat as described for cross streaking. This test, in addition to providing a good method for examination of a large number of donor strains, could be adopted for quantitative comparisons by using dilutions of Hfr cultures.

ACRIDINE ORANGE CURING

The technique was essentially that described by Hirota (1960), except that the medium employed was tryptone yeast extract broth adjusted to pH7.6. An initial inoculum of 10^2 to 10^3 cells/ml was incubated (24 hr) in the presence or absence acridine orange (50μg/ml). Survivors were plated on nutrient agar and 20 to 100 from each tube were examined for auxotrophic characteristics, sensitivity to male-specific phage, and sensitivity to the HfrAB259 by the lethal zygosis plate test.

FILTRATES AND OTHER PREPARATIONS FROM Hfr CELLS

Filtrates were prepared from early logarithmic-phase cultures by using a Sartorius membrane filter (SM11306; pore size, 0.45μm). The cells on the membrane were resuspended gently and adjusted to the desired volume with fresh medium. A sample at 60 min from a standard liquid lethal zygosis test was filtered as described above to provide a lethal zygosis filtrate.

Supernatant fluids were prepared by centrifugation (5,000 x g, 20 min) of early logarithmic-phase cultures, removing the upper fraction only to reduce pellet resuspension (viable count was about 5×10^5 cells/ml). Streptomycin-treated cells were logarithmic-phase Hfr cultures incubated for 60 min at 37C in the presence of streptomycin (1,000μg/ml), centrifuged, washed once, and resuspended in fresh nutrient broth. Heat-treated cells were incubated at 56C for 60 min. All filtrates etc. were used on the day of preparation.

MATING CHAMBER STUDIES

A cylindrical perspex chamber (3.5cm diameter by 2.6cm width) was divided by a Sartorius membrane filter (SM11306; pore size 0.45μm). To one side of the chamber, 5ml of an early logarithmic-phase donor culture (about 2×10^8 cells/ml) was added while the other contained 5ml of a diluted stationary-phase recipient culture (about 1×10^7 cells/ml). The chamber was agitated at 37C in a shaking water bath, and samples were removed at intervals for survivor and recombinant counts.

INCORPORATION OF ^{3}H-THYMINE AND ^{3}H-THYMIDINE

Glucose-minimal liquid medium supplemented with 0.2% (w/v) casein hydrolysate, tryptophan, and growth requirements was employed for prior growth of donor and recipient cultures, and during mating. With Thy^{-} strains the mating mixture was supplemented with thymine (final concentration of 1μg/ml). ^{3}H-thymine or ^{3}H-thymidine (Radiochemical Centre, Amersham, England) was added to give 5μCi/μg/ml or 0.5μCi/ml, respectively. Duplicate samples (0.1ml) of the culture were collected on 2cm discs of glass-fibre paper (Whatman GF83) and the extent of ^{3}H incorporation into acid insoluble material was estimated as described by Hull and Reeves (1971).

INDUCTION OF β-GALACTOSIDASE

Two media were employed for the study of the induction of β-galactosidase (EC3.2.1.23) in recipient cells. In the first experiments, cells were grown and mated in minimal liquid medium supplemented with casein hydrolysate (0.2%, w/v), tryptophan, and growth requirements of the strain involved. Glycerol (0.4%, v/v) was used as a carbon source to limit catabolite repression.

In later experiments, tryptone yeast extract broth was employed for the growth of early stationary-phase F^{-} cultures and for logarithmic-phase donor cells. When F^{-} cells were diluted into fresh broth a lag in induction was observed which was less marked when dilution was into a 20-fold excess of Tra^{-} Hfr cells or into preconditioned broth. This lag

was probably associated with a utilizable substrate which gave catabolite repression of induction. Therefore, prior to mating, all cells were centrifuged and resuspended in preconditioned tryptone yeast extract broth (see above) to ensure uniformity of preconditioning.

Induction in both media was by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

ASSAY FOR β-GALACTOSIDASE ACTIVITY

Samples (1.0ml) were removed at intervals into 4.0ml of ice-cold sodium phosphate buffer (pH6.8, 0.03M) containing 0.05ml of toluene. The samples were incubated at 37C for 30 min with occasional vigorous agitation and assayed for β-galactosidase activity as follows: 1.0ml of orthonitrophenyl-β-D-galactopyranoside (ONPG) at 1mg/ml in phosphate buffer was added, and incubation at 37C was continued until a colour developed approximating that of 10^{-4}M orthonitrophenol (ONP). The reaction was stopped by the addition of 1.0ml of 1M sodium carbonate, and the period of incubation was noted. The optical density was measured at 420nm, and a correction for turbidity was applied (Pardee, Jacob and Monod, 1959). One unit of β-galactosidase activity was defined as the amount of enzyme that liberated 1 nanomole of ONP per min.

UPTAKE OF TMG

^{14}C-methyl-thio-β-D-galactopyranoside (^{14}C-TMG; New England Nuclear Corp., Boston, U.S.A., NEC316) was added to the mating mixture, and samples (0.25ml) were removed and rapidly filtered in a prewarmed membrane-

filter apparatus (HA filter, 0.45μm pore size; 35mm in diameter; Millipore Corp., Boston, U.S.A.). The sample was washed with three 5ml volumes of tryptone yeast extract broth (37C). The operation was completed in 20 sec. The filtration and washing were performed at 37C to avoid rapid loss of accumulated substrate (Leder, 1972). The filters were dried and added to 5ml of scintillation fluid (toluene, 1 litre; 2, 5-diphenyloxazole, 4g; 1, 4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, 0.5g) and counted in a Packard liquid scintillation counter.

ONPG HYDROLYSIS

ONPG hydrolysis was used as a measure of ONPG transport with intact cells. ONPG was added to the mating mixture (3×10^{-3}M final concentration) and, at intervals, 2.5ml samples were added to 0.5ml of ice cold 1M sodium carbonate to stop the reaction. In an alternative method, samples (2.5ml) of mating culture were added to 0.2ml of ONPG (3×10^{-2}M) and incubated (37C) for 10 min. The reaction was stopped by the addition of 0.5ml of ice-cold 1M sodium carbonate. With both methods, the ONP produced was determined as follows: the majority of cells were lysed by the addition of 1 drop of 20% sodium dodecyl sulphate, the absorbance was measured at 420nm, and corrections were made for turbidity (Pardee et al., 1959).

Toluene treatment of cultures was as follows: toluene (0.01ml/ml) was added to broth and incubated at 37C for 30 min in a stoppered flask. F^- cells were suitably diluted into this and incubated with agitation for

10 min.

ELECTRON MICROSCOPY OF CONJUGATION

An early logarithmic-phase culture of the F^- strain AB1133, at about 5 x 10^8 cells/ml, was mixed with a similar culture of the HfrH $T6^r$ strain P332 (or the Tra^-Hfr strain P333), and media where required, to give the desired Hfr:F^- ratio (a range of 1:5 to 5:1 was covered). The F^- cell concentration was about 1 x 10^8 cells/ml in all experiments, and tryptone yeast extract broth was used throughout. The mating mixture was gently agitated in a "Gyrotory Water Bath" (New Brunswick Scientific Co., Inc., U.S.A.) and samples were taken, from 2 min after mixing, and MS2 or T6 phage was added to give a final concentration of 10^{11} pfu/ml or 10^9 pfu/ml, respectively. When T6 was employed, KCN (0.025M final concentration) was also added to the sample to limit lysis. After allowing 2 min at 37C for phage adsorption, specimens were prepared immediately by adding drops of the phage labelled mating culture to a carbon-coated grid, allowing 2 min for cells to settle, washing with KCL solution (0.1M), and staining with 1% (w/v) uranyl acetate. The specimens were then examined with a Siemens Elmiskop 1 electron microscope.

Grid preparation, mounting, staining, and electron microscopy were skillfully performed by Miss P. Dyer, The Department of Biochemistry, The University of Adelaide.

PREPARATION OF CELL ENVELOPE FRACTIONS AND TRITON X-100 EXTRACTION

Procedures employed for the preparation of envelope fractions and Triton extraction, were essentially as described by Schnaitman (1970, 1971a). Early logarithmic-phase cultures grown in nutrient broth, were harvested by centrifugation (10,000 x g, 10 min) and suspended in 0.05M tris (hydroxymethyl) aminomethane (Tris) buffer (pH7.8). The cells were ruptured by passage (2X) through a French pressure cell and the cell debris removed from the suspension by centrifugation (5,000 x g, 15 min). The supernatant fluid was made 2mM in Mg^{2+} (with $MgCl_2$) and then centrifuged (78,640 x g, 60 min) again. The pellet was suspended in Tris buffer + Mg^{2+} (as above), and centrifugation (78,640 x g, 60 min) was repeated. The final pellet was resuspended in deionized distilled water and the protein concentration of this whole envelope fraction was determined by the method of Schacterle and Pollack (1973).

The Triton-insoluble envelope fraction was prepared as follows. A sample of the unfractionated envelope preparation, at a concentration of approximately 10mg/ml in 10mM N-2-hydroxyethylpiperazine-N'-2'-ethane sulphonic acid (HEPES) buffer (pH7.4), was mixed with an equal volume of 4% Triton X-100 in the same buffer. After incubation at 18C for 20 min, the suspension was centrifuged (150,000 x g, 60 min) and the pellet containing the Triton-insoluble fraction of the envelope was suspended

in deionized distilled water, to give approximately the same protein concentration as the whole envelope preparation. Both preparations were stored at 4C.

SOLUBILIZATION AND POLYACRYLAMIDE GEL ELECTROPHORESIS OF ENVELOPE PROTEINS

The solubilization of cell envelope proteins and the subsequent analysis of those proteins by gel electrophoresis was carried out essentially as described by Neville (1971). Samples of whole envelope preparations or Triton-insoluble extracts were made 50mM in sodium carbonate, and sodium dodecyl sulphate (SDS) was added at 4mg/mg of protein, followed by the addition of 10% by volume β-mercaptoethanol. The maximum time for this whole step was 2 min. This solubilized protein preparation was diluted to 4µg protein/µl in upper gel buffer (Neville, 1971) + sucrose (2%) + bromophenol blue (0.02%) (the latter component acted as a tracking dye); samples containing up to 200µg protein were loaded onto gels (10 x 0.6cm) with a Hamilton microsyringe. The gels, in a SDS borate-sulphate discontinuous buffer system (Neville, 1971), were run at 0.5-1.0mA per tube at 25C until the sample entered the upper gel then the current was increased to 1.5mA tube. After electrophoresis, the gels were stained for 16-20 hr with 0.02% (w/v) Coomassie brilliant blue in fixative (methanol 50%, acetic acid 7.5%, water 42.5%), then destained with an aqueous solution containing 20% methanol and 7.5% acetic acid. The stained protein bands were recorded by photography and by densitometry (Joyce-Loebel Chromoscan MKII).

CHAPTER 3

CHARACTERIZATION OF LETHAL ZYGOSIS ASSOCIATED WITH CONJUGATION IN ESCHERICHIA COLI K-12

INTRODUCTION

Studies of conjugation in Escherichia coli usually make use of mating cultures in which donor cells are mixed with an excess of recipient cells, the ratios ranging from one male to twenty females (0.05:1) up to one to one (1:1). If, however, recipient cells are mixed with an excess of Hfr cells (20:1), there may follow a lethal event which results in a decrease in the number of F^- survivors. This phenomenon, first described by Alfoldi et al. (1957), was named lethal zygosis, and further reports extended and clarified the nature of the effect (Alfoldi et al., 1958; Nagel de Zwaig et al., 1962; Ben-Gurion, 1963).

As the Hfr concentration was increased, the yield of recombinants (Gross, 1963b) and the survival of F^- cells (Clowes, 1963) decreased. These observations led both authors to suggest that the lethality observed could have resulted from multiple conjugation involving simultaneous mating between one F^- cell and several Hfr cells. As pointed out in Chapter one, there have been comments on the phenomenon, but no further detailed studies have been reported.

Characterization of lethal zygosis was therefore undertaken, and in this chapter conditions required for a high and reproducible lethal effect are described, as are the changes brought about when the parameters involved are altered.

DEVELOPMENT OF CONDITIONS FOR LETHALITY

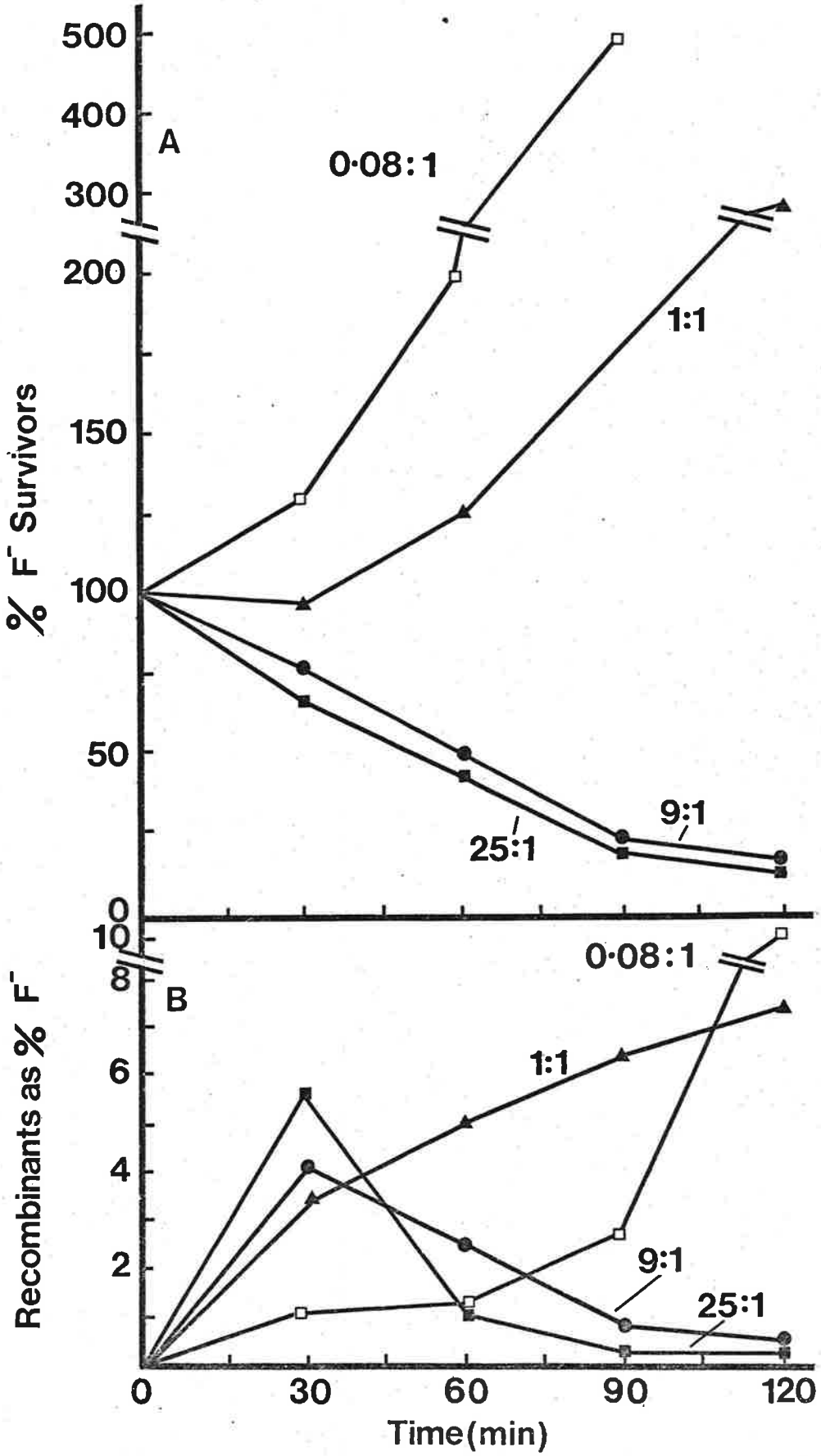
Initially, experiments were based on the conditions commonly employed in this laboratory for conjugation. Aerated logarithmic-phase donor cells and overnight nonaerated cultures of recipient cells, both adjusted to approximately 2 x 10^8 cells/ml by dilution with fresh pre-warmed broth, were mixed at the desired ratio (usually 20:1) in flasks or bottles maintained at 37C. The results obtained with a series of F^- and Hfr strains did not, in general, show any decrease in F^- cell viable count, the most common observation being complete inhibition of recipient growth. The only exception involved P882, an F^- strain which was killed by some Hfr strains, but to an extent which varied between 10 to 100% survivors from day to day. Similar results have been reported for other recipients (Nagel de Zwaig et al., 1962; Clowes, 1963).

Once continued aeration had been shown to be a requirement for consistent lethality (see below), a number of experiments were performed using overnight nonaerated F^- cultures. One such experiment (Fig. 3-1A) confirmed the effect of progressively increasing the number of Hfr cells per F^- cell, and, although rate and final extent of kill were much lower than found by Clowes (1963), it is interesting to note the inhibition of F^- growth at a ratio of 1:1 compared to 0.08:1. The results in Fig. 3-1B show that with the high ratio of 25:1 there was initially a good yield of recombinants; this value reached a peak and then fell, an effect not observed at the ratio of 1:1 or lower.

Further experiments showed that growth conditions of both F^- and

FIG. 3-1

Effect of concentration of RC759 (HfrP4X) on survival of the F^- strain P882 (A) and on Pro^+Str^r recombinant formation (B). With the exception of overnight nonaerated F^- cultures, standard lethal zygosis conditions were employed (see Materials and Methods). The figures (1:1) indicate the ratio of Hfr cells to F^- cells at commencement.


500
400
A
300
200
150
100
50
0
% F⁻ Survivors
0·08:1
1:1
9:1
25:1
10
8
B
6
4
2
0
Recombinants as % F⁻
0·08:1
1:1
9:1
25:1
0
30
60
90
120
Time(min)

Hfr cultures prior to mixing were critical in obtaining rapid lethal effects, and led to the adoption of the standard conditions as described in Materials and Methods. These conditions were employed in some of the following studies to examine the effect of varying single parameters, while otherwise maintaining optimum conditions.

As a control for physiological and genetic studies of conjugation and lethal zygosis, Tra^- (transfer-defective) mutants of the Hfr under examination were employed where possible. This was preferred to an F^- x F^- control, as the Tra^-Hfr had a similar growth rate to its parent (Fig. 3-2), and therefore any metabolic changes in the F^- cells, due to competition for nutrients with a large number of growing Hfr cells, would be similar. The Tra^-Hfr cells did not appear to affect the F^- cells in any way, for the same lag was observed when stationary phase F^- cultures were diluted into a Tra^-Hfr culture or into fresh media. Also, as reported in Chapter 5, recipient cell DNA synthesis (as measured by incorporation of ^{3}H-thymine) and induction of β-galactosidase were unchanged in the presence of large numbers of Tra^-Hfr cells.

Both liquid and solid media tests (Materials and Methods) made use of streptomycin to prevent the growth of one of the cell types; however, the extent of lethality observed was equal, or greater, when contra-selection was on the basis of auxotrophy. These results, together with those in the next chapter (Table 4-2), show that transfer of streptomycin sensitivity, and the expression of that sensitivity in a recipient, was not responsible for lethal zygosis. The plating of samples for F^- viable

counts on supplemented minimal agar (with or without streptomycin) increased the apparent extent of kill over that observed with nutrient agar, which was commonly employed. This suggests that some damaged cells could go on to survive under more favourable nutrient conditions.

AERATION AND LETHALITY

In the initial series of experiments, mating cultures were aerated intermittently by gentle swirling or by bubbling air. It was only when continously aerated cultures were examined and found to show higher lethality that it became apparent that aeration affected the extent of kill. Fig. 3-2 shows the effect of varying aeration conditions during lethal zygosis using otherwise standard conditions. With the continuously aerated culture there was a rapid and extensive kill: 5% survivors at 120 min compared with 60% survivors under poor aeration. The total cell count (Fig. 3-2B), which may be regarded as an indication of Hfr or Tra$^-$ Hfr growth, increased eight- to nine-fold when aerated, compared with an approximate two- to three-fold increase with poor aeration. In this experiment, as in others, lethal zygosis was accompanied by an initial rise and then a fall in the number of recombinants (Fig. 3-2C).

The results of an experiment in which mating cells were aerated for various times after mixing are shown in Table 3-1. No attempt was made to interrupt pairs previously formed or to prevent further pair formation throughout the experiment. The results show that even though aeration was stopped at a given time there was further killing but to a lesser

FIG. 3-2

Effect of aeration on lethal zygosis. AB261 (HfrP4X) and the recipient P882 were mixed at a ratio of 20:1 under standard conditions for lethal zygosis and aeration as described in Materials and Methods (■). Nonaerated culture conditions were identical except that flasks stood in a 37C water bath and received agitation only at the time of sampling (□). Samples were diluted and plated for survival of F^- cells (A), total cell count (B), and Pro^+Str^r recombinants (C). Controls with the Tra^- mutant P729 were included (aerated, ▲ ; non-aerated, △).

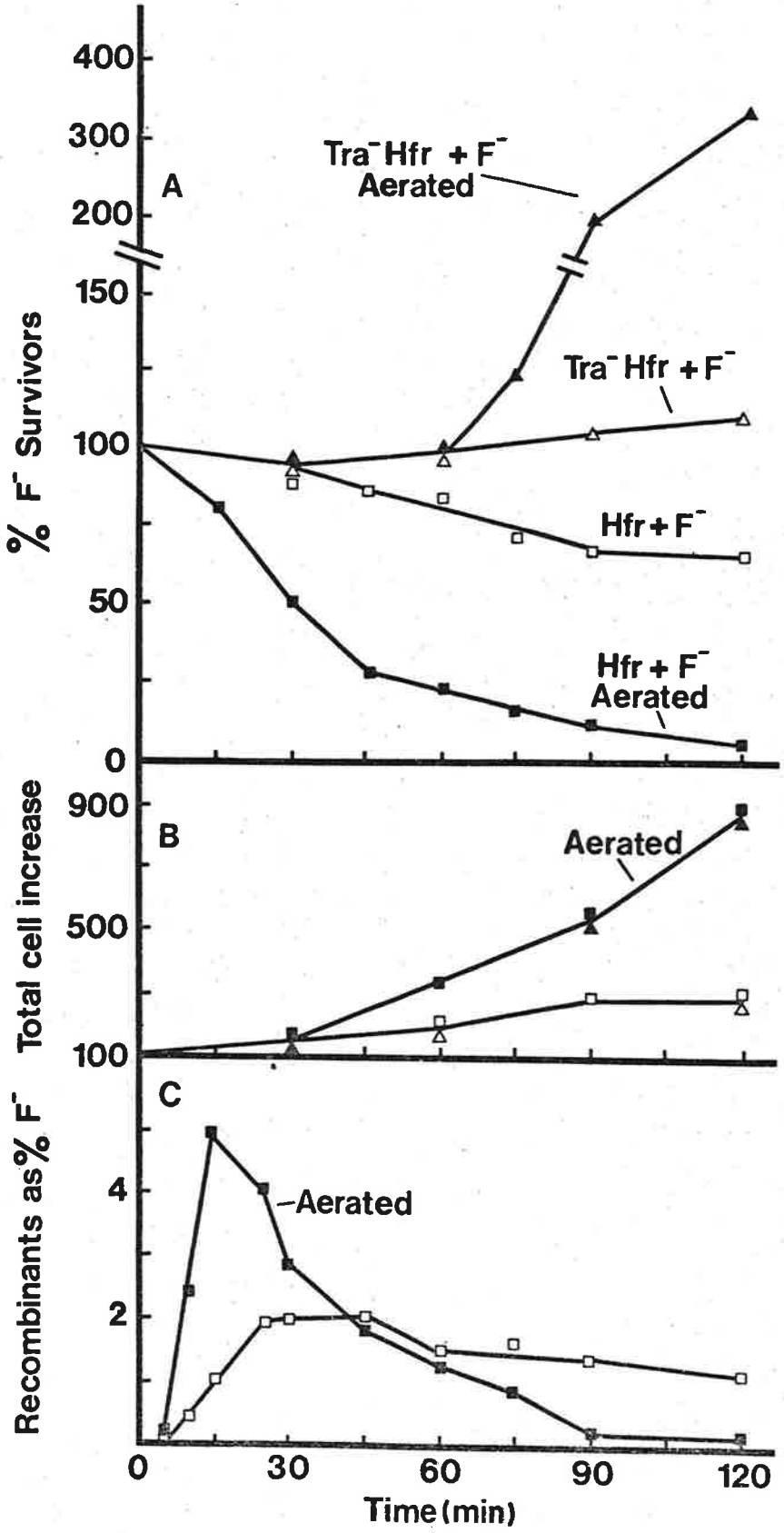

400
300
200
150
100
50
0
A
Tra⁻ Hfr + F⁻
Aerated
Tra⁻ Hfr + F⁻
Hfr + F⁻
Hfr + F⁻
Aerated
% F⁻ Survivors
900
500
100
B
Aerated
Total cell increase
C
4
2
Aerated
Recombinants as % F⁻
0
30
60
90
120
Time (min)

TABLE 3-1

Effect of varying the period of aeration on the extent of lethal zygosis[a].

Period of aeration (min)[b]	Survivors at time aeration ceased (%)	Survivors at 120 min (%)	Total count increase at 120 min
0	100	83	185
15	85[c]	47	268
30	55[c]	29	285
60	20	20	370
120	7	7	810

[a] AB259 (HfrH) mixed with the F^- strain P882 at ratio of 20:1. (Standard cultures and lethal zygosis conditions).

[b] Aeration was continuous from time of mixing until time indicated.

[c] Approximate values taken from curves constructured from viable counts at 0, 20, 40, 60, 90, and 120 min.

extent than if full aeration was allowed.

These results, obtained by varying the degree of aeration, suggest that lethal zygosis is correlated with recombinant formation, and with Hfr growth; however, aeration will also alter the state of the recipient, a parameter which is examined in the next section.

EFFECT OF PRIOR GROWTH CONDITIONS OF RECIPIENT

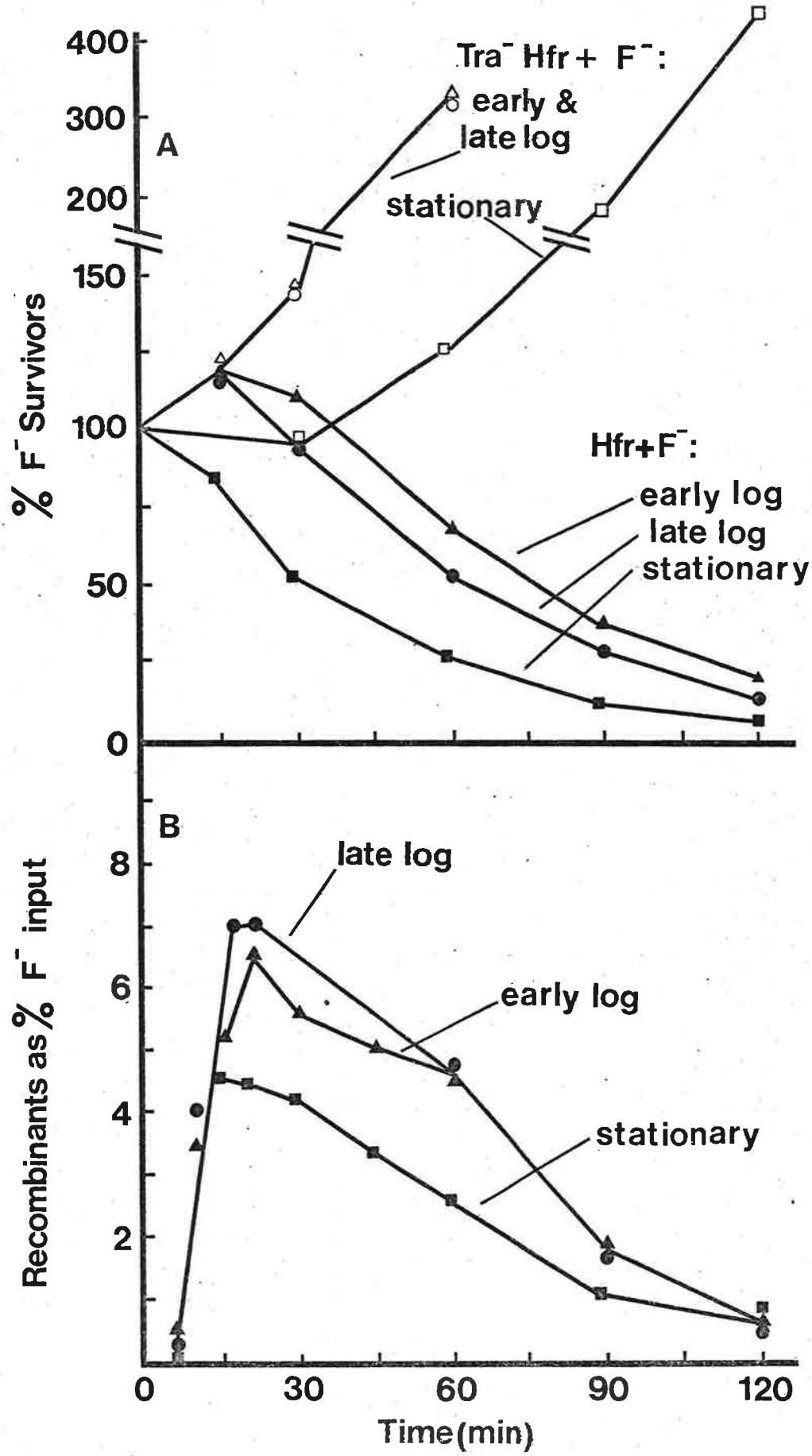
Clowes (1963 and personal communication) made use of F^- cultures that were either in logarithmic phase or were dilutions of an overnight aerated culture. He found that higher multiplicities of Hfr cells per F^- cell were required to produce a similar lethal effect on logarithmic cultures, compared to overnight cultures. Stationary-phase F^- cultures were also reported to be more sensitive to lethal zygosis than logarithmic-phase cultures [Clowes quoted by Gross (1963b)].

As previously mentioned wide variations in extent of kill were observed with nonaerated overnight cultures; however, these cells were, in general, more sensitive than logarithmic-phase F^- cells. In order to establish the relationship between phase of the growth cycle and sensitivity to lethal zygosis, three culture types were compared (Fig. 3-3). With early and late logarithmic-phase F^- cells, an initial increase in viable count was seen when mixed with the Hfr strain AB261 or its Tra^- mutant, followed by a decrease only with the Hfr, the rate and extent depending on the physiological state of the culture.

Stationary-phase cells were more sensitive to lethal zygosis but

FIG. 3-3

Effect of recipient growth stage on lethal zygosis. Cultures of the F^- strain P882, in nutrient broth, were grown to early logarithmic (▲, △), late logarithmic (●, ○), and stationary phase (■, □), diluted, and tested for lethal zygosis (see Materials and Methods) by mixing with an early logarithmic-phase culture of the Hfr strain AB261 (closed symbols) or with a similar culture of the Tra^- mutant P729 (open symbols). Samples were diluted and plated for survivors (A) and Pro^+Str^r recombinants (B).


400
300
200
150
100
50
0
A
% F⁻ Survivors
Tra⁻ Hfr + F⁻:
early &
late log
stationary
Hfr+F⁻:
early log
late log
stationary
B
Recombinants as % F⁻ input
8
6
4
2
late log
early log
stationary
0
30
60
90
120
Time(min)

this did not appear to be related to an altered ability to undergo conjugation, for the early kinetics of zygote formation were identical with all three culture types, suggesting identical rates of pair formation and chromosome transfer. However, differences were observed once the stage was reached where lethal events reduced F^- survivors, for this would also affect the subpopulation of cells which had already received donor DNA and may have gone on to form recombinants. The recipient ability of the three culture types of strain P882 was also examined at a ratio of 1:1 but again no great difference existed between stationary and logarithmic-phase cultures in this regard.

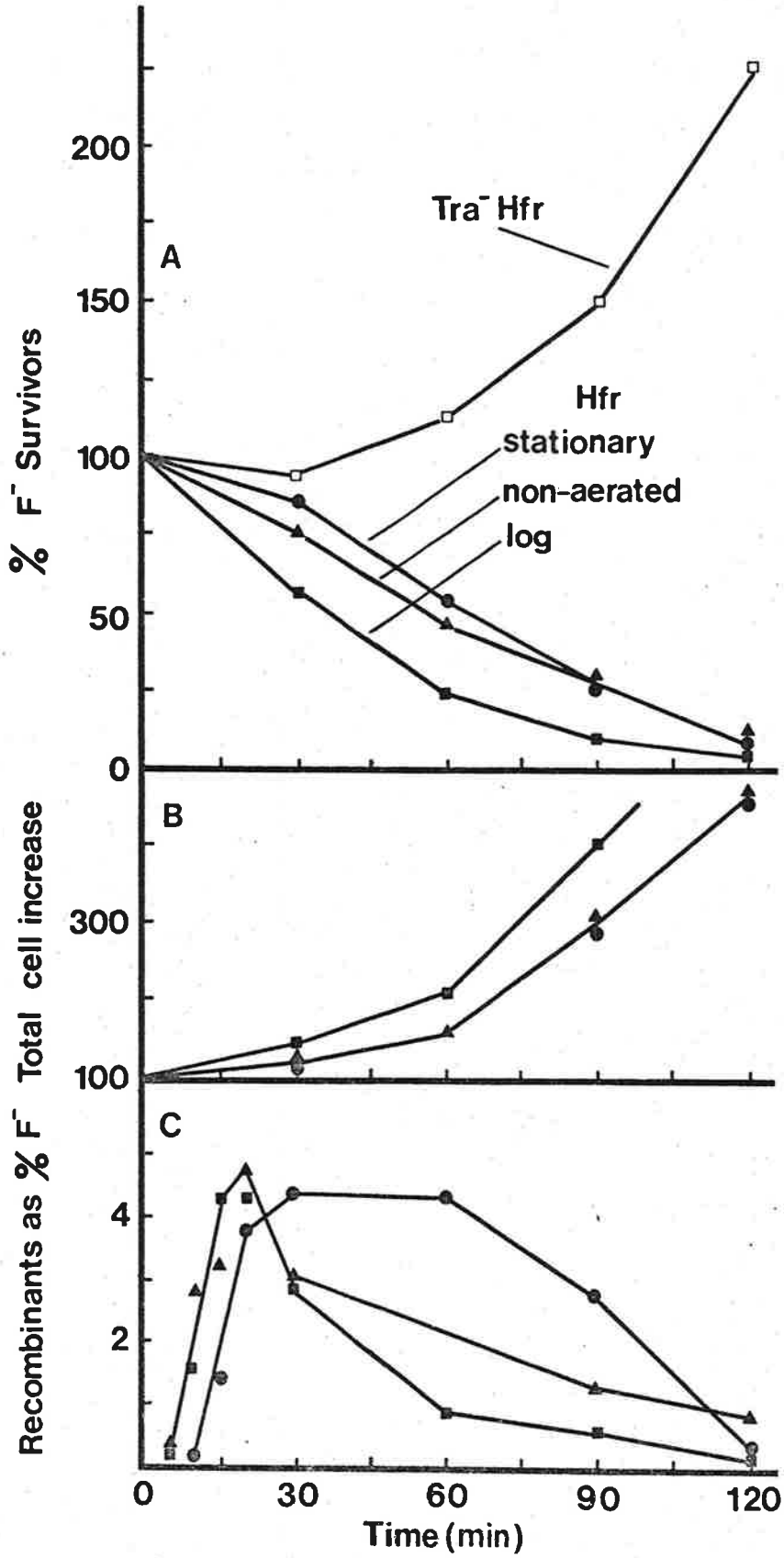
It can be concluded from these experiments that increase in F^- cell age, from early logarithmic through to the late stationary phase, is accompanied by an increasing sensitivity to lethal zygosis.

EFFECT OF PRIOR GROWTH CONDITIONS OF DONOR

The ability of a donor strain to yield recombinants can be affected by the conditions of growth, for cultures grown without aeration were reported to be more proficient than aerated cultures (Curtiss et al., 1968, 1969). The effect of varying donor growth conditions was therefore examined in terms of lethal zygosis and recombinant formation ability (Fig. 3-4). In contrast to the results of Curtiss et al. (1968, 1969), aerated (logarithmic-phase) and nonaerated Hfr cultures had similar rates of zygote formation under standard conditions; however, the logarithmic cells were more lethally active in the early stages following mixing.

FIG. 3-4

Effect on lethal zygosis of prior growth conditions of the donor. AB261 (HfrP4X) early logarithmic-phase (■), stationary-phase (●), and nonaerated cultures (▲) and a logarithmic-phase culture of the Tra^- mutant P729 (□) were prepared as described (Materials and Methods) and tested for lethal zygosis with a stationary-phase culture of the F^- strain P882. Samples were diluted and plated for survivors (A), total cell count (B), and Pro^+Str^r recombinant formation (C).


200
150
100
50
0
% F⁻ Survivors
A
Tra⁻ Hfr
Hfr
stationary
non-aerated
log
B
300
100
C
4
2
Recombinants as % F⁻ Total cell increase
0
30
60
90
120
Time (min)

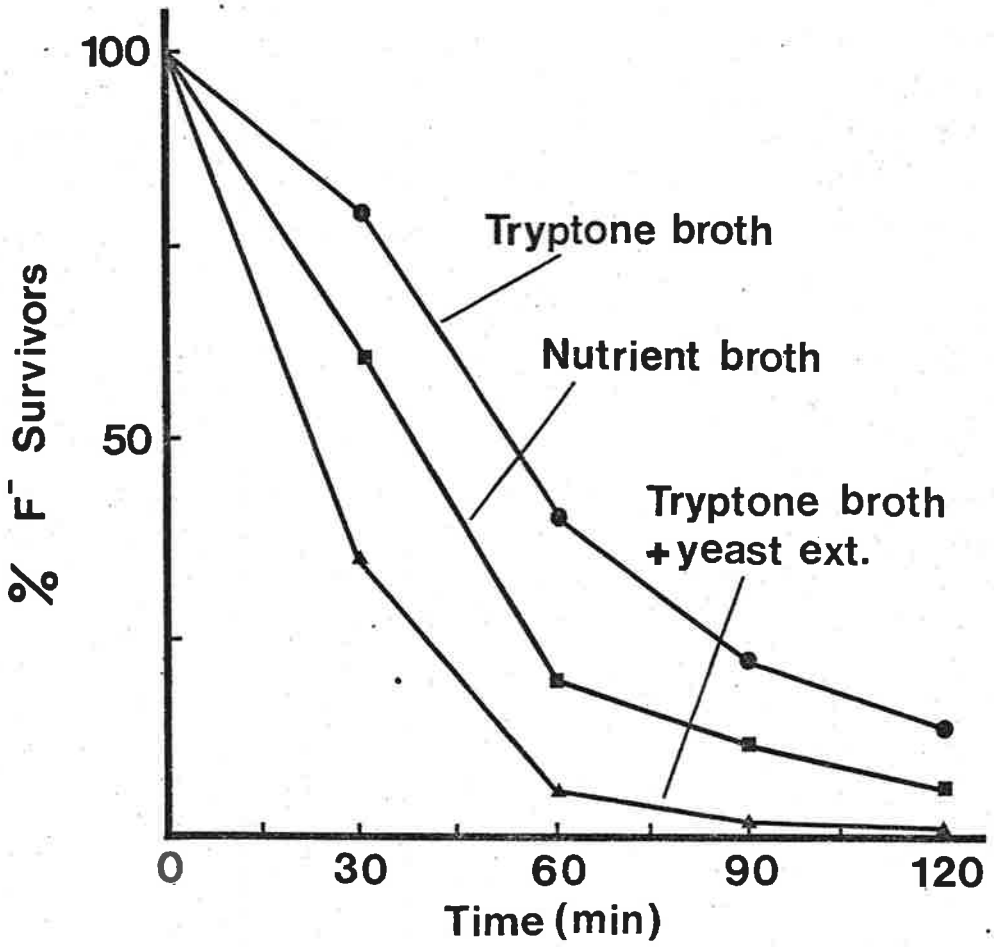
Results with late logarithmic-phase cultures were identical to those shown for early logarithmic-phase cells.

Stationary-phase aerated donor cultures have been shown to temporarily lose their donor ability but gain recipient ability (Lederberg et al., 1952). Such F^- phenocopies lack F pili (Brinton, 1965) but on subculture into fresh medium show rapid restoration of donor function. Recombinant formation with stationary-phase Hfr cells (Fig. 3-4) showed a lag of 5 min, followed by an increase similar to that observed with logarithmic and nonaerated cells. These results can be explained in terms of restoration of pili and donor function (Curtiss et al., 1969). The decrease of recombinants with the three culture types mirrored the pattern of lethal zygosis, as shown in Fig. 3-4A.

There was in this experiment, as in the aeration studies, a relationship between rate of growth and the kinetics of lethality. Such a correlation was also observed in experiments employing different culture media. The latter studies were initiated to establish conditions that could be used to examine metabolic functions during the lethal event, and it was found in general that rich media, which allowed rapid growth, also produced more rapid lethal zygosis (Fig. 3-5). With tryptone broth there was a two- to three-fold increase in total count during the experiment, compared with a nine-fold increase when yeast extract (0.5%) was added, an addition which halved the number of F^- survivors at 30 min.

FIG. 3-5

Effect of media on lethal zygosis. An overnight culture of AB259 (HfrH) in nutrient broth was diluted 1:50 in tryptone broth (●), 1:100 in nutrient broth (■), and 1:100 in tryptone broth + yeast extract (0.5% w/v) (▲). These were incubated with aeration to early logarithmic phase (about 2×10^8 cells/ml). A diluted early (6 hr) stationary-phase culture of the recipient P882 ($OD_{650} = 1.75$) in tryptone broth was added to give a ratio of 20:1. Samples were diluted and plated for survivors as described in Materials and Methods.


100
50
% F⁻ Survivors
Tryptone broth
Nutrient broth
Tryptone broth
+yeast ext.
0
30
60
90
120
Time (min)

REQUIREMENT FOR CELL TO CELL CONTACT FOR LETHAL ZYGOSIS

One way in which Hfr cells could exert their effect is by the release of an extracellular substance or particle into the medium which then binds to F^- cells at a given receptor and leads to the death of that cell, a situation analogous to colicin action. An alternative is that Hfr strains produce these effects by direct cell to cell contact with recipients, as occurs in conjugation (Tatum and Lederberg, 1947; Davis, 1950). In order to test these possibilities experiments were performed with a number of Hfr culture preparations. The results in Fig. 3-6 show that there was no inhibition of F^- cell growth by filtrates from the Hfr strain AB259, the lethal activity remaining with the re-suspended donor cells. Similarly, filtrates from matings in which lethal zygosis had occurred showed no inhibition (Table 3-2).

Using chambers (Materials and Methods) in which Hfr and F^- cells were separated by a bacterial filter, it was shown that there was no lethal zygosis or recombination when contact was prevented, growth of both mating types being normal under these conditions. It therefore appears that whole cells in direct contact, rather than filtrates or supernatant fluids, are required. Streptomycin-treated, or heat-killed Hfr cells were unable to kill (Table 3-2) indicating that the lethal event was not simply due to the addition of a large amount of male material to the F^- culture.

Gross (1963b) examined the effect of blending mating mixtures containing high Hfr:F^- ratios. He concluded that the damage to F^- cells

FIG. 3-6

Effect of filtrates from donor cells on lethal zygosis. Samples (0.25ml) of a stationary-phase culture of the F^- strain AB1133 diluted to about 2×10^8 cells/ml were added to 5ml of a filtrate (□) or to 5ml of the resuspended culture (■) (about 2×10^8 cells/ml) from AB259 (HfrH). Samples were also added to a filtrate (△) and a resuspended culture from the Tra^- mutant P980 (▲). At the times indicated, samples were diluted and plated for survivors as described in Materials and Methods.

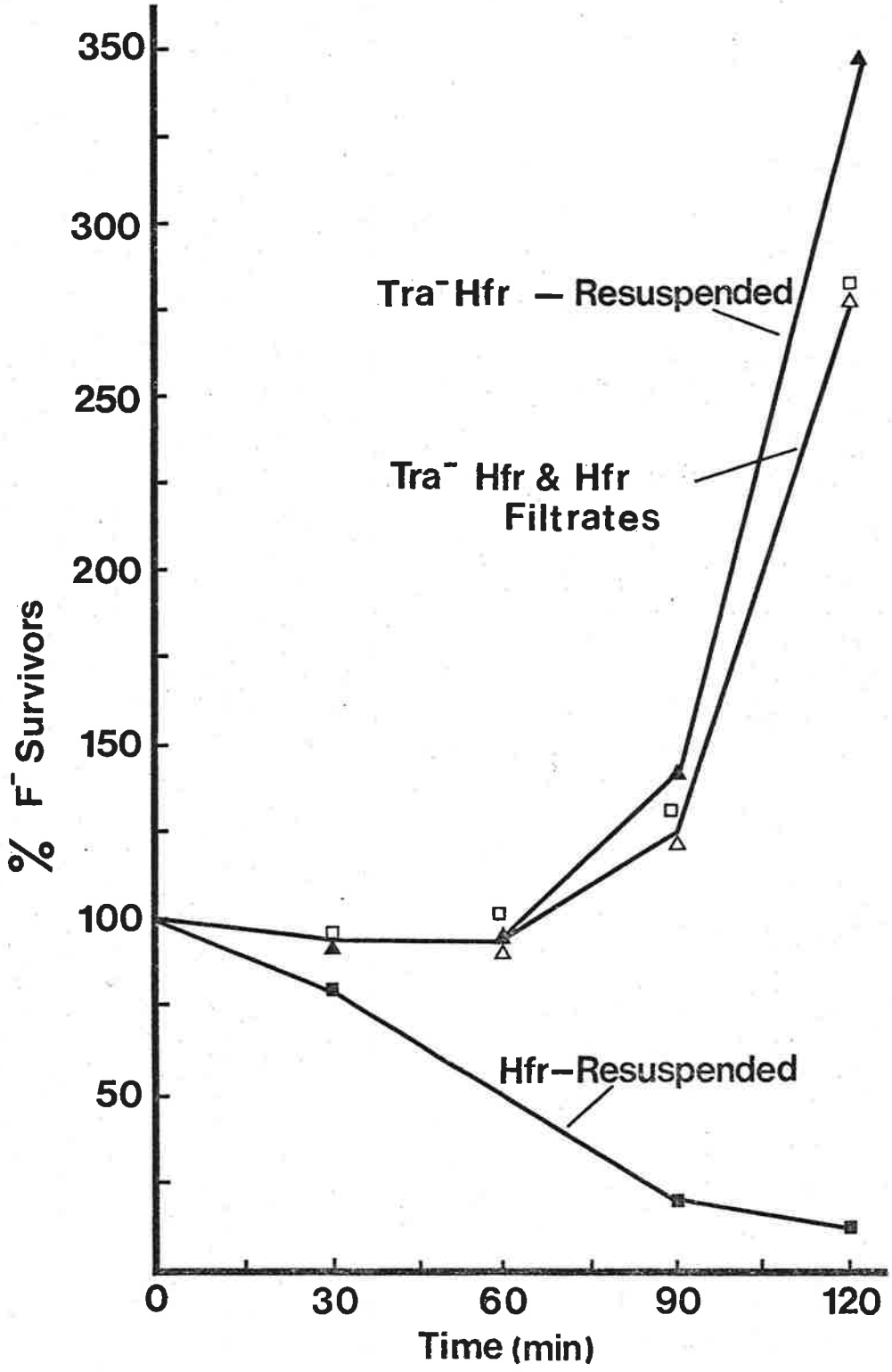

350
300
250
200
150
100
50
% F⁻ Survivors
Tra⁻ Hfr — Resuspended
Tra⁻ Hfr & Hfr
Filtrates
Hfr—Resuspended
0
30
60
90
120
Time (min)

TABLE 3-2

Ability of preparations from AB259 (HfrH) to produce lethal zygosis in liquid media with two recipient strains.

Hfr pretreatment or preparation used[a]	Survivors at 120 min (%)	
	P882	AB1133
Log phase (no treatment)	1	13
Log phase Tra$^-$ (P980) (no treatment)	500	400
Supernatant fluid	ND[b]	300
Streptomycin-treated cells	480	355
Heated 56C, 60 min	480	460
Lethal zygosis filtrate	430	300

[a]See materials and methods for preparations.

[b]Not done.

resulted from conjugation involving more than one Hfr cell (multiple contact). Evidence in support of the existence of multiple mating is provided by a triparental (Hfr x Hfr x F^-) cross (Fischer-Fantuzzi and di Girolamo, 1961). In addition there is the observation made by Curtiss et al. (1969) that more than one F pilus can contact a single F^- cell.

Examination of conjugation by electron-microscopy was therefore undertaken in an attempt to establish if multiple contacts occurred, and whether their numbers increased proportionally as the Hfr:F^- ratio increased. Hfr$HT6^r$(P332) and F^- (AB1133) cells could be distinguished (on the basis of size, number of common pili, and adsorption of phage T6 to F^- cells or phage MS2 to Hfr pili) in mating mixtures when low ratios were examined. As the ratio increased from 1:5 to 5:1 resolution became more difficult, for two minutes after mixing the majority of cells were to be found in large clumps. These clumps consisted of up to 20 cells in close wall to wall or pili to wall contact and, although examples of multiple Hfr-F^- contact were observed, statistical analysis was impossible.

The clumps were also observed when mating mixtures were examined by phase contrast microscopy, the number per field depending upon the ratio of Hfr:F^- cells. In the absence of F^- cells, Hfr cultures did show clump formation, but to a lesser extent, whereas Tra^-Hfr cells, even when mixed with F^- cells, showed little tendency to form clumps.

These results, while inconclusive, do suggest that clump formation

is dependent on the presence of F pili, and it might be that Hfr-F^- associations act as a nucleus for the development of an entangled mass of closely associated cells, the number of which increase as the Hfr:F^- ratio increases.

SUMMARY AND CONCLUSIONS

Conditions which allow a high and reproducible lethal effect are described. The data indicate that such requirements include (i) use of stationary or late logarithmic-phase F^- cultures, (ii) use of logarithmic-phase Hfr cells capable of continued metabolic activity, (iii) prior growth and mating in rich media, and (iv) continuous but gentle aeration throughout the period of the experiment. Use of these procedures with a number of Hfr and F^- strains resulted in lethal zygosis with kills of up to 99% of the initial F^- population.

The absence of lethal zygosis with filtrates and supernatant fluids from donors suggests a dependence on direct cell to cell contact as found for conjugation. Tra^- Hfr cells, which lack F pili, were unable to produce lethal zygosis, indicating that this specific contact between Hfr and F^- cells is mediated by F pili.

CHAPTER 4

THE SPECIFICITY OF DONOR AND RECIPIENT CELLS INVOLVED IN LETHAL ZYGOSIS

INTRODUCTION

Clowes (1963) found that recipient strains differed in their sensitivity to a given Hfr donor. For example, the F^- strain RC748 was consistently more sensitive to HfrH than the F^- strains W677, W1, C600, and P678 were. Gross (1963b) also employed the F^- strain RC748 (called W1:M^-P^-), and found it to be the only F^- strain to show marked loss of viability and lowered yields of recombinants when mixed with high numbers of Hfr donors.

At the commencement of the studies reported here, the only recipient strain found to be sensitive to lethal zygosis was the F^- strain P882 (derived from RC748). Once standard conditions for lethal zygosis were established a number of F^- strains were re-examined.

The Hfr strains examined by Gross (1963b) fell into two groups. Cells of HfrH, HfrC, and HfrR4 all had a marked effect on recipients, whereas those of HfrP10, HfrR1, and HfrR3 had little or no effect. These results confirmed the observations of Clowes (1963); he had also found that HfrP10 (called J4) lacked killing ability. Studies to determine the nature of the differences between these groups of Hfr strains are described in this chapter.

Curtiss (1969) has discussed the effects of genetic inhomology on recombinant formation and pointed out the problems which may arise in the

use of nonisogenic parents in conjugation studies. Series of closely related (isogenic) strains have therefore been examined.

COMPARISON OF LETHAL ZYGOSIS IN LIQUID AND ON SOLID MEDIA

The cross streak plate test and the spot test (Fig. 2-2) were developed in order to allow more rapid screening of strains. These tests showed a good correlation with lethal zygosis in liquid (Tables 4-1 and 4-5). Tra^- Hfr x F^- and F^- x F^- crosses did not show inhibition on agar and, as in standard liquid conditions, acted as convenient controls. RC749, the HfrP10 strain employed by Clowes (1963) and Gross (1963b), gave a low level of recombinants (10^{-2} to 10^{-3} per F^- cell), and did not inhibit any F^- strains on solid media. χ57 (an HfrH strain) was interesting in that it appeared to be a normal Hfr giving good yields of recombinants, but nonetheless gave much lower lethal zygosis in liquid and in the plate test did not inhibit any F^- strain examined. This suggests that the plate test is not as sensitive as lethal zygosis performed in liquid.

SPECIFICITY OF DONOR CELL TYPES INVOLVED IN LETHALITY

The results in Table 4-1 confirmed the observations of Clowes (1963) and Gross (1963b) that no unique origin or direction of transfer is required for an Hfr strain to be lethal. These experiments made use of strains with widely different genetic backgrounds. In order to determine if the low extent or absence of lethal zygosis with some Hfr strains was due to a particular genetic background, a series of Hfr

TABLE 4-1

Lethal zygosis produced by various strains in liquid or on solid media.

Strain No.	Mating type	Effect on P882	
		Survivors in liquid at 120 min (%)	Solid media[a]
AB259	HfrH	1.5	++
P980	Tra⁻HfrH	500	-
P969	HfrH	4	++
χ57	HfrH	45	-
P722	HfrC	5	+
P721	Tra⁻HfrC	300	-
AB261	HfrP4X	3	++
P729	Tra⁻HfrP4X	320	-
RC749	HfrP10	220	-
KL16	Hfr	5	+
χ503	HfrOR21	3	+
KL99	Hfr	20	ND[b]
Ra-2	Hfr	10	ND
B7	Hfr	5	ND
P703	HfrR1	100	ND
P300	HfrR2	20	ND
P301	HfrR3	90	ND
P702	HfrR4	15	ND
P816	HfrR5	200	ND

[a]Cross streak plate test as described in Materials and Methods.

[b]Not done.

strains derived from a single F^+ strain (χ209) were examined (Table 4-2). Whereas the Hfr strains χ869, χ876, χ883, χ884, χ886, χ895, χ896, and χ900 all showed lethal zygosis, the strains χ437 and χ897 did not. Therefore, the previously observed variations were not due to genetic background. Significantly all six strains (HfrP10, HfrR1, HfrR3, HfrR5, χ437, and χ897) which gave little or no lethal zygosis, also gave reduced recombinant yields, indicating a correlation with fertility. The molecular basis for this variation is not understood.

A number of strains of the other two donor types, F^+ and F-prime (F'), were examined in liquid and on solid media. Unlike the majority of isogenic Hfr strains derived from it, the F^+ strain χ209 did not show lethal zygosis (Table 4-2), nor did the F^+ strains χ15 and KL20, from which Hfr, F', and F^- strains were derived (Tables 4-3, 4-4; Fig. 4-1).

Variations in ability to produce lethal zygosis were observed among the F-prime strains examined. The F<u>lac</u> strains χ818 (Table 4-3), P995, and JC6583 and the F<u>gal</u> strain P601, produced no lethal zygosis in solid media tests and, at the most, only slight inhibition of F^- growth in liquid tests (similar to F^+ χ15 in Fig. 4-1) even though high levels of transfer were observed. Of the survivors (90 min) examined from the latter liquid tests, up to 95% had become sensitive to the male-specific phage MS2. This suggests that the majority of surviving recipients had received and maintained the F factor. In contrast, the F' strains χ517 and χ594 (ORF-1) initially produced some lethality, and the strain χ573

TABLE 4-2

Ability of a series of donor strains, mostly derived from χ209, to produce lethal zygosis on solid media.

Streptomycin-sensitive strains		Streptomycin-resistant strains			
		AB1133	P882	AB312	χ1088[a]
		F^-	F^-	Hfr	F^-
AB259	HfrH	++	++	-	+
P980	Tra^- HfrH	-	-	-	-
χ900[a]	HfrOR80	++	++	-	+
χ884[a]	HfrOR64	++	++	-	+
χ896[a]	HfrOR76	++	++	-	+
χ437[a]	HfrOR7	-	-	-	-
χ886[a]	HfrOR66	++	++	-	+
χ876[a]	HfrOR56	+	ND[b]	ND	+
χ895[a]	HfrOR75	++	++	-	+
χ897[a]	HfrOR77	-	-	-	-
χ883[a]	HfrOR63	+	ND	ND	+
χ869[a]	HfrOR49	++	++	-	+
χ209[c]	F^+	-	-	-	-
χ42	F^+	ND	-	ND	ND

[a]Closely related strains derived from χ209: provided by R. Curtiss III.

[b]Not done.

[c]χ209 is derived from χ42.

TABLE 4-3

Comparison of mating types mostly derived from χ15, for ability to produce lethal zygosis on solid media.

Streptomycin-sensitive strains		Streptomycin-resistant strains			
		AB1133	P882	AB312	χ545[a]
		F^-	F^-	Hfr	F^-
AB259	HfrH	++	++	-	++
P980	Tra^-HfrH	-	-	-	-
χ15	F^+	-	-	-	-
χ493[a]	HfrOR11	++	+	-	+
χ503[a]	HfrOR21	+	++	-	+
χ573[a]	F'ORF-4	+	++	-	+
χ818[a]	F'ORF-207	-	ND[b]	-	-
χ289[a]	F^-	-	-	-	-

[a]Closely related strains descended from χ15; provided by R. Curtiss III.

[b]Not done.

TABLE 4-4

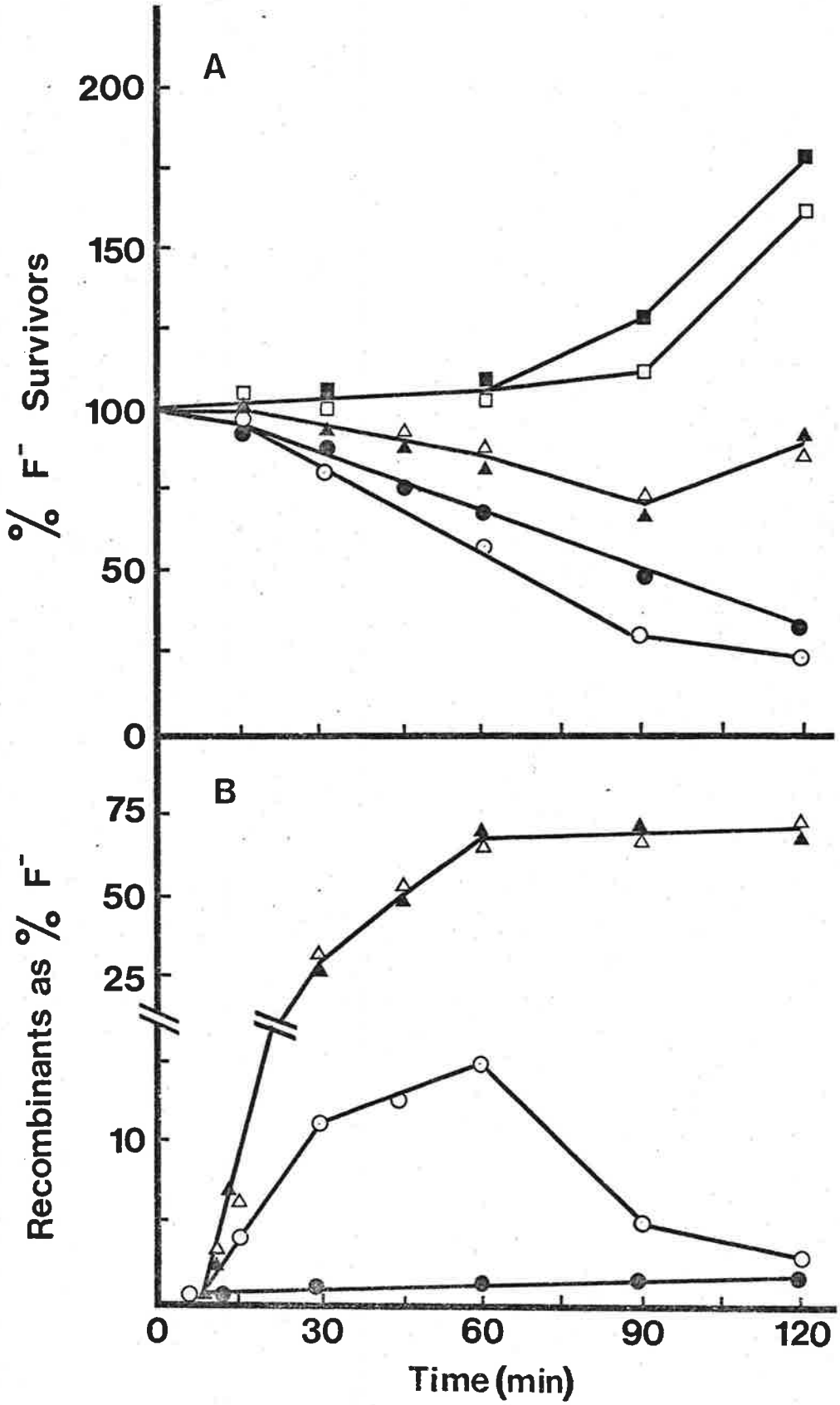
Comparison of mating types derived from KL20 for ability to produce lethal zygosis on solid media.

Streptomycin-sensitive strains		Streptomycin-resistant strains		
		KL184[a]	AB1133	AB312
		F^-	F^-	Hfr
KL20	F^+	-	-	-
KL96[a]	Hfr	+	+	-
KL174[a]	Hfr	+	+	-
KL175[a]	F^+	-	-	-
KL182[a]	F^-	-	-	-
KL84[a]	Hfr	+	+	-
KL16[a]	Hfr	+	+	-

[a]Closely related strains derived from KL20; provided by K. Brooks Low.

FIG. 4-1

Comparison of mating types derived from χ15 for ability to produce lethal zygosis. A stationary-phase culture of the F^- strain χ985, in nutrient broth, was diluted and tested (see Materials and Methods) for sensitivity to early logarithmic-phase cultures of the following strains; χ15(F^+)(□), χ517(F'0RF-1)(▲), χ594(F'0RF-1)(△), χ573(F'0RF-4)(●), χ503(Hfr0R21)(○), and χ289(F^-)(■). Samples were diluted and plated for survivors (A) and Pur^+Str^r recombinants (B).


A
200
150
100
50
0
% F⁻ Survivors
B
75
50
25
10
Recombinants as % F⁻
0
30
60
90
120
Time (min)

(ORF-4) killed to the same extent as the HfrOR21 strain χ503 (Table 4-3, Fig. 4-1). None of the Pur^+Str^r recombinants or F^- survivors examined from crosses with these strains (χ517, χ594, χ573) had gained sensitivity to phage MS2, a result which is in agreement with the observation of Berg and Curtiss (1967). The low level of Pur^+Str^r recombinants with the F'ORF-4 (Fig. 4-1) suggested this strain had reverted to an Hfr state with a transposed orientation of transfer; however, no attempt has been made to clarify this situation.

RELATIONSHIP BETWEEN MATING TYPE AND SENSITIVITY TO LETHAL ZYGOSIS

Only F^- cells were found to be sensitive to Hfr cells, a specificity shown in Table 4-5. Even when shaken overnight to produce F^- phenocopies, the Hfr strain AB313 and the F^+ strain P201 lacked sensitivity to the Hfr strain AB261 although recombinant formation occurred. The lack of sensitivity of these donor strains suggests that there is an immunity mechanism associated with the presence of the F factor (see Chapter 6). In general, all F^- strains examined have been shown to be sensitive, but there are variations in the extent of lethal zygosis observed.

ROLE OF RECOMBINATION IN LETHAL ZYGOSIS

The observation that aeration led to an increased extent of lethal zygosis as well as a higher yield of recombinants, together with the inability of F^+ and most F-prime strains to kill, and the correlation between Hfr killing ability and fertility, all suggested a close relationship between lethal zygosis and recombination. Recipient strains,

TABLE 4-5

Sensitivity of various strains to lethal zygosis on solid media.

Streptomycin-sensitive strain	Streptomycin-resistant strain							
	AB1133	P122	AB313	PA309	RC12	AB312	AB1157	JC2921[a]
	F^-	F^-	Hfr	F^-	F^-	Hfr	F^-	F^- (recA1)
AB259 HfrH	++[b]	++[b]	-	++[b]	++[b]	-	++[b]	++
P980 Tra^-HfrH	-[c]	-	-	-	-	-	-[c]	-
P722 HfrC	ND[d]	++	-	++	++	ND	ND	ND
P721 Tra^-HfrC	ND	-	-	-	-	ND	ND	ND
RC749 HfrP10	ND	-	-	-	-	ND	ND	ND
KL16 Hfr	++	+	-	++	++	-	+	ND
P107 F^-	-	-	-	-	-	-	-	ND

[a] Similar results with the following strains carrying the mutations shown in brackets: JC5743(recB21), JC5489(recC22), JC2917(recA12) JC2926(recA13), JC2929(recA65).

[b] <5% survivors in liquid lethal zygosis.

[c] >100% survivors in liquid lethal zygosis.

[d] Not done (generally due to λ^+ donor x λ^- recipient).

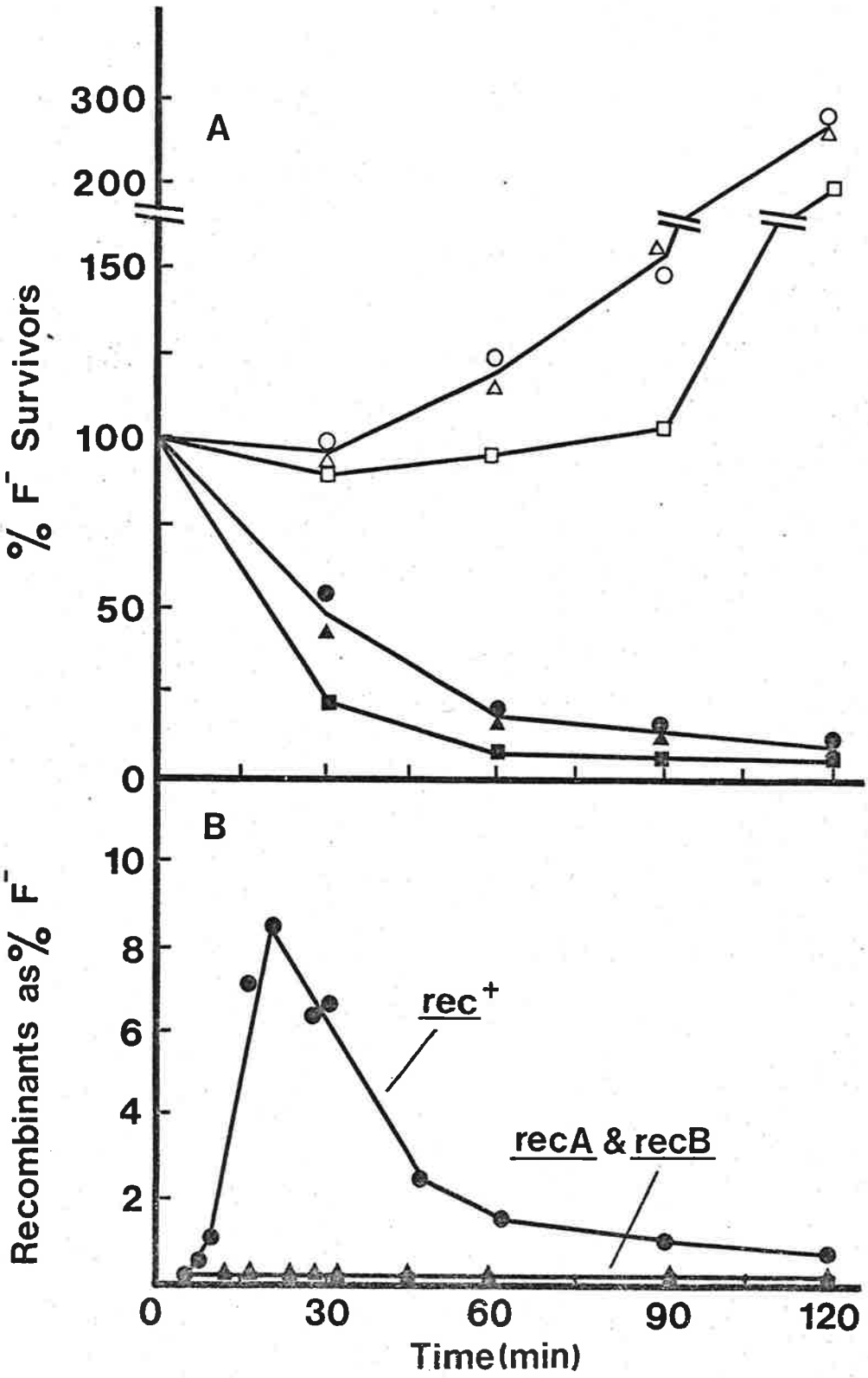
altered in their ability to form recombinants, were therefore tested for their sensitivity to lethal zygosis.

The majority of recombination-deficient (Rec^-) mutants fall into one of two phenotypic groups (Clark, 1967, 1971). Those strains with mutations in recA are characterized by extensive breakdown of DNA following ultraviolet (UV)-irradiation and are highly recombination deficient. The rare recombinants which have arisen from Hfr x F^-/recA crosses, appear to be either F-prime strains or nondonor merodiploids (Low, 1968). The second phenotypic group includes mutations in recB and recC, and strains carrying either of these are moderately UV-sensitive and have reduced recombination proficiency. Extracts of recB and recC mutants lack an adenosine triphosphate (ATP)-dependent nucleolytic activity (Oishi, 1969; Barbour and Clark, 1970). This enzyme, exonuclease V (Wright, Buttin and Hurwitz, 1971), specified by the recB and recC genes, has been purified, and possesses both endo- and exonucleolytic deoxyribonuclease activities as well as ATPase activity (Goldmark and Linn, 1972; Nobrega, Rola, Pasetto-Nobrega and Oishi, 1972).

As shown in Table 4-5, both phenotypic classes of mutants were sensitive to lethal zygosis on solid media. In liquid media (Fig. 4-2) the recombination-deficient strains showed sensitivity equal to or greater than the Rec^+ parent, even though recombinant formation was not detected ($<10^{-4}$ of Rec^+ level) in the case of the recA mutant and was

FIG. 4-2

Sensitivity of Rec$^-$ recipients to lethal zygosis. Stationary-phase cultures of the recipient strains AB1157(*rec*$^+$)(●, ○), JC2921(*recA1*)(▲, △), and JC5743(*recB21*)(■, □) were diluted and mixed with an early logarithmic-phase culture of the Hfr strain AB259 (closed symbols) or with a similar culture of the Tra$^-$ mutant P980 (open symbols). Samples were diluted and plated for survivors (A) and for Thr$^+$ Leu$^+$ Str^r recombinants (B). Results with the strain JC5489(*recC22*) were identical to those with JC5743.


A
300
200
150
100
50
0
% F⁻ Survivors
B
10
8
6
4
2
0
Recombinants as % F⁻
rec⁺
recA & recB
30
60
90
120
Time (min)

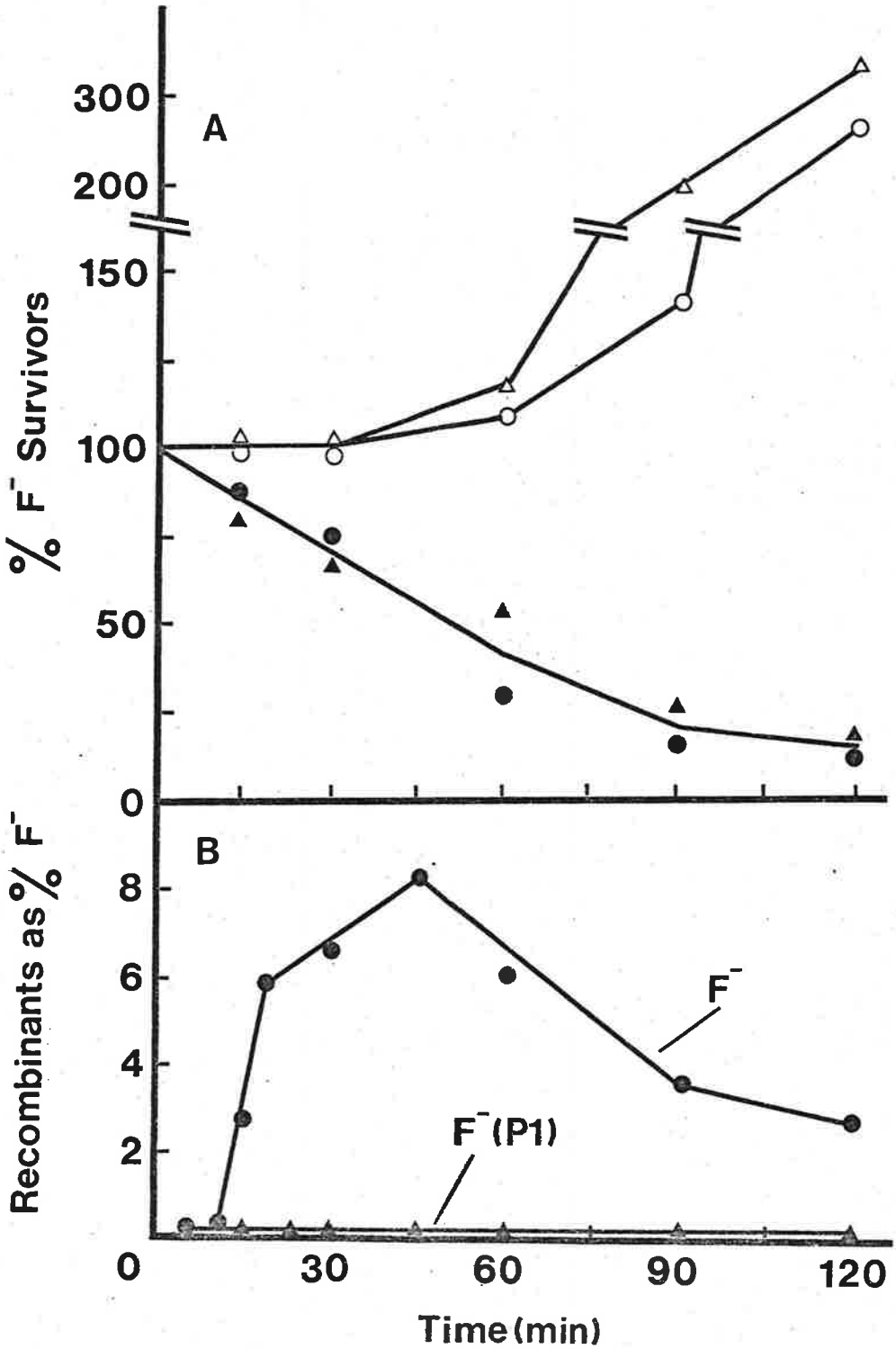
greatly reduced (10^{-3} to 10^{-4} of Rec^+ level) with strains carrying recB and recC. These results indicate either that recombination events are not involved in lethal zygosis or that the products of recA, recB, and recC genes are involved in a stage of the recombination process subsequent to that at which lethal events arise.

UV-sensitive strains which are deficient in excising and repairing the UV-induced pyrimidine dimers (Uvr^-), undergo normal recombination (Howard-Flanders and Boyce, 1966). The Uvr^+ parent strain AB1157 and three Uvr^- strains, with mutations located at widely separated loci [AB1884(uvrC4), AB1885(uvrB5), AB1886(uvrA6)], were found to be sensitive to lethal zygosis on solid media with the Hfr strain AB259. It would appear, therefore, that neither the excision (Uvr) nor the recombination (Rec) pathway is required for lethal zygosis.

The formation of recombinants has also been shown to be considerably reduced when the recipient strain employed was "restrictive" for the DNA of the donor strain (Arber, 1962; Boice and Luria, 1963; Pittard, 1964; Boyer, 1964; Copeland and Bryson, 1966). Arber and Morse (1965) found the recombinant frequency to be 100- to 1,000-fold lower in crosses between an Hfr and a recipient lysogenic for P1 phage [$F^-(P1)$] than in crosses between Hfr x F^-, Hfr(P1) x F^-, and Hfr(P1) x $F^-(P1)$. In addition, these authors showed that the acceptance of a number of transmissible plasmids by the $F^-(P1)$ was reduced. Results from an experiment employing recipients that were either lysogenic

FIG. 4-3

Lethal zygosis sensitivity of a recipient lysogenic for phage P1. Stationary-phase cultures of the F^- strain 102 (●, ○) and the $\bar{F}(P1)$ strain 342 (▲, △) were diluted and mixed with an early logarithmic-phase culture of the Hfr strain AB259 (closed symbols) or with a similar culture of the Tra^- mutant P980 (open symbols), as described in Materials and Methods. Samples were plated for survivors (A) and $Thr^+Leu^+Str^r$ recombinants (B).


A
300
200
150
100
50
0
% F⁻ Survivors
B
Recombinants as % F⁻
8
6
4
2
0
F⁻
F⁻(P1)
0
30
60
90
120
Time (min)

or nonlysogenic for phage P1 are shown in Fig. 4-3. Although the F^-(P1) strain formed recombinants at a level less than 10^{-4} of that observed with the nonlysogenic F^-, both recipients were equally sensitive to lethal zygosis with the HfrH(λ^-) strain AB259.

SUMMARY AND CONCLUSIONS

A number of strains have been compared using optimum conditions for lethal zygosis and the results showed that in general, crosses between Hfr and F^- strains which gave normal yields of recombinants demonstrated lethal zygosis. There was, with Hfr strains, a correlation with fertility rather than origin or genetic background.

The phenomenon was also observed on solid media, and these two methods were used to examine strains of different mating types. Within groups of isogenic strains it was found that an F^- strain was sensitive to a number of closely related Hfr strains but not to the isogenic F^+ or F^- strains. Only one F-prime strain (of doubtful F' status) produced lethal zygosis to the extent observed with killing Hfr strains; the others produced either intermediate or slight effects on F^- cell growth. These results suggest that lethal zygosis may be associated with an event which occurs at higher frequency in, or is unique to, matings with Hfr donors.

Results with recipient strains altered in recombinant forming ability (Rec^- or "restricting" P1 lysogens) indicate that recombination per se is not required for lethal zygosis.

CHAPTER 5

ALTERED RECIPIENT CELL FUNCTIONS ASSOCIATED WITH CONJUGATION

INTRODUCTION

The results of experiments described in the two previous chapters support the hypothesis (Clowes, 1963; Gross, 1963b) that cellular contact, as required for conjugation, can lead to the death of the F^- cell, a phenomenon dependent upon the Hfr cell concentration. Even at a ratio of 1:1, division of F^- cells was inhibited, although a continued rise in recombinant numbers was observed. Could it therefore be that lethal zygosis is an extreme manifestation, at high donor to recipient ratios, of events that occur during conjugation involving only a single donor cell?

There have been few reports of changes in the physiology or metabolism of the recipient cell during conjugation, although the metabolic requirements for conjugation have received considerable attention (see Introduction). An investigation into possible conjugation-induced perturbations was therefore undertaken and in this chapter alterations in F^- cell macromolecular syntheses and membrane-associated functions are described.

Overnight stationary-phase cultures of recipient cells, employed in standard conditions (Materials and Methods), showed a lag in recovery of metabolic activities (DNA synthesis and β-galactosidase induction), on dilution. Consequently, early stationary-phase cultures

were used in the studies described in this chapter.

DNA SYNTHESIS

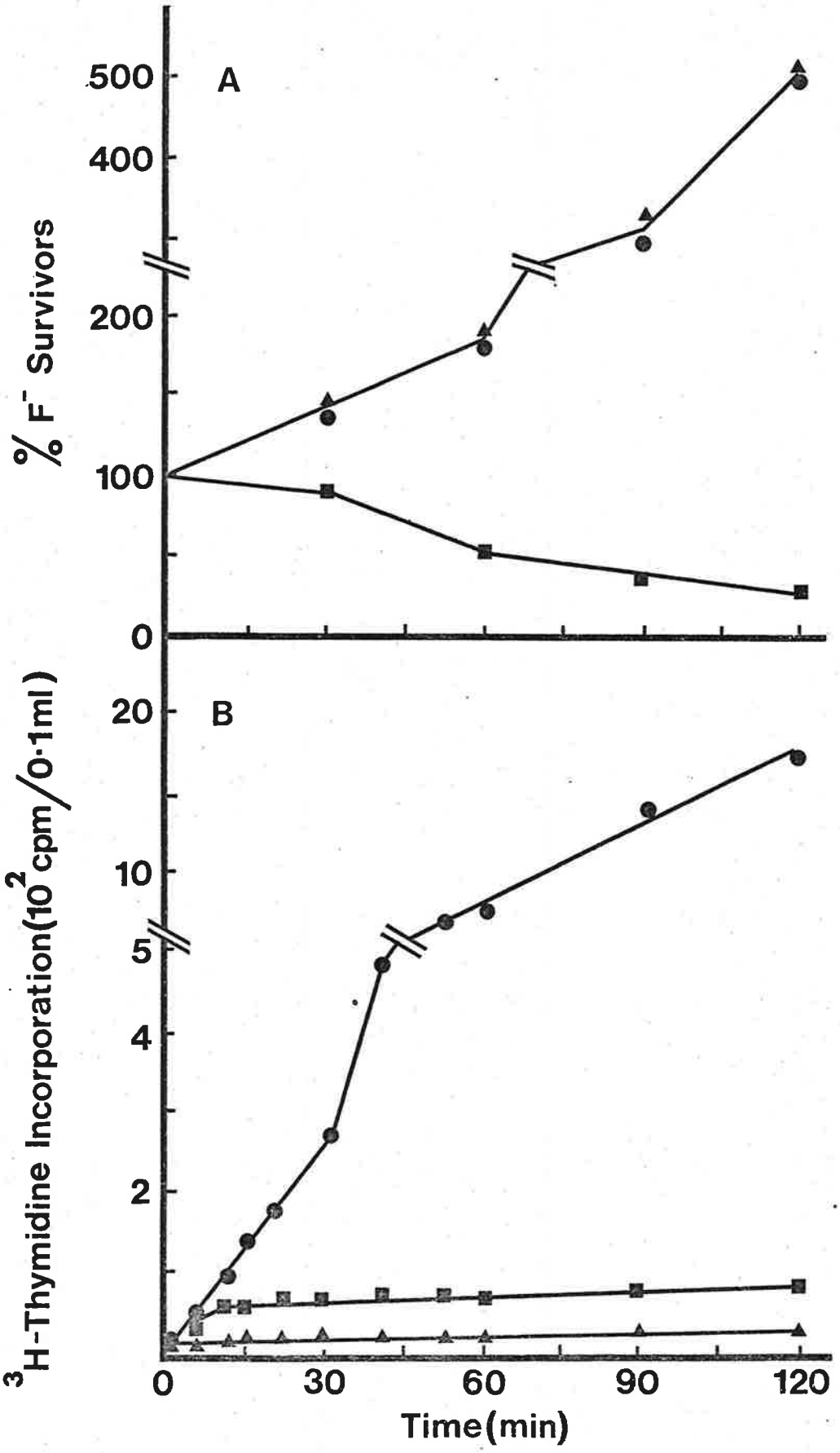
In order to selectively incorporate radioactive precursors into the DNA of recipient cells, thymidine kinase deficient mutants (Hiraga et al., 1967; Igarashi et al., 1967) of Hfr and Tra$^-$Hfr cells were employed. Incorporation of exogenous thymine or thymidine by these Tdk$^-$ cells was approximately 0.05% of that incorporated by Tdk$^+$ recipient cells and permitted the use of high donor:recipient ratios. This system, which has the advantage of allowing specific labelling of newly synthesized recipient DNA without the necessity to inhibit DNA synthesis in the donor, has been used in previous conjugation studies (Vapnek and Rupp, 1970; Wilkins et al., 1971).

Initially, the incorporation of ^{3}H-thymidine into Thy$^+$ recipient cells was examined, but it was found that an excess of F$^-$Tdk$^-$ cells totally prevented this incorporation, while acting as a non-mating control (Fig. 5-1). This inhibition, which was dependent upon the concentration of Tdk$^-$ cells present, was presumably due to the presence of high levels of thymidine phosphorylase (EC2.4.2.4; Rachmeler, Gerhart and Rosner, 1961), for chromatographic analysis showed degradation of thymidine to thymine to be rapid in the presence of cells of the Tdk$^-$ strain P948.

The incorporation of ^{3}H-thymine into cultures of a low level thymine requiring strain was therefore measured as an estimate of recipient

FIG. 5-1

Effect of lethal zygosis on recipient cell thymidine incorporation. Samples of a diluted culture of the recipient strain P882 were mixed, at a ratio of 25:1, with a culture of the Tdk^- HfrP4X strain P727(■), with a culture of the Tdk^-F^- strain P948(▲), and with media (●) (for details see Materials and Methods). ^{3}H-thymidine (0.5μCi/ml) was added at the commencement of mating and incorporation into acid-insoluble material was measured as described in Materials and Methods (B). Samples were also diluted and plated for F^- survivors (A).


A
500
400
200
100
0
% F⁻ Survivors
B
20
10
5
4
2
0
30
60
90
120
Time(min)
³H-Thymidine Incorporation(10² cpm/0·1ml)

cell DNA synthesis. Incorporation by these cells was similar, whether they were diluted into preconditioned media or into a culture of a $Tdk^{-}Tra^{-}$ donor (Hfr) strain, the latter providing an adequate non-conjugating control. The results from one such experiment (Fig. 5-2) show the inhibition in incorporation, which was observed as early as 20 min following mixing, when mating and non-mating F^{-} cells were compared. If equal numbers of Hfr and F^{-} cells were employed, inhibition was observed although it was less pronounced than at the higher ratio of 25:1. In this and in all other experiments described in this chapter, recombinant ($Pro^{+}Str^{r}$) formation was examined at intervals and found to be comparable to that previously observed (Chapter 3).

It can be concluded from these results that inhibition of DNA synthesis, like reduction in F^{-} viable count, resulted from an event that was dependent upon the multiplicity of Hfr cells employed.

β-GALACTOSIDASE INDUCTION

To further characterize metabolic changes associated with conjugation the induction of the enzyme β-galactosidase was examined as a measure of protein synthesis. This system was particularly amenable for such studies, as Hfr strains carrying a complete deletion of the *lac* operon provided a low background of activity, against which recipient synthesis could be readily examined.

With cells grown and mated in supplemented glycerol minimal media (Fig. 5-3), there was a 40-50% reduction in the rate of enzyme synthesis

FIG. 5-2

Effect of lethal zygosis on recipient cell thymine incorporation. Cultures of the donor strain P727 (HfrP4X) and the recipient strain P978 were mixed (for details see Materials and Methods) at zero time, to give the ratios indicated (1:1, ▲; 25:1, ■). ^{3}H-thymine (5μCi/μg/ml) was added at the commencement of mating, and incorporation into acid insoluble material was measured. Controls with the Tra^{-} strain P766 were included (1:1, Δ; 25:1, □). Incorporation by the donor strain P727, at the cell density used in 25:1 mating, is also shown (●). The insert shows F^{-} survival in the four flasks.

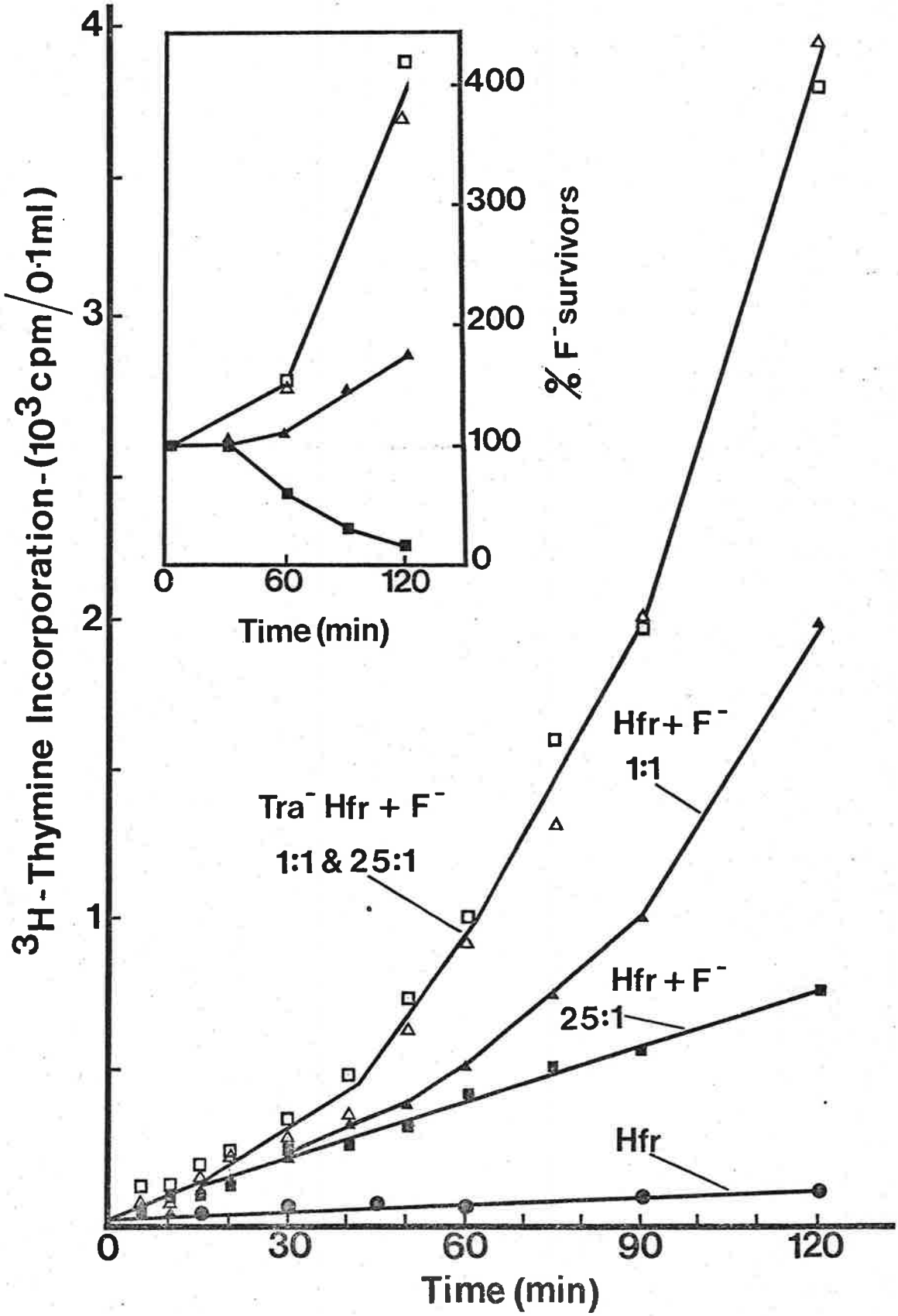
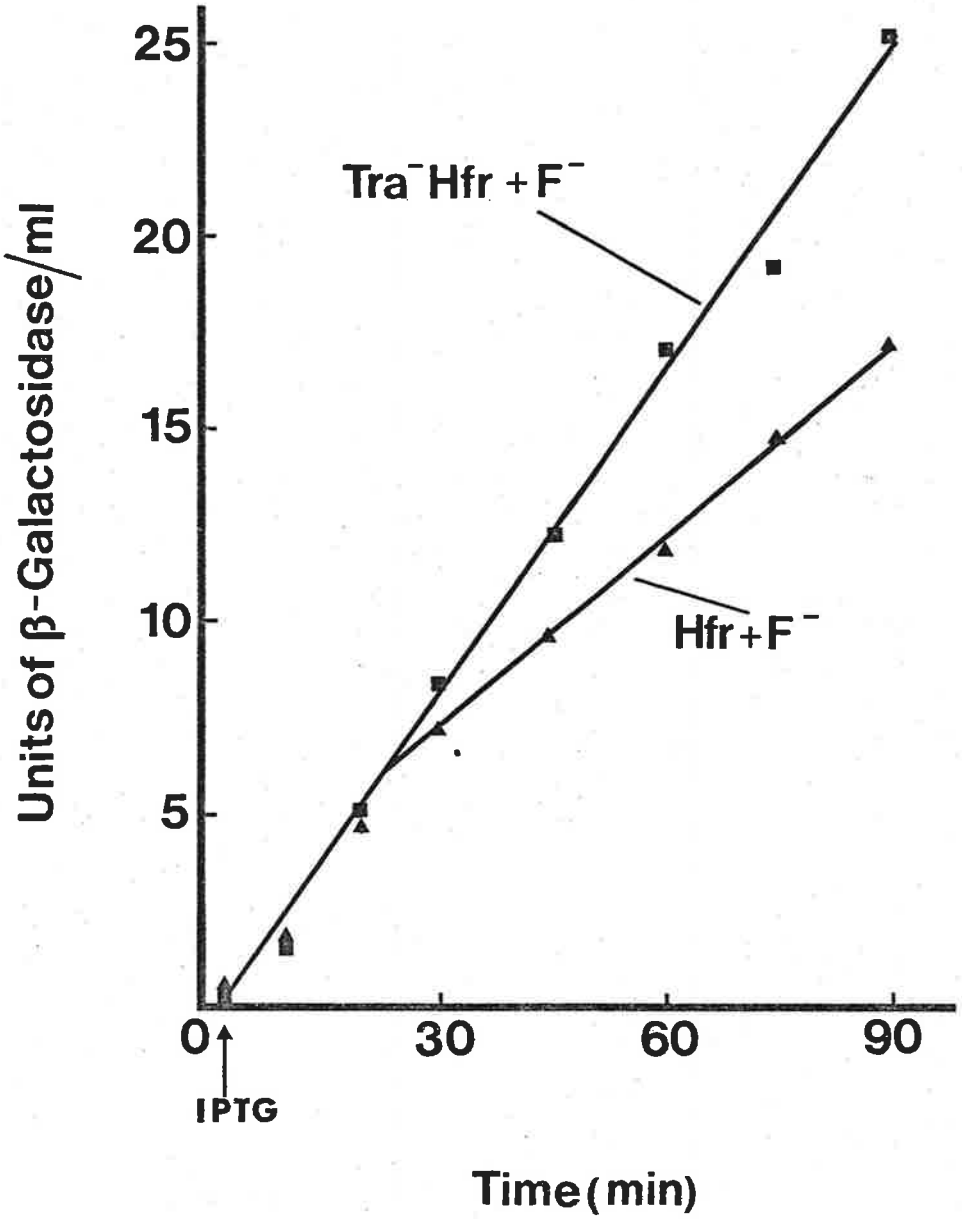

4
3
2
1
0
30
60
90
120
Time (min)
^{3}H-Thymine Incorporation-(10^3cpm/0·1ml)
Tra$^-$ Hfr + F$^-$
1:1 & 25:1
Hfr+ F$^-$
1:1
Hfr + F$^-$
25:1
Hfr
400
300
200
100
0
% F$^-$ survivors
0
60
120
Time (min)

FIG. 5-3

Effect of lethal zygosis on recipient cell β-galactosidase induction. Supplemented minimal liquid medium cultures of P311 (HfrH) and the recipient P882 were mixed at zero time, to give a ratio of 25:1 (▲), in a final volume of 20ml. IPTG (5×10^{-4}M final concentration) was added and, at the indicated times, samples (1.0ml) were taken and assayed for β-galactosidase activity (Materials and Methods). A control with the Tra^- mutant P310 was included (■).


Units of β-Galactosidase/ml
25
20
15
10
5
0
30
60
90
IPTG
Time (min)
Tra⁻ Hfr + F⁻
Hfr + F⁻

by F^- cells in the presence of Hfr cells, the deviation from the control ($Tra^-Hfr + F^-$) occurring 25 to 30 min after mixing. This inhibition, with a ratio of 25:1, was accompanied by a 50% reduction in the F^- viable count at 120 min, and the kinetics of kill resembled those at a ratio of 1:1 with tryptone yeast extract broth (Fig. 5-4).

The use of the latter media allowed an examination of β-galactosidase induction under conditions where high levels of lethal zygosis were observed (Fig. 5-4). When Hfr and F^- cells were mixed in equal numbers there was a noticeable reduction in rate of induction, compared with the non-mating control (1:1), and as the ratio of Hfr:F^- was increased to 5:1 and 10:1 there was a corresponding increased effect. With a further increase to 25:1, deviation from the control (25:1) was observed by about 25 min, and by 65 min after mixing, synthesis was completely arrested.

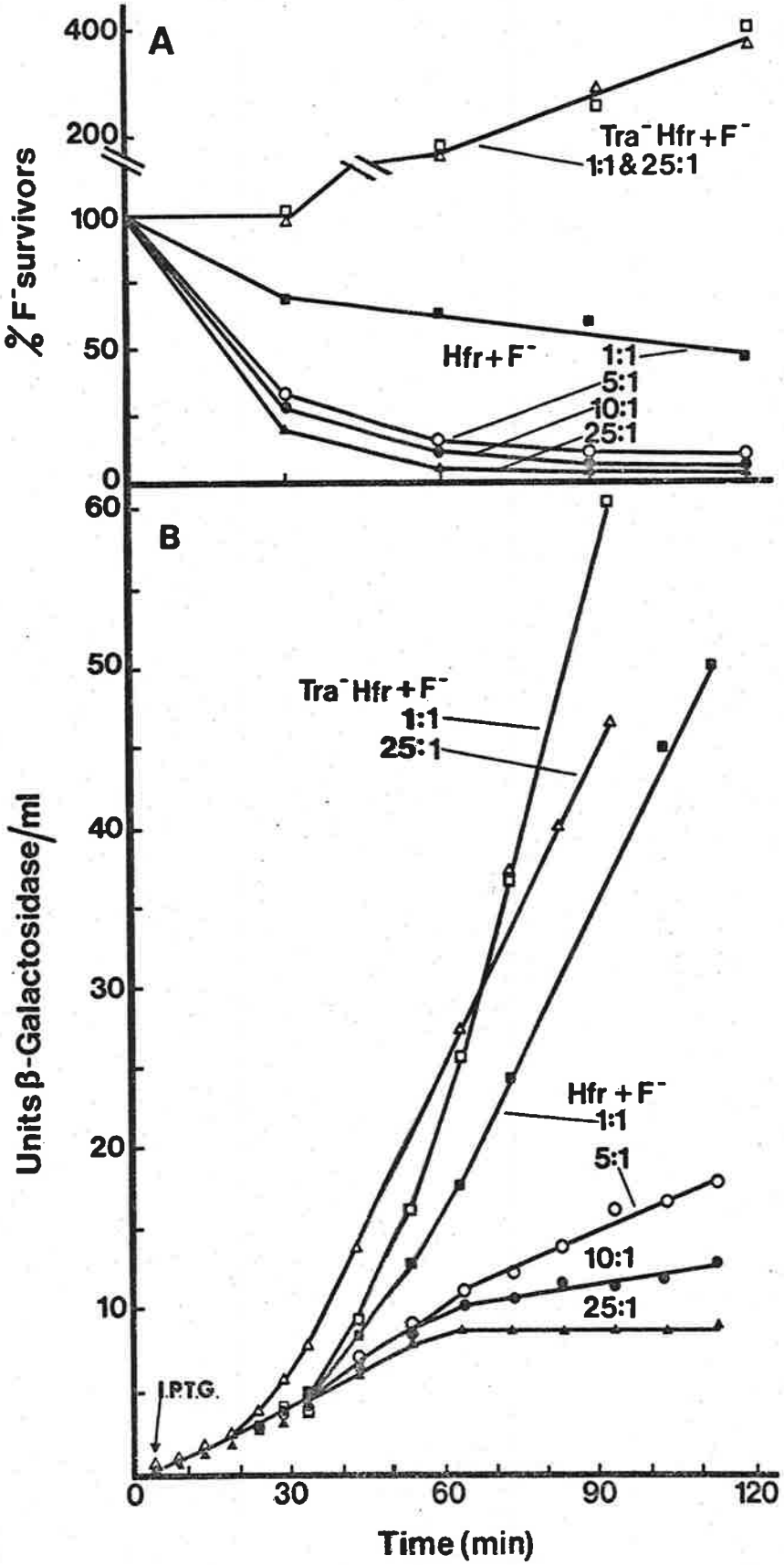
The initial difference in rate of induction between controls at 25:1 and 1:1 probably depends upon the presence of a low level of utilizable substrate, even in preconditioned media. This would be more rapidly exhausted in the presence of high cell numbers (25:1), but once removed, the culture with the lower cell density (1:1) could support a more rapid rate of synthesis.

TMG ACCUMULATION

A primary lesion that could have led to the observed arrest of both DNA synthesis and β-galactosidase induction, would be inhibition of energy metabolism. The accumulation by *E. coli* Lac^+ cells of non-

FIG. 5-4

Effect of concentration of the donor strain P311 (HfrH) on β-galactosidase induction in the recipient strain P882 (B). Cells grown in tryptone yeast extract broth were resuspended in preconditioned media, and donor and recipient cultures were mixed at zero time to give the following ratios: 1:1(■), 5:1(○), 10:1(●), 25:1(▲), in a final volume of 30ml. IPTG (5×10^{-4}M final concentration) was added and, at the indicated times, 1.0ml samples were taken and assayed for β-galactosidase activity. Induction in the presence of the Tra^- strain P310 was examined at ratios of 1:1(□) and 25:1(△). All enzyme activities were similar prior to 20 min; some data have been omitted for clarity. Percent F^- survival in each flask is shown (A).


A
400
200
100
50
0
% F⁻ survivors
Tra⁻ Hfr + F⁻
1:1 & 25:1
Hfr + F⁻
1:1
5:1
10:1
25:1
B
60
50
40
30
20
10
0
Units β-Galactosidase/ml
Tra⁻ Hfr + F⁻
1:1
25:1
Hfr + F⁻
1:1
5:1
10:1
25:1
I.P.T.G.
30
60
90
120
Time (min)

utilizable galactosides against a concentration gradient requires a continuing supply of metabolic energy (Kennedy, 1970), and therefore uptake of ^{14}C-TMG by the lactose permease system provides a convenient measure of energetic changes associated with lethal zygosis.

The result of one such experiment (Fig. 5-5) shows that accumulation by the recipient cells was rapid and, in the presence of Tra^- Hfr cells, continued to rise over the period of the experiment. When mixed with Hfr cells, by 15 min there was a loss of the ability to retain the accumulated galactoside against a concentration gradient. The Hfr (or Tra^-Hfr) cells which carry a lac deletion, and which were in 10-fold excess over the F^- cells, showed an uptake per cell of about 1% of that accumulated by the Lac^+ recipient cells. This indicates that, as expected, the lac deletion cells have only the same concentration of TMG internally as is present outside.

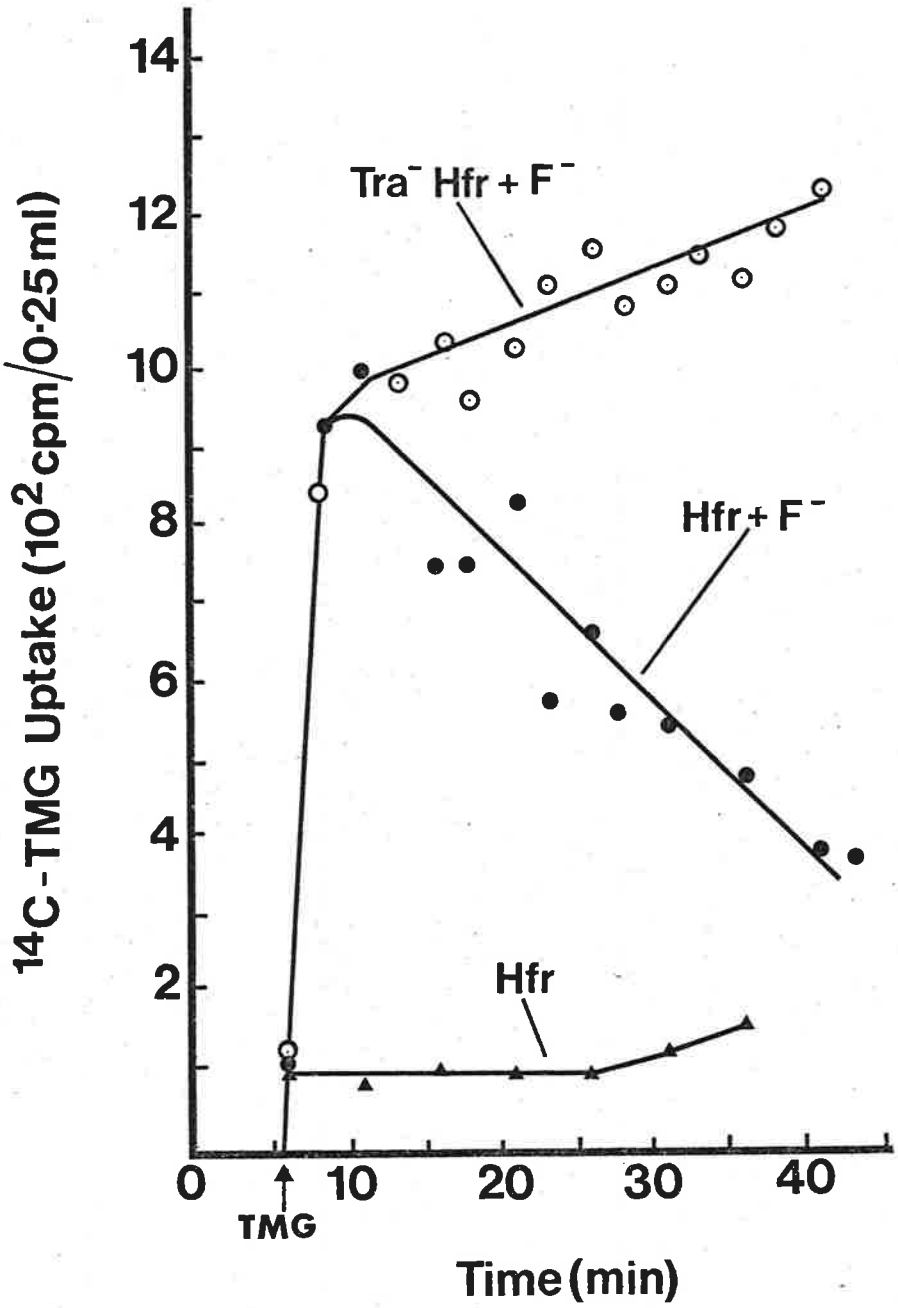
ONPG HYDROLYSIS

With intact cells, the rate-limiting step in the hydrolysis of ONPG to ONP by the enzyme β-galactosidase is the passage of this substrate across the membrane (Rickenberg, Cohen, Buttin and Monod, 1956). This hydrolysis has been used as a measure of ONPG transport either by the lactose permease-mediated system, in Lac^+ cells, or by the diffusion-like process which exists in cryptic cells (Herzenberg, 1959; Koch, 1964).

F^- (lacY) cells, in the presence of Tra^-Hfr cells, hydrolyzed ONPG

FIG. 5-5

Effect of lethal zygosis on recipient cell accumulation of TMG. The recipient strain P882 was induced by growth in tryptone yeast extract broth plus IPTG (10^{-3}M final concentration), and the cells were centrifuged, resuspended, and diluted in media without IPTG. A culture of the donor strain P311 (HfrH) was mixed at zero time with this preinduced F^- culture to give a ratio of 10:1(●). The initial F^- viable count was 3×10^7 cells/ml. ^{14}C-TMG was added (0.4μCi/ml, 4×10^{-5}M final concentration) and, at the indicated times, samples (0.25ml) were taken and the uptake of TMG was measured as described (Materials and Methods). As a control, F^- cells were mixed with the Tra^- strain P310 at the same ratio (○). The uptake by the donor strain P311 is also shown (▲). F^- survival was similar to that in Fig. 5-4.


14
12
10
8
6
4
2
0
10
20
30
40
TMG
Time (min)
^{14}C-TMG Uptake (10^2 cpm/0·25 ml)
Tra$^-$ Hfr + F$^-$
Hfr + F$^-$
Hfr

at a rate 20 times slower than toluene-treated cells (Fig. 5-6); however, when mixed with the donor strain P311 (HfrH), the hydrolysis increased as early as 15 to 20 min and eventually reached a rate which was 25 to 30% of that observed with toluene-treated cells.

As the long period of contact with ONPG produced high although reproducible levels of ONP in the control situation, the extent of hydrolysis over a 10 min period (Fig. 5-7) was also examined. With the ratio of 1:1, an increase in hydrolysis was observed by 50 min, and as the multiplicity of the Hfr strain employed was increased the extent of hydrolysis also increased, with deviation from the control occurring at progressively earlier times.

The latter method also allowed a comparison of the change in rate of hydrolysis with the extent of leakage of β-galactosidase from F^- cells (Fig. 5-7A). The initial background may have been partially due to the presence of a small number of cells remaining in the supernatant fluid. In the presence of the donor strain P311, the levels of β-galactosidase in the supernatant fluid increased to a level three times greater than the control culture and after 50 min accounted, in part, for the increase in hydrolysis observed in Fig. 5-7B.

The effect of lethal zygosis on the leakage of intracellular potassium (^{42}K) from prelabelled F^- cells was examined, but these experiments were complicated by the presence of an excess of donor cells.

FIG. 5-6

Effect of lethal zygosis on ONPG hydrolysis by recipient cells. The F^- strain P322 was grown in tryptone yeast extract broth in the presence of 10^{-3}M IPTG. These cells were harvested by centrifugation, resuspended, and diluted in IPTG free media; they were then mixed, at zero time, with either the donor strain P311 (HfrH, Δ) or the Tra^- strain P310 (▲) to give the indicated ratio. ONPG (3×10^{-3}M final concentration) was added and, at the indicated times, samples were examined for ONP produced (Materials and Methods). The hydrolysis by a toluene preparation of the F^- cells is shown (●). F^- survival was similar to that in Fig. 5-4.

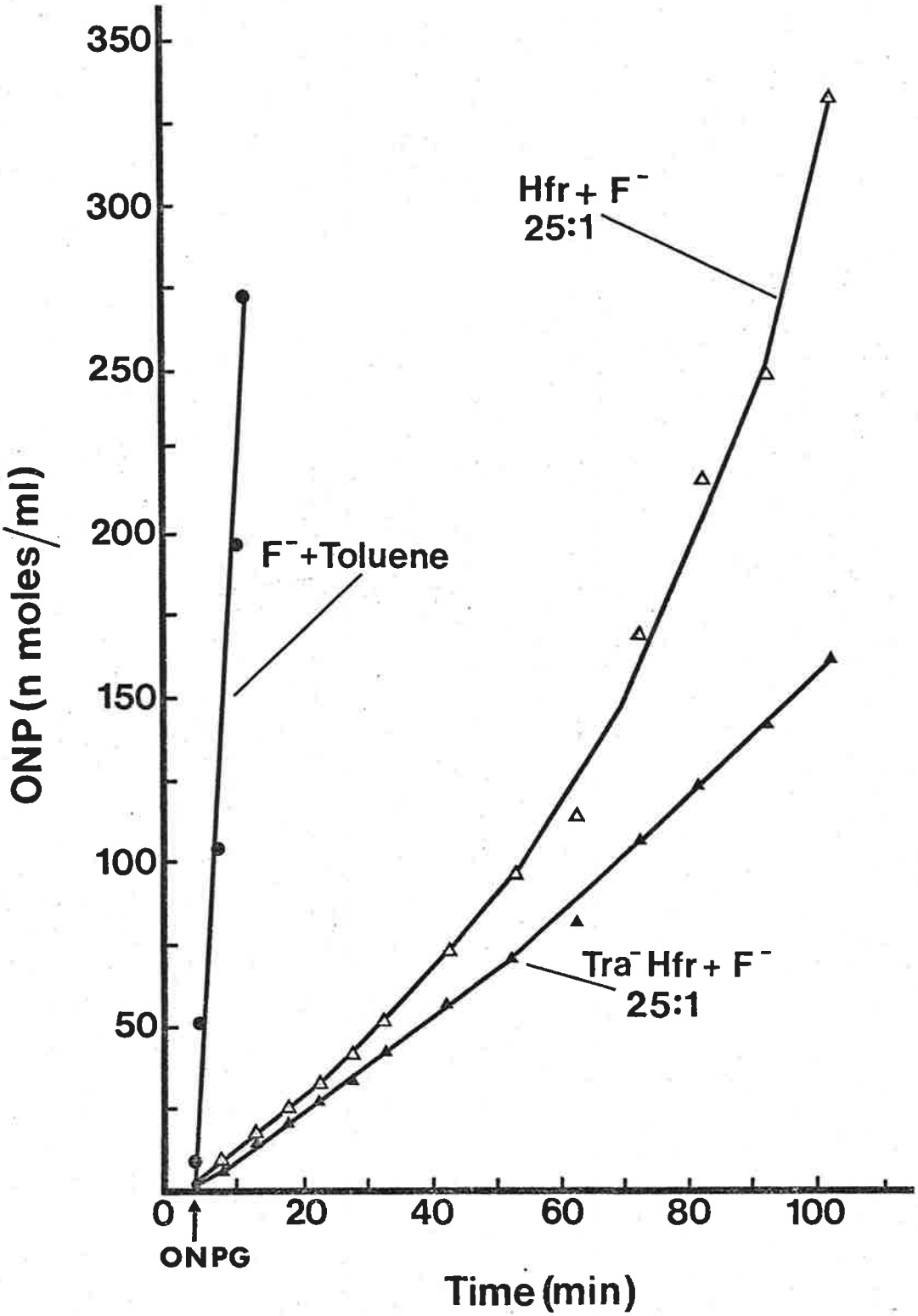
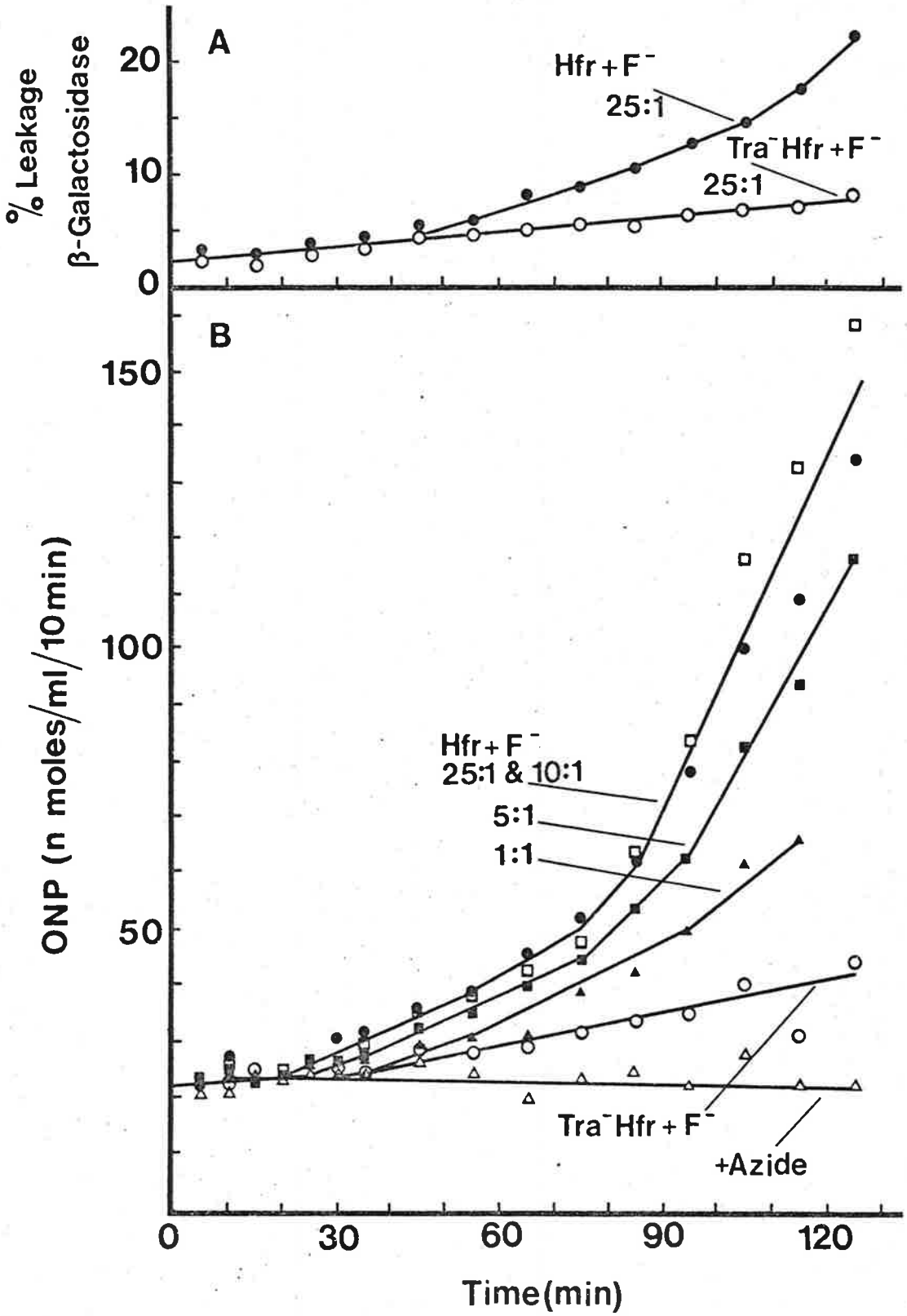

350
300
250
200
150
100
50
ONP (n moles/ml)
Hfr + F⁻
25:1
F⁻+Toluene
Tra⁻ Hfr + F⁻
25:1
0
ONPG
20
40
60
80
100
Time (min)

FIG. 5-7

Effect of concentration of the donor strain P311 (HfrH) on hydrolysis of ONPG by cells of the recipient strain P322. The F^- culture was preinduced in tryptone yeast extract broth in the presence of 10^{-3}M IPTG. The cells were centrifuged, resuspended in media free of IPTG, and mixed with donor cells and media where required to give the following ratios: 1:1(▲), 5:1(■), 10:1(□), and 25:1(●). Samples of the preinduced F^- culture were also mixed with a culture of the Tra^- strain P310(○) and with this same strain in the presence of 10^{-2}M sodium azide (△) to give, in each case, a ratio of 25:1. At the indicated times after mixing, samples were removed and added to ONPG (~2 x 10^{-3}M final concentration), incubated for 10 min, and examined for production of ONP (B).

Samples (1.5ml) were also removed from mating (●) and control flasks (○), centrifuged, and assayed for β-galactosidase activity in the supernatant fluid as described (Materials and Methods) with the omission of toluene treatment (A). Activity in the supernatant fluid is expressed as a percentage of the total enzyme activity. F^- survival was similar to that in Fig. 5-4.


A
% Leakage
β-Galactosidase
20
10
0
Hfr + F⁻
25:1
Tra⁻ Hfr + F⁻
25:1
B
150
100
50
ONP (n moles/ml/10 min)
Hfr + F⁻
25:1 & 10:1
5:1
1:1
Tra⁻ Hfr + F⁻
+Azide
0
30
60
90
120
Time (min)

SUMMARY AND CONCLUSIONS

Evidence is presented showing that conjugation was accompanied by a number of changes in the physiology of recipient cells. Both DNA and protein syntheses (measured respectively by ^{3}H-thymine incorporation and β-galactosidase induction) were found to be inhibited, as was the energy dependent accumulation of ^{14}C-TMG.

The observed leakage of β-galactosidase and the increased rate of ONPG hydrolysis both suggest that recipient cells may lose membrane integrity during conjugation. The extent of changes in macromolecular syntheses and ONPG hydrolysis, like F^{-} cell death, were found to be dependent upon the number of Hfr cells employed.

CHAPTER 6

F FACTOR-MEDIATED IMMUNITY TO LETHAL ZYGOSIS

INTRODUCTION

The studies reported in Chapter 4 showed that all F^- strains examined were sensitive to lethal zygosis, whereas Hfr and F^+ strains were insensitive. These results led to the suggestion that there was an immunity to lethal zygosis associated with the presence of the F factor.

A closer re-examination of this apparent immunity was prompted by the isolation of derivatives of an F^- strain that were insensitive to lethal zygosis but had gained sensitivity to male-specific phages. In this chapter, the selection of these strains (the P190 series) is described and their properties are compared with strains known to carry the sex factor F.

In order to further characterize the immunity to lethal zygosis possessed by Hfr and F^+ strains, and to locate the gene(s) responsible for such on the F factor map, a number of donor strains with point mutations or deletions in their F factor were examined and these results are described.

ISOLATION OF THE P190 SERIES OF STRAINS

When a culture of an F^- strain is cross-streaked against a culture of a Hfr strain, as in the plate test, or when the Hfr culture is spotted onto a recently spread lawn of F^- cells, there is inhibition of F^- growth in the area of direct Hfr contact (Fig. 2-2). If these plates are incu-

bated further there arises, within the zone of inhibition, a number of small colonies. When 32 such survivors, from a test between the Hfr strain AB259 and the F^- strain AB1133, were tested against AB259, all but one survivor (P190) showed sensitivity, indicating that the majority of survivors did not make up a genetically more resistant population; approximately 50% of the survivors were recombinant for donor chromosomal markers. The strain, P190, had not only gained resistance to AB259 but to all other Hfr strains tested and, unlike the other survivors, had become sensitive to the male-specific phage MS2. In the case of liquid lethal zygosis with these strains, recombinants made up 5 to 10% of the surviving population, and all survivors tested remained sensitive to the Hfr strain AB259. Heterogeneity with respect to competence to form mating pairs, within a population of recipient cells (Walmsley, 1973), could account for the few percent of F^- cells that survive lethal zygosis.

In a further attempt to isolate mutants resistant to lethal zygosis (see Chapter 7), the solid media selection procedure was repeated. Of a total of 250 survivors examined, 36 showed resistance to AB259 (Table 6-1). Again approximately 50% of the survivors were recombinants for the markers tested, including 33 of the 36 resistant survivors. In addition, all of the resistant survivors had, like P190, become sensitive to the male-specific phages MS2 and fd (Table 6-2) which adsorb to the sides and to the tip of the F pilus, respectively. These strains are

TABLE 6-1

P190 series; derivatives of the recipient strain AB1133, selected from solid media plate tests[a] with the Hfr strain AB259.

Representative strains	No. of each type isolated	Donor chromosomal markers inherited	Sensitivity to AB259 on solid media[a]
AB1133			++
P190[b]	1	None	-
P323	3	None	-
P325	1	*thr*$^+$	-
P324	2	*thr*$^+$ *leu*$^+$	-
P358	24	*thr*$^+$ *leu*$^+$ *pro*$^+$	-
P329	4	*thr*$^+$ *leu*$^+$ *pro*$^+$ *lac*$^+$	-
P359	2	*thr*$^+$ *leu*$^+$ *pro*$^+$ *lac*$^+$ *his*$^+$	-

[a]Solid media lethal zygosis as described in Materials and Methods.

[b]Isolated in an independent series of tests from the remainder.

TABLE 6-2

Relative efficiencies of plating of male- and female-specific phages on various strains.

Strain	Efficiency of Plating					
	MS2[a]	fd[a]	W31[b]	T7[b]	ØII[b]	ØW[b]
AB1133	$<1x10^{-9}$	$<1x10^{-8}$	1	1	1	1
AB259	1	1	$*10^{-2}$	*0.3	$*7.1x10^{-3}$	$*3x10^{-5}$
P190	0.8	0.8	ND[c]	0.7	0.9	ND
P323, P324, P325, P329	0.8-1.2	0.6-0.9	0.5	0.6-1.1	0.8-1.0	0.5
χ209	1.1	1.1	$*4x10^{-4}$	$*1.1x10^{-2}$	ND	ND
P347	1.1	1.1	*0.2	*0.24	ND	ND
KL20	1.0	0.9	$*2x10^{-4}$	$*7x10^{-2}$	$*8.3x10^{-2}$	$<1x10^{-5}$
P348	1.1	1.0	*0.2	*0.23	*0.36	$*3x10^{-3}$

[a]Hfr strain AB259 was used as a reference for the male-specific phages MS2 and fd.

[b]F^- strain AB1133 was used as a reference for the female-specific phages W31, T7, ØII, and ØW.

*Marked reduction in plaque size compared with AB1133.

[c]Not done.

referred to as the P190 series.

PROPERTIES OF THE P190 SERIES OF STRAINS

The sensitivity to male-specific phages indicated that the P190 series had acquired, during the plate mating, at least the region of the F factor coding for pilus production. The lack of sensitivity to Hfr donors, observed with these strains, might therefore have been due to the proposed F factor-mediated immunity to lethal zygosis. To test this hypothesis the properties of the P190 series were compared with those of AB1133 carrying known F factors.

With all strains examined, the efficiency of plating the male-specific phages was similar; however, response to the so called female-specific (F restricted) phages differed widely. The Hfr strain AB259 plated the phages W31, T7, ØII, and ØW with a much reduced efficiency and plaque size than did the F^- strain AB1133 (Table 6-2). The two F^+ strains χ209 and KL20 also demonstrated this restriction, as did P347 and P348, the latter to a lesser extent. In contrast to these results, the P190 series plated the phages with an efficiency approaching the F^- parent, and in no case was a marked reduction in plaque size or loss of halo observed.

The ability of the P190 series to act as donors was examined in a number of crosses with the recipient strain P882. With both P190 and P324, Met^+ recombinants were formed at a frequency of 10^{-6} per input

donor cell, whereas transfer of sensitivity to the male-specific phage, MS2, occurred at a frequency of 30 to 50%. P323 and P325 also transferred sensitivity to MS2 phage at this high frequency. This transfer of MS2 phage sensitivity was always accompanied by transfer of immunity to lethal zygosis, as in the formation of the strain P353 (Table 6-3). The transfer of MS2 phage sensitivity also occurred at a high frequency (80 to 90%) in a cross between P324 and P107, yet recombinants ($T^+L^+Str^s$) were formed at a frequency of 1 in 10^6. The strains that received MS2-sensitivity (e.g., P350) were themselves capable of acting as donors in crosses with the recipient AB1133 but were unable to inhibit this strain on solid media plate tests. Thus, it is apparent that the P190 series arose as a result of F factor transfer, and that the presence of that sex factor rendered them immune to lethal zygosis.

Since it had been reported (Linial and Malamy, 1970) that F restriction of female-specific phage depended upon the host strain employed, the transfer of the factors of P190 series strains out of the AB1133 background, into P107 and P882, allowed further examination of the apparent inability of these cells to restrict. The results (Table 6-3) show that in both of these strains, as in AB1133, the sex factor of P324 had no effect on plating efficiency or plaque size of phage ØII-T. In contrast, introduction of the known sex factors from χ209 and KL20 into these strains led to restriction of ØII-T. Similar results were obtained with ØII supplied by D. Monner.

TABLE 6-3

Transfer of immunity to lethal zygosis and the sensitivity of various strains to ØII-T.

Strain	Sex and derivation[a]	Sensitivity to AB259 on solid media	ØII-T EOP[b]	ØII-T plaque morphology
AB1133	F^-	++	1.0	Large centre; v. large halo
AB259	HfrH	ND[c]	2.8×10^{-3}	Small centre: small halo
P324	$MS2^s$(AB259xAB1133)	-	1.1	As AB1133
P107	F^-	ND	1.25	Large centre; v. large halo
P350	$MS2^s$(P324xP107)	ND	1.0	As P107
P882	F^-	++	1.25	Large centre; large halo
P353	$MS2^s$(P324xP882)	-[d]	1.2	As P882
χ209	F^+	ND	5×10^{-4}	
P347	F^+(χ209xAB1133)	-	0.2	V.small centre; no halo
P357	F^+(χ209xP882)	-	0.5	V.small centre; v. small halo
KL20	F^+	ND[e]	1.2×10^{-4}	V.small centre; no halo
P348	F^+(KL20xAB1133)	-	0.24	As KL20
P356	F^+(KL20xP882)	-	0.5	V.small centre; v. small halo

[a] Selected following conjugation (parents shown in brackets).

[b] Efficiency of plating relative to the female strain AB1133, as described in Materials and Methods.

[c] Not done.

[d] Similar results with recombinants from crosses between other $MS2^s$ strains (P190, P323, P325) and the F^- strain P882.

[e] P314, a F^+/His^- Str^r derivative of KL20, is resistant to AB259.

ACRIDINE ORANGE TREATMENT

It has been shown (Hirota, 1960) that F^+ donor populations are converted to recipients (cured) by treatment with acridine orange, whereas the sex factor is not removed from Hfr strains by this dye. Like the known F^+ strains, the P190 series of strains grown in the presence of acridine orange lost the ability to plate male-specific phage (Table 6-4). This loss, which had occurred in 95 to 100% of survivors examined, was always accompanied by sensitivity to lethal zygosis. In addition, in those colonies tested there was also loss of donor ability and increased recipient ability. The strains which had, during selection, received donor chromosomal markers (Table 6-1) were unchanged in these chacteristics after acridine orange treatment.

RECIPIENT ABILITY AND IMMUNITY DURING LETHAL ZYGOSIS IN LIQUID MEDIA

Cells harbouring functional F factors are poor recipients in crosses with other donor cells carrying isogenic or closely related sex factors. This exclusion (Meynell, 1969; Novick, 1969) can be temporarily overcome by growth of donor bacteria to saturation density in aerated broth (Lederberg *et al.*, 1952), the cells so produced behaving as recipients. These F^- phenocopies retain their sex factor, for on subculture into fresh media full donor ability is restored (Curtiss *et al.*, 1969).

The results in Table 6-5, obtained from crosses with exponentially growing cells as recipients, indicate that the P190 series have surface exclusion characteristics comparable with F^+ strains. Furthermore, when

TABLE 6-4

Acridine orange treatment of various strains.

Strain	Sensitivity to MS2 phage[a]	Sensitivity to AB259 on solid media[b]
AB1133 (F^-)	R	++
P347 (F^+)	S	-
P347+AO[c]	R	++
P348 (F^+)	S	-
P348+AO	R	++
P190[d]	S	-
P190+AO[d]	R	++
P314 (F^+)	S	-
P314+AO	R	++
P315 (Hfr)	S	-
P315+AO	S	-

[a] By spot test (Materials and Methods). R, resistance to phage; S, sensitivity.

[b] As described in Materials and Methods.

[c] Acridine orange treatment as described in Materials and Methods.

[d] Similar results with the strains P323, P324, P325, and P329.

TABLE 6-5

Surface exclusion characteristics of various donor strains in matings with the Hfr strain KL96[a].

Recipient strains	Logarithmic-phase recipients		Stationary-phase recipients	
	Recombinants[b] (%)	Surface exclusion index[c]	Recombinants (%)	Surface exclusion index
AB1133	1.07	1	0.66	1
P190	0.003	360	0.1	6.6
P323	0.004	270	0.09	7.3
P324	0.004	270	0.03	22.0
P347	0.004	270	0.28	2.4
P348	0.002	540	0.03	22
P314	0.005	210	0.09	7.3
P315	0.002	540	0.03	22

[a] As described in Materials and Methods.

[b] His^+Str^r recombinants as percent input recipient.

[c] Surface exclusion index is essentially as described by Achtman et al. (1971), and is defined as the ratio of the number of recombinants obtained with the F^- (in this case AB1133) to the number obtained with the strain under test (for details see Materials and Methods).

stationary-phase, aerated cultures were employed as recipients, there was a gain in recipient ability, indicating the formation of F^- phenocopies.

Standard liquid lethal zygosis conditions (Materials and Methods) make use of aerated stationary-phase recipient cultures, and in this state there was a rapid and continuous fall in the number of AB1133 survivors when this recipient strain was mixed with an excess of the Hfr strain AB259 (Fig. 6-1). In contrast, P323 and the F^+ strain P348 showed an initial sensitivity, yet by 90 min the total population of both had begun to increase and eventually reached a level above the starting titre. Also, both strains yielded more recombinants than did AB1133, while demonstrating similar initial kinetics of transfer. The three strains grew equally well in the presence of the transfer defective (Tra^- Hfr) strain P980, which provided a suitable nonmating control. This immunity to liquid lethal zygosis has also been demonstrated with the strain P190 and with P356, a derivative of the recipient strain P882 carrying the same F factor as P348.

Logarithmic-phase recipient cells are less susceptible to lethal zygosis than are stationary-phase cells (Chapter 3). However, such a culture of AB1133 was markedly inhibited in the presence of Hfr cells, whereas similar cultures of P323 and P328, which formed 2,000-fold fewer recombinants than AB1133, were unaffected (Fig. 6-2).

FIG. 6-1

Lethal zygosis in liquid media: sensitivity of cells carrying an F factor. Aerated, stationary-phase cultures of the F^- strain AB1133 (●,○), the F^+ strain P348 (■,□), and the $MS2^s$ strain P323 (▲,△) were diluted and used as recipients in crosses with either logarithmic-phase cultures of the Hfr strain AB259 (closed symbols) or with a similar culture of the Tra^- Hfr strain P980 (open symbols). The ratio of Hfr cells to recipient cells was 25:1. Standard lethal zygosis conditions (Materials and Methods) were employed, and samples were diluted and plated for survivors (A) and for $Thr^+Leu^+Str^r$ recombinants (expressed as a percentage of the input recipient cells) (B).

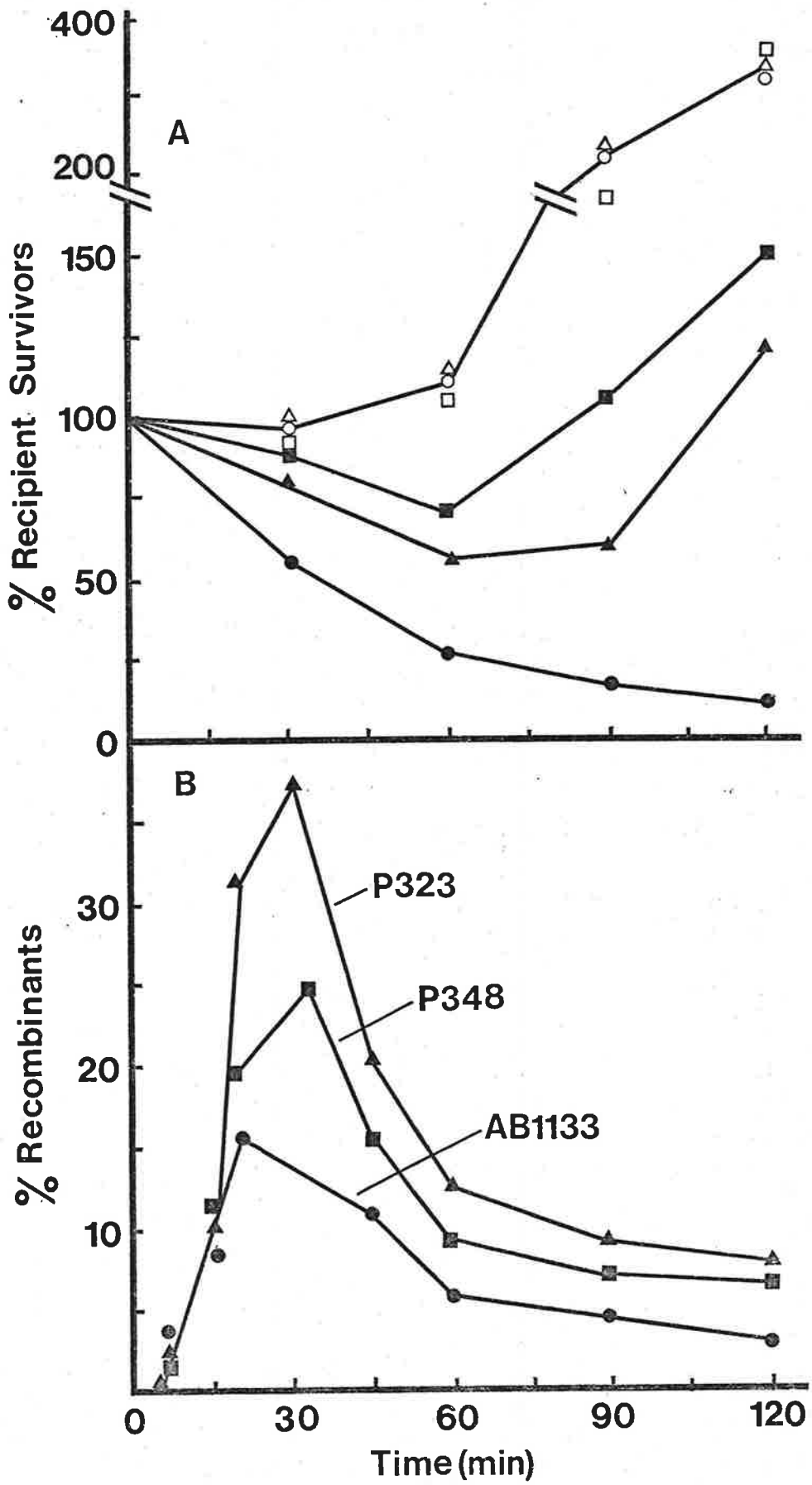
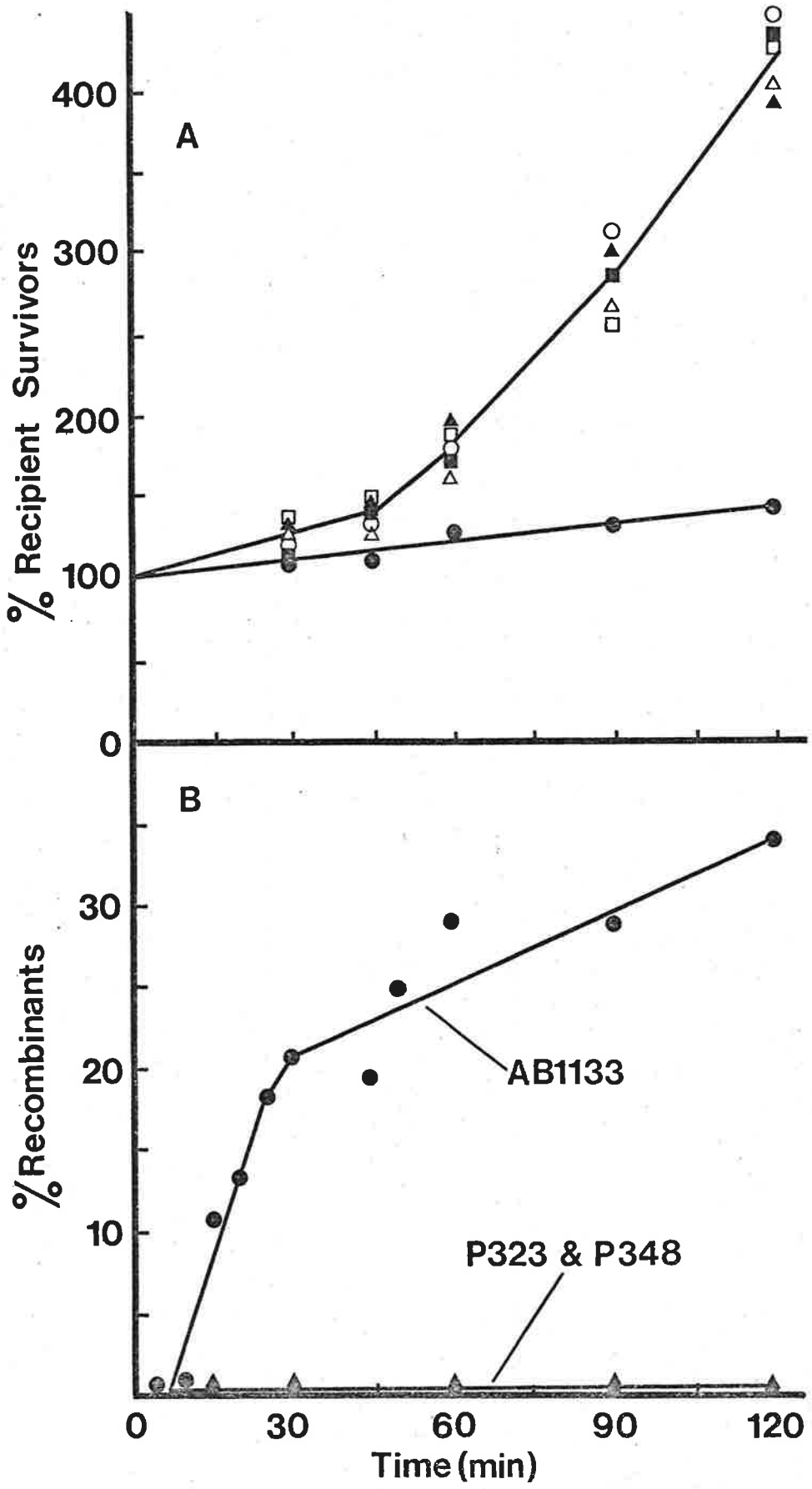

400
200
150
100
50
0
A
% Recipient Survivors
B
30
20
10
% Recombinants
P323
P348
AB1133
0
30
60
90
120
Time (min)

FIG. 6-2

Lethal zygosis in liquid media: sensitivity of cells carrying an F factor. Except for the use of logarithmic-phase cultures of the strains (AB1133, P323, and P348) to be employed as recipients, all procedures and symbols were as described in Fig. 6-1.


A
% Recipient Survivors
400
300
200
100
0
B
%Recombinants
30
20
10
AB1133
P323 & P348
0
30
60
90
120
Time (min)

ROLE OF *tra* GENES IN IMMUNITY TO LETHAL ZYGOSIS

The results in the previous sections confirm that there is an F factor-mediated immunity to lethal zygosis (Ilz^+). There was also a correlation between surface exclusion and immunity, for it was only when the exclusion barrier was broken down (Fig. 6-1) that any degree of sensitivity was induced in a donor cell culture. Since both donor ability and exclusion are known to depend upon genes within the *tra* region of the F factor and are thought to be in one operon under *J* control (Willetts, 1972a; Achtman, 1973a and personal communication), it could be that immunity is also under the same control and might even be due to surface exclusion mediated by the *traS* product(s).

In order to test this hypothesis, the properties of donor strains carrying an F*lac* episome, mutant in one of a number of *tra* cistrons, were compared with those of a F*lac* *tra*$^+$ donor strain (Table 6-6). Although P372, carrying JCFL90, had lost transfer ability (Tra^-) and surface exclusion (Sex^-), the cells carrying this episome retained immunity to lethal zygosis in a solid media test, as did the Tra^+ Sex^- strain ED2177 and the pililess *traA* mutant P371.

These results were confirmed by liquid lethal zygosis tests with logarithmic-phase cultures of the F*lac* strains as recipients (Fig. 6-3). The Sex^- strains, like the F^- parent JC3272, formed high levels of recombinants although there was a slight lag with the piliated strain ED2177; however, unlike the F^- strain which was inhibited, these Sex^-

TABLE 6-6

Properties of transfer-defective (*tra*) mutants of F*lac*[a].

Strain no.	Episome no.	*tra* mutation	Phage sensitivities[b]		Surface exclusion index[c]	Transfer efficiency[d]	Sensitivity to AB259 on solid media[e]
			MS2	ØII-T			
JC3272			R	S	1.0		++
P373	JCFL0	*tra*$^+$	S	Rs	500	50	-
P371	JCFL1	*traA1*	R	Rs	150	$<1\times10^{-6}$	-
P372	JCFL90	*traJ-90*	R	Rs	1.0	$<6\times10^{-5}$	-
ED2177	EDFL33[f]	*tra*$^+$(Sex$^-$)	S	Rs	1.0	70	-
P370	JCFL4	*tra-4*	R	Rs	1.3	$<1\times10^{-6}$	-

[a] All episomes were transferred to the F$^-$Su$^-$ strain JC3272, for direct comparison.

[b] By spot test. R, resistance to the phage; S, sensitivity; Rs, restriction (markedly reduced plaque numbers and size).

[c] Surface exclusion index, as described in Table 6-5, was calculated relative to JC3272. Logarithmic-phase recipient cells were mixed with the Hfr strain KL96, as described in Materials and Methods.

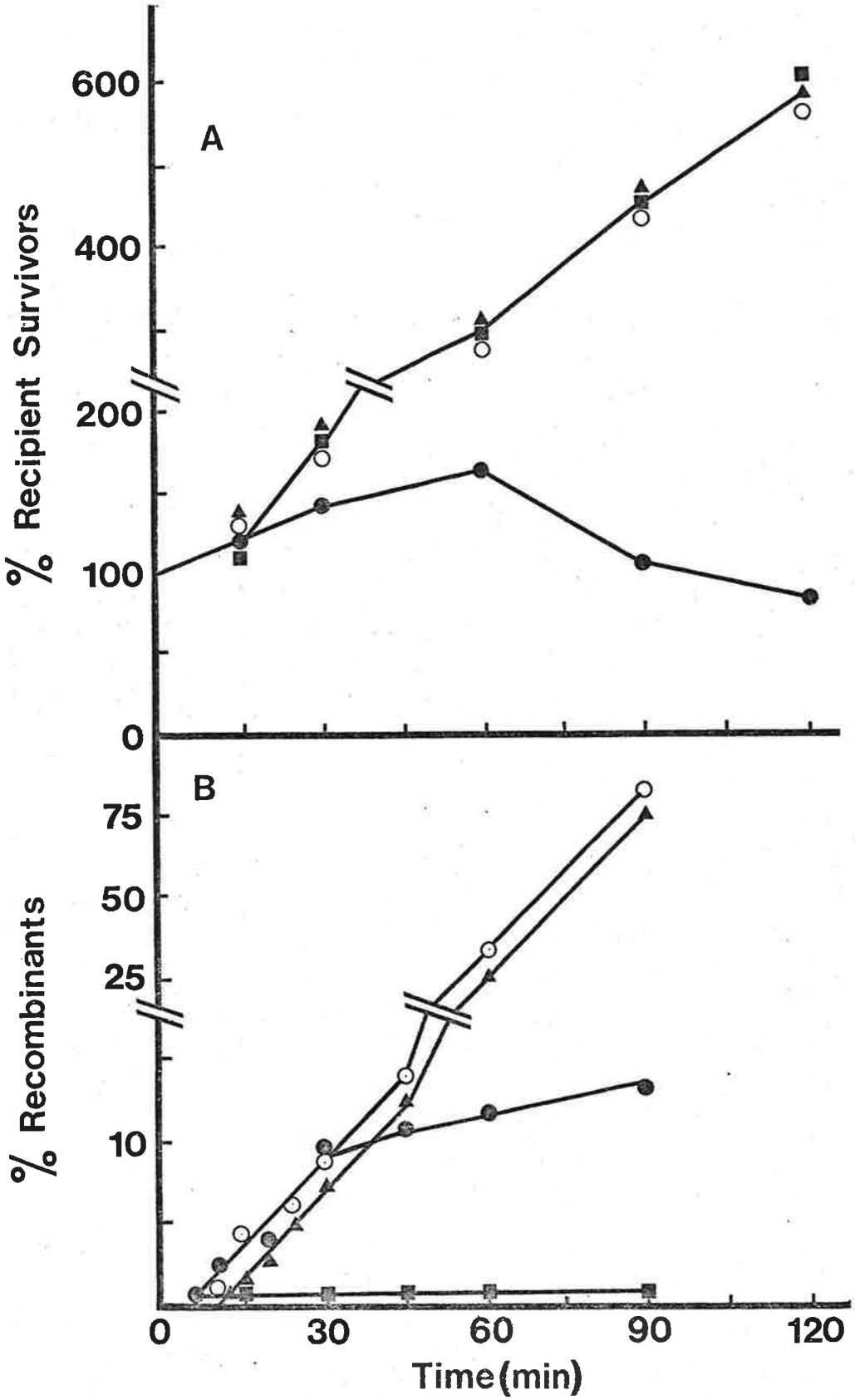
[d] Transfer efficiency was measured (as described in Materials and Methods) with ED267 as recipient and selection for Lac$^+$Lys$^+$ recombinants. The results are expressed as a percentage of the input donor cells.

[e] Similar immunity to lethal zygosis was observed when these episomes were examined after transfer to the F$^-$Su$^-$ strain ED267.

[f] EDFL33 is a Tra$^+$ revertant of JCFL3 (which carries *traC3* and a second mutation leading to Sex$^-$; N. Willetts, personal communication).

FIG. 6-3

Sensitivity of transfer-defective (*tra*) mutants of F*lac* to lethal zygosis. Late logarithmic-phase cultures of the F*lac* strains P373 (Tra^+Sex^+, ■), ED2177 (Tra^+Sex^-, ▲), and P372 (Tra^-Sex^-, ○) and the F^- strain JC3272 (●), were diluted and mixed with an early logarithmic-phase culture of the Hfr strain KL96 (for details see Materials and Methods). Samples were diluted and plated for survivors (A) and for His^+Str^r recombinants (B).


A
600
400
200
100
0
% Recipient Survivors
B
75
50
25
10
% Recombinants
0
30
60
90
120
Time (min)

strains grew as well as the Tra$^+$ strain P373 which exhibited normal surface exclusion under these conditions.

The episome JCFI4 carries an amber mutation, tra-4, which is located near traK and is thought to be polar on the postulated traS cistron as well as on traK, traB, traC, traF, traH, and traG. (Willetts and Achtman, 1972). Cells carrying this episome were also found to be fully immune to lethal zygosis on solid (Table 6-6) and in liquid media (similar to P372; Fig. 6-3).

It would appear, therefore, that a cell need not possess either F pili or a surface exclusion system to be immune to lethal zygosis.

LOCATION OF ilz ON THE F FACTOR GENETIC MAP

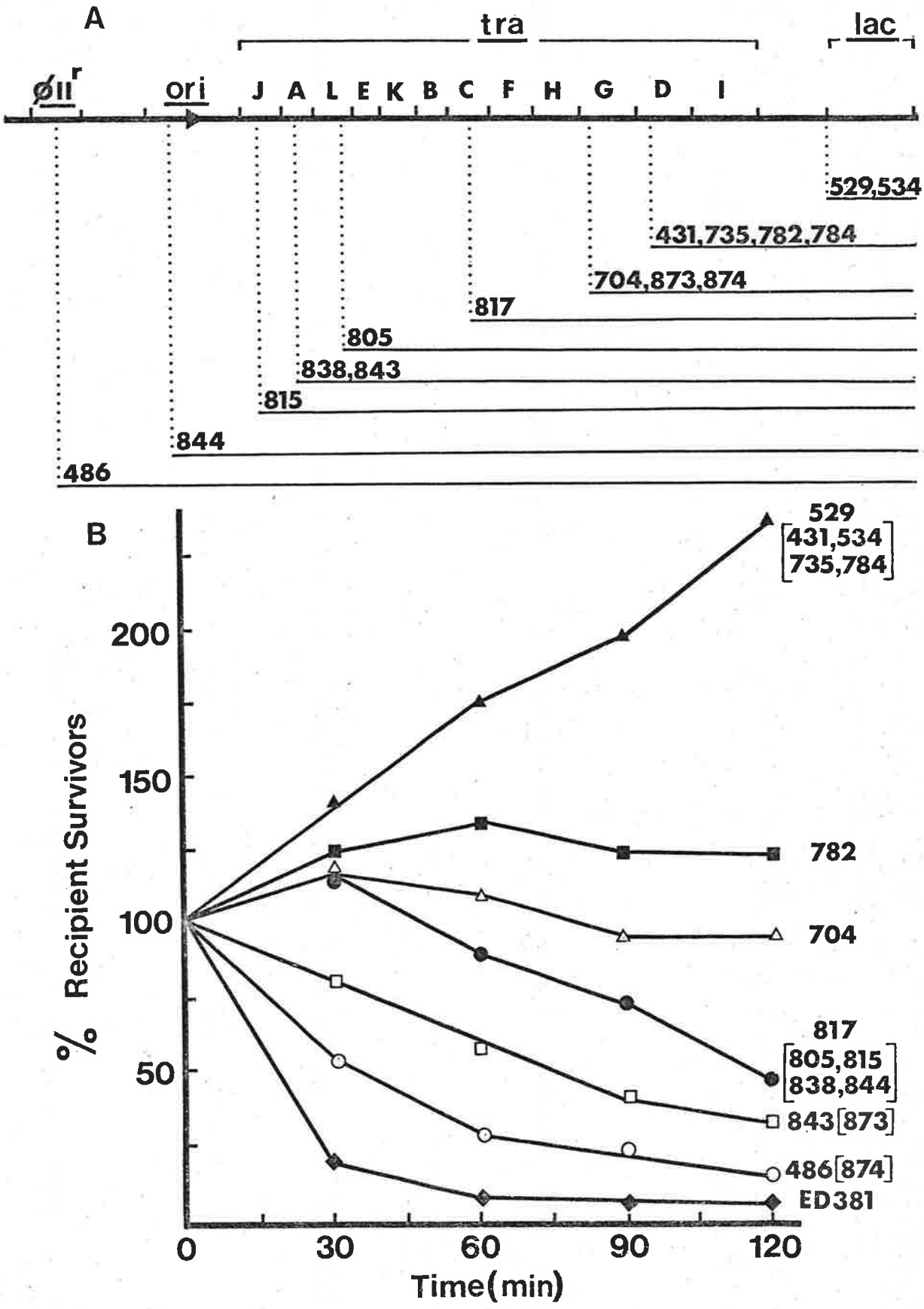
The results with the traJ-90 strain P372, in the previous section, indicate that the postulated ilz region(s) on the F factor, that codes for immunity to lethal zygosis, is in a different operon from the tra genes or is, at least, independent of J control.

In order to establish the location of the ilz gene(s) on the F factor map in relation to the known tra genes, strains of the KI series of Hfr strains (Ippen-Ihler et al., 1972) were examined. These strains, which carry deletions extending for various lengths into an integrated Flac episome (as shown in Fig. 6-4), were employed as recipients in a series of liquid lethal zygosis tests. It would be expected that deletions extending into the ilz region would render the host cell sensitive to lethal zygosis.

FIG. 6-4

A. A map of Flac. The indicated cistrons have been described in the Introduction. Map distances are arbitrary and lines underneath represent the extent of deletions carried by the KI series of Hfr strains (Ippen-Ihler et al., 1972; Willetts, 1972b and personal communication).

B. Lethal zygosis sensitivity of the KI series of Hfr strains. Late logarithmic-phase cultures of the KI strains 529 (▲), 782 (■), 704 (△), 817 (●), 843 (□) and 486 (○), and the F⁻ strain ED381 (◆) were mixed with an early logarithmic-phase culture of the Hfr strain AB261 (lethal zygosis conditions as described in Materials and Methods). Samples were diluted and plated for survivors on selective media at the indicated times. The results obtained are representative of patterns of sensitivity of these and other KI strains (shown in brackets) in more than one experiment.


A
tra
lac
øII
ori
J A L E K B C F H G D I
529,534
431,735,782,784
704,873,874
817
805
838,843
815
844
486
B
% Recipient Survivors
200
150
100
50
0
30
60
90
120
Time(min)
529
[431,534
735,784]
782
704
817
[805,815
838,844]
843[873]
486[874]
ED381

As shown in Fig. 6-4B, some deletions (KI431, KI735, and KI784) ending in traD retained immunity to lethal zygosis, whereas one other deletion ending in traD (KI782) and all longer deletions had become sensitive to lethal zygosis to some extent. This suggests that ilz is located between traG and traD. Wide variations in the extent of lethality were observed among the sensitive strains, and it could be said that, in general as the deletion got longer so sensitivity to lethal zygosis increased. However, a strain carrying a deletion into traG (KI874) was found to be as sensitive as one in which the deletion extended beyond the ØII^r locus (KI486).

The KI strains 431, 486, and 805 grew equally well in the presence of the Tra$^-$Hfr strain P727, whereas the F$^-$ strain ED381 grew at a faster rate. This F$^-$ strain is a derivative of XA7012, the F$^-$ parent of the transposition Hfr strain from which the Hfr deletions were made (Willetts, 1972b); its different growth rate may be reflected in its apparent increased sensitivity to lethal zygosis over the KI deletion strains.

SUMMARY AND CONCLUSIONS

It is clear from the results in this chapter that there is an immunity to lethal zygosis (Ilz$^+$) associated with the F factor. Temporary sensitivity to lethal zygosis can be induced in a cell by the formation of F$^-$ phenocopies, a result which suggested a relationship between surface exclusion (Sex$^+$) and immunity (Ilz$^+$). However, it was shown that Sex$^-$ Flac donor strains were Ilz$^+$, as were strains carrying Flac

with a traJ or traA mutation. It appears therefore that while Ilz is not under J control, its expression, like Sex, is affected by starvation of donor cells. The results with a series of Hfr deletion strains indicate that a gene(s) for immunity to lethal zygosis (ilz^+) is located on the F factor map between traG and traD.

CHAPTER 7

CONJUGATION-DEFECTIVE MUTANTS OF RECIPIENT CELLS AND RESISTANCE TO LETHAL ZYGOSIS

INTRODUCTION

It has been shown (Chapter 4) that F^- strains defective in recombinant formation (Rec^-) retained sensitivity to lethal zygosis as did a F^- P1 lysogen which was "restrictive" for donor DNA. It could therefore be argued that lethal zygosis resulted from conjugational events prior to the formation of recombination complexes. In addition, F*lac* Sex^- mutants were fully immune to lethal zygosis yet formed recombinants at high levels (Chapter 6), an observation which tends to preclude the hypothesis that lethal zygosis resulted from excess recombination events. Furthermore, the metabolic and physiological changes observed during conjugation (Chapter 5) would be unlikely to have all resulted from the recombination event per se.

To obtain further understanding of those primary physiological events necessary for lethal zygosis but presumably occurring in the recipient prior to the processes mediated by the Rec gene products, the selection of lethal zygosis-resistant F^- mutants was attempted. Among such cells one might expect to find conjugation-defective mutants altered in pair forming ability, analogous to bacteriophage receptor mutants, and mutants altered in any other subsequent step necessary for lethal zygosis.

Reeves (1972) has proposed that receptors for colicin molecules are

in fact surface structures on the cell, primarily concerned with conjugational events. It was further suggested that the initial contact between donor and recipient cells involved these receptors and that the subsequent events leading to chromosome transfer were triggered by the receptor's response to such contact. If this hypothesis is correct then some classes of colicin-resistant mutants of recipient cells might also be defective in conjugation. In this chapter the screening of such mutants is described.

Since cross-resistance between colicins and bacteriophages has been described (Fredericq and Gratia, 1949), bacteriophage-resistant mutants were also examined.

ATTEMPTS TO ISOLATE LETHAL ZYGOSIS-RESISTANT MUTANTS

In an attempt to select conjugation-defective mutants, a large number of survivors from liquid and solid media lethal zygosis tests were screened for sensitivity to an Hfr strain (Chapter 6). As yet no such mutant has been detected by this technique, for in all cases the "resistance" observed resulted from transfer of an F factor to the recipient, so rendering it immune.

In an effort to improve this selection procedure, mutagenesis (Adelberg, Mandel and Chen, 1965) combined with sib-selection in fluid culture (Cavalli-Sforza and Lederberg, 1956) was employed. A culture of the recipient strain AB1133 was treated with nitrosoguanidine, and diluted samples of this mutagenized culture (2-5 cells/ml) were dis-

pensed in 10ml amounts. After overnight incubation each sample was tested for sensitivity to the Hfr strain AB259 by the lethal zygosis plate test. It might have been expected from such a fluctuation test that any sample containing a higher proportion of resistant cells would be detected by this test, and that progressive enrichment would yield fully resistant cultures. However, no change in lethal zygosis sensitivity was observed among 250 samples examined.

SCREENING OTHER MUTANTS FOR LETHAL ZYGOSIS RESISTANCE

In addition to the attempted isolation, described above, a number of recipient strains were screened for resistance to lethal zygosis by the solid media plate test. Included were the following F^- strains that are resistant to a wide variety of colicins: P114, P115, P116, P117, P118, P119, P120, P125, P134, and P138 derived from AB1133 (Reeves, 1966, 1972); tolA-9, tolP-305, and tolP-507 derived from AB1133 (Bernstein, Rolfe and Onodera, 1972); ASH102 derived from ASH10 (Holland and Threlfall, 1969). All of these mutants retained the sensitivity to the HfrAB259 demonstrated by their colicin-sensitive parents. As well as these documented strains, a large series of recently isolated colicin-resistant mutants (Tables 7-1 and 7-2) were examined. Whereas the majority of strains were found to be fully sensitive to lethal zygosis those strains (P212, P657) tolerant to colicins K and L (Tol XIV; Table 7-1) were resistant.

Among the bacteriophage-resistant mutants examined (Table 7-3)

TABLE 7-1

Lethal zygosis sensitivity[a] of Group A colicin resistant-mutants[b] derived from AB1133.

Phenotypic class	Type strain	Number tested	Colicin resistance
Bfe	P525	10	E1,E2,E3,A
Tsx	P209	3	K
Rcx	P224	3	X
Tol Ia	P218	2	K,L,A,S4
Tol Ib	P210	3	K,L,A,S4,N
Tol IIb	P651	6	E1,E2,E3,K,L,A,S4,N
Tol IIc	P555	5	E1,E2,E3,K,L,A,S4,N
Tol III	P660	2	E2,E3,K,L,A,S4,N
Tol IV	P692	1	E2,E3,L,A,N
Tol VII	P689	1	E2,L,A,S4
Tol VIII	P602	1	E1,A
Tol IX	P663	1	E1,E3,K,L,A,S4,N
Tol X	P207	2	L,A,N
Tol XI	P661	1	L,A,N,S4
Tol XII	P653	1	L,p[c]A,S4
Tol XIII	P220	1	L,pA,pS4
Tol XIV	P212	2	K,L
Tol XV	P520	1	E2,E3,K,L,A,S4
Tol XVI	P530	1	E2,E3,K,L,A,N,X
Tol XVII	P686	2	E2,E3,K,L,A,N,S4,X
Tol XVIII	P516	1	E2,L,A
Tol XIX	P652	1	E1,K,L,A,S4,N

[a]By solid media plate test with the HfrH strain AB259.

[b]Details of colicin resistance and phenotypes were provided by Mr. J. Davies. Strains are tolerant to the colicins indicated with the exception of Bfe and Tsx strains which are resistant. Some of the characterization is provisional; final results to be published soon (Davies and Reeves, to be submitted to J. Bacteriol.)

[c]Partial resistance.

TABLE 7-2

Lethal zygosis sensitivity[a] of Group B colicin-resistant mutants[b] derived from AB1133.

Phenotypic class	Type strain	Number tested	Colicin resistance
I[c] (TonB)	P585	7	B,D,G,H,I,M,V,S1
II and III	P540	27	B,D,G,H,I,M,V,S1
IV	P535	1	B,D,G,H,M
V	P618	4	B,D
VI	P623	5	I^r,V,$S1^r$
VII	P628	11	I,V,S1
IX	P632	1	V

[a]By solid media plate test with the HfrH strain AB259.

[b]Details of colicin resistance and phenotypes were provided by Mr. J. Davies. Strains are tolerant to the colicins indicated, with the exception of those in Class VI which are resistant (r) to colicins I and S1. Final results to be published soon (Davies and Reeves, to be submitted to J. Bacteriol.).

[c]Strains of Class I are distinguished from those of Class II and III by their resistance to phage T1.

TABLE 7-3

Lethal zygosis sensitivity[a] of bacteriophage-resistant mutants[b] derived from P400.

Phenotypic class	Type strain	Number tested	Bacteriophage resistance
TonA	P417	5	T1,T5,E21,E25,D,K22,K26,K27,K28,K30
Bar	P402	19	B,F,G,J,T3,E4S,E7,0x2,0x4,0x5,K16, K20,K21,H^+,V,F27
TonA+Bar	P410	4	
TonB	P442	1	T1,E25,K22,K26,K27,K28,K30,Ø80
Tsx	P438	2	T6,H1,H3,H8,K9,K18,K31,0x1
Tsx+Bar	P434	2	
Tsx+TonA	P485	1	
Bfe	P445	5	BF23,K6,K8,K11,K12,Ac4,M3,E15
Tsx+Bfe	P463	1	
Kox	P460	3	K3,K4,K5,0x2,0x3,0x4,0x5,M1,Ac3
Kox+Bfe	P462	1	
Ktn	P466	2	K10
Ttk	P423	8	T2,K16,K19
Ktw	P456	5	K2,K20,K21,K29
Bar+Ktw	P488	2	
multigroup	P479	5	Bar+Ktw+Ktn+0x3,M1,Ac3,T7,Ø1,H,W31, E11

[a]By solid media plate test with the HfrH strain AB259.

[b]Details of bacteriophage resistance and phenotypes were provided by Mr. R. Hancock. Final results to be published soon (Hancock and Reeves, to be submitted to J. Bacteriol.).

those selected as resistant to phages K3 or K5 were found to be resistant to lethal zygosis (Table 7-4). These included the strains in the phenotypic classes Kox (P459, P460, P461) and Kox + Bfe (P462), but it was clear that this resistance to lethal zygosis was associated with Kox^r alone as Bfe mutants (Tables 7-1 and 7-3) retained sensitivity. Whole cells of the Kox^r strains were unable to adsorb the phages (Hancock and Reeves, unpublished data), indicating that these strains probably have altered receptor activity for Kox phages.

It has now been established that there is cross-resistance between colicins K and L and the bacteriophages of the Kox group (Davies, Hancock and Reeves, unpublished data), for the Tol XIV class of mutants were found to be Kox^r and vice versa. It would therefore seem that resistance to lethal zygosis and to the phages of the Kox group, as well as tolerance to colicins K and L, are closely related and that Kox^r and Tol XIV are perhaps identical. Kox^r strains were found to be fully resistant to lethal zygosis in liquid media (e.g., P460; Fig. 7-1A), as were the Tol XIV strains P212 and P657 which, like P460, grew equally well whether in the presence of an excess of cells of the Hfr strain AB259 or of the Tra^- Hfr strain P980.

A further group of F^- strains examined for lethal zygosis-resistance included the ampicillin-resistant (Amp^r) mutants described by Boman, Eriksson-Grennberg, Normark, and Matsson (1968) and Monner et al. (1971). These authors found that some F^- strains (e.g., D31) with high levels of

TABLE 7-4

Properties of strains resistant to lethal zygosis

Strain	Phenotypic class	Sensitivity to AB259 on solid media	Recipient ability[a]	Ampicillin resistance[b] (μg/ml)	EOP with phage[c]	
					ØW	Ø3
P400	Kox^s	++	1	1.5	0.5	0.7
P459	Kox^r	-	$<10^{-3}$	0.5	0.6	0.8
P460	Kox^r	-	$<10^{-3}$	1.0	0.6	0.9
P461	Kox^r	-	$5x10^{-3}$	0.5	0.6	0.9
P462	Kox^r+Bfe	-	$2.5x10^{-3}$	0.5	0.6	0.9
D11	Amp^s	++	1	1.5	$<10^{-7}$	0.7
D21	Amp^r-I	++	1	25	$<10^{-7}$	0.6
D31	Amp^r-III	±	0.5	100	1	1
D31m4	Amp^r-III+ $ØW^r$	-	$4x10^{-3}$	100	$<10^{-7}$	*10^{-1}-10^{-2}

[a]As described in Materials and Methods. Recipient ability is defined as the ratio of the number of recombinants obtained with the mutant strain to the number obtained with the parent (P400 or D11). With Kox^r strains $T^+L^+St^r$ recombinants were measured in a cross with AB259; with ampicillin-resistant strains Pro^+Str^r recombinants were measured after mixing with AB261.

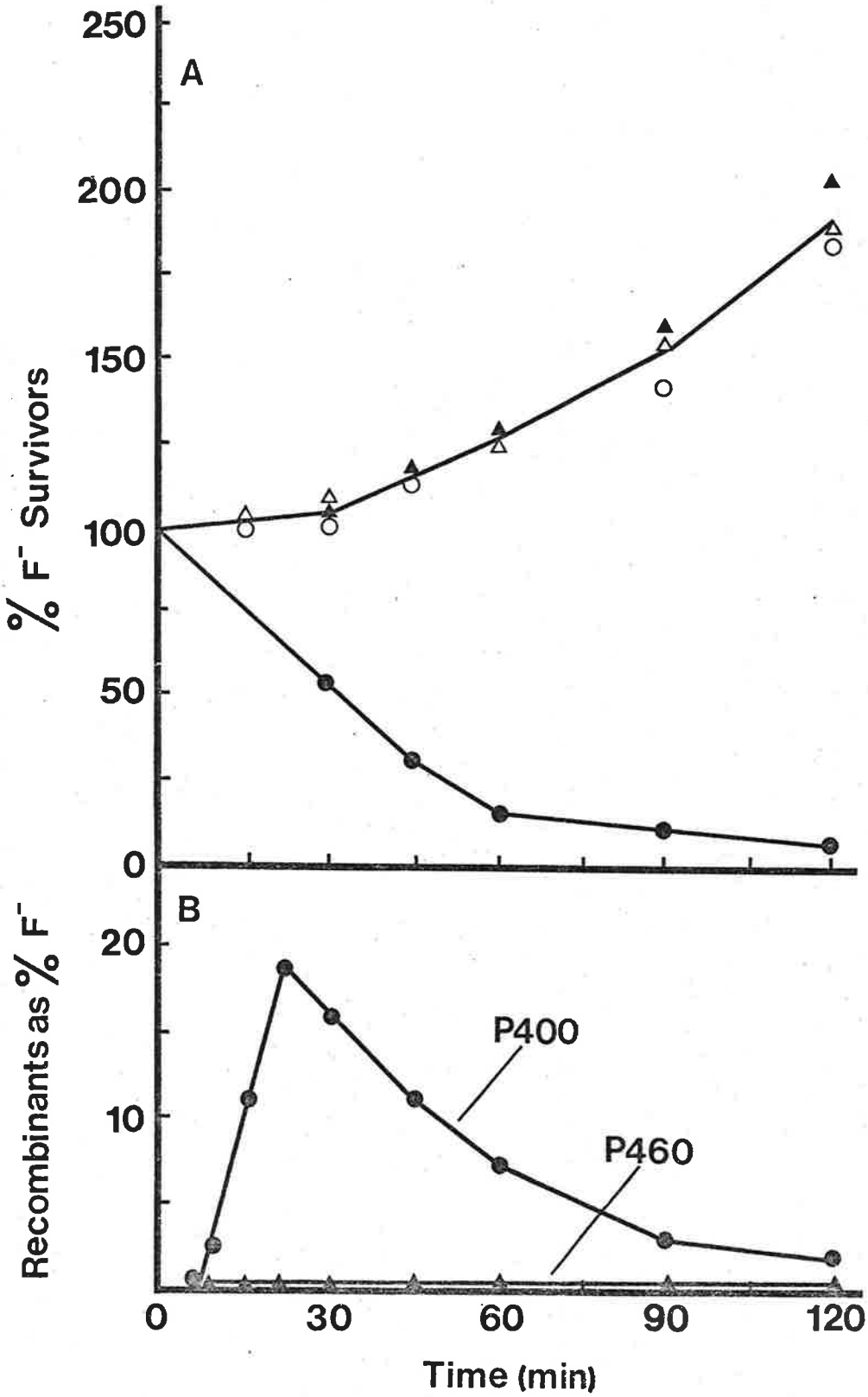
[b]Maximum concentration (μg/ml) of D-ampicillin in agar plates which permitted 100% survival of single cells (Monner et al., 1971).

[c]Efficiency of plating relative to E. coli B.

*Marked reduction in plaque size and halo compared with D31.

FIG. 7-1

Sensitivity of the Kox^r mutant P460 to lethal zygosis. Stationary-phase cultures of P400 (●, ○) and P460 (▲, △) were diluted and mixed with an early logarithmic-phase culture of the HfrH strain AB259 (closed symbols) or with a similar culture of the Tra^- Hfr strain P980 (open symbols). Samples were diluted and plated for survivors (A) and for $T^+L^+Str^r$ recombinants (B)(for details see Materials and Methods).


A
B
250
200
150
100
50
0
% F⁻ Survivors
20
10
Recombinants as % F⁻
P400
P460
0
30
60
90
120
Time (min)

ampicillin-resistance (Amp^r-III; 75-100μg/ml) also had reduced ability to form mating pairs [as estimated by the de Haan and Gross (1962) dilution technique]. Strain D31 had also gained sensitivity to the phage ØW, and spontaneous revertants to $ØW^r$ (e.g., D31m4) were reduced even further in pair forming ability (Monner et al., 1971). These strains, when tested on solid media (Table 7-4) or in liquid media (Fig. 7-2A), were found to be either partially (D31) or fully (D31m4) resistant to lethal zygosis, whereas the strains from which they were derived (D11 and D21) were lethal zygosis-sensitive.

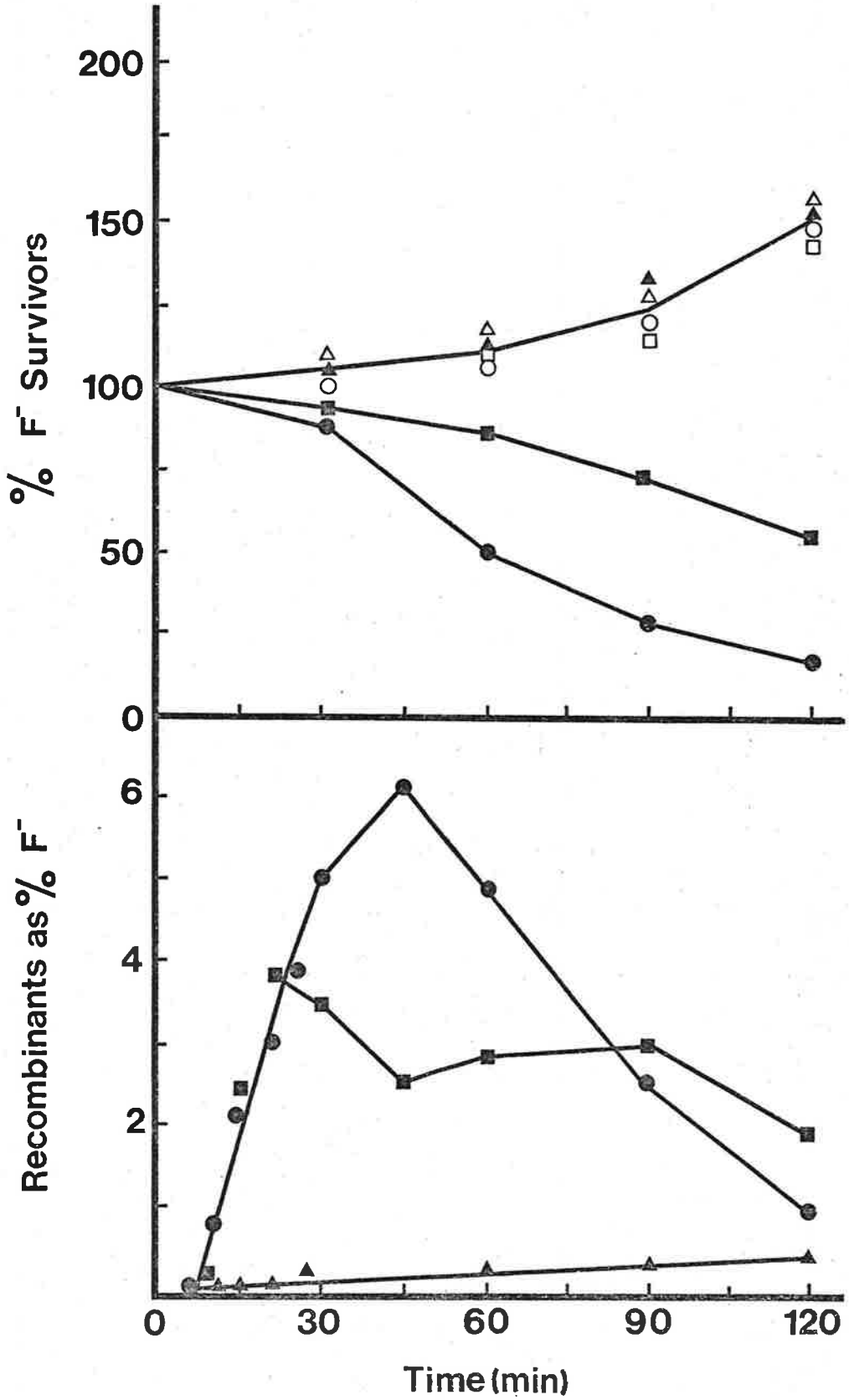
COMPARISON OF SOME PROPERTIES OF STRAINS RESISTANT TO LETHAL ZYGOSIS

Since lethal zygosis resistance was found to be a property common to the Kox^r strains and the Amp^r strain D31m4, further tests were carried out in order to determine the extent of relatedness between these two classes. It should be noted that Kox^r strains were employed since they were detected prior to the Tol XIV strains.

Like the class III ampicillin-resistant mutant D31m4 (Fig. 7-2B), the Kox^r strains were found to be defective in conjugation producing 100- to 1,000-fold fewer recombinants than P400 when crossed with an Hfr donor strain (Fig. 7-1B, Table 7-4). Furthermore, the ability of the Kox^r strains to produce progeny by repliconation (Clark, 1967), in matings with the Flac strain JC6583 and the Fgal strain P601, was also reduced approximately 1,000-fold. Mating pair formation, as measured by the

FIG. 7-2

Sensitivity of ampicillin-resistant mutants of F^- strains to lethal zygosis. Stationary-phase cultures of the recipient strains D11 (Amp^s; ●, ○), D31 (Amp^r-III; ■, □), and D31m4 (Amp^r-III + $\emptyset W^r$; ▲, △) were diluted and mixed with an early logarithmic-phase culture of the Hfr strain AB261 (closed symbols) or with a similar culture of the Tra^- mutant P729 (closed symbols) (for details see Materials and Methods). Samples were diluted and plated for survivors (A) and for Pro^+Str^r recombinants (B). Results with the strain D21 were similar to those with D11.


200
150
100
50
0
% F⁻ Survivors
6
4
2
Recombinants as % F⁻
0
30
60
90
120
Time (min)

dilution technique of deHaan and Gross (1962), was greatly impaired with P460 (Fig. 7-3) and also with P459, P461, and P462.

In contrast to the ampicillin-resistant mutants D31 and D31m4, the Kox^r strains were, if anything, supersensitive to ampicillin, and in addition retained sensitivity to the phages ØW and Ø3 (Table 7-4). The Kox^r strains also remained sensitive to the female-specific phages ØII, W31, and T7 and resistant to the male-specific phage MS2.

These results indicate that the Kox^r strains (and the Tol XIV strains which were also found to be defective in crosses with Hfr strains) constitute a unique class of conjugation-defective mutants, and that such a defect renders them resistant to lethal zygosis.

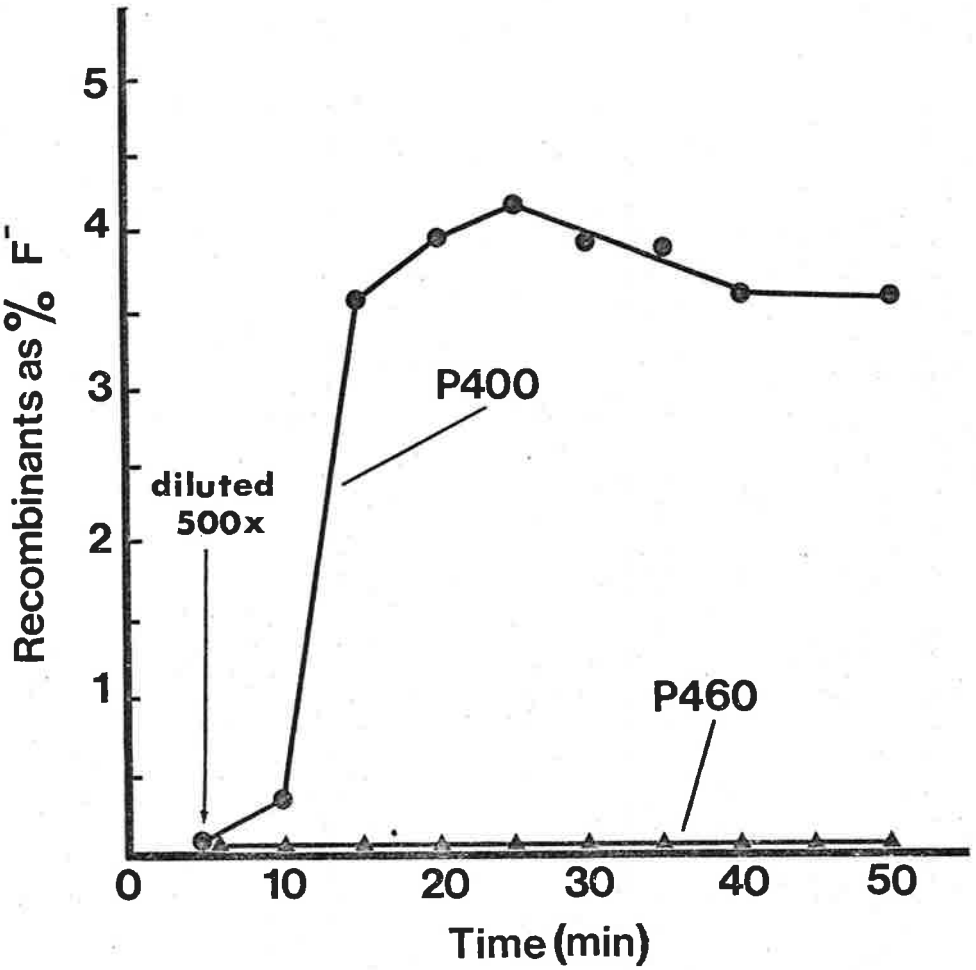
EXAMINATION OF THE CELL ENVELOPE OF CONJUGATION-DEFECTIVE MUTANTS

Since the loss of receptor activity for phages would be expected to be associated with structural alterations near the surface of the cell, the cell envelope of Kox^r strains was examined. The envelope of *E. coli* contains, in addition to the cytoplasmic (inner) membrane, a cell wall composed of a rigid peptidoglycan (murein), a membrane bilayer (outer membrane), and the outermost region, the lipopolysaccharide (LPS) (Schnaitman, 1971b).

Gas liquid chromatography analysis (Hancock and Reeves, unpublished data) of the LPS from strains P459 and P460 has shown that the relative molar ratios of the sugars glucose, galactose, heptose, and peak X were

FIG. 7-3

Mating pair formation by the recipient strains P400 (●) and P460 (○) with the HfrH strain AB259, estimated as described by de Haan and Gross (1962) (see Materials and Methods). At the indicated times after mixing samples were diluted and plated for $T^+L^+Str^r$ recombinants.


Recombinants as % F⁻
5
4
3
2
1
0
10
20
30
40
50
Time (min)
diluted
500x
P400
P460

unchanged in these strains compared with the parent strain P400. This suggests that the mutation to Kox^r and conjugation defectiveness was not associated with any significant alteration in LPS carbohydrate composition. In contrast, the ampicillin-resistant mutants D31 and D31m4 have both been shown to have major deficiencies in the carbohydrate composition of their LPS (Monner et al., 1971).

The receptors for some colicins and bacteriophages are now known to be associated with the E. coli cell wall and to be at least partly proteinaceous in nature (Sabat and Schnaitman, 1971, 1973; Weltzien and Jesaitis, 1971). Therefore, the protein composition of the cell envelope of the Kox^r mutant P460 was examined. Polyacrylamide gel electrophoresis (Neville, 1971) of whole envelope preparations showed that a major protein (peak 25), present in the parent strain P400, was either absent or greatly reduced in the mutant P460 (Fig. 7-4).

Schnaitman (1971a) has shown that Triton X-100 treatment was specific in solubilizing proteins of the cytoplasmic membrane from unfractioned E. coli envelope preparations. Proteins of the outer membrane remained among the Triton-insoluble fraction and could be readily separated by centrifugation. The Triton-insoluble fractions from whole envelope preparations of P400 and P460 were therefore compared by gel electrophoresis. As shown in Fig. 7-5, the protein of peak 25 was found to be a major protein component of the outer membrane of P400 yet was

FIG. 7-4

Comparison of the protein profiles, on acrylamide gels (see Materials and Methods), of whole envelope preparations from the Kox^r strain P460 and the parent strain P400. The peaks have been numbered from 1 to 33. The arrows indicate the positions at which the following proteins of known molecular weight (MW) banded in this gel system: A, bovine serum albumin (MW, 69,000); B, ovalbumin (MW, 43,000); C, carbonic anhydrase (MW, 29,000); D, lysozyme (MW, 13,900).

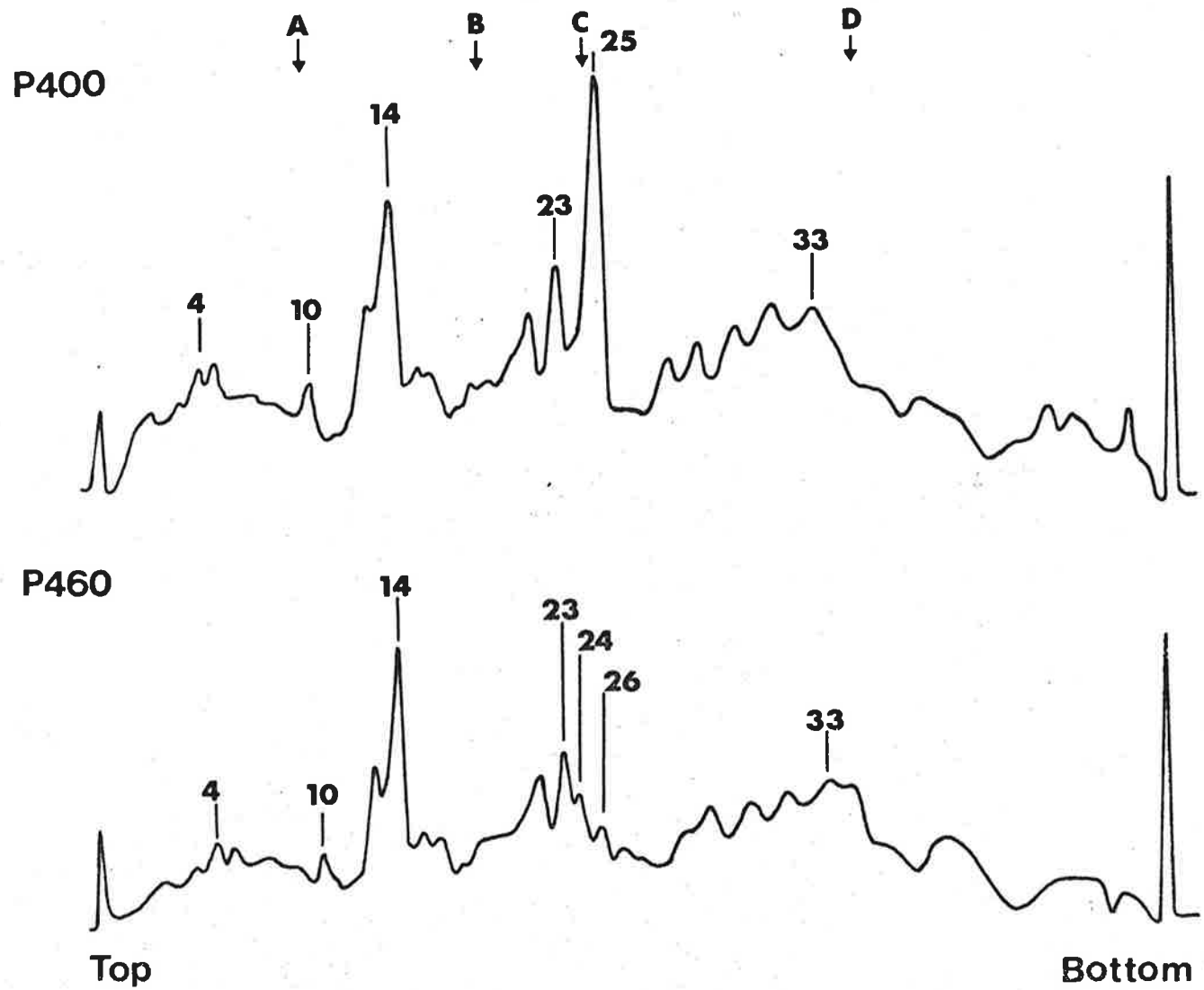
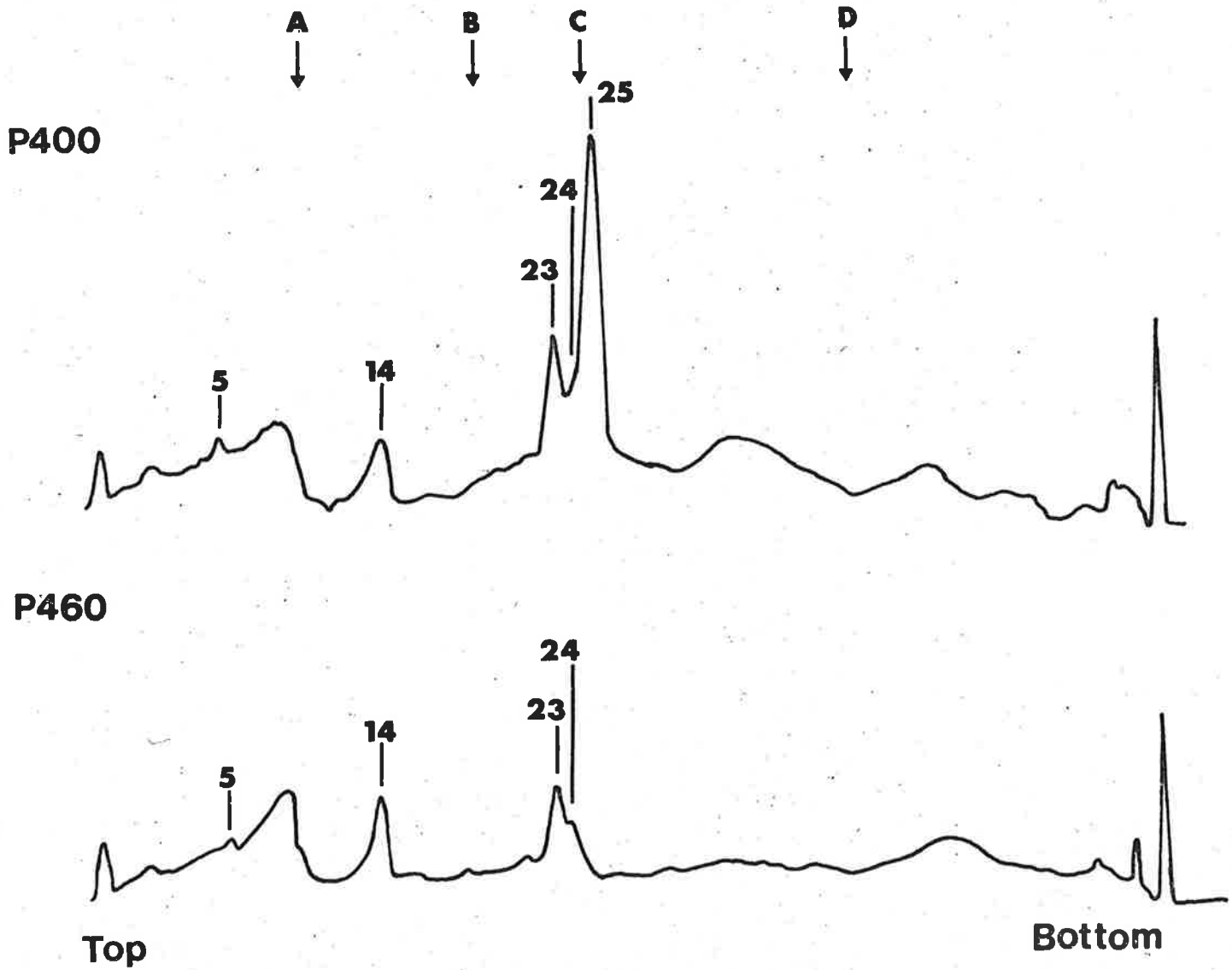

P400
A
B
C
D
25
14
23
33
4
10
P460
14
23
24
26
33
4
10
Top
Bottom

FIG. 7-5

Comparison of the protein profiles, on acrylamide gels (see Materials and Methods), of Triton-insoluble envelope proteins, from the Kox^r strain P460 and the parent strain P400. The numbering of the peaks and the standard proteins employed are as in Fig. 7-4.


A
B
C
D
P400
25
24
23
5
14
P460
24
23
14
5
Top
Bottom

missing in a similar preparation from the Kox^r mutant P460. The apparent molecular weight of this outer membrane protein was estimated to be 28,000 using the relative mobility values (Rf) obtained with bovine serum albumin (A), ovalbumin (B), carbonic anhydrase (C), and lysozyme (D) as standards (Shapiro, Vinuela and Maizel, 1967).

SUMMARY AND CONCLUSIONS

The lethal zygosis resistance observed with conjugation-defective mutants further supports the hypothesis that lethal zygosis results from an exaggerated conjugational event.

The defective mutants described in this chapter were examined in an attempt to clarify the early events that occur in the recipient cell during conjugation. The step (or steps) that was blocked in this process, by such a mutation, has not yet been fully resolved; however, the observation that the Kox^r mutants were as defective in inheriting F-prime elements (F*lac*, F*gal*) as they were in inheriting chromosomal markers indicates that the block was prior to recombination.

It is possible that the defect in these recipient cells prevented effective contact with donor cells or that if such contact did occur then transfer of the DNA into the cell was impaired. The observed absence of, or alteration in, a major outer membrane protein in these conjugation-defective cells is in agreement with such a suggestion.

The concomitant loss of sensitivity to Kox bacteriophages, colicins K and L, and lethal zygosis by these mutants, associated with the con-

jugation defects observed, lends support to the hypothesis proposed by Reeves (1972).

CHAPTER 8

DISCUSSION

In this thesis the effects of conjugation on recipient cell physiology and metabolism have been examined. Lethal zygosis, a major conjugation-induced abberation, has been characterized in terms of parameters affecting the phenomenon and the specificity of cells involved. Immunity to lethal zygosis, a newly recognized F factor-specified function, has been described, and a unique class of conjugation-defective mutants of F^- cells have been detected. Evidence is also presented showing altered macromolecular syntheses and membrane associated functions in F^- cells as a result of mixing with Hfr cells. A detailed discussion of these results is provided in the following sections.

LETHAL ZYGOSIS: THE PHENOMENON AND ITS CHARACTERIZATION

The studies described in Chapter 3 indicate that direct cell to cell contact, mediated by F pili, is required for lethal zygosis. Isolated F pili, released into the medium, would be unlikely to produce the observed effects for the filtrates and supernatant fluids from Hfr cultures that were examined would have been rich in F pili (Brinton and Beer, 1967; Valentine *et al.*, 1969). The results therefore support the hypothesis (Clowes, 1963; Gross, 1963b) that cellular contact, as required for conjugation, can lead to death of the F^- cell.

The parameters affecting the extent of lethal zygosis have been

described (Chapter 3) and these include the cultural conditions of both donor and recipient cells prior to and during mixing. Stationary-phase F^- cells, which were depressed in their rate of metabolism (Chapter 5), were killed by lethal zygosis far more readily than early logarithmic-phase cells, whereas the converse applied to the ability of Hfr cells to effect lethal zygosis. These opposite effects of growth phase of Hfr and F^- cells on lethal zygosis, suggest that metabolic activity in the Hfr cell is necessary for lethal zygosis but that metabolic activity by the F^- cell may prevent it. The condition which most affected the extent of lethal zygosis was agitation of the mixed culture. This may produce its effect either by increasing the chance of collision between Hfr and F^- cells (Walmsley, 1973), so allowing greater effective pair formation or, alternatively, by increasing aeration and hence energy availability.

The correlation between fertility of Hfr donor strains and lethal zygosis (Chapter 4) further supports the hypothesis that some stage of conjugation is required for F^- cell death. However, of the F^+ and F' strains examined, only one strain, χ573 (of doubtful F-prime status), produced lethal zygosis to the extent observed with Hfr strains, and as pilus synthesis would be similar in all donor strains, it is suggested that contact alone is not sufficient for recipient death. Lethal zygosis then must be associated with an event which occurs at higher frequency in, or is unique to, matings with Hfr donors.

The extent to which haploid recombinants are formed is an obvious

difference between Hfr and F^+ or F' donors, and aberrations associated with recombination (Curtiss, 1969; Clark, 1971) may lead to lethal zygosis. The F-prime strains χ517 and χ594 did produce some lethality, but these strains carry the episome F'-ORF1 which is 7 to 8 min long, and a large percentage of F^- recipients receiving the purE marker from this episome have been found to be haploid recombinants (Berg and Curtiss, 1967). These strains should be contrasted with χ818, and other short F-prime strains; the majority of recipients receiving the latter episomes would be diploid (Curtiss et al., 1968).

Despite the correlations between recombination and lethal zygosis, there were strains (rec mutants and a P1 lysogen) which were fully sensitive to lethal zygosis but did not show high recombinant yields. In addition, cells which were either phenotypically or genotypically surface exclusion deficient (Sex^-) (Chapter 6) were found to be fully functional as recipients yet showed either little or no sensitivity to lethal zygosis. As suggested previously (Chapter 7), these results preclude the hypothesis that lethal zygosis resulted from excess recombination events.

A further difference between Hfr and F^+ or F' donors may lie in the amount of DNA transferred, and Silver (1963) obtained some evidence for this. However, the difference may be much less than one would at first think because it has been shown that multiple lengths of F^+ and F' DNA are transferred during conjugation. (Matsubara, 1968; Ohki and Tomizawa, 1968).

IMMUNITY TO LETHAL ZYGOSIS

The results presented in Chapter 6 show that there is an immunity to lethal zygosis (Ilz^+) associated with the presence of the sex factor F; loss of the sex factor, by acridine orange curing, rendered a cell fully sensitive. Study of the immunity of the P190 series, and standard F^+ strains, to lethal zygosis showed that such immunity was absolute if logarithmic-phase cultures were used, when the phenomenon of lethal zygosis was in any case less marked even for F^- cells. If stationary-phase cultures were used, the immunity was only partial, and killing occurred although to a lesser extent than the killing of a similar culture of F^- cells. However, recombinants were only formed at high frequency if stationary-phase (F^- phenocopy) cultures were used, and under these conditions F^+ recipients gave more recombinants than F^- recipients, supporting the conclusion that recombination per se does not cause lethal zygosis.

There was good correlation between immunity to lethal zygosis and surface exclusion; procedures that break down surface exclusion also induced sensitivity to lethal zygosis in a donor culture. Despite this correlation, it was found that F*lac* strains deficient in surface exclusion (Sex^-) formed high numbers of recombinants but were fully immune to lethal zygosis, as were those strains carrying F*lac* episomes with either *traJ* or *traA* mutations. These results indicate, as pointed out in Chapter 6, that a cell need not possess either a surface exclusion system

or F pili to be immune to lethal zygosis, although all three F factor properties, like donor ability, are affected by starvation of donor cells. Curtiss *et al.* (1969) have shown that on regrowth of starved Hfr cells there was a return of F pili in normal numbers and lengths by 20 to 35 min, but that full donor ability was not restored until 40 to 50 min, and they argued from these results for the existence of donor-specific components other than F pili. The results (Fig. 6-1) from an experiment in which F^- phenocopies were employed are fully compatible with this hypothesis, since it was within a period of 30 to 60 min after dilution that the surviving population of F^+ cells had begun to multiply, a result which could be accounted for by synthesis of donor components, specified by *ilz*$^+$, preventing further lethal events.

Analysis of the sensitivity of a series of Hfr deletion strains indicates that at least one *ilz* gene is located between *traG* and *traD* on the F factor map. There is, however, an anomaly with this location of *ilz* for it is thought that all genes within the *tra* region (*A* to *I*) are in one polycistronic operon (Achtman, personal communication), presumably under the control of the *traJ* product, yet the results with an episome carrying the *traJ-90* mutation indicate that *ilz* is not under *J* control. The location of *traS* is also thought to be between *traG* and *traD* (Willetts, 1972a); however, the physical distance between these two cistrons is approximately 8 kb (from Achtman, 1973a) and would provide sufficient genetic information for up to 8 genes. The immunity observed with cells carrying a *tra-4* mutation in their episome [the

polarity of *tra-4* extends from *traK* to the postulated *traS* cistron (Willetts and Achtman, 1972)] indicates that the order of the genes is *traG traS ilz traD*.

The origin of transfer (*ori*) is located between the proximal $\emptyset II^r$ locus and the distal *traJ* locus on the F*lac* map (Willetts, 1972b) and, in accordance with this observation, it has been shown that the majority of Hfr strains do not transfer *tra* genes early (Broda, Meacock and Achtman, 1972; Walker and Pittard, 1972). Since at least one *ilz* gene is located within the *tra* region it would be transferred to a recipient, by most Hfr strains, too late to provide immunity to lethal events. In contrast, F^+ and F-prime strains would be less likely to produce lethal zygosis (as observed) for rapid transfer and expression of the immunity region would render recipients immune. Valentine (1966) has shown the sex conversion of F^- to F^+ to begin as early as 30 min after mixing, a time at which expression of immunity may also commence.

Unlike normal F^+ strains, and the Hfr strain from which they were derived, the P190 strains were unable to restrict female-specific phage, and it might be that the sex factor present lacks some or all of the genes coding for female specific phage inhibition, as do the mutants previously described (Morrison and Malamy, 1971; Ippen-Ihler *et al.*, 1972). Such defective sex factors may have arisen during the Hfr x F^- plate mating by a mechanism similar to that proposed by Scaife (1967)

for the formation of F-primes, and may well be F-primes carrying chromosomal determinants we have not yet detected. A type-1 excision (Scaife, 1967) could, of course, lead to a deletion [deletions in F have been described (Sharp et al., 1972)] covering the $\emptyset II^r$ region located proximally to the origin of F transfer (Willetts, 1972b).

MUTANTS DEFECTIVE IN RECIPIENT ABILITY

The inhibition of mating pair formation by lipopolysaccharide (LPS) preparations from either male or female cells (Reeves, 1959; Kern, 1962; Yura, 1962; Lancaster, Goldschmidt and Wyss, 1965) and the almost total absence of carbohydrates in the LPS from class III - Amp^r strains, which are defective in conjugation (Monner et al., 1971), suggest the recipient ability of a cell is closely linked to the availability of sites within its LPS. This is further supported by studies with mutants defective in LPS biosynthesis. Whereas mutations resulting in loss of O-specific side chains in "smooth" strains of Salmonella (Watanabe, Arai and Hattori, 1970) and E. coli (Wiedemann and Schmidt, 1971, 1972) led to an increased ability to accept R factors, those mutations which affected the biosynthesis of the core polysaccharides to any great extent, left the cells with the reduced recipient ability of their "smooth" parent. It should be remembered that the fertile E. coli K-12 is a phenotypically "rough" strain (Ørskov and Ørskov, 1962) although rhamnose, a common component of O-specific side chains, has been detected in the K-12 LPS (for review see Jones, Koeltzow and Stocker, 1972).

In contrast to these conjugation defects associated with the LPS of the cell wall, the alteration in the Kox^r strains, which leads to reduced recipient ability, does not appear to involve the LPS (Chapter 7). These strains are, however, altered in the protein composition of their outer envelope; a major protein (peak 25) of the Triton-X100 insoluble fraction is either missing or altered. Although this protein accounts for up to 20% of the total cell wall protein, cells carrying this mutation appear to have normal morphological characteristics and their growth rates are comparable to the parental strain (data not shown). These results suggest other than a structural role for this protein and, while the defect has not yet been fully resolved, it is suggested that such a protein might be involved in a nonessential receptor or transport system. Further comments on the role of this protein can only be speculative and will be dealt with in a later section.

The missing (or altered) protein of peak 25 has an apparent molecular weight (MW) of 28,000 daltons in the Neville (1971) system. This gel procedure had not been previously employed for the examination of E. coli envelope proteins and therefore comparison with literature data is difficult; however, a major outer membrane protein of E. coli of similar molecular weight (28,500) has been described by Bragg and Hou (1972). The latter protein was found to migrate more slowly (apparent MW, 33,400) when the membrane preparation was heated (100C) prior to electrophoresis.

Furthermore, when run in an alkaline SDS gel system this single band could be resolved into three proteins A1, A2, and B with molecular weights of 44,000, 38,100, and 33,400, respectively. It might be that the proteins of peaks 23, 24, and 25 (Fig. 7-5) represent a similar resolution of this "heat modifiable" protein in the Neville system. Schnaitman (1973a,b) has also noted that the extent to which samples are heated prior to electrophoresis can greatly modify the apparent molecular weight of the E. coli outer envelope proteins. A comparison of the outer envelope proteins of the Kox^r strains and their parent is currently being undertaken with the systems employed by Schnaitman (1973a,b) and preliminary results (Hancock and Reeves, unpublished data) suggest that the protein of peak 25 is equivalent to the reported peak C in those systems.

RECIPIENT CELL PHYSIOLOGY DURING CONJUGATION

The inhibition of F^- cell division at a ratio of 1:1 (Fig. 3-1) and the previous observation (Riley et al., 1960) that exconjugant cell division is inhibited suggest, as pointed out in Chapter 5, that lethal zygosis may be an extreme manifestation, at high donor:recipient ratios, of a phenomenon which occurs during conjugation involving only a single donor cell. This hypothesis is further supported by the observed conjugation-induced changes in recipient cell physiology, the extent of which were dependent upon the number of Hfr cells per F^- cell in the mating mixture, as was F^- cell death. Such physiological alterations

included (i) inhibition of DNA synthesis, (ii) inhibition of β-galactosidase induction, (iii) loss of ability to accumulate ^{14}C-TMG, (iv) altered ONPG transport, and (v) leakage of β-galactosidase into the supernatant fluid. These changes, with the exception of the latter, were manifested by 15 to 20 min after mixing, a time also noted by Gross (1963b) as that at which a decrease in recombinant numbers occurs.

The role of DNA synthesis during conjugation has been the subject of a number of studies (see Chapter 1). While recent evidence (Vapnek and Rupp, 1970, 1971; Wilkins et al., 1971) suggests the occurrence of mating-dependent DNA synthesis in both donor and recipient cells, no reports are available which compare total DNA synthesis in conjugating and non-conjugating cells. The results (Fig. 5-2) from such a comparison indicate that F^- cell DNA synthesis is partially inhibited during conjugation, the extent of inhibition depending upon the multiplicity of Hfr cells employed.

As stated earlier (Chapter 5), an event that could lead to the arrest of both DNA synthesis and induction of β-galactosidase would be inhibition of energy metabolism, a mechanism that is supported by the observed inhibition of the energy-dependent active transport of TMG by 15 min. It is possible that the loss of ability to accumulate this galactoside could result from interactions at the cytoplasmic membrane, producing either one or both of the following lesions: (i) a specific interference with energy generating mechanisms, such as ATP production,

or (ii) a non-specific disruption of the permeability barrier. The increased rate of ONPG hydrolysis with conjugating F^- cells does suggest an early loss of membrane integrity, as no such change was observed with non-mating F^- cells in the presence of sodium azide, an uncoupler of oxidative phosphorylation.

While the changes described could have all resulted from alterations in the cytoplasmic membrane, the paucity of knowledge regarding the attachment of the donor to the recipient prohibits precise comments on the mechanism of such conjugation-induced damage. Nevertheless, whether this attachment occurs by wall to wall contact with a conjugation bridge (Anderson et al., 1957) or via the F pilus (Brinton, 1965), chromosome transfer requires the passage of DNA through the recipient cytoplasmic membrane. This structure, which normally opposes entry of all but the simplest molecules, must in some way change to accommodate the entry of the very large DNA molecule, and while such an alteration may be readily restored with conjugation involving a single Hfr cell, at high ratios, multiple hits may produce cells incapable of recovery.

Anderson (1958) and Ou and Anderson (1970), who studied conjugating bacteria by micromanipulation, observed a number of non-viable exconjugant F^- cells, many of which formed filaments or possessed abnormal division patterns. These results could well be explained by the inhibition of DNA synthesis reported in this thesis, as could the inhibition of exconjugant cellular division (Riley et al., 1960), and it

may be that biosynthetic perturbations induced during conjugation need not always be associated with gross membrane damage. Such metabolically altered cells might also be highly susceptible to a period of unbalanced growth induced by shift down from a nutrient-rich to a synthetic medium. This is suggested by the increased extent of lethality observed when broth-mated F^- cells were plated on minimal agar (Chapter 3) and could account in part for the loss in recombinants that follows such a shift (Gross, 1963a). It therefore appears that some time after contact a proportion of the F^- cells are damaged without being committed to death or survival, and that the outcome depends upon subsequent treatment (dilution and plating).

CONJUGATION INDUCED CHANGES IN RECIPIENT CELLS: A UNIFIED HYPOTHESIS

Any model for the early physiological events that occur during conjugation must take into account the observations discussed in the previous sections and, in particular, immunity to lethal zygosis and the resistance to lethal zygosis associated with the Kox^r (Tol XIV) cells.

It is therefore proposed that there exist complementary sites on the surface of donor and recipient cells capable of limited interaction, and that at these sites areas of adhesion between the cell wall and the cytoplasmic membrane occur, such as observed by Bayer (1968a). These sites are LPS-protein complexes so constructed as to provide (i) receptors at the surface of the cell for bacteriophage, colicins, and also cells of the opposite mating type and (ii) pores or "holes" for the

transmission of not only DNA from bacteriophage, as proposed by Bayer (1968a), but also DNA from donor cell to recipient cell, across the respective outer envelopes. Such multicompetent pores would also provide a mechanism for colicin transmission.

It is also suggested that the outer envelope protein missing from, or altered in, Kox^r (Tol XIV) recipient cells is either directly or indirectly associated with this receptor-transmission complex. The complex in these Kox^r mutants is affected such that the Kox phage are unable to adsorb, colicins K and L can no longer reach their site of action, and either donor cells are unable to make effective contact or, if they can, transfer of DNA is inhibited.

In this model, the F pilus, which is sited at or near a wall-membrane adhesion (Bayer, 1968b), contacts the recipient and, by retraction (Curtiss, 1969; Marvin and Hohn, 1969), draws the mating partners together such that "conduction pores" in each cell are in direct apposition. The "conduction pore" is then able to undergo conformational change to accommodate the large DNA molecule which enters the recipient cell. The transferred DNA, at least in the case of F^+ or F' transfer, is replicated at an inner membrane site which is located near the pore.

At low Hfr:F^- ratios, when a F^- cell contacts only one Hfr cell, the conformational changes in the pore would have little effect on the cell. By being transmitted to the inner membrane such changes may, of course, act as a signal to control vegetative chromosomal replication

and so limit lethal replication complexes (Curtiss, 1969; Clark, 1971). If, however, a number of Hfr cells simultaneously contact the F^- cell (and there must be some steric limitations to the possible number) the conformational changes in the recipient are amplified, producing membrane perturbations and in some cases leakage. Metabolically active cells are more able to repair such damage and are less sensitive to lethal zygosis than are stationary-phase cells.

It is further suggested that donor cells also possess the same receptor-transmission sites as F^- cells, however, the sites are modified by the following two F factor-specified components: (i) the traS product, which alters the site so as to limit effective contact; (ii) the ilz product, which stabilizes the outer envelope against gross conformational change which accompanies multiple mating. Both mechanisms would provide an evolutionary advantage to a cell in contact with a number of other donor cells within a population. In this model, DNA from F^+ and F' donors would be rapidly transferred, undergo circularization, and express ilz^+, so rendering the recipient immune.

Within the framework of this speculative model, it is, of course, possible to find alternative explanations for the primary lesion that produces the physiological changes observed. The formation of abnormal chromosome complexes (Curtiss, 1969; Clark, 1971), as a result of concomitant vegetative replication and transfer, would also be expected to terminate DNA synthesis.

EPILOGUE

There are examples in *E. coli* (Chernin, Goldberg and Goldfarb, 1970), and higher organisms such as *Saccharomyces cerevisiae* (Woods and Bevan, 1968; Throm and Duntze, 1970) and *Paramecium aurelia* (Beale, 1954; Beale, Jurand and Preer, 1969), of extracellular substances, produced by cells of one mating type, which are able to inhibit DNA synthesis or cell division in cells of the opposite mating type; excreted substances do not appear to be involved in lethal zygosis. However, mating type inhibition dependent on cell to cell contact, similar to lethal zygosis, has been observed with a strain of *Vibrio El Tor* (Iyer and Bhaskaran, 1969). There are also analogies between conjugation and events during fertilization in higher cells, for the entrance of more than one sperm into a monospermic egg (polyspermy) can lead to abnormal or lethal development (Wilson, 1937); such a phenomenon, like lethal zygosis, may be best considered as an example of pathological communication between sexually differentiated cells.

A P P E N D I X

PUBLISHED MATERIAL

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1. SKURRAY, R.A., and P. REEVES. 1973. Characterization of lethal zygosis associated with conjugation in Escherichia coli K-12. J. Bacteriol. 113 : 58-70. (Reprint enclosed).

2. SKURRAY, R.A., and P. REEVES. 1973. Physiology of Escherichia coli K-12 during conjugation; altered recipient cell functions associated with lethal zygosis. J. Bacteriol. 114 : 11-17. (Reprint enclosed).

3. SKURRAY, R.A., and P. REEVES. 1974. F factor-mediated immunity to lethal zygosis in Escherichia coli K-12. J. Bacteriol. 117 : 100-106.

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Characterization of Lethal Zygosis Associated with Conjugation in *Escherichia coli* K-12

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When F^- cells are mixed with an excess of Hfr cells there is a lethal event which results in a decrease in the number of F^- survivors. We have described and discussed the parameters affecting this phenomenon of lethal zygosis, and these include the cultural conditions of both donor and recipient cells prior to mixing and the use of aeration throughout the period of the experiment. The absence of lethal zygosis with filtrates and supernatant fluids from donors suggests a dependence on direct cell-cell contact as found in conjugation. The phenomenon, which is normally observed in liquid media, also occurs on solid media, and use of these two methods has allowed examination of strains of different mating types. Whereas most Hfr strains capable of producing normal yields of recombinants showed killing activity, no F^+ and only one F' donor produced lethal zygosis. Only F^- strains were sensitive to this phenomenon. The relationship between lethal zygosis and the various stages of conjugation is discussed.

Studies of conjugation in *Escherichia coli* usually make use of mating cultures in which donor cells are mixed with an excess of recipient cells, the ratios ranging from one male to twenty females (0.05:1) up to one to one (1:1). If, however, recipient cells are mixed with an excess of Hfr cells (20:1), there may follow a lethal event which results in a decrease in the number of F^- survivors. This phenomenon, first described by Alfoldi, Jacob, and Wollman (1), was named lethal zygosis, and further reports extended and clarified the nature of the effect (2, 30, 31).

As the Hfr concentration was increased, the yield of recombinants (24) and the survival of F^- cells (8) decreased. These observations led to the suggestion that the lethality observed could result from multiple conjugation involving simultaneous mating between one F^- cell and several Hfr cells (8, 24). There have been comments on the phenomenon in a number of reviews and reports (25, 27, 32, 37, 41), but no further detailed studies have been reported, and as a preliminary to examining recipient cells for metabolic changes associated with the lethal event, the phenomenon was characterized.

This report describes conditions required for a high and reproducible lethal effect and the changes brought about when the parameters involved are altered. We also report a technique for demonstrating lethal zygosis on agar and on the specificity of mating types involved in this phenomenon.

MATERIALS AND METHODS

Media. Nutrient broth (Difco-0003) was prepared double strength plus 0.5% (w/v) sodium chloride; nutrient agar was blood-agar base (Difco-0045) prepared as directed, without the addition of blood. Minimal liquid medium is that described by Davis and Mingioli (17). Minimal agar was prepared by addition of 2% (w/v) agar (Difco-0140) to minimal liquid medium. Glucose was added as a carbon source at a final concentration of 0.5% (w/v) to minimal agar and minimal liquid medium. Tryptone broth was prepared as 0.8% (w/v) tryptone (Difco-0123) with addition of 0.5% (w/v) sodium chloride. Yeast extract (Difco-0127) and casein hydrolysate (acid) (Oxoid-L41) were used as supplements in some experiments. Other growth supplements were added at the following concentrations: purines, pyrimidines, and amino acids, 20 μg/ml; vitamins, 1 μg/ml.

Bacterial strains. The strains of *E. coli* K-12 used are listed in Table 1 with their source or derivation. The origins and directions of transfer for the Hfr strains are shown in Fig. 1. Note that some Hfr strains are omitted from Table 1. All strains were stored as freeze-dried cultures, and working stocks were maintained on nutrient agar slopes at 4 C.

RC748, a W1 recombinant isolated by Gross (24),

TABLE 1. *Bacterial strains*[a]

Strain no.	Mating type	Genotype[b]	Source,[c] reference,[d] derivation
RC748	F⁻	*met⁻pro⁻tsx str*ʳ	a. (8, 24) W1:M⁻P⁻ [e]
P513	F⁻	*met⁻tsx str*ʳ	RC748
P882	F⁻	*met⁻proA⁻tsx str*ʳ	P1*kc* AB1133 → P513
RC759	HfrP4X(J2)	*met⁻*	a.
RC749	HfrP10(J4)	*thr⁻leu⁻thi⁻mal⁻lac⁻gal⁻ton* λʳ	a.
RC12	F⁻	*thr⁻leu⁻thi⁻lac⁻mal⁻gal⁻ara⁻xyl⁻mtl⁻ str*ʳ*azi*ʳ	a. W677 *azi*ʳ*str*ʳ [e]
P201	F⁺	*thr⁻leu⁻thi⁻lac⁻mal⁻gal⁻ara⁻xyl⁻mtl⁻ str*ʳ*ton*	W677 *str*ʳ [e]
PA309	F⁻	*thr⁻leu⁻thi⁻his⁻trp⁻arg⁻str*ʳ	b. (30)
P107	F⁻	*thr⁻leu⁻lac⁻gal⁻ara⁻mal⁻xyl⁻mtl⁻*	Cavalli 42[e]
AB1133	F⁻	*thi⁻argE⁻his⁻proA⁻thr⁻leu⁻mtl⁻xyl⁻ara⁻ galK⁻lacY⁻str*ʳ λ⁻ sup-37 (amber)	c.
P122	F⁻	As AB1133 but λ⁺	AB1133
P190	?	As AB1133	AB1133′
AB1157	F⁻	*thi⁻argE⁻his⁻proA⁻leu⁻thr⁻lacY⁻galK⁻ ara⁻xyl⁻mtl⁻str*ʳ*tsx* λ⁻ sup-3 (amber)	d.
JC5743	F⁻	As AB1157 but *recB21*	i.
JC5489	F⁻	As AB1157 but *recC22*	i.
JC2921	F⁻	As AB1157 but *recA1*	i.
JC2917	F⁻	As AB1157 but *recA12*	i.
JC2926	F⁻	As AB1157 but *recA13*	i.
JC2929	F⁻	As AB1157 but *recA65*	i.
AB313	Hfr	*thi⁻thr⁻leu⁻lacZ⁻str*ʳ	f. CGSC313
AB312	Hfr	*thi⁻thr⁻leu⁻lacZ⁻str*ʳ λ⁻	f. CGSC312
AB259	HfrH	*thi⁻*λ*⁻rel*	f. CGSC259
P980	HfrH	*thi⁻*λ*⁻rel tra⁻*	AB259
P969	HfrH	*thi⁻rel* λ⁺ lysogen	AB259
KL16	Hfr	*thi⁻*λ*⁻rel*	f. CGSC4245
AB261	HfrP4X-6	*metB⁻*	f. CGSC261
P729	HfrP4X-6	*metB⁻ tra⁻*	AB261
KL20	F⁺	Prototroph λ⁻	e.
KL96	Hfr	*thi⁻* λ⁻	f. CGSC4243
KL174	Hfr	*his⁻* λ⁻	e.
KL175	F⁺	*his⁻* λ⁻	e.
KL182	F⁻	*his⁻* λ⁻	e.
KL184	F⁻	*his⁻ str*ʳ λ⁻	e.
KL84	Hfr	*thi⁻* λ⁻	f. CGSC4238 (may have point of origin as P4X)
P601	F′	F′ *gal⁺/met⁻* λᴿ	
P722	HfrC	*met⁻*	g. (9)
P721	HfrC	*met⁻ tra⁻*	g. (9)
χ57	HfrH	*thi⁻* λ⁻	h. (14)
χ15	F⁺	Prototroph λ⁻	h. (3)
χ493	Hfr OR11	Prototroph λ⁻	h. (3)
χ503	Hfr OR21	Prototroph λ⁻	h. (14)
χ289	F⁻	Prototroph λ⁻	h. (14)
χ545	F⁻	*proB-lac*^{del} *str*ʳ λ⁻	h. (14)
χ818	F′ORF-207	F′*lac⁺/proB-lac*^{del} λ⁻	h. (41)
χ573	F′ORF-4	F′*lac⁺proC⁺purE⁺/lac-purE*^{del} λ⁻ *ser/gly⁻*	h. (3)
χ985	F⁻	*thr⁻pyrA⁻lacY⁻proC⁻tsx purE⁻* λ⁻ *pdxC⁻ pyrC⁻trp⁻his⁻str*ʳ*ilv⁻met⁻his⁻*	h. (41)
χ42	F⁺		h. (10)
χ209	F⁺	Prototroph λ⁻	h. (16)
χ1088	F⁻	*thr⁻leu⁻pro⁻lac⁻tsx trp⁻his⁻lys⁻str*ʳ *xyl⁻ ilv⁻*	h.

[a] The abbreviations and nomenclature are essentially that of Demerec et al. (18) with exceptions noted by Curtiss (11).

[b] Genotypes are those provided with strain or obtained from references cited.

[c] Strains were kindly provided by: a, R. Clowes; b, R. Nagel de Zwaig; c, A. L. Taylor; d, P. Howard-Flanders; e, K. Brooks Low; f, B. Bachmann, Coli Genetic Stock Center (CGSC numbers shown); g, E. Meynell; h, R. Curtiss III; i, A. J. Clark.

[d] Numbers in parentheses are references in Literature Cited.

[e] Synonym.

[f] Arose from solid media lethal zygosis with AB259. MS2ˢ.

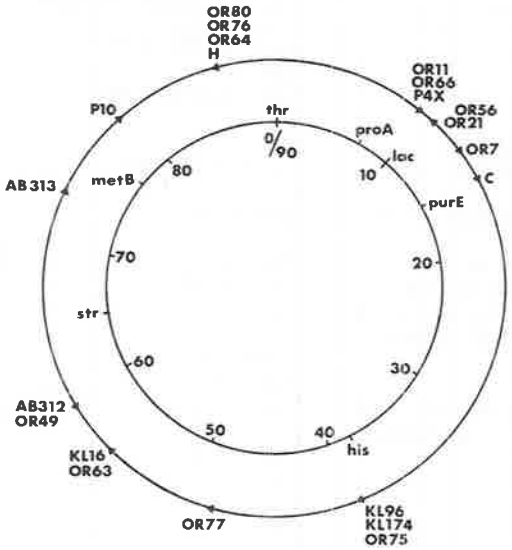

FIG. 1. *Chromosome map of Escherichia coli. Arrows indicate origins and direction of transfer of Hfr donors used (from Curtiss and Stallions [16], Stallions and Curtiss [41], Taylor and Trotter [42], and from Coli Genetic Stock Center information). The Hfr strains with the origins OR80 (χ900), OR64 (χ884), OR76 (χ896), OR7 (χ437), OR66 (χ886), OR56 (χ876), OR75 (χ895), OR77 (χ897), OR63 (χ883), and OR49 (χ869) were provided by R. Curtiss III. They are all derivatives of χ209 (16).*

was recovered as Met$^-$, having apparently lost proline auxotrophy, and a *proA* marker was introduced from AB1133 by P1*kc* transduction to give P882. Transfer-defective (*tra*$^-$) mutants of Hfr strains were obtained by isolating colonies resistant to the ribonucleic acid donor-specific phage, MS2 (kindly supplied by E. Meynell), and testing for the presence of the F factor by ability to give recombinants for proximal markers at low frequency (10^{-6} to 10^{-7} per cell) and for continued resistance to bacteriophage T3 (39). These mutants were also resistant to the deoxyribonucleic acid (DNA) male-specific phage, fd, and probably lack F pili (9, 35).

Preparation of bacterial cultures. All cultures were incubated at 37 C, the temperature used in all experiments. Nonaerated overnight (16–20 hr) cultures were obtained by subculturing, from a slope or single colony, to 10 ml of media (nutrient broth unless otherwise indicated). These generally had an optical density (OD) at 650 nm between 0.5 and 0.8. Stationary-phase cultures were inoculated in a similar way (10-ml volumes in 100-ml flasks or bottles) and aerated by agitation on a reciprocating shaker overnight (120 strokes/min; 32-mm amplitude). These had an OD_{650} between 2.0 and 3.5 depending upon media employed. Logarithmic cultures were prepared by diluting an overnight nonaerated culture 50- to 100-fold in fresh media (50 ml in 100-ml bottles) and then aerating with a reciprocating shaker as above. Early logarithmic cultures were collected at 2.5 to 3 hr ($OD_{650} \sim 0.3$ and a viable count of about 2×10^8 cells/ml), and late logarithmic cultures were taken at OD_{650} between 1.0 and 1.5 after 4 to 5 hr of incubation. All cultures, where required, were diluted immediately before use with fresh prewarmed media to an OD_{650} of 0.2 to 0.3 and then had a viable count of about 2×10^8 to 3×10^8 cells/ml.

Standard conditions for lethal zygosis. The following conditions were used unless otherwise stated. A stationary-phase culture of the F$^-$ strain (or other streptomycin-resistant strain under test) was diluted with fresh media to about 2×10^8 cells/ml. A 0.25-ml amount of this was mixed with an early logarithmic culture of a streptomycin-sensitive strain, and media where required, to give the desired ratio (usually 20:1) in a final volume of 5 ml. The mixed culture, in a 100-ml flask or bottle, was gently aerated with a reciprocating water bath shaker (60 strokes/min; 20-mm amplitude). In all experiments, the F$^-$ strain viable count at commencement was about 1×10^7 cells/ml. At intervals, 0.1-ml samples were diluted in minimal liquid medium supplemented with glucose and either 0.04% (w/v) casein hydrolysate or 1% (v/v) nutrient broth and vigorously agitated with a "Whirlimixer" (Fisons Scientific Apparatus Ltd.) to separate mating pairs. Viable counts were estimated by adding 0.1-ml volumes of suitably diluted samples to a 3-ml overlay of "soft agar" (equal parts of nutrient broth and nutrient agar) and pouring onto nutrient agar plates. The plates were incubated overnight at 37 C. In the case of survivor viable counts, streptomycin (100 μg/ml) was added to the overlay to prevent growth of streptomycin-sensitive strains, or in some cases contraselection against the Hfr strain was made on selective minimal agar without streptomycin. Survivors and total cell counts were expressed as a percentage of the input number of the respective cell type. Viable counts were similar whether dilutions were performed in nutrient broth or the dilution media described above.

Recombination frequency was estimated by plating, immediately after agitation, 0.1-ml volumes of the diluted samples on selective media containing streptomycin, and incubating for 24 to 36 hr at 37 C. The inclusion of supplements in the dilution medium allows traces of nutrient broth or amino acids to be plated with samples on glucose minimal agar and so limits metabolic imbalance which can lead to reduced recombinant yields (23). Because the F$^-$ strain is the minority cell type and is present at approximately the same initial titer in all experiments described, recombinants are expressed as a percentage of input F$^-$ cells.

Lethal zygosis on solid media. Volumes (0.1 ml) of overnight nonaerated cultures of streptomycin-sensitive strains were streaked on nutrient agar plates and allowed to dry. A 1:100 dilution of a culture of a streptomycin-resistant strain was then streaked across this at right angles and allowed to dry. The plate was incubated at 37 C for 2.5 to 3 hr and then sprayed (De Vilbiss atomizer-15) with streptomycin at 10^4 units/ml to prevent further

growth of the streptomycin-sensitive strain under test. After a further 18 hr of incubation, a zone of inhibition is observed if the streptomycin-sensitive strain can kill the other by lethal zygosis. We have distinguished two levels of inhibition: ++, complete inhibition; +, inhibition with slight growth.

Filtrates and other preparations from Hfr cells. Filtrates were prepared from early logarithmic cultures by using a Sartorius membrane filter (SM11306; pore size, 0.45 μm). The cells on the membrane were resuspended gently and adjusted to the desired volume, with fresh medium. A sample at 60 min from a standard lethal zygosis was filtered as described to provide a lethal zygosis filtrate.

Supernatant fluids were prepared by centrifugation (5,000 × *g*, 20 min) of early logarithmic cultures, removing the upper fraction only to reduce pellet resuspension (viable count was about 5×10^5 cells/ml). Streptomycin-treated cells were logarithmic-phase Hfr cultures incubated for 60 min at 37 C in the presence of streptomycin (1,000 μg/ml), centrifuged, washed once, and resuspended in fresh nutrient broth. Heat-treated cells were incubated at 56 C for 60 min. All preparations were used on the same day.

Mating chamber studies. A cylindrical perspex chamber (3.5-cm diameter by 2.6-cm width) was divided by a Sartorius membrane filter (SM11306; pore size, 0.45 μm). To one side of the chamber, 5 ml of an early logarithmic-phase donor culture (about 2×10^8 cells/ml) was added while the other contained 5 ml of a diluted stationary-phase recipient culture (about 1×10^7 cells/ml). The chamber was agitated at 37 C in a shaking water bath, and samples were removed at intervals for survivor and recombinant counts.

RESULTS

Development of conditions for lethality. Our first experiments were based on the conditions commonly employed in this laboratory for recombination. Aerated logarithmic-phase donor cells and overnight nonaerated cultures of recipient cells, both adjusted to approximately 2×10^8 cells/ml by dilution with fresh prewarmed broth, were mixed at the desired ratio (usually 20:1) in flasks or bottles maintained at 37 C. The results obtained with a series of F^- and Hfr strains did not, in general, show any decrease in F^- cell viable count, the most common observation being complete inhibition of recipient growth. The only exception involved P882, an F^- strain which was killed by some Hfr strains, but to an extent which varied between 10 to 100% survivors from day to day. Similar results have been reported for other recipients (8, 30).

Once continued aeration had been shown to be a requirement for consistent lethality (*see below*), a number of experiments were performed with overnight nonaerated F^- cultures. One such experiment (Fig. 2A) confirmed the effect of progressively increasing the number of Hfr cells per F^- cell, and, although rate and final extent of kill are much lower than found by Clowes (8), it is interesting to note the inhibition of F^- growth at a ratio of 1:1 compared to 0.08:1. The results in Fig. 2B show that with the high ratio of 25:1 there is initially a good yield of recombinants; this value reaches a peak and then falls, an effect not observed at a ratio of 1:1 and lower.

Further experiments showed that growth conditions of both F^- and Hfr cultures prior to mixing were critical in obtaining rapid lethal effects and led to the adoption of our standard conditions (see Materials and Methods). These conditions were employed in some of the fol-

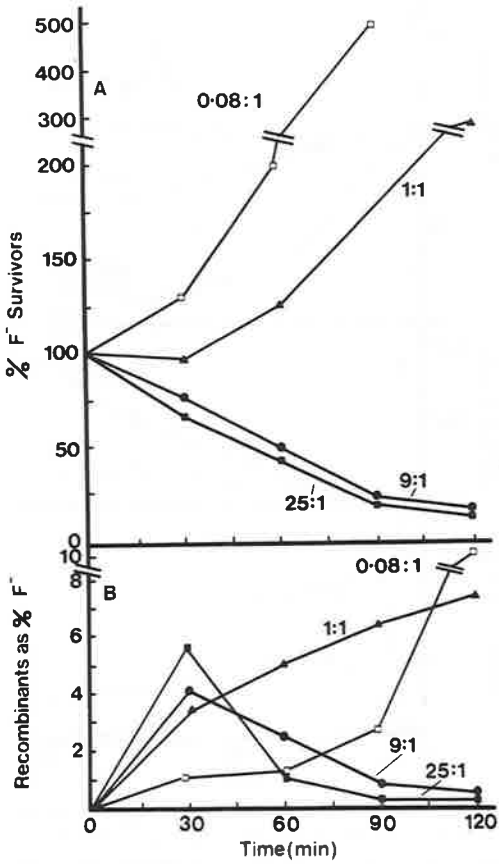

FIG. 2. *Effect of concentration of RC759 (Hfr P4X) on survival of the F^- strain P882 (A) and on pro^+ str^r recombinant formation (B). With the exception of overnight nonaerated F^- cultures, standard lethal zygosis conditions were employed (see Materials and Methods). The figures (1:1) indicate the ratio of Hfr cells to F^- cells at commencement.*

lowing studies to examine the effect of varying single parameters while otherwise maintaining optimal conditions.

As a control for physiological and genetic studies of conjugation and lethal zygosis we have made use of *tra*⁻ (transfer-defective) mutants of the Hfr under examination. This is preferred to an F⁻ × F⁻ control, as the Tra⁻ Hfr has a similar growth rate to its parent (Fig. 3B), and therefore any metabolic changes in the F⁻ strain, due to competition for nutrients with a large number of growing Hfr cells, will be similar. The Tra⁻ Hfr cells do not appear to affect the F⁻ cells in any way, for the same lag is observed on dilution of stationary-phase F⁻ cultures either into a Tra⁻ Hfr culture or fresh media. Likewise, both DNA synthesis (as measured by incorporation of ^{3}H-thymine) and induction of β-galactosidase are unchanged in the presence of large numbers of Tra⁻ Hfr cells (Skurray and Reeves, *unpublished data*).

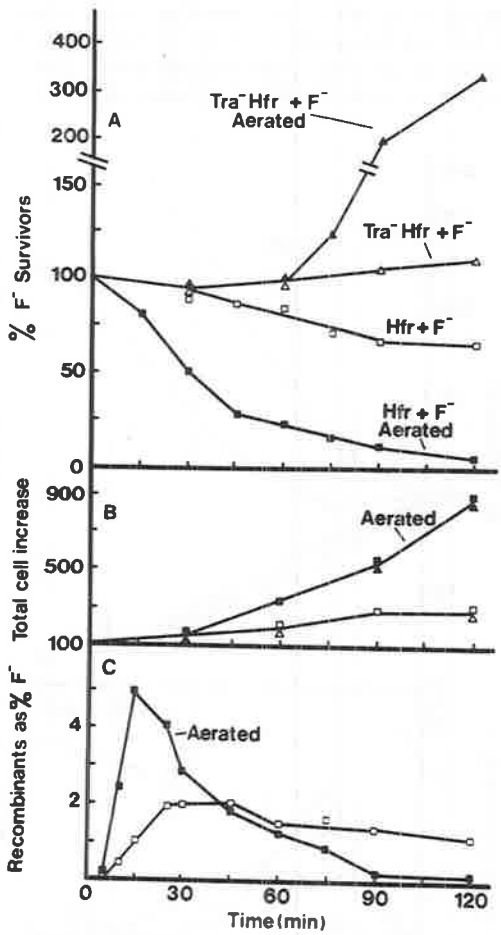

FIG. 3. *Effect of aeration on lethal zygosis. AB261 (Hfr P4X) and the recipient P882 were mixed at a ratio of 20:1 under standard conditions for lethal zygosis and aeration as described* (■). *Nonaerated culture conditions were identical except that flasks stood in a 37 C water bath and received agitation only at the time of sampling* (□). *Samples were diluted and plated for survival of F⁻ cells (A), total cell count (B), and pro⁺ str^r recombinants (C). Controls with the tra⁻ mutant P729 were included (aerated, ▲; nonaerated, △).*

Aeration and lethality. In our first experiments, mating cultures were aerated by gentle swirling or by bubbling air, and it was only when continuously aerated cultures were examined and found to show higher lethality that it became apparent that aeration affected the extent of kill. Figure 3 shows the effect of various aeration conditions during lethal zygosis under otherwise standard conditions. With the continuously aerated culture there is a rapid and extensive kill: 5% survivors at 120 min compared with 60% survivors under poor aeration. The latter result is affected by the number of samples taken; the flask showing 83% survivors in Table 2 was sampled only half as frequently and therefore received less agitation. The results of an experiment in which mating cells were aerated for various times after mixing are shown in Table 2. No attempt was made to interrupt pairs previously formed or to prevent further pair formation throughout the experiment. The results show that even though aeration was stopped at a given time there was further killing but to a lesser extent than if full aeration was allowed. The total cell count (Fig. 3B and Table 2), which may be regarded as an indication of Hfr or Tra⁻ Hfr growth, increased eight- to ninefold when aerated, compared with an approxi-

TABLE 2. *Effect of varying the period of aeration on the extent of lethal zygosis*[a]

Period of aeration (min)[b]	Survivors at time aeration ceased (%)	Survivors at 120 min (%)	Total count increase at 120 min
0	100	83	185
15	85[c]	47	268
30	55[c]	29	285
60	20	20	370
120	7	7	810

[a] AB259 (HfrH) mixed with the F⁻ strain P882 at ratio of 20:1 (standard cultures and lethal zygosis conditions).

[b] Aeration was continuous from time of mixing until time indicated.

[c] Approximate values taken from curves constructed from viable counts at 0, 20, 40, 60, 90, and 120 min.

mate two- to threefold increase with poor aeration. In this experiment, as in others, lethal zygosis was accompanied by an initial rise and then a fall in the number of recombinants (Fig. 3C).

These results, obtained by varying the degree of aeration, show that lethal zygosis is correlated with recombinant formation and with Hfr growth, and it might be argued that one or both are required for F^- cell death; however, aeration will also alter the state of the recipient, a parameter which is examined in the next section.

Effect of prior growth conditions of recipient. Clowes (reference 8 and *personal communication*) made use of F^- cultures that were either in logarithmic phase or were dilutions of an overnight aerated culture and found that higher multiplicities of Hfr cells per F^- cell were required to produce a similar lethal effect on logarithmic cultures, compared to overnight cultures. With nonaerated overnight cultures we found wide variations in extent of kill, but these cells were generally more sensitive than logarithmic F^- cells.

Since stationary-phase F^- cultures were more sensitive to lethal zygosis than logarithmic cultures (Clowes quoted by Gross [24]), we examined F^- cells taken from various phases of the growth cycle while otherwise maintaining standard conditions. With early and late logarithmic F^- cells, an initial increase in viable count was seen when mixed with the Hfr strain AB261 or its *tra*$^-$ mutant, followed by a decrease only with the Hfr, the rate and extent depending on the physiological state of the culture (Fig. 4).

The increased sensitivity of the stationary-phase culture does not appear to be related to an altered ability to undergo conjugation, as measured by recombinant formation (Fig. 4B). The early kinetics of zygote formation are identical with all three culture types, suggesting identical rates of pair formation and chromosome transfer, but when the stage is reached where lethal events reduce F^- survivors, this also affects the subpopulation of cells which have already received donor DNA and would have gone on to form recombinants. We also examined the recipient ability of the three cultures at a ratio of 1:1 but found again that no great difference existed between stationary- and logarithmic-phase cultures in this regard, although previous authors have claimed that either logarithmic-phase (22) or stationary-phase cultures (14) made better recipients. We can offer no explanation for these conflicting results.

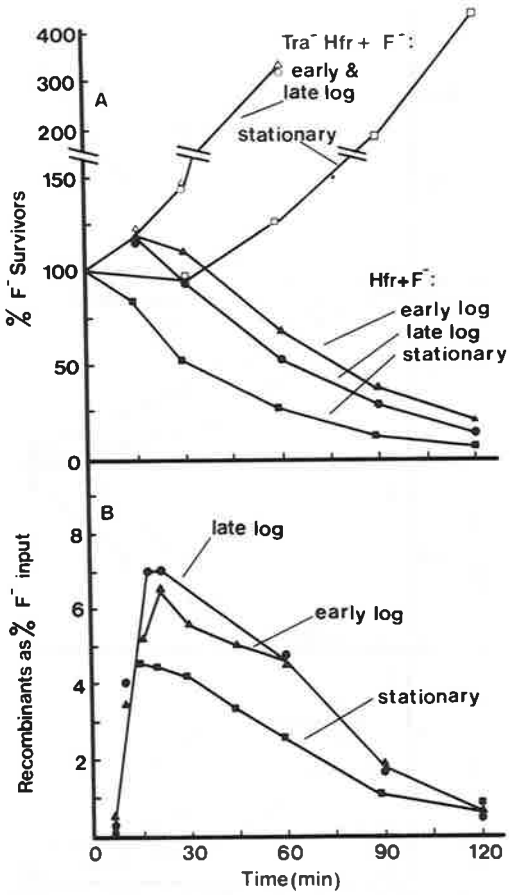

FIG. 4. *Effect of recipient growth stage on lethal zygosis. Cultures of the F⁻ strain P882, in nutrient broth, were grown to early logarithmic (▲, △), late logarithmic (●, ○), and stationary phase (■, □), diluted, and tested for lethal zygosis by mixing with an early logarithmic-phase culture of the Hfr strain AB261 (closed symbols) or with a similar culture of the tra⁻ mutant P729 (open symbols). Samples were diluted and plated for survivors (A) and pro⁺ str^r recombinants (B).*

It can be concluded from these experiments that increase in F^- cell age, from early logarithmic through to the late stationary phase, is accompanied by an increasing sensitivity to lethal zygosis.

Effect of prior growth conditions of donor. As we had noted similar lethality and recombinant formation with Hfr cultures taken throughout the logarithmic phase, we compared donor cells from various stages of the growth cycle. Since Curtiss et al. (14) found the yield of recombinants to be higher when donor cultures were grown without aeration, we also compared aerated and nonaerated logarithmic-phase Hfr cells. However, Fig. 5

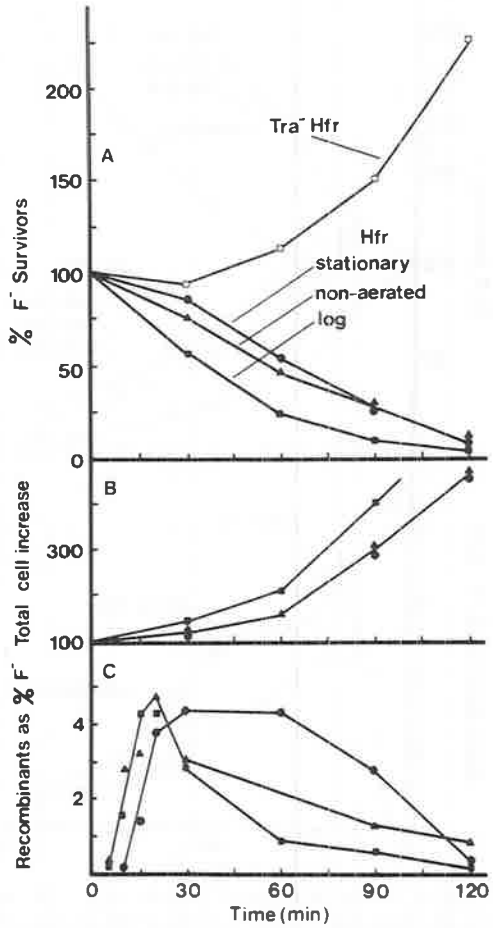

FIG. 5. *Effect on lethal zygosis of prior growth conditions of the donor. AB261 (Hfr P4X) early logarithmic-phase (■), stationary-phase (●), and nonaerated cultures (▲) and a logarithmic-phase culture of the tra⁻ mutant P729 (□) were prepared as described (see Materials and Methods) and tested for lethal zygosis with a stationary-phase culture of the F⁻ strain P882. Samples were diluted and plated for survivors (A), total cell count (B), and pro⁺ str^r recombinant formation (C).*

shows that in our hands aerated and nonaerated Hfr cultures have similar rates of zygote formation under standard conditions, but that logarithmic cells are more lethally active in the early stages after mixing. Identical results were found with aerated early and late logarithmic cultures.

Stationary-phase aerated donor cultures temporarily lose their donor ability but gain recipient ability (28). These F⁻ phenocopies lack F pili (4) but on subculture into fresh medium show rapid restoration of donor function. Recombinant formation with stationary-phase Hfr cells (Fig. 5C) shows a lag of 5 min which can be explained in terms of restoration of pili and donor function (4, 13, 33) and is followed by an increase similar to that observed with logarithmic and nonaerated cells. The decrease of recombinants with the three culture types mirrors the pattern of lethal zygosis as shown in Fig. 5A.

There is, as in the aeration studies, a relationship between rate of growth and the kinetics of lethality which is also shown in experiments with different culture media. These studies were initiated to establish conditions that could be used to examine metabolic functions during the lethal event, and we found in general that rich media, which allow rapid growth, also produce more rapid lethal zygosis (Fig. 6). With tryptone broth there is a two- to threefold increase in total count during the experiment, compared with a ninefold increase when yeast extract (0.5%) is added, an addition which halves the number of F⁻ survivors at 30 min.

Requirement for donor cell contact. One way in which Hfr cells could exert their effect is by the release of an extracellular substance or particle into the medium which then binds to F⁻ cells at a given receptor and leads to the death of that cell, a situation analogous to colicin action. An alternative is that Hfr strains produce these effects by direct cell-cell contact with recipients, as might occur in conjugation. To test these possibilities experiments were

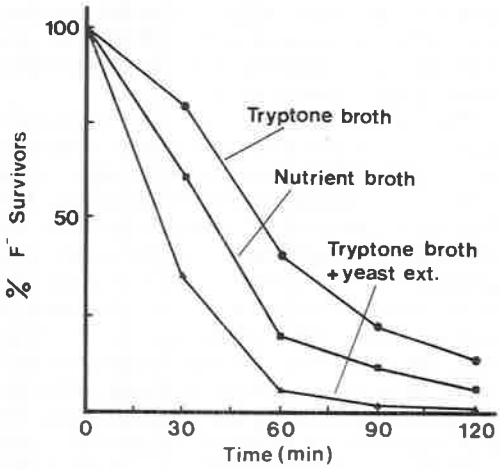

FIG. 6. *Effect of media on lethal zygosis. An overnight culture of AB259 (HfrH) in nutrient broth was diluted 1:50 in tryptone broth (●), 1:100 in nutrient broth (■), and 1:100 in tryptone broth plus yeast extract (0.5%, w/v) (▲). These were incubated at 37 C with aeration to early logarithmic phase (about 2 × 10^8 cells/ml). A diluted early (6 hr) stationary-phase culture of the recipient P882 (OD_{650} = 1.75) in tryptone broth was added to give a ratio of 20:1.*

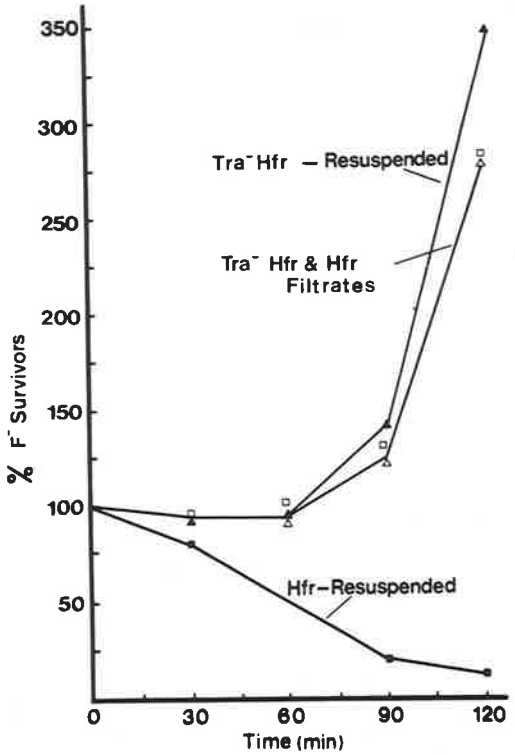

FIG. 7. *Effect of filtrates from donor cells on lethal zygosis. Samples (0.25 ml) of a stationary-phase culture of the F⁻ strain AB1133 diluted to about 2 × 10⁸ cells/ml were added to 5 ml of a filtrate (□) or to 5 ml of the resuspended culture (■) (about 2 × 10⁸ cells/ml) from AB259 (HfrH). Samples were also added to a filtrate (△) and a resuspended culture from the tra⁻ mutant P980 (▲).*

performed with a number of Hfr culture preparations. Figure 7 shows that there is no inhibition of F⁻ cell growth by filtrates from the Hfr strain AB259, the lethal activity remaining with the resuspended donor cells. Similarly, filtrates from matings in which lethal zygosis has occurred show no inhibition (Table 3).

By using chambers (see Materials and Methods) in which Hfr and F⁻ cells were separated by a bacterial filter, we have shown that there is no lethal zygosis or recombination when contact is prevented, growth of both mating types being normal under these conditions. It therefore appears that whole cells in direct contact, rather than filtrates or supernatant fluids, are required. Streptomycin- or heat-killed Hfr cells are unable to kill (Table 3), indicating that the lethal event is not simply due to the addition of a large amount of male material to the F⁻ culture.

Comparison of lethal zygosis in liquid and on solid media. A number of strains have been compared by using optimal conditions for lethal zygosis (Tables 4 and 5), and the results show that, in general, crosses between Hfr and F⁻ strains which give normal yields of recombinants demonstrate lethal zygosis, the extent varying with the strains employed.

The cross-streak plate test (Fig. 8) was developed to allow more rapid screening of strains and shows good correlation with lethal zygosis in liquid (Tables 4 and 5), inhibition of the F⁻ strain growth occurring only where Hfr and F⁻ cells are in direct contact. Tra⁻ Hfr × F⁻ and F⁻ × F⁻ crosses do not show inhibition on agar and, as in standard liquid conditions, act as convenient controls. RC749, an Hfr strain which gives a low level of recombinants (10^{-2} to 10^{-3} per F⁻ cell), does not inhibit any F⁻ strains on solid media. χ57 (an HfrH strain) was interesting in that it appeared to be a normal Hfr giving good yields of recombi-

TABLE 3. *Ability of preparations from AB259 (HfrH) to produce lethal zygosis in liquid media with two recipient strains*

Hfr pretreatment or preparation used[a]	Survivors at 120 min (%)	
	P882	AB1133
Log phase (no treatment)	1	13
Log phase *tra*⁻ (P980) (no treatment)	500	400
Supernatant fluid	ND[b]	300
Streptomycin-treated cells	480	355
Heated 56 C, 60 min	480	460
Lethal zygosis filtrate	430	300

[a] See materials and methods for preparations.
[b] Not done.

TABLE 4. *Lethal zygosis produced by various strains in liquid or on solid media*

Strain no.	Mating type	Effect on P882	
		Survivors in liquid 120 min (%)	Solid media
AB259	HfrH	1.5	++[a]
P980	Tra⁻ HfrH	500	−[a]
P969	HfrH	4	++
χ57	HfrH	45	−[a]
P722	HfrC	5	+
P721	Tra⁻ HfrC	300	−
AB261	Hfr P4X	3	++
P729	Tra⁻ Hfr P4X	320	−
RC749	Hfr P10	220	−
KL16	Hfr	5	+[a]
χ503	Hfr OR21	3	+[a]

[a] Similar results with AB1133.

TABLE 5. *Sensitivity of various strains to lethal zygosis on solid media*

Streptomycin-sensitive strain		Streptomycin-resistant strain								
		AB1133	P122	P882	AB313	PA309	RC12	AB312	AB1157	JC2921[a]
		F^-	F^-	F^-	Hfr	F^-	F^-	Hfr	F^-	F^-
AB259	HfrH	++[b]	++[b]	++[b]	−	++[b]	++[b]	−	++[b]	++
P980	Tra^- HfrH	−	−	−[c]	−	−	−	−	−	−
P722	HfrC	ND[d]	++	+[b]	−	++	++	ND	ND	ND
P721	Tra^- HfrC	ND	−	−[c]	−	−	−	ND	ND	ND
RC749	Hfr P10	ND	−	−[c]	−	−	−	ND	ND	ND
KL16	Hfr	++	+	+[b]	−	++	++	−	+	ND
P107	F^-	−	−	−[c]	−	−	−	−	−	ND

[a] Similar results with the following *rec*$^-$ strains: JC5743, JC5489, JC2917, JC2926, JC2929.
[b] <5% survivors in liquid lethal zygosis.
[c] >100% survivors in liquid lethal zygosis.
[d] Not done (generally due to λ^+ donor × λ^- recipient).

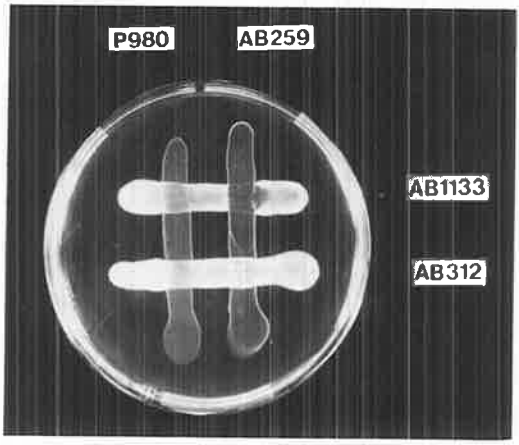

FIG. 8. *Lethal zygosis on solid media with AB259, an HfrH strain, and the recipient AB1133. Controls included are the tra*$^-$ *mutant P980 which does not produce inhibition, and the Hfr strain AB312, which, like other donors, is insensitive to the phenomenon.*

nants, but nonetheless gave much lower lethal zygosis in liquid and in the plate test did not inhibit any F^- strain examined. This suggests that the plate test is not as sensitive as lethal zygosis performed in liquid.

An alternative method for demonstrating lethal zygosis on solid media is to spot Hfr cultures directly onto a diluted F^- culture which had been spread on a nutrient agar plate and to incubate and treat with streptomycin as described for cross-streaking. This test, in addition to providing a good method for examination of a large number of donor strains, may be adopted for quantitative comparisons by using dilutions of Hfr cultures.

Specificity of cell types involved in lethality. The results in Tables 4 and 5 confirm the observations of Clowes (8) and Gross (24) that no unique origin or direction of transfer is required for an Hfr strain to be lethal. These experiments made use of Hfr and F^- strains with widely different genetic backgrounds, and in order to determine if the absence of lethal zygosis in Hfr strains such as RC749 and χ57 is due to the particular genetic background, we have examined a series of Hfr strains derived from a single F^+ strain, χ209 (16). Whereas the Hfr strains with origins OR80, OR64, OR76, OR66, OR56, OR75, OR63 and OR49 all showed lethal zygosis, strains with OR7 and OR77 did not (Table 6), and in this resemble RC749, for all three strains give reduced recombinant yields indicating a correlation with fertility rather than origins or backgrounds.

We have also examined a number of strains of the other two donor types, F^+ and F′, in liquid and on solid media. Unlike the isogenic Hfr strains derived from it, the F^+ strain χ209 does not show lethal zygosis nor do the F^+ strains, χ15 and KL20, from which Hfr, F′, and F^- strains were derived (Tables 7 and 8). These sets of isogenic strains demonstrate the sensitivity of an F^- strain to a number of closely related Hfr strains but not to the isogenic F^+ or F^-. In addition to the F′ crosses shown in Tables 7 and 8, solid and liquid medium tests between the nonisogenic strains P601 (F′*gal*) and P122 (F^-) showed no lethal zygosis. Thus, from a total of three F′ and five F^- strains, only χ573 (F′ORF-4) demonstrated lethal zygosis.

Only F^- cells are sensitive to the Hfr cells, a specificity shown in Table 5. Even when shaken overnight to produce F^- phenocopies, the Hfr strain AB313 and the F^+ strain P201 showed no sensitivity with the killing Hfr

strain AB261 even though recombinant formation occurred. In general all F⁻ strains including the recombination-deficient strains (Table 5) have been shown to be sensitive, but there are variations in the extent of lethal zygosis observed.

We have examined a large number of AB1133 survivors from lethal zygosis, on solid or in liquid media, and found that 5 to 10% were recombinants, the same as in the total mating population. When re-examined on solid media, all but one survivor showed sensitivity, indicating that they do not make up a genetically more resistant population. The one survivor strain, P190, has been shown to be resistant to all other Hfr strains when tested on solid media, and in addition has become sensitive to male-specific phage, although stationary-phase cultures of this strain form recombinants at rates similar to the parent AB1133.

TABLE 6. *Ability of a series of donor strains, mostly derived from χ209, to produce lethal zygosis on solid media*

Streptomycin-sensitive strains		Streptomycin-resistant strains			
		AB1133	P882	AB312	χ1088[a]
		F⁻	F⁻	Hfr	F⁻
AB259	HfrH	++	++	−	+
P980	Tra⁻ HfrH	−	−	−	−
χ900[a]	Hfr OR80	++	++	−	+
χ884[a]	Hfr OR64	++	++	−	+
χ896[a]	Hfr OR76	++	++	−	+
χ437[a]	Hfr OR7	−	−	−	−
χ886[a]	Hfr OR66	++	++	−	+
χ876[a]	Hfr OR56	+	ND[b]	ND	+
χ895[a]	Hfr OR75	++	++	−	+
χ897[a]	Hfr OR77	−	−	−	−
χ883[a]	Hfr OR63	+	ND	ND	+
χ869[a]	Hfr OR49	++	++	−	+
χ209[c]	F⁺	−	−	−	−
χ42	F⁺	ND	−	ND	ND

[a] Closely related strains derived from χ209, provided by R. Curtiss III.
[b] Not done.
[c] χ209 is derived from χ42.

TABLE 7. *Comparison of mating types mostly derived from χ15, for ability to produce lethal zygosis on solid and in liquid media*

Streptomycin-sensitive strains		Streptomycin-resistant strains tested on solid media				Survivors of χ985[a] at 120 min in liquid media (%)
		AB1133	P882	AB312	χ545[a]	
		F⁻	F⁻	Hfr	F⁻	
AB259	HfrH	++	++	−	++	ND[b]
P980	Tra⁻ HfrH	−	−	−	−	ND
χ15	F⁺	−	−	−	−	140
χ493[a]	Hfr OR11	++	+	−	+	15
χ503[a]	Hfr OR21	+	++	−	+	28
χ573[a]	F′ ORF-4	+	++	−	+	22
χ818[a]	F′ORF-207	−	ND	−	−	180
χ289[a]	F⁻	−	−	−	−	156

[a] Closely related strains descended from χ15, provided by R. Curtiss III.
[b] Not done.

TABLE 8. *Comparison of mating types derived from KL20 for ability to produce lethal zygosis on solid media*

Streptomycin-sensitive strains		Streptomycin-resistant strains		
		KL184[a]	AB1133	AB312
		F⁻	F⁻	Hfr
KL20	F⁺	−	−	−
KL96[a]	Hfr	+	+	−
KL174[a]	Hfr	+	+	−
KL175[a]	F⁺	−	−	−
KL182[a]	F⁻	−	−	−
KL84[a]	Hfr	+	+	−
KL16[a]	Hfr	+	+	−

[a] Closely related strains derived from KL20, provided by K. Brooks Low.

The presence or absence of λ prophage in either mating type does not affect lethal zygosis, for AB259 (λ⁻) is effective against AB1133 (λ⁻) and P122 (λ⁺) (Table 5). With AB261 (λ⁺), we have observed similar extents of kill of both AB1133 and P122 up to 60 min. Zygotic induction could not have influenced F⁻ cell numbers at this stage of conjugation, for λ would be transferred late by this strain.

DISCUSSION

The studies with preparations from Hfr cultures, and with chambers separating the two mating types, indicate that direct cell-cell contact is required for lethal zygosis. An active factor, if released, may be bound by the filter rather than passing into the filtrate, but this is unlikely on the basis of the pore size employed (0.45 μm). This interpretation is confirmed by the observation that the supernatant fluid of centrifuged Hfr cells lacked activity (Table 3).

The *tra*⁻ mutant (P721) which is known to lack pili (9) and those we have isolated in a similar fashion, which probably also lack pili, are unable to produce lethal zygosis. This suggests a requirement for a specific contact between Hfr and F⁻ cells mediated by F pili. However, it is unlikely that isolated pili released into the medium produce the observed effects, for the filtrates and supernatant fluids

of Hfr cultures examined would be rich in pili (5, 43). Our results therefore support the hypothesis (8, 24) that cellular contact, as required for conjugation, can lead to death of the F^- cell, a phenomenon dependent upon the Hfr cell concentration employed. Evidence for multiple conjugation between one F^- cell and a number of Hfr cells is provided by a triparental (Hfr $\times$ Hfr $\times$ F^-) mating (19). Ou and Anderson (36) studied conjugating bacteria by micromanipulation and distinguished between "loose" pairs (F pilus contact only) and "close" pairs (cells in intimate contact), the latter being approximately twice as fertile as the former. It is of interest that a proportion of the F^- cells were killed during conjugation, and with "close" pairs there was a significantly larger number of nonviable F^- exconjugants, many of which formed filaments.

We have described conditions which allow a high and reproducible lethal effect, and we have examined changes when critical parameters are altered. Our data indicate that such requirements include (i) use of stationary or late logarithmic-phase F^- cultures, (ii) use of logarithmic-phase Hfr cells capable of continued metabolic activity, (iii) prior growth and mating in rich media, and (iv) continuous but gentle aeration throughout the period of the experiment. Use of these procedures with a number of Hfr and F^- strains results in lethal zygosis with kills of up to 99% of the initial F^- population.

Stationary-phase F^- cells are killed by lethal zygosis far more readily than are early logarithmic-phase cells, and the converse applies to the ability of Hfr cells to effect lethal zygosis. Our logarithmic-phase cells are able to grow and divide immediately on dilution into fresh broth, whereas the stationary-phase cells on dilution lose their internal potassium pool and do not start DNA or β-galactosidase synthesis for 10 to 15 min, and the turbidity remains unchanged for 10 min (Skurray and Reeves, *unpublished data*). Cell division of stationary-phase cells is of course delayed for 30 to 60 min after dilution. The stationary-phase cells in our mixture are thus much less active in metabolism than early logarithmic-phase cells, and the opposite effects of growth phase of Hfr and F^- cells, on lethal zygosis, suggest that metabolic activity in the Hfr cell is necessary for lethal zygosis but that metabolic activity by the F^- cell may prevent it. Perhaps the lesion which causes cell death can be repaired if the recipient is metabolically active. It is known that energy is not necessary for specific pair formation (15), and hence the metabolic activity is presumably required for events subsequent to the first step in conjugation. Stationary-phase males lack pili (4), but these regenerate rapidly (4, 13, 33). The inefficiency of stationary-phase males in lethal zygosis is presumably not due to this requirement to produce pili, as it is much more marked than is the delay in zygote formation (Fig. 5).

The condition which most affects the extent of lethal zygosis is agitation or aeration of the mixed culture. This may produce its effect by increasing the chance of collison between Hfr and F^- cells, so allowing greater effective pair formation and in turn multiple contact. Alternatively, the rapid lethal zygosis with aeration may be due to increased oxygen absorption and hence energy availability for either parent. It should be noted that conjugation (20), and in particular chromosome transfer, are endergonic (14, 21). The apparent requirement for metabolic activity of donor cells is also supported by the increased lethality when a rich medium is employed. The conditions which give higher levels of lethal zygosis are also those where total viable counts rise more rapidly; it is unlikely that the increased lethal zygosis is due solely to this increased cell number, for the enhanced early lethal effects associated with the use of aeration, rich media, or logarithmic-phase Hfr cells are seen at a stage (15 to 30 min) when Hfr numbers have only slightly increased.

The correlation between fertility of donor strains and lethal zygosis further supports the hypothesis that some stage of conjugation is required for F^- cell death. Of the F^+ and F′ strains examined only one produced lethal zygosis, and, as pilus synthesis would be similar in all donor strains, it is suggested that contact alone is not sufficient for recipient death. Lethal zygosis then must be associated with an event which occurs at higher frequency or is unique to matings with Hfr donors. The extent to which haploid recombinants are formed is an obvious difference between Hfr and F^+ or F′ donors, and aberrations associated with recombination may lead to lethal zygosis. The difference between Hfr and F^+ or F′ donors may also lie in the amount of DNA transferred, and Silver (40) obtained some evidence for this. However, the difference may be much less than one would at first think because it has been shown that multiple lengths of F^+ and F′ DNA are transferred during conjugation (29, 34). χ573, an F′ strain which does show lethality, carries an episome 7 to 8 min long, and a large percentage of F^- recipients receiving the *purE* marker are found to be

haploid recombinants (3). This should be contrasted with χ818 (F'-ORF207), for 99% of recipients receiving the short F'*lac* are diploid (14). The correlation between increased recombinant formation and lethality when aeration is employed also supports the involvement of recombination, an event which could well provide the observed lethal effects when associated with concomitant vegetative replication (7, 12). However, despite these correlations between recombination and lethal zygosis, there are individual donor and recipient strains which are fully functional in recombination but do not show lethal zygosis. We have described the properties of the Hfr strain, χ57 in the Results section, and we have obtained a single recombinant, P190, which is fully functional as a recipient but has some donor properties and is not killed by Hfr strains on the plate test. We are continuing studies with this strain, but it and χ57 show that, if lethal zygosis results from excessive chromosomal recombination, then these lethal events can be obviated in certain strains. The sensitivity of *recA*, *recB*, and *recC* mutants indicates either that recombination events are not implicated in lethal zygosis or that the gene products of these strains are involved in a stage of the recombination process subsequent to that at which lethal events arise.

Results with the strain P190 do suggest that there exists some immunity mechanism associated with the presence of the F episome, for in this case, at least, the area coding for production of pili has been introduced during conjugation. The inability of most F^+ and F' strains tested to produce lethal zygosis may be due to early transfer and expression of an F factor immunity gene in the recipient, whereas Hfr strains which produce good lethality may transfer such a gene too late to provide an immune recipient. Broda et al. (6) have recently shown that most Hfr strains do not transfer *tra* genes early, and it might be that the postulated immunity gene is located near the *tra* genes. An F factor-mediated immunity would also account for the lack of sensitivity of F^+ and Hfr strains to lethal zygosis, even when F^- phenocopies are produced.

Lethal zygosis has properties in common with colicinogeny, including specificity associated with an extrachromosomal element and the possibility of immunity of cells producing the effect; however, lethal zygosis has not been demonstrated in the absence of live cells. An observation similar to lethal zygosis has been made with a strain of *Vibrio el tor* (26).

The inhibition of F^- cell division does appear to occur even at ratios of 1:1 (Fig. 2A) although a continued rise in recombinant number is observed. This and the observation that exconjugant cell division is inhibited (38) suggest that lethal zygosis may be an extreme manifestation, at high donor to recipient ratios, of a phenomenon which occurs during conjugation involving only a single donor cell.

As yet no studies have been reported describing the metabolic changes which accompany lethal zygosis; however, we have found that recipient DNA and protein synthesis are markedly inhibited 20 to 30 min after mixing (Skurray and Reeves, *in preparation*). Such changes are unlikely to have both resulted from perturbations in the recombination event per se, and it may be that a single primary metabolic lesion produces substantial F^- cell "switch-off." Damage to cell integrity, analogous to phage lysis from without, was suggested as a possible mechanism (24), and we are currently investigating this possibility as well as extending metabolic studies in order to establish the mechanisms by which Hfr cells produce F^- cell death.

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Physiology of *Escherichia coli* K-12 During Conjugation: Altered Recipient Cell Functions Associated with Lethal Zygosis

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The number of viable F^- cells decreases when *Escherichia coli* recipient cells are mixed with an excess of Hfr cells. Evidence is presented showing that lethal zygosis was accompanied by changes in the physiology of the recipient cells, including (i) inhibition of deoxyribonucleic acid synthesis, (ii) inhibition of β-galactosidase induction, (iii) altered transport and accumulation of galactosides, and (iv) leakage of β-galactosidase into the supernatant fluid. The results are discussed in terms of possible conjugation-associated changes that, at high Hfr to F^- ratios, lead to lethal zygosis.

When recipient (F^-) cells of *Escherichia coli* K-12 are mixed with an excess of donor (Hfr) cells, there is a decrease in the number of viable F^- cells and an associated fall in recombinant numbers (8, 13, 22). The early reports clarified this phenomenon of lethal zygosis, as it was termed by Alfoldi et al. (2), but there have been no reported attempts to describe the metabolic lesions associated with the lethal event.

As a preliminary to investigating the physiology of the recipient cell during conjugation, the phenomenon of lethal zygosis was further characterized (30). It was found that the ideal conditions for lethal zygosis involved metabolically active Hfr cells and stationary-phase F^- cells. The results of this study also confirmed that lethal zygosis was mediated by events associated with conjugation.

Since division of F^- cells was inhibited when a ratio of 1:1 was employed, it was suggested that lethal zygosis may be an extreme manifestation, at high donor to recipient ratios, of events that occur during conjugation involving only a single donor cell (30). There have been few reports of changes in the metabolism or physiology of the recipient cell accompanying conjugation (3, 24, 29), and we have commenced an investigation into such perturbations and their relationship to lethal zygosis. Alterations in macromolecular syntheses and membrane-associated functions which occur in F^- cells after mixing with Hfr cells are described in this paper.

MATERIALS AND METHODS

Bacterial strains. The strains of *E. coli* K-12 used, together with their source or derivation, are listed in Table 1.

Strain P978 is a low-level thymine (1 μg/ml)-requiring mutant of strain P882, selected after the introduction of a *thyA* mutation by the method of Stacey and Simson (31). Strain P322 was selected as a lactose-negative colony on tetrazolium agar (21) after P1kc transduction of a *lacY* mutation from strain PA309 to strain P882. The absence of the lactose permease was confirmed by the inability to utilize the α-galactoside melibiose at 42 C (26). The strain produced normal levels of β-galactosidase on induction.

Strain P727 was derived as follows. A *trp* marker from strain PA309 was transferred by P1kc transduction to a T6-resistant mutant (P726) of strain AB261. The *tdk-1* allele (15, 17) was cotransduced with trp^+ from strain BW40 into this *trp* mutant. Selection of Tdk^- cells was based on their inability to utilize thymidine in the presence of 5-fluorouracil (15). The absence of deoxythymidine kinase (EC 2.7.1.21) was confirmed by the inability of Tdk^- cells to incorporate ^{3}H-thymidine to levels greater than 0.1% of the incorporation by Tdk^+ strains.

The transfer-defective (Tra^-) mutants P766 and P310 were isolated from colonies resistant to male-specific phage (MS2) and possessed the phenotype described previously (30).

Media. The minimal medium was that described by Davis and Mingioli (10). For preparation of cultures, this was supplemented with casein hydrolysate-acid (Oxoid; 0.2%, wt/vol), tryptophan (20 μg/ml), growth factors (amino acids, 20 μg/ml; thymine, 5 μg/ml; vitamins, 1 μg/ml), or glucose (0.5% wt/vol).

TABLE 1. *Bacterial strains*[a]

Strain no.	Mating type	Genotype	Source[b] or derivation
P882	F⁻	*met proA tsx str* (λ)	(30)
P978	F⁻	*met proA tsx str thyA* (λ)	P882[c]
P322	F⁻	*met proA tsx str lacY* (λ)	P882
BW40	F⁻	*proA leu thr arg his thi ara uvrB mtl xyl gal lacY tsx str tdk*	Wilkins (34)
PA309	F⁻	*thr leu thi his arg lacY str trp* (λ)	Nagel de Zwaig
AB261	Hfr P4X	*metB* (λ)	Bachman
P727	Hfr P4X	*metB tsx tdk* (λ)	AB261
P766	Hfr P4X	*metB tsx tdk tra* (λ)	P727
CA241	HfrH	*thi lac*^{del}	Scaife
P311	HfrH	*thi lac*^{del} (λ)	λ lysogen of CA241
P310	HfrH	*thi lac*^{del} *tra* (λ)	P311

[a] The abbreviations and nomenclature are essentially that of Demerec et al. (11) and Taylor (32). Mutations in the F factor leading to transfer deficiency have been termed *tra* (1, 23).

[b] Strains were kindly donated by B. M. Wilkins, R. Nagel de Zwaig, B. Bachmann, and J. Scaife. Numbers in parentheses refer to the Literature Cited.

[c] In addition to *thyA*, P978 carries second mutation (*dra* or *drm* [5]) allowing growth in low levels of thymine.

Tryptone-yeast extract broth was prepared as 1.0% (wt/vol) tryptone (Difco) with the addition of 0.5% (wt/vol) sodium chloride and 0.25% (wt/vol) yeast extract (Difco). Nutrient broth, nutrient agar, and minimal agar were as previously described (30).

Preconditioning of media was by growth of the appropriate transfer-defective (Tra⁻) donor strain to an optical density at 650 nm of ~0.3 (see below), followed by centrifugation to remove the majority of cells and filtration of the supernatant fluid (Sartorius membrane filter SM11306; pore size, 0.45 μm). The filtrate, which was used on the day of preparation, was prewarmed to 37 C immediately before use.

Preparation of bacterial cultures and mating procedures. All cultures were incubated at 37 C. Details of conditions employed for the preparation of bacterial cultures, conjugation, and lethal zygosis have been described elsewhere (30).

Although 20-h aerated stationary-phase cultures of recipient cells were most sensitive to lethal zygosis (30), they showed a lag in recovery of metabolic activities (deoxyribonucleic acid [DNA] synthesis and β-galactosidase induction [unpublished data]). Consequently, we used early stationary-phase cultures in the studies described here. These were prepared by dilution (5- to 10-fold) of a nonaerated overnight (16 to 20 h) culture, followed by incubation with aeration for 4 to 5 h, and they had an optical density at 650 nm of 1.5 to 2.5, depending on the medium employed. After dilution to about 2×10^8 cells/ml, suitable volumes of the recipient culture were mixed with early logarithmic-phase donor cultures, and media were required, to give the desired ratio (expressed as donor cells per F⁻ cell, e.g., 25:1). The initial F⁻ viable count in the mating mixture was about 10^7 cells/ml, apart from the exception noted. In all experiments, recombinant (Pro⁺ Str^r) formation was examined at intervals and found to be comparable to that previously observed (30).

Incorporation of ^{3}H-thymine. Glucose-minimal liquid medium supplemented with casein hydrolysate, tryptophan, and growth requirements was employed for prior growth of donor and recipient cultures, and during mating. The mating mixture was supplemented with thymine (final concentration of 1 μg/ml), and ^{3}H-thymine (Radiochemical Centre, Amersham, England) was added to give 5 μCi/μg. Duplicate samples (0.1 ml) of the culture were collected on 2-cm discs of glass-fiber paper (Whatman GF83), and the extent of ^{3}H-thymine incorporation into acid-insoluble material was estimated as described by Hull and Reeves (16).

Assay for β-galactosidase activity. Samples (1.0 ml) were removed at intervals into 4.0 ml of ice-cold sodium phosphate buffer (pH 6.8, 0.03 M) containing 0.05 ml of toluene. The samples were incubated at 37 C for 30 min with occasional vigorous agitation and assayed for β-galactosidase (EC 3.2.1.23) activity as follows: 1.0 ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at 1 mg/ml in phosphate buffer was added, and incubation at 37 C was continued until a color developed approximating that of 10^{-4} M *o*-nitrophenol (ONP). The reaction was stopped by the addition of 1.0 ml of 1 M sodium carbonate, and the period of incubation was noted. The optical density was measured at 420 nm, and a correction for turbidity was applied (25). We have defined 1 unit of β-galactosidase activity as the amount of enzyme that liberated 1 nmol of ONP per min.

Uptake of TMG. ^{14}C-methyl-thio-β-D-galactopyranoside (^{14}C-TMG; New England Nuclear Corp., Boston, Mass.) was added to the mating mixture, and samples (0.25 ml) were removed and rapidly filtered in a prewarmed membrane-filter apparatus (HA filter, 0.45 μm pore size, 35 mm in diameter; Millipore Corp., Boston, Mass.). The sample was washed with three 5-ml volumes of tryptone-yeast extract broth (37 C). The operation was completed in 20 s. The filtration and washing were performed at 37 C to avoid rapid loss of accumulated substrate (20). The filters were dried and added to 5 ml of scintillation fluid (toluene, 1 liter; 2,5-diphenyloxazole, 4 g; 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, 0.5 g) and counted in a Packard liquid scintillation counter.

ONPG hydrolysis. Two methods of ONPG hydrolysis were employed. ONPG was added to the mating mixture (3×10^{-3} M final concentration) and, at intervals, 2.5-ml samples were added to 0.5 ml of ice cold 1 M sodium carbonate to stop the reaction. With the alternative method, samples (2.5 ml) of mating culture were added to 0.2 ml of ONPG

(3×10^{-2} M) and incubated (37 C) for 10 min. The reaction was stopped by the addition of 0.5 ml of ice-cold 1 M sodium carbonate. With both methods, the ONP produced was determined as follows: the majority of cells were lysed by the addition of 1 drop of 20% sodium dodecyl sulfate. The absorbance was measured at 420 nm, and corrections were made for turbidity (25).

Toluene treatment of cultures was as follows: toluene (0.01 ml/ml) was added to broth and incubated at 37 C for 30 min in a stoppered flask. F^- cells were suitably diluted into this and incubated with agitation for 10 min.

RESULTS

DNA synthesis. For selective incorporation of radioactive precursors into the DNA of recipient cells, we employed thymidine kinase-deficient (15, 17) mutants of Hfr and Tra^- Hfr cells. Incorporation of exogenous thymine or thymidine by these Tdk^- cells was approximately 0.05% of the incorporation by Tdk^+ recipient cells and permitted the use of high donor to recipient ratios. This system, which has the advantage of allowing specific labeling of newly synthesized recipient DNA without the necessity to inhibit DNA synthesis in the donor, has been used in previous conjugation studies (33, 34).

Initially, we examined the incorporation of ^{3}H-thymidine into Thy^+ recipient cells and found that an excess of F^- Tdk^- cells totally prevented incorporation, while acting as a mating control. This was presumably due to the presence of high levels of thymidine phosphorylase (EC 2.4.2.4 [27]), for we also observed a rapid degradation of thymidine to thymine (Skurray and Reeves, unpublished data). We have therefore examined the incorporation of ^{3}H-thymine into cultures of a low-level thymine-requiring mutant, as an estimate of recipient cell DNA synthesis. Incorporation by these cells was similar, whether they were diluted into preconditioned media or into a culture of a Tdk^- Tra^- donor (Hfr) strain, the latter providing an adequate nonconjugating control.

The results from one such experiment (Fig. 1) show the inhibition of incorporation, which was observed as early as 20 min after mixing, when mating and nonmating F^- cells were compared. When equal numbers of Hfr and F^- cells were employed, the inhibition was less pronounced than at the higher ratio of 25:1.

It can be concluded from these results that inhibition of DNA synthesis, like reduction in F^- viable count, results from an event that is dependent upon the multiplicity of Hfr cells employed.

β-Galactosidase induction. To characterize further the metabolic changes associated with

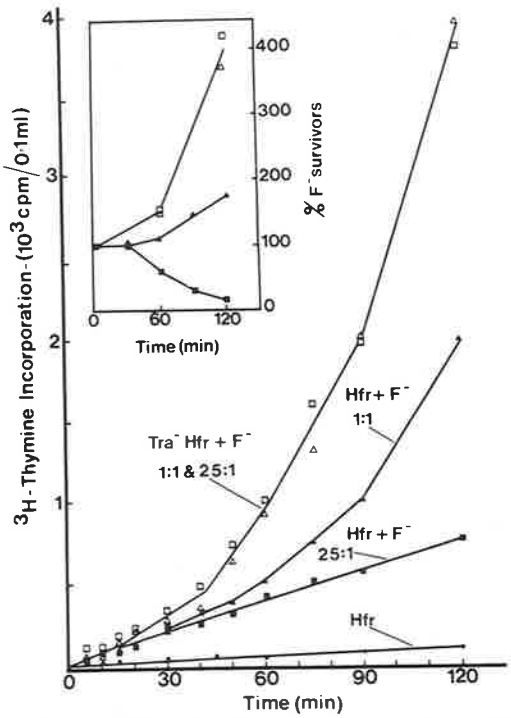

FIG. 1. *Effect of lethal zygosis on recipient cell thymine incorporation. Cultures of the donor strain P727 (Hfr P4X) and the recipient strain P978 were mixed (for details, see Materials and Methods) at zero time, to give the ratios indicated (1:1, ▲; 25:1, ■). ^{3}H-thymine (5 μCi/μg) was added at the commencement of mating, and incorporation into acid-insoluble material was measured. Controls with the Tra^- strain P766 were included (1:1, △; 25:1, □). Incorporation by the donor strain P727, at the cell density used in 25:1 mating, is also shown (●). The insert shows F^- survival in the four flasks.*

conjugation, we examined the induction of the enzyme, β-galactosidase, as a measure of protein synthesis. This system is particularly amenable for such studies, as Hfr strains carrying a complete deletion of the *lac* operon provide a low background of activity against which recipient synthesis can be readily examined.

When Hfr and F^- cells were mixed in equal numbers, there was a noticeable reduction in the rate of synthesis, compared with the nonmating control (1:1; Fig. 2B), and as the ratio of Hfr to F^- was increased to 5:1 and 10:1 there was a corresponding increased effect. With a further increase to 25:1, deviation from the control (25:1) was observed by about 25 min, and by 65 min after mixing synthesis was completely arrested.

It should be noted that when F^- cells were diluted into fresh tryptone-yeast extract broth a lag in induction was observed which was less marked when dilution was into a 20-fold excess

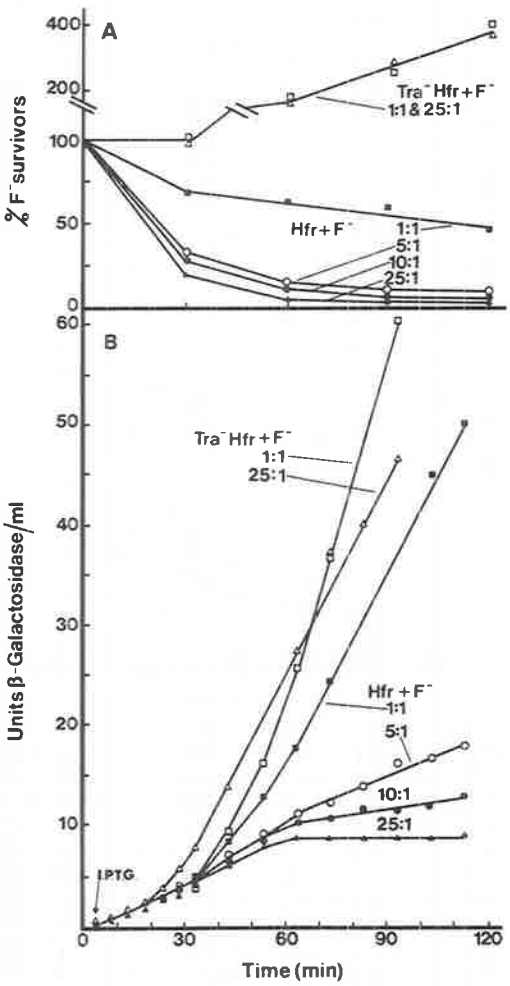

FIG. 2. *Effect of concentration of the donor strain P311 (HfrH) on β-galactosidase induction in the recipient strain P882 (B). Cells grown in tryptone-yeast extract broth were resuspended in preconditioned media, and donor and recipient cultures were mixed at zero time to give the following ratios: 1:1 (■), 5:1 (○), 10:1 (●), 25:1 (▲), in a final volume of 30 ml. Isopropyl-β-D-thiogalactopyranoside (5 × 10⁻⁴ M final concentration) was added and, at the indicated times, 1.0-ml samples were taken and assayed for β-galactosidase activity. Induction in the presence of the Tra⁻ strain P310 was examined at ratios of 1:1 (□) and 25:1 (△). All enzyme activities were similar prior to 20 min; some data have been omitted for clarity. Percent F⁻ survival in each flask is shown (A).*

of Tra⁻ Hfr cells or into preconditioned broth (unpublished data). This could be associated with a utilizable substrate giving catabolite repression of induction. The initial difference in rate of induction between controls at 25:1 and 1:1 (Fig. 2B) could also depend upon the presence of such a substrate, even in preconditioned media. This would be more rapidly exhausted in the presence of high cell numbers (25:1), but, once removed, the culture with the lower cell density (1:1) could support a more rapid rate of synthesis.

TMG accumulation. A primary lesion that could lead to the observed arrest of both DNA and β-galactosidase synthesis would be inhibition of energy metabolism. The accumulation by *E. coli* Lac⁺ cells of nonutilizable galactosides against a concentration gradient requires a continuing supply of metabolic energy (see review by Kennedy [18]), and therefore uptake of ^{14}C-TMG by the lactose permease system provides a convenient measure of energetic changes associated with lethal zygosis. The result of one such experiment (Fig. 3) shows that accumulation by the recipient cell was rapid and, in the presence of Tra⁻ Hfr cells, continued to rise over the period of the experiment. When mixed with Hfr cells, by 15 min there was a loss of the ability to retain the accumulated galactoside against a concentration gradient. The Hfr (or Tra⁻ Hfr) cells which carry the *lac* deletion, and which were in 10-fold excess over the F⁻ cells, showed an uptake per cell of about 1% of that accumulated by the Lac⁺ recipient cells. This indicates that, as expected, the *lac* deletion cells have only the same concentration of TMG internally as is present outside.

ONPG hydrolysis. With intact cells, the rate-limiting step in the hydrolysis of ONPG to ONP by the enzyme β-galactosidase is the passage of this substrate across the membrane (28). This hydrolysis has been used as a measure of ONPG transport either by the lactose permease-mediated system, in Lac⁺ cells, or by the diffusion-like process which exists in cryptic cells (14, 19).

We found that F⁻ (*lacY*) cells, in the presence of Tra⁻ Hfr cells, hydrolyzed ONPG at a rate 20 times slower than toluene-treated cells (Fig. 4); however, when mixed with the donor strain P311 (HfrH), the hydrolysis increased as early as 15 to 20 min and eventually reached a rate which was 25 to 30% of that observed with toluene-treated cells.

As the long period of contact with ONPG produced high although reproducible levels of ONP in the control situation, we also examined the extent of hydrolysis over a 10-min period (Fig. 5B). With the ratio of 1:1, an increase in hydrolysis was observed by 50 min, and as the multiplicity of the Hfr strain employed was increased the extent of hydrolysis also increased, with deviation from the control occur-

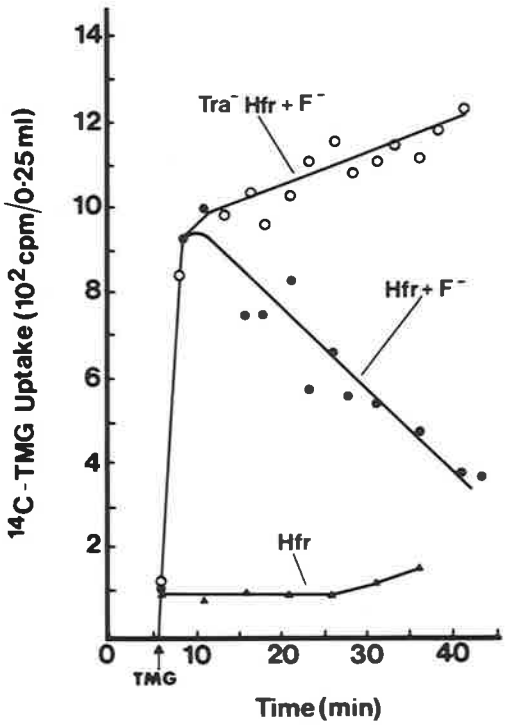

FIG. 3. *Effect of lethal zygosis on recipient cell accumulation of TMG. The recipient strain P882 was induced by growth in tryptone-yeast extract broth plus isopropyl-β-D-thiogalactopyranoside (IPTG, 10^{-3} M final concentration), and the cells were centrifuged, resuspended, and diluted in media without IPTG. A culture of the donor strain P311 (HfrH) was mixed at zero time with this preinduced F⁻ culture to give a ratio of 10:1 (●). The initial F⁻ viable count was 3×10^7 cells/ml. ^{14}C-TMG was added (0.4 μCi/ml, 4×10^{-5} M final concentration) and, at the indicated times, samples (0.25 ml) were taken and the uptake of TMG was measured as described (Materials and Methods). As a control, F⁻ cells were mixed with the Tra⁻ strain P310 at the same ratio (○). The uptake by the donor strain P311 is also shown (▲). F⁻ survival was similar to that in Fig. 2A.*

ring at progressively earlier times.

The latter method also allowed a comparison of the change in rate of hydrolysis with the extent of leakage of β-galactosidase from F⁻ cells (Fig. 5A). The initial background may be partially due to the presence of a small number of cells remaining in the supernatant fluid. In the presence of the donor strain P311, the levels of β-galactosidase in the supernatant fluid increased to a level three times greater than in the control culture and after 50 min accounted, in part, for the increase in hydrolysis observed in Fig. 5B.

DISCUSSION

In this report, we have demonstrated that lethal zygosis is accompanied by the following changes in recipient cell physiology: (i) inhibition of DNA synthesis, (ii) inhibition of β-galactosidase induction, (iii) loss of ability to accumulate ^{14}C-TMG, (iv) altered ONPG transport, and (v) leakage of β-galactosidase into the supernatant fluid.

The role of DNA synthesis during conjugation has been the subject of a number of studies (see review by Curtiss [9]). Although recent evidence (33, 34) suggests the occurrence of mating-dependent DNA synthesis in both donor and recipient cells, no reports are available which compare total DNA synthesis in conjugating and nonconjugating cells. Our re-

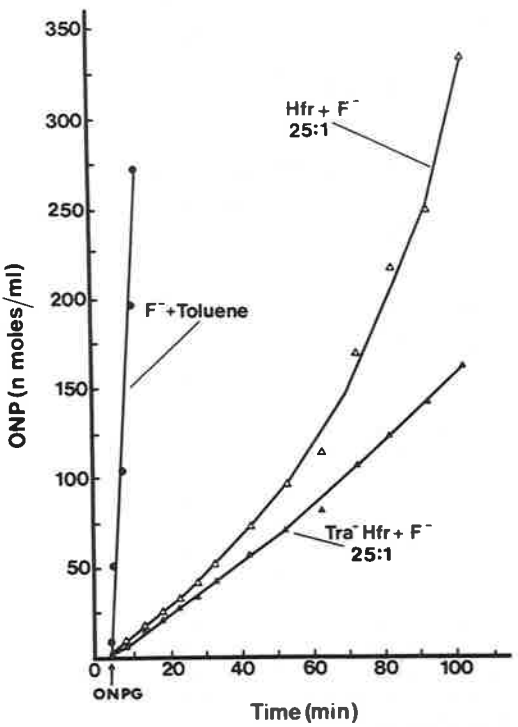

FIG. 4. *Effect of lethal zygosis on ONPG hydrolysis by recipient cells. The F⁻ strain P322 was grown in tryptone-yeast extract broth in the presence of 10^{-3} M isopropyl-β-D-thiogalactopyranoside (IPTG). These cells were harvested by centrifugation, resuspended, and diluted in IPTG-free media; they were then mixed, at zero time, with either the donor strain P311 (HfrH, △) or the Tra⁻ strain P310 (▲) to give the indicated ratio. ONPG (3×10^{-3} M final concentration) was added, and at the indicated times samples were examined for ONP produced (Materials and Methods). The hydrolysis by a toluene preparation of the F⁻ cells is shown (●). F⁻ survival was similar to that in Fig. 2A.*

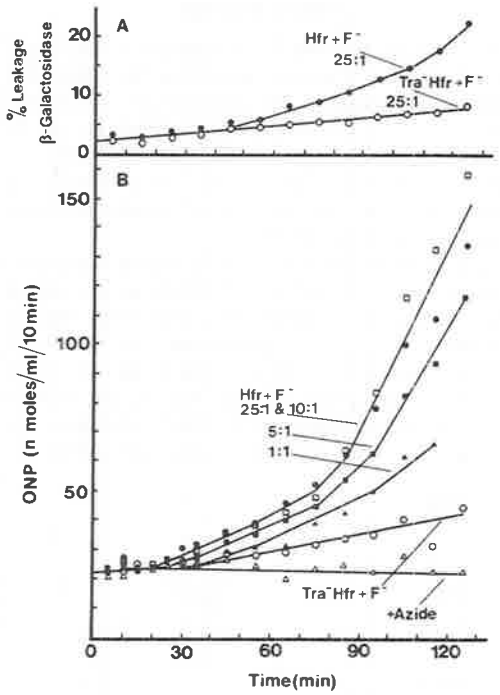

FIG. 5. *Effect of concentration of the donor strain P311 (HfrH) on hydrolysis of ONPG by cells of the recipient strain P322. (B) The F⁻ culture was preinduced in tryptone-yeast extract broth in the presence of 10^{-5} M isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were centrifuged, resuspended in media free from IPTG, and mixed with donor cells and media where required to give the following ratios: 1:1 (▲), 5:1 (■), 10:1 (□), and 25:1 (●). Samples of the preinduced F⁻ culture were also mixed with a culture of the Tra⁻ strain P310 (○) and with this same strain in the presence of 10^{-2} M sodium azide (△) to give, in each case, a ratio of 25:1. At the indicated times after mixing, samples were removed and added to ONPG (~2×10^{-3} M final concentration), incubated for 10 min, and examined for production of ONP. (A) Samples (1.5 ml) were also removed from mating (●) and control flasks (○), centrifuged, and assayed for β-galactosidase activity in the supernatant fluid as described (Materials and Methods) with the omission of toluene treatment. Activity in the supernatant fluid is expressed as a percentage of the total enzyme activity. F⁻ survival was similar to that in Fig. 2A.*

sults (Fig. 1) from such a comparison indicate that F⁻ cell DNA synthesis is partially inhibited during conjugation, the extent of inhibition depending upon the multiplicity of Hfr cells employed, as does the number of F⁻ survivors.

As stated earlier, an event that could lead to the arrest of both DNA synthesis and induction of β-galactosidase would be inhibition of energy metabolism, a mechanism that is supported by the observed inhibition of the energy-dependent active transport of TMG by 15 min. It is possible that the loss of ability to accumulate this galactoside could result from interactions at the cytoplasmic membrane, producing either one or both of the following lesions: (i) a specific interference with energy-generating mechanisms, such as adenosine triphosphate production, or (ii) a nonspecific disruption of the permeability barrier. The increased rate of ONPG hydrolysis with conjugating F⁻ cells does suggest an early loss of membrane integrity, as no such change was observed with nonmating F⁻ cells in the presence of sodium azide, an uncoupler of oxidative phosphorylation.

Although the changes described could have all resulted from alterations in the cytoplasmic membrane, the paucity of knowledge regarding the attachment of the donor to the recipient prohibits precise comments on the mechanism of such conjugation-induced damage. Nevertheless, whether this attachment occurs by wall-wall contact with a conjugation bridge (4) or via the F pilus (6), chromosome transfer requires the passage of DNA through the recipient cytoplasmic membrane. This structure, which normally opposes entry of all but the simplest molecules, must in some way change to accommodate the entry of the very large DNA molecule. Although such an alteration may be readily restored with conjugation involving a single Hfr cell, at high ratios multiple hits may produce cells incapable of recovery.

Anderson (3) and Ou and Anderson (24), who studied conjugating bacteria by micromanipulation, observed a number of nonviable exconjugant F⁻ cells, many of which formed filaments or possessed abnormal division patterns. These results could well be explained by the inhibition of DNA synthesis we have observed, as could the inhibition of exconjugant cellular division (29), and it may be that biosynthetic perturbations induced during conjugation need not always be associated with gross membrane damage. Such metabolically altered cells might also be highly susceptible to a period of unbalanced growth induced by shift down from a nutrient-rich to a synthetic medium. This is suggested by the increased extent of lethality observed when broth-mated F⁻ cells are plated on minimal agar (Skurray and Reeves, unpublished data) and could account in part for the loss in recombinants that follows such a shift (12). It therefore appears that sometime after contact a proportion of the F⁻ cells are damaged without being committed to death or survival, and that the outcome depends upon

subsequent treatment (dilution and plating).

Although we are continuing these studies, to establish the primary lesion associated with lethal zygosis, our present observations are interesting to consider in terms of the sex virus model of genetic transfer, proposed by Brinton (7), for it appears that infection of a recipient by donor DNA can, in some cases, be associated with death of the invaded cell.

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