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An Upper Limit on β-Galactosidase Transfer in Bacterial Conjugation[1]

J. L. ROSNER,[2] EDWARD A. ADELBERG, AND MICHAEL B. YARMOLINSKY

Department of Microbiology, Yale University, New Haven, Connecticut, 06510, and Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

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An upper limit for β-galactosidase transfer between mating F^+ and F^- *Escherichia coli* has been determined by a new technique which relies on selective lysis of the donor strain by heat induction of a thermo-inducible strain of λ, accompanied by chymotryptic digestion of the released β-galactosidase. No significant transfer of β-galactosidase during mating between F^+ and F^- cells has been observed: 0.05 ± 0.05% of the enzyme originally present in the male cells is found in the female cells after 1 hr of mating at 37 C.

Conjugation in the procaryote *Escherichia coli* involves the transfer of nuclear material between two cells which are in direct contact. The nature of the union between the conjugating partners and the extent of material transfer are not entirely clear. Bridges consisting of cytoplasmic extentions, 1,000 to 3,000 A in width, have been observed repeatedly (2, 11). Brinton, Gemski, and Carnahan (3) argued that these cytoplasmic extensions are artifacts of preparation and that the true conjugal bridges are male specific F-pili. The F-pili are thin tubular structures resembling the non-sex specific type I pili which have an inner diameter of about 25 A. If Brinton et al. (3) are correct, only molecules which are not too large to pass through this structure could be transferred during conjugation.

The transfer of deoxyribonucleic acid (DNA) during conjugation of Hfr or F^+ cells with F^- cells has been inferred from genetic experiments. Direct measurement of the amount and rate of transfer of radioactively labeled DNA has confirmed the genetic estimates (9, 11, 26, 27). The transfer of materials other than DNA, if it occurs, appears to be relatively minor. During crosses between Hfr and F^- bacteria, no physiologically significant transfer of such diverse substances as arginine, maltose, inducers and repressors of β-galactosidase, or λ repressor has been observed (17, 24, 25). Moreover, Silver (26) has found that the transfer of radioactively labeled ribonucleic acid (RNA) or protein from Hfr to F^- does not exceed the lower limit of detection of about 1%. However, the possibility of an exchange of DNA precursor pools between donor and recipient cells has been suggested by Gross and Caro (11) and again by Bonhoeffer (4).

At the time this study was begun, there had been no quantitative studies on cytoplasmic transfer in $F^+ \times F^-$ crosses. Moreover, Fisher (8) had reported that λ immunity could be transferred from F^+ cells but not Hfr cells. More recently, however, Fisher (*personal communication*) has found that this effect is not phage-specific and probably does not represent cytoplasmic transfer.

We report here an improved method for selectively eliminating one class of cells in a mixed population, and its application to the study of material transfer in mating. The method makes use of the fact that heat induction of a thermo-inducible prophage (28) can be used to lyse selectively and completely the lysogenic cells. Such "lysis from within" has proven useful in other experiments requiring quantitative release of radioactivity from (abortively) lysogenic cells (Yarmolinsky and Korn, *unpublished data*). The present experiments take advantage of both lysis from within and lysis from without.

The choice of substances suitable for the study of material transfer is greatly limited by the need to avoid even a small amount of nonconjugal transfer via the growth medium. We have chosen to study the transfer of an enzyme, β-galactosidase, which is unlikely to be transferred from cell to cell in the absence of conjugation. β-Galac-

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[2] Present address: Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md. 20014.

tosidase has the advantages of stability, of being detectable by a simple, accurate, and sensitive assay, and of possessing a rate of synthesis subject to genetic and physiological control.

MATERIALS AND METHODS

Reagents and media: *o*-Nitrophenyl β-D-galactoside (ONPG) and isopropyl β-D-galactoside (IPTG) were purchased from Mann Research Laboratories, New York, N.Y. Deoxyribonuclease I and α-chymotrypsin were purchased from Worthington Biochemical Corp., Freehold, N.J.

L broth was that of Luria and Burrous (22) except that the medium contained 10^{-3} M $MgSO_4$ and no glucose. The minimal medium, M56 (23), was used diluted and appropriately supplemented with amino acids, sugars, vitamins, and streptomycin as described by Adelberg and Burns (1). Media were solidified with 2% agar for plates or 0.7% agar for top agar. Indicator agar for the detection of sugar fermentation contained 0.0012% bromothymol blue and 1% sugar; 4% lactose was used for detection of fermentation by Y^- Z^+ cells. (For explanation of symbols, see footnote to Table 1.)

Bacteria and phages. Bacterial strains used are listed in Table 1. All of the F^+ strains contain the wild-type sex factor, F1, introduced by mating with strain AB264. The *lacZ* mutation used was selected for its stability and for the absence of leakiness, and is presumed to be due to a deletion. The mutation does not alter the expression of the neighboring *lacI* or *lacY* genes (J. L. Rosner, *unpublished data*).

Two strains of λ were used throughout this work. The wild-type strain, which will be referred to simply as λ, is the "λ^{++}" strain of Kaiser (19), obtained from M. Meselson. The thermo-inducible strain, which will be referred to as λct, is λCI857 described by Sussman and Jacob (28) and was obtained from F. Jacob. Both λ phages were grown and titered on AB1157. High-titer stocks of 5×10^{12} T4 or T6 plaque-forming units (PFU)/ml were prepared by alternate low- and high-speed centrifugations of stocks grown in L broth on *E. coli* B under conditions of lysis inhibition (6). The concentrated phage were resuspended in T2 buffer (13).

Growth and mating conditions. Bacteria were grown as shake cultures in L broth at 34 C. In growing (λct) strains, care was taken to minimize the number of nonlysogenic segregants by using as the inoculum a fresh, small, single colony grown on an L plate for about 17 hr at 30 C. For all other strains, the inoculum consisted of a small amount of a culture aerated overnight at 34 C. Cells with a fully induced level of β-galactosidase were prepared by growth for several generations in L broth supplemented with 10^{-3} M IPTG. The culture was grown to about 2×10^7 cells/ml, chilled, and harvested by centrifugation at 13,000 × *g* for 5 min; the pellet was washed once with an equal volume of L broth to prevent further induction during subsequent growth.

Matings were carried out at 34 C with the Z^- partner in fivefold excess, at 10^8 cells/ml. Incubations were for 1 hr without agitation in 2-liter flasks containing 300 ml of culture. F transfer was measured by testing individual colonies for ability to recombine with AB59 or other appropriate tester females on a selective medium (1).

β-Galactosidase release, assay, and digestion. Intracellular β-galactosidase was released from bacteria by "lysis from without." Bacteria harboring λct prophage were lysed from within by vigorously shaking broth cultures at 40 C. Clearing was observed within 60 to 90 min, at which point deoxyribonuclease at 10 μg/ml was added; shaking was continued for an additional 30 min. In mating experiments, the extent of lysis of the (λct) cells is obscured by the presence of the nonlysing (λ) cells. Therefore, a control flask containing

TABLE 1. *List of strains*

Strain no.	Mating type	Prophages[a]	Pertinent phenotypic properties[b]	Use
AB59	F^-	—	Thr^-, Leu^-, Sm^r	F^- tester strain
AB264	F^+	—	Prototrophic, Sm^s	Source of sex factor
AB1157	F^-	—	Thr^-, Leu^-, Sm^r, $T6^r$, Y^-	Growth of λ
AB1630	F^-	λct	Thr^-, Leu^-, Sm^r, $T6^r$, λ^r, Y^-	F^- Z^+
AB1631	F^+	λct	Thr^-, Leu^-, Sm^r, $T6^r$, λ^r, Y^-	F^+ Z^+
AB1632	F^-	λ	Ilv^-, Sm^r, λ^r, $T6^s$, Z^-	F^- Z^-
AB1633	F^+	λ	Ilv^-, Sm^r, λ^r, $T6^s$, Z^-	F^+ Z^-
AB1634	F^-	λ	Ilv^-, Sm^r, λ^r, $T6^s$, Y^-	β-Galactosidase recovery
AB1635	F^-	λct	Ilv^-, Sm^r, λ^r, $T6^s$, Z^-	Z^- lysate
NS21[c]	Hfr	—	Z^- (transfer order *thr*, *leu*, *proB*. . .)	Source of *lacZ* mutation
AC2501	F^-	—	(*Escherichia coli* B)	Growth of T4 and T6

[a] Prophages: λ, wild-type lambda; λct, λ carrying the mutation CI857 (λct is thermo-inducible); (—), no λ prophages present.

[b] Abbreviations: Thr^-, requirement for threonine; Leu^-, requirement for leucine; Ilv^-, requirement for isoleucine and valine; Sm^r and Sm^s, resistance or sensitivity to streptomycin; $T6^r$ and $T6^s$, resistance or sensitivity to phage T6; λ^r, resistance to phage λ; Y^-, lacking β-galactosidase permease activity; Z^-, lacking β-galactosidase activity; I^-, constitutive synthesis of β-galactosidase.

[c] Provided by N. Schwartz.

only (λct) cells was used to judge the time of lysis in the flasks also containing (λ) cells. Lysis from without (5) was accomplished by adding in excess of 300 T4 or T6 phage per sensitive bacterium and incubating at 40 C for 30 min in the presence of 10 μg of deoxyribonuclease per ml.

Intracellular β-galactosidase was rendered accessible to substrate without cell disruption by exposure of the cells to isoamyl alcohol. Cells were gently shaken for 30 min at 34 C in stoppered tubes with one-twentieth volume of isoamyl alcohol.

β-Galactosidase was assayed spectrophotometrically by a modification of the procedure of Pardee et al. (24). Samples to be assayed were diluted in the following buffer: 0.1 M sodium phosphate (*p*H 7.0), 10^{-3} M magnesium sulfate, and 2×10^{-4} M manganese chloride. All reagents were prewarmed to 28 C. Two tubes, one containing 0.1 ml of 0.013 M ONPG and the other without ONPG, each received 0.5-ml portions of the diluted sample. The tubes were incubated at 28 C for varying periods of time. Upon the appearance of color, 0.25 ml of 1 M Na_2CO_3 was then added to each tube to terminate the reaction, and immediately 0.1 ml of 0.013 M ONPG was added to the control tube. Suspended material was removed by centrifugation at room temperature, and, if necessary, by filtration through a membrane filter (0.45 μ pore size; Millipore Corp., Bedford, Mass.). One enzyme unit is defined as that which produces an increase in absorbance at 420 mμ (over absorbance in the control) of 0.001 per minute of incubation at 28 C under the conditions described. Microcuvettes with a 1-cm light path were used. Under these conditions, the assay of intracellular or extracellular β-galactosidase is linear over a 750-fold concentration range, requiring incubation periods of 10 min to 10 hr. When cells have been diluted to an enzyme concentration of only 0.20 units/ml, the error is about 10%.

The digestion of crude extracellular β-galactosidase was carried out in the present experiments by gentle shaking of the sample with 1 mg of α-chymotrypsin per ml in 0.1 M tris(hydroxymethyl)aminomethane chloride buffer, *p*H 8.0, at 40 C for 3 hr (10). Chymotrypsin was removed by centrifugation at 13,000 × *g* for 5 min, and two successive washes of the pelleted material with equal volumes of the β-galactosidase assay buffer.

Experimental plan. Measurements of the transfer of β-galactosidase from F^+ to F^-, from F^- to F^+, and from F^- to F^- are provided by these experiments. In each case, the Z^+ cells which are being tested as donors of β-galactosidase are $I^+Y^-T6^r\lambda^r(\lambda ct)$ and the potential Z^- recipients are $I^+Y^+T6^s\lambda^r(\lambda)$.

A fully induced log-phase culture of the Z^+ strain, washed free from inducer, is mated at 34 C with the Z^- strain which contains no detectable β-galactosidase activity. After allowing 1 hr for mating, the incubation temperature is raised to 40 C, selectively lysing the Z^+ population by induction of the λct prophage. The β-galactosidase of the Z^+ cells is released into the medium upon lysis and is separated from the remaining cells by centrifugation.

The activity remaining in the pellet may be derived from (i) Z^- cells containing transferred enzyme, (ii) unlysed Z^+ cells, or (iii) β-galactosidase adsorbed to cells or debris. The source of the activity is analyzed by means of "lysis from without" (see Table 2). The washed pellet of cells plus debris is resuspended in three portions. Portion I is not treated with phage. Portion II is treated with T6 at a high multiplicity of infection which selectively lyses the Z^- cells. Portion III is similarly treated with T4 which lyses both Z^+ and Z^- cells. All three portions are then treated with chymotrypsin to destroy extracellular β-galactosidase, centrifuged, washed, concentrated 50-fold and assayed for β-galactosidase. The distribution of β-galactosidase activity in unlysed Z^+ cells, Z^- cells, or in an unidentified form which is chymotrypsin-resistant is estimated from differences in activity of the three portions.

RESULTS

Lack of interference between cells and extracts. The measurements of transferred β-galactosidase are valid if (i) the Z^- cells do not inactivate transferred β-galactosidase and (ii) the β-galactosidase detected in the Z^- cells represents enzyme transferred by mating, and not acquired from the medium. The results of Table 3 support the first assumption, since β-galactosidase activity in a crude extract is not affected either by whole Z^- cells or by a lysate of such cells. The second assumption is supported by the finding that Z^- cells (AB1632) fail to acquire β-galactosidase from a filtered lysate of fully induced strain AB1630 in 1 hr at 34 C, although an uptake of 0.01% of the activity present in the medium is detectable (data not shown).

Efficiency and selectivity of phage-induced lysis. The efficiency of lysis from without by phage T6 has been measured by the release of β-galactosidase after infection of $T6^s$, β-galactosidase induced cells (AB1634). At the multiplicity of infection used in these experiments (300 to 500 PFU per cell), 90% of the cellular β-galactosidase is released. Certain T6 preparations cause lysis of up to 15% of the $T6^r$ strains, presumably owing to contamination by phage lysozyme. The error introduced by this nonspecificity is negligible, since the T6 is added after the bulk of the Z^+T6^r cells have been removed by lysis from within (see below). Comparable results have been obtained with phage T4.

The efficiency of lysis from within, i.e., lysis by heat induction of a λct lysogen, is about 99.9% (see column under I, Table 4). Cells which are lysogenic for λ do not release β-galactosidase as a result of incubation at 40 C.

Efficiency and selectivity of digestion by chymotrypsin. Under conditions where the activity of extracellular β-galactosidase in filtered crude extracts is destroyed rapidly and completely by chymotrypsin, the activity of intracellular β-galactosidase remains unaffected (Fig. 1).

Measurements of β-galactosidase transfer during

TABLE 2. *Distribution of β-galactosidase among cells of a mating mixture after lysis from within*[a]

Treatment	Possible source of β-galactosidase present after treatment with phage
I: no phage	Z^- $T6^s$ $T4^s$ cells, Z^+ $T6^r$ $T4^s$ cells, and Unidentified
II: T6	Z^+ $T6^r$ $T4^s$ cells, and Unidentified
III: T4	Unidentified

[a] β-Galactosidase in Z^- cells = I − II; β-galactosidase in Z^+ cells = II − III.

mating. The results of all transfer experiments are summarized in Table 4. Experiment 1 is a nonconjugal control in which both participants are F^-. Experiment 2 measures the transfer of β-galactosidase in the direction opposite from the direction of DNA transfer. Experiments 3, 4, and 5 measure β-galactosidase transfer in the same direction as DNA transfer. The efficiency of conjugation in the mating mixtures is high, as indicated by the frequency of F transfer and recombinant formation.

In each case, heat-induced lysis of the Z^+ strain, washing, and chymotrypsin treatment eliminate about 99.9% of the β-galactosidase. The majority of the remaining β-galactosidase is shown to be present in unlysed Z^+ cells. Since the small residue of β-galactosidase which cannot be eliminated by the successive phage and chymotrypsin treatments (portion III) is observed in the nonconjugal F^- to F^- experiment, this activity does not represent material transferred by conjugation. In the three F^+ to F^- transfer experiments, an average value of 0.01 ± 0.01% of the β-galactosidase originally in Z^+ cells is found associated with the Z^- cells.

Recovery of β-galactosidase in reconstruction experiments. Our estimated upper limit for β-galactosidase transfer in these experiments is raised by the finding of incomplete recovery of enzyme activity from small amounts of F^-Z^+ cells added at the onset of mating. The data from two reconstruction experiments are shown in Fig. 2. The slope of the line fitted to these data is 0.9; the y-intercept is −0.03%. The reason for the constant loss of 0.03% is not known. Nevertheless the values observed in the transfer experiments may be underestimated by the incomplete recovery. It is estimated from Fig. 2 that a reliable upper limit for β-galactosidase transfer in these experiments is 0.10% of the β-galactosidase initially present in the F^+ strain.

TABLE 3. *Effect of intact or lysed Z^- cells on β-galactosidase activity*

Sample	Units of β-galactosidase after incubation[a] in the presence of		
	L broth	Intact cells[b]	Lysed cells[c]
1	100	108	110
2	109	119	115
3	116	124	122

[a] Samples of extracellular β-galactosidase (See legend to Fig. 1) were diluted 10-fold into broth, culture, or lysate as indicated. After 1 hr at 34 C, samples were diluted 10-fold into β-galactosidase assay buffer and assayed as described in the text.

[b] AB1635 at 2×10^8 cells/ml in L broth.

[c] AB1635 at 2×10^8 cells/ml lysed by heat induction at 40 C for 1 hr.

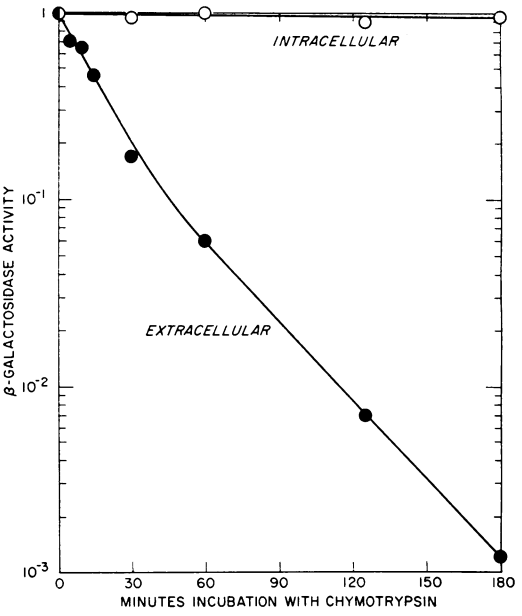

FIG. 1. *Effect of chymotrypsin on intracellular and extracellular β-galactosidase. Intracellular β-galactosidase: fully induced AB1634 was concentrated to 2×10^9 cells per ml in L broth. Extracellular β-galactosidase: fully induced AB1630 was incubated at 40 C and concentrated to 2×10^9 cells per ml prior to lysis. After lysis at 40 C, the liberated β-galactosidase was passed through a membrane filter (0.45 μ pore size). The reaction mixture contained in a final volume of 1 ml: 0.1 ml of β-galactosidase, 1 mg of chymotrypsin, 0.1 M tris(hydroxymethyl)aminomethane chloride, pH 8.0 at 40 C. The reaction was terminated by a 100-fold dilution into the β-galactosidase assay buffer and assayed as described in the text.*

TABLE 4. *β-Galactosidase transfer experiments*

Expt. no.	Participants		Percentage of initial β-galactosidase[a] recovered in					Proportion of cells relative to minority parent initially present which	
			Portion[b]						
	Z^+ strain (minority parent)	Z^- strain (majority parent)	I	II	III	Z^+ cells (II − III)	Z^- cells (I − II)	Receive F^-[c]	Form recombinants[d]
1	F^- (AB1630)	F^- (AB1632)	0.10	0.10	0.03	0.07	0.00	—	—
2	F^- (AB1630)	F^+ (AB1633)	0.13	0.11	0.03	0.08	0.02	1.0	8×10^{-5}
3	F^+ (AB1631)	F^- (AB1632)	0.13	0.11	0.06	0.05	0.02	3.1	—
4	F^+ (AB1631)	F^- (AB1632)	0.12	0.11	0.05	0.06	0.01	2.3	13×10^{-5}
5	F^+ (AB1631)	F^- (AB1632)	0.08	0.08	0.04	0.04	0.00	2.6	—

[a] Expressed as the percentage of the total β-galactosidase activity in the Z^+ strain at the onset of mating.

[b] B-Galactosidase activity remaining after treatment with chymotrypsin (I), T6 and chymotrypsin (II), or T4 and chymotrypsin (III). See text for details.

[c] After mating for 1 hr, a sample was diluted, blended for 30 sec, and plated on media selective for the F^- partner. Individual colonies were then tested for ability to transfer chromosomal markers to F^- tester strains on selective media.

[d] After mating for 1 hr, a sample was diluted, blended for 30 sec, and plated on media selective for *thr*$^+$ *leu*$^+$ *ilv*$^+$ recombinants.

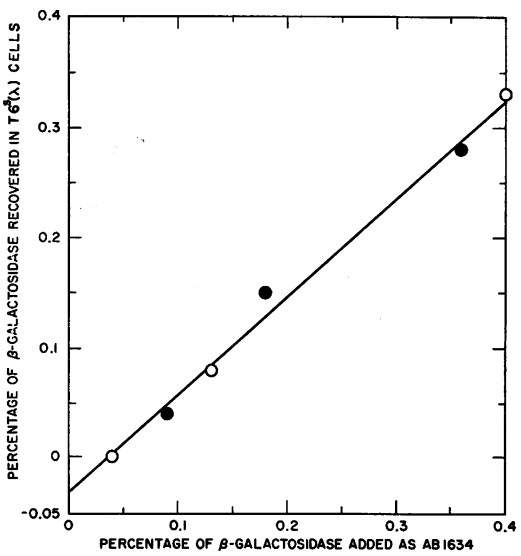

FIG. 2. *Recovery of β-galactosidase in reconstruction experiments. Various measured amounts of fully induced AB1634 cells (washed free from IPTG) were added at the onset of mating between the Z^+ strain, AB1631, and the Z^- strain, AB1632. The amounts of β-galactosidase recovered in T6^s (λ) cells were determined as described in Materials and Methods. The values obtained were corrected for the β-galactosidase found in T6^s (λ) cells in the control flasks which contained no added AB1634. The data for the control flasks were presented in experiments 4 and 5 of Table 4, which correspond to the experiments designated here by open and closed circles, respectively. All values are percentages of the β-galactosidase present at the onset of mating in AB1631.*

DISCUSSION

The principal technical concern of this work is a refinement in the methods for selectively eliminating the material of one partner in a mating mixture of *E. coli*. The method presented here relies upon the highly selective and efficient lysis of λct lysogens by thermal induction followed by selective lysis from without by use of virulent phages.

We have used these methods to study the transfer of an enzyme, β-galactosidase, from cell to cell during conjugation. We conclude that there is no significant transfer of β-galactosidase during mating between F^+ and F^- cells: 0.05 ± 0.05% of the β-galactosidase originally present in the male cells is found in the female cells. Silver et al. (27) found that 0.06 ± 0.19% of the radioactively labeled protein present in F^+ cells was transferred to F^- cells during mating. Their data, however, were not corrected for losses in recovery. Thus, the measurements reported here place a further refinement on the limits of general cytoplasmic mixing which occurs between mating cells of *E. coli*. If we assume that a fully induced cell contains roughly 10,000 molecules of β-galactosidase (15), the upper limit of β-galactosidase transfer in mating corresponds to no more than two active enzyme molecules per F present in the donor strain.

The transfer of DNA to the exclusion of other materials may be variously explained. The transfer of DNA is an energy-requiring reaction (7) which Jacob, Brenner, and Cuzin (14) suggest may be driven by the process of DNA synthesis. There is

no reason to assume that the same driving force would transport any other cellular constituent. Moreover, the attachment of F-episomal DNA to the cell membrane (14, 16) may bring this DNA into a favored position for transfer relative to other substances. Finally, if the male-specific sex pili form the unique connections between mating *E. coli*, the inside diameter of these tubes (about 25 A) would not permit the passage of molecules as large as β-galactosidase, whose smallest dimension is greater than 70 A (20).

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