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Inducible System for the Utilization of β-Glucosides in *Escherichia coli*

II. Description of Mutant Types and Genetic Analysis

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ABSTRACT

Two types of mutants obtained by treating β-*gl*$^+$ cells with nitrosoguanidine are described. One type, β-*gl*$^+$ *c*, is constitutive for the biosynthesis of the aryl β-glucoside splitting enzyme(s) and for the β-glucoside permease; the other (β-*gl*$^+$ *sal*$^-$) has lost the capacity to ferment salicin, but has retained the capacity to ferment arbutin and other aryl β-glucosides. By two successive mutational steps, β-*gl*$^+$ *sal*$^-$ *c* double mutants can be obtained. Determinations of the enzymatic splitting of salicin and *p*-nitrophenyl β-glucoside by β-*gl*$^+$ *sal*$^-$ cells and extracts showed that these mutants have lost the capacity to split salicin but do split *p*-nitrophenyl β-glucoside; they possess the β-glucoside permease, and in them salicin is a gratuitous inducer for enzyme and permease biosynthesis. Studies on a β-*gl*$^+$ strain, which splits salicin as well as *p*-nitrophenyl β-glucoside, have shown that the splitting of salicin is more temperature-sensitive than that of *p*-nitrophenyl β-glucoside and other β-glucosides. Other properties of the two activities are similar. Interrupted mating experiments and cotransduction with P1kc phage showed that the genetic determinants of the β-glucoside system map between the *pyrE* and *ile* loci. Three distinct mutational sites were found and are presumed to have the following functions: β-*glA*, a structural gene for an aryl β-glucoside splitting enzyme; β-*glB*, either the structural gene for the β-glucoside-permease or a regulatory gene; and β-*glC*, a regulatory gene (or site). *Escherichia coli* wild-type strains are of the genotype A$^+$ B$^-$ C$^+$. The β-*gl*$^+$ mutation determining the ability to ferment β-glucosides is considered to be a permease or regulatory mutation, and the resulting genotype is A$^+$ B$^+$ C$^+$. The β-*gl*$^+$ *sal*$^-$ phenotype results from a mutation in the β-*glA* gene (genotype A′ B$^+$ C$^+$), and the constitutive phenotype results from a mutation in the β-*glC* gene, the genotypes A$^+$ B$^+$ C_a and A′ B$^+$ C_a corresponding to the phenotypes β-*gl*$^+$*c* and β-*gl*$^+$ *sal*$^-$ *c*.

As described in the preceding paper (11), spontaneous β-glucoside-fermenting mutants (β-*gl*$^+$) can be isolated from *Escherichia coli* K-12 wild type and several of its auxotrophic derivatives. Further studies on such mutants demonstrated the presence of an inducible system for the accumulation and utilization of β-glucosides. This system consists of one or more aryl β-glucoside-splitting enzymes and a β-glucoside permease. It was found that the induction of the enzyme and permease occurs simultaneously. These data indicate the existence of a regulon (9) consisting of at least two genes. It was shown in a preliminary note (Schaefler and Maas, Federation Proc. **24**:417, 1965) that the mutation β-*gl*$^-$ → β-*gl*$^+$, determining the ability to ferment β-glucosides, maps close to the *pyrE* locus. The presence of several other markers in the same region permitted further genetic analysis by interrupted mating and by transduction.

The present paper deals with the isolation from β-*gl*$^+$ strains of mutants with altered fermentation pattern for salicin and the isolation of mutants constitutive for the β-glucoside-splitting enzyme(s) and the β-glucoside-permease. The mapping on the *E. coli* K-12 chromosome of several sites controlling β-glucoside fermentation is also discussed.

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MATERIALS AND METHODS

Media. The media A, AY, ABsal, and ABarb have been described previously (12). Medium A − N consists of medium A without $(NH_4)_2SO_4$. Complex media used were LB [20 g of tryptone (Difco), 10 g of yeast extract, 1 g of NaCl, 1 ml of 1 M NaOH, and 1,000 ml of water], LB + A (10 ml of 10 times concentrated A medium added to 100 ml of LB medium), Neopeptone (Difco) agar and broth, and nutrient agar (Difco) with one of the following sugars: lactose, maltose, xylose, or mannitol.

Strains. In addition to the strains described in the previous paper (11), the strain AB 1255 (*ile*$^-$, *met*$^-$, *thi*$^-$, *mal*$^-$, *xyl*$^-$, *F*$^-$), obtained from E. A. Adelberg, was used.

Designation of phenotypes. The phenotypes β-*gl*$^-$ and β-*gl*$^+$ were described in the preceding paper. Mutants with altered fermentation pattern which do not ferment salicin but retain the ability to ferment arbutin and other aryl β-glucosides are designated as β-*gl*$^+$ *sal*$^-$; strains constitutive for both the β-glucoside permease and the aryl β-glucoside-splitting enzyme(s) are designated as β-*gl*$^+$ *c*; constitutive strains lacking the capacity to ferment salicin are called β-*gl*$^+$ *sal*$^-$ *c*. The isolation of these mutants will be described under Results.

Designation of genotypes. The loci (sites) controlling the fermentation of β-glucosides are called β-*gl*; the individual loci are designated by capital letters, β-*glA*, β-*glB*, β-*glC*.

Interrupted mating experiments. Hfr and F$^-$ cultures growing exponentially in Neopeptone broth at 10^9 cells per milliliter were mixed as follows: 5 ml of Neopeptone broth, 4.5 ml of F$^-$ cells, and 0.5 ml of Hfr cells. The mating mixture was maintained at 37 C with gentle shaking; 0.5-ml samples were withdrawn at zero-time and at times specified, and were added to 0.5 ml of T6 phage preparation (1.5×10^{11} particles per milliliter); after 30 min of incubation with shaking at 37 C, dilutions ranging from 2×10^{-3} to 2×10^{-4} prepared in medium A − N were plated on selective media. After 36 hr, isolated colonies were picked, plated in patch form on the same selective media, and tested by replica-plating for unselected markers. Controls included the plating of the parental strains on selective media at the lowest dilution used and plating of the mating mixture with T6 phage onto medium complete for the Hfr strain to test for male survivors.

Transduction with P1kc phage. The transduction experiments (6) were performed by adding $CaCl_2$ (final concentration, 0.005 M) to the recipient culture in LB broth in the early logarithmic phase (3×10^8 cells per milliliter), followed by addition of 0.5 ml of culture to 0.5 ml of phage suspension (5×10^{10} to 3×10^{11} particles per milliliter). The mixture was incubated for 15 min at 37 C, centrifuged, and resuspended in medium A − N. Dilutions of 5×10^{-2} and 10^{-2} were plated on selective media.

Figure 1 shows the *E. coli* chromosome region analyzed by interrupted mating and transduction experiments (12).

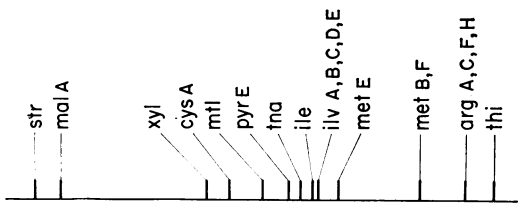

FIG. 1. *Chromosome region of Escherichia coli analyzed by interrupted mating and transduction with P1kc phage. Symbols: str = streptomycin; mal = maltose; xyl = xylose; cys = cysteine; mtl = mannitol; pyr = pyrimidine; tna = tryptophanase, ile = isoleucine, ilv = isoleucine-valine, met = methionone, arg = arginine, thi = thiamine.*

RESULTS

Mutant types. Treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 10) of spontaneous β-*gl*$^+$ mutants isolated from the K-12 wild-type strain and from some of its derivatives gave several types of secondary mutants. Two of these groups of mutants were selected for further studies. The first, β-*gl*$^+$ *sal*$^-$, is characterized by the fermentation of arbutin and lack of fermentation of salicin; the second, β-*gl*$^+$ *c*, is constitutive for both the aryl β-glucoside-splitting enzyme and β-glucoside permease.

β-*gl*$^+$ *sal*$^-$ *mutants.* In attempts to obtain β-*gl*$^-$ revertants, β-*gl*$^+$ cultures treated with NTG were streaked on ABsal and ABarb plates. Colonies appearing phenotypically β-*gl*$^-$ were routinely retested on both media. All the β-*gl*$^-$ revertants isolated on medium ABarb were β-*gl*$^-$ on both media, whereas, among those isolated on medium ABsal, some fermented arbutin, though to different degrees. Further investigations showed that, among the β-*gl*$^-$ revertants of different β-*gl*$^+$ strains isolated on ABsal medium, 8 to 12% were β-*gl*$^+$ *sal*$^-$. Such partial revertants were obtained from β-*gl*$^+$ mutants of strains K-12, AB1450, AT1243, AB2071, AT12, and AB673.

Several β-*gl*$^+$ *sal*$^-$ strains (mainly K-12 β-*gl*$^+$ *sal*$^-$/*6*, K-12 β-*gl*$^+$ *sal*$^-$ c_1, and AT12 β-*gl*$^+$ *sal*$^-$/*1*) were compared with their β-*gl*$^+$ parents in regard to the induction of the aryl β-glucoside-splitting enzyme, the splitting of aryl β-glucosides by cells and sonic extracts, and the uptake of C^{14}-thioethyl β-glucoside (TEG) and salicin.

It was found that alkyl and aryl β-glucosides (including salicin) induce in β-*gl*$^+$ *sal*$^-$ strains the biosynthesis of the aryl β-glucoside splitting enzyme and of the β-glucoside permease. The induced enzyme splits *p*-nitrophenyl β-glucoside and other aryl β-glucosides but not salicin or 1-naphthyl β-glucoside. Owing to the failure of β-*gl*$^+$ *sal*$^-$ cells to split salicin, this compound becomes a gratuitous inducer. Comparative data

on the splitting of *p*-nitrophenyl β-glucoside, *o*-nitrophenyl β-glucoside, phenyl β-glucoside, and salicin by strains K-12 β-*gl*$^+$/2 and K-12 β-*gl*$^+$ *sal*$^-$/6 are summarized in Table 1.

The data show that neither the β-*gl*$^+$ *sal*$^-$ cells nor their crude sonic extracts split salicin, but, in comparison with the β-*gl*$^+$ strain, no major change was found in the splitting of the other compounds. Experiments with other strains showed that the splitting of *p*-nitrophenyl β-glucoside by fully induced β-*gl*$^+$ *sal*$^-$ cells is 40 to 90% of that by the corresponding β-*gl*$^+$ cells. Various other parameters of the splitting of *p*-nitrophenyl β-glucoside were studied in β-*gl*$^+$ and β-*gl*$^+$ *sal*$^-$ strains, and were found to be similar (Table 2). These characteristics include: K_m value for *p*-nitrophenyl β-glucoside, competitive inhibition by salicin, temperature sensitivity, and loss of activity after sonic treatment, toluene treatment, and freezing and thawing.

Induced or constitutive β-*gl*$^+$ *sal*$^-$ cells take up C^{14}-TEG and tritium-labeled salicin through the β-glucoside permease (11). The active uptake of salicin by β-*gl*$^+$ *sal*$^-$ cells, and the lack of enzymatic splitting of salicin by induced cells and extracts, indicate that the mutation to β-*gl*$^+$ *sal*$^-$ blocks the enzymatic splitting of salicin. It appears that, except for a lowered specific activity and lack of splitting of salicin and 1-naphthyl β-glucoside, this genetic block has only minor effects on the splitting of other aryl β-glucosides.

Comparison of the splitting of p-nitrophenyl β-glucoside and salicin by β-gl$^+$ *strains.* Since the site of the β-*gl*$^+$ *sal*$^-$ mutation is apparently at the level of the structural gene of an aryl β-glucoside-splitting enzyme, a possible explanation for the nature of this mutation is that salicin and *p*-nitrophenyl β-glucoside are split by different enzymes, the mutation β-*gl*$^+$ → β-*gl*$^+$ *sal*$^-$ occurring at the level of a salicin-splitting enzyme.

In attempts to obtain further information on the possible existence of the two enzymes, the enzymatic splitting of salicin by β-*gl*$^+$ cells and sonic extracts was compared with that of *p*-nitrophenyl β-glucoside and other aryl β-glucosides. Data on splitting of salicin and *p*-nitrophenyl β-glucoside are summarized in Table 2.

It appears that the two activities differ in thermal lability. Whereas the enzymatic splitting of salicin is greatly diminished after incubation for 5 min at 48 C, most of the activity toward the splitting of *p*-nitrophenyl β-glucoside is preserved (when *o*-nitrophenyl β-glucoside and phenyl β-glucoside were used as substrates the results were similar to those found with *p*-nitrophenyl β-glucoside). It was found, however, that the two activities behave similarly with regard to other characteristics, such as inactivation by toluene and freezing and thawing, the particulate nature of the enzyme and cofactor requirements (Table 2 and Schaefler, *unpublished data*). It was also found that in β-*gl*$^+$ extracts *p*-nitrophenyl β-glucoside and salicin are reciprocal competitive inhibitors. Noninduced β-*gl*$^+$ cells and extracts do not split salicin. Induction experiments with the strains AT12 β-*gl*$^+$/5 and K-12 β-*gl*$^+$/2, with 10^{-4} M thiomethyl and thiophenyl β-glucoside as inducers, showed that the rate of enzyme biosynthesis as determined with salicin as substrate is similar to that described previously for the same strains (11) with *p*-nitrophenyl β-glucoside as substrate.

Constitutive mutants. Constitutive mutants (β-*gl*$^+$ *c* and β-*gl*$^+$ *sal*$^-$ *c*) of strains K-12 β-*gl*$^+$ and AB2071 β-*gl*$^+$ *sal*$^-$ were obtained as follows: after treatment with NTG and incubation for 8 hr in Neopeptone broth, the cultures were plated onto Neopeptone agar to yield approximately 500 colonies per plate. The colonies were replicated on plates with medium A with 0.1% yeast extract, 0.5% Na succinate, and the required growth factors. The replica plates were sprayed with an aqueous solution of 2×10^{-2} M *p*-nitrophenyl β-glucoside and 0.2% chloramphenicol (8). Constitutive colonies turned yellow after 4 to 10 min, and were reisolated from the master plates. Two constitutive mutants were obtained from each strain. From one of the K-12 β-*gl*$^+$ *c* mutants, β-*gl*$^+$ *sal*$^-$ *c* mutants have been obtained by a second treatment with NTG.

When grown in patch form (incubation, 12 to 16 hr at 37 C) on LB agar or medium A with 0.1% yeast extract and 0.5% succinate, cultures of these

TABLE 1. *Splitting of p-nitrophenyl β-glucoside, o-nitrophenyl β-glucoside, phenyl β-glucoside, and salicin*[a]

Substrate (2×10^{-3} M)	K-12 β-*gl*$^+$/2		K-12 β-*gl*$^+$ *sal*$^-$/6	
	Cells	Extracts	Cells	Extracts
p-Nitrophenyl β-glucoside	50	39.5	41	35
o-Nitrophenyl β-glucoside	63.5	50.2	38	35
Phenyl β-glucoside	37	36	35	29.5
Salicin	35	31	<0.5	<0.5

[a] Cells were grown overnight in medium A + 2% yeast extract and 0.5% salicin. Extracts were prepared by sonic treatment for 5 min. Cells, 0.5 mg of protein per ml; extracts, 5 mg of protein per ml. Activity is expressed in millimicromoles of aglycon liberated per minute.

TABLE 2. *Comparative data on β-gl⁺ and β-gl⁺ sal⁻ strains*

Determination	K-12 β-gl⁺ c_1		K-12 β-gl⁺ sal⁻ c_1
	Splitting of *p*-nitrophenyl β-glucoside	Splitting of salicin	Splitting of *p* nitrophenyl β-glucoside
Specific activity of crude sonic-treated extracts[a]	5.6	4.3	4.2
Specific activity of the 20 to 35% ammonium sulfate fraction of sonic-treated extracts[b]	21.6	16	17.1
Loss of activity after 5-min sonic treatment of cells	92%	92.3%	93.1%
Loss of activity after shaking cells for 30 min with toluene at 37 C	87.7%	91.3%	92.5%
Loss of activity after single freezing and thawing of cells	73%	78%	71%
K_m	6.9×10^{-4} M	1.34×10^{-4} M	8.4×10^{-5} M
Salicin as competitive inhibitor	2.96×10^{-4} M[c]	—	2.66×10^{-4} M[c]
p-Nitrophenyl β-glucoside as competitive inhibitor	—	1.06×10^{-4} M[c]	—
Temperature sensitivity at 48 C of enzyme in cells, 0.5 mg of protein/ml, zero-time = 100%			
5 min	82%	5%	78%
10 min	54%	<2%	51%
20 min	49%	<2%	43%
Temperature sensitivity at 48 C of enzyme in sonic-treated extracts, 5 mg of protein/ml, zero-time = 100%			
5 min	58%	4%	51%
10 min	38%	<2%	39%
20 min	24%	<2%	28%

[a] Sonic extracts were obtained from cell suspension of 100 to 130 mg of cells per ml, by sonic treatment at 2 C with a Raytheon 10-kc sonic oscillator for 5 min. Enzyme determinations were made in 0.075 M phosphate buffer (*p*H 7.5) with 10^{-3} M Mg^{++}, with extract samples containing 5 mg of protein per ml.

[b] The following additions are required for activity: 45 to 60% ammonium sulfate fraction from β-gl⁻ or β-gl⁺ extracts, 10^{-3} M Mg^{++}, and 0.1 ml of boiled (10 min) sonic extract (thermostable cofactor). The 20 to 35% and 45 to 60% ammonium sulfate fractions contain 2.5 mg of protein per ml each.

[c] K_i.

strains give a positive color reaction with *p*-nitrophenyl β-glucoside after 2 to 5 min. In liquid media, the highest rate of enzyme biosynthesis was observed in AY and LB + A media.

It was found that the strains K-12 β-gl⁺ c_1 and AB β-gl⁺ sal⁻ c_1 are constitutive for both the biosynthesis of the aryl β-glucoside-splitting enzyme(s) and the β-glucoside permease. When grown on LB + A medium, the specific activities of the constitutive strains, with *p*-nitrophenyl β-glucoside as substrate, were 58 to 67 units.

Data on the use of constitutive β-gl⁺ sal⁻ and glucose permease-negative strains for the determination of the uptake of labeled salicin and TEG through the β-glucoside permease were reported in the preceding paper.

Genetics. The results reported in this and the accompanying paper suggest the following loci connected with the fermentation of β-glucosides: two loci for the structural genes of permeases (one of them identical with the structural gene of the glucose permease), one or several loci for the structural gene(s) of the aryl β-glucoside-splitting enzyme(s), and a regulatory gene. Genetic analysis by interrupted mating and transduction with P1kc phage with the available types of mutants gave similar results, indicating a cluster of at least three mutational sites (*β-glA*, *β-glB*, and *β-glC*) located between the *pyrE* and *ile* loci.

Interrupted mating experiments. Crosses of the strains AB1450 β-gl⁻ × AB673 β-gl⁺ (origin between *thi* and *arg*, direction of entry *arg*, *met*, *ilv* . . .), with *arg* and *ilv* as selected markers (Fig. 2), showed that the site of the β-gl⁻ → β-gl⁺ mutation is between the *ilv* and *mtl* loci. Interrupted mating with two other pairs of F⁻ ×

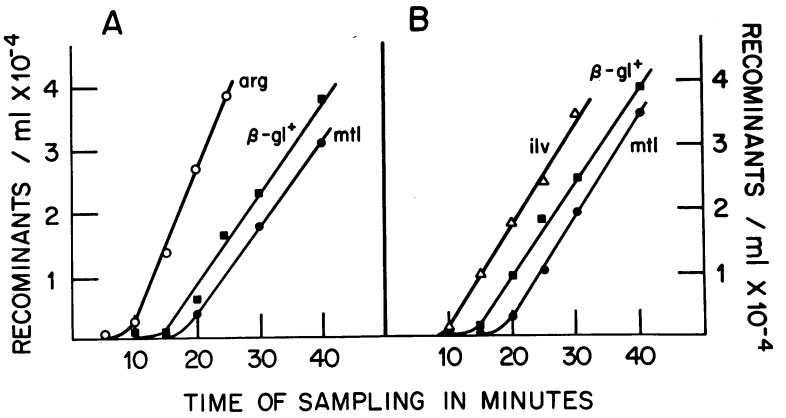

FIG. 2. *Interrupted mating AB1450 β-gl⁻ F⁻ × AB673 β-gl⁺ Hfr. (A) Selection for arg⁺. (B) Selection for ilv⁺. β-gl⁺ and mtl are unselected markers.*

Hfr strains indicated the same location of the *β-gl⁻ → β-gl⁺* mutation.

Cotransduction with pyrE as selected marker. The recipient strain was AT1243 *pyrE⁻* and the donor was K-12 *pyrE⁺*. The results of the cotransduction experiments are shown in Fig. 3.

Figure 3 shows that, in the *pyrE⁺ β-gl⁺ sal⁻ × pyrE⁻ β-gl⁻* cross, 1.62% of the *pyrE⁺* recombinants are of the donor type, but in addition there appear 0.3% salicin-fermenting recombinants with the phenotype of the spontaneous *β-gl⁺* mutants. In the reciprocal cross *pyrE⁺ β-gl⁻ × pyrE⁻ β-gl⁺ sal⁻*, the frequency of *pyrE⁺ β-gl⁺* recombinants increased to 2.3%. No cotransduction of β-glucoside fermentation and mannitol fermentation was observed (cotransduction of *pyrE* and *mtl* was 12.8%).

These results can be explained by assuming the existence of two adjacent loci called *β-glA* and *β-glB*, located between the *pyrE* and *ile* loci. It is presumed that the *β-glB* gene is the gene for the β-glucoside permease or a regulatory site, at whose level the *β-gl⁻ → β-gl⁺* mutation occurs. The *β-glA* gene is considered to be a gene for an aryl β-glucoside-splitting enzyme. The active allele of the *β-glA* gene (*β-glA⁺*) exists in *β-gl⁻* as well as in *β-gl⁺* strains, but is phenotypically expressed only in *β-gl⁺* cells possessing the β-glucoside permease. The *β-glA⁺ → β-glA′* mutation affecting the gene *β-glA* results in the phenotype *β-gl⁺ sal⁻*. *E. coli β-gl⁻* wild-type strains are of the genotype *β-glA⁺ β-glB⁻*, *β-gl⁺* strains are *β-glA⁺ β-glB⁺*, and *β-gl⁺ sal⁻* strains are of the genotype *β-glA′ β-glB⁺*.

Assuming the sequence of loci *pyrE β-glA β-glB*, the appearance of *pyrE⁺ β-gl⁺ sal⁻* recombinants in the cross *pyrE⁺ β-gl⁺ sal⁻ × pyrE⁻ β-gl⁻* requires a single crossover (Fig. 3a), whereas the appearance of *pyrE⁺ β-gl⁺* recombinants requires a double crossover (Fig. 3b). In the reciprocal cross, *pyrE⁺ β-gl⁻ × pyrE⁻ β-gl⁺ sal⁻*, the appearance of both *pyrE⁺ β-gl⁻* and *pyrE⁺ β-gl⁺* requires a single crossover (Fig. 3c, d). The observed frequency of *β-gl⁺* recombinants in both types of transduction is consistent with the above hypothesis.

Experiments with *β-gl⁺ sal⁻* donors derived from the strains AB673 and AT12 gave results similar to those with the K-12 *β-gl⁺ sal⁻* donor. The transduction K-12 *pyrE⁺ β-gl⁺ ×* AT1243 *pyrE⁻ β-gl⁻* gave 1.87% *pyrE⁺ β-gl⁺* recombinants and no *β-gl⁺ sal⁻* recombinants, presumably due to the presence of the *β-glA⁺* allele in both parental strains.

Cotransduction with ilvD and ilvD + metE as selected markers. Strains AB1450 *ilvD⁻* and AB-2071 *ilvD⁻ metE⁻* were used as recipients, and the strain K-12 *ilvD⁺ metE⁺* was used as donor. The results are shown in Fig. 4.

The much higher frequency of cotransduction with the *ilvD* locus than with the *pyrE* locus indicates that both the *β-glA* and *β-glB* loci are closer to the *ilvD* locus than to the *pyrE* locus. The observed ratio of *β-gl⁺ sal⁻/β-gl⁺* recombinants can be explained by single crossovers, including in one case both *β-glA* and *β-glB* loci (Fig. 4a) and in the other only the *β-glB* locus (Fig. 4b).

In the reciprocal cross, the appearance of *ilvD⁺ β-gl⁺* recombinants requires a double crossover. This is consistent with the observed frequency of such recombinants (Fig. 4d).

In the K-12 *ilvD⁺ metE⁺ β-gl⁺ sal⁻ × ilvD⁻ metE⁻ β-gl⁻* transduction, only *ilvD⁺ metE⁺ β-gl⁺* β-glucoside-fermenting recombinants were obtained. This may be attributed to the limits of size of the chromosome fragment that can be transduced (Fig. 4e).

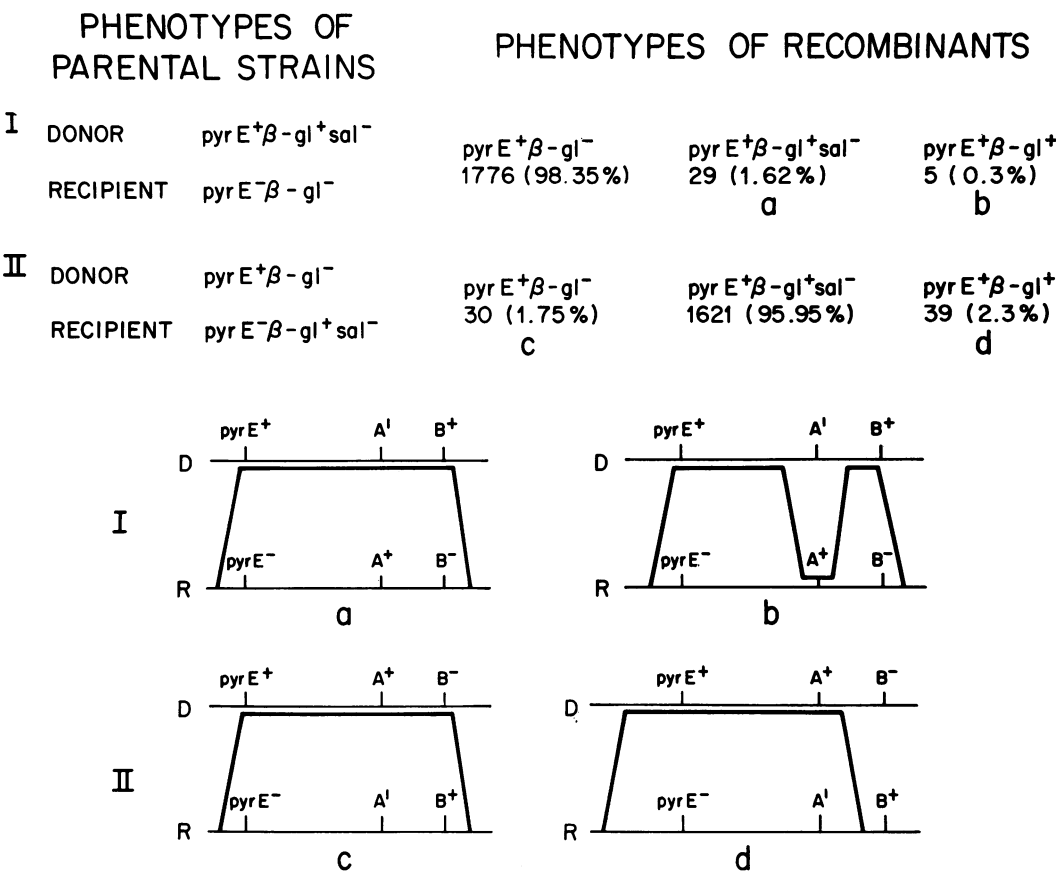

FIG. 3. *Cotransduction of β-gl loci with pyrE as selected marker. (I) K-12 pryE+ β-gl+ sal− × AT1243 pyrE− β-gl−. (II) K-12 pyrE+ β-gl− × AT1243 pyrE− β-gl+ sal−.*

A K-12 *ilvD+ β-gl+* × AB1450 *ilvD− β-gl−* cross gave 34.1% *β-gl+* cotransductants; no *β-gl+ sal−* cotransductants were obtained.

The cross K-12 *β-gl+ sal−* × AB1255 *β-gl−* with *ile* as selected marker gave 20.8% *ile+ β-gl+ sal−* recombinants and 14.1% *ile+ β-gl+* recombinants, a ratio similar to that obtained by selecting for *ilvD*. This result can be explained by the nearness of the *ile* and *ilv* loci to each other (Fig. 1).

Transduction with constitutive mutants. Attempts were made to map the pleiotropic genetic determinant for constitutive enzyme and permease biosynthesis. It was found that most of the β-glucoside-fermenting recombinants obtained with the strains K-12 *β-gl+ sal− c*1 and AB2071 *β-gl+ sal− c*1 as donors are constitutive. This indicates that a mutational site determining constitutivity (*β-glC*) lies close to the loci *β-glA* and *β-glB*. It was considered that the wild-type allele *β-glC+* is present in *β-gl−*, *β-gl+*, and *β-gl+ sal−* strains, and its mutant allele $\beta\text{-}glC_a$ is present in the analyzed constitutive strains. The ratio of constitutive and inducible recombinants, using *pyrE* and *ilvD* as selected markers, is given in Fig. 5. The above data can be explained by assuming that the *β-glC* locus maps between *β-glA* and *β-glB*. According to this hypothesis, in the cross K-12 *pyrE+ β-gl+ sal− c*1 × AT1243 *pyrE− β-gl−*, the appearance of the phenotype *pyrE+ β-gl+ sal− c* (Fig. 5a) requires a single crossover, whereas the appearance of the phenotypes *pyrE+ β-gl+ sal−* (Fig. 5b), *pyrE+ β-gl+ c* (Fig. 5c), and *pyrE+ β-gl+* (Fig. 5d) requires a double crossover. In the cross K-12 *ilvD+ β-gl+ sal− c*1 × AB1450 *ilvD− β-gl−*, the appearance of the phenotypes *ilvD+ β-gl+ sal− c* (Fig. 5e) and *ilvD+ β-gl+* (Fig. 5h) requires a single crossover, whereas the appearance of the phenotype *ilvD+ β-gl+ sal−* (Fig. 5f) requires a double crossover. Similar data have been obtained with the strain K-12 *β-gl+ sal− c*2 as donor.

		PHENOTYPES OF PARENTAL STRAINS	PHENOTYPES OF RECOMBINANTS		
I	DONOR	ilv D$^+$ β - gl$^+$sal$^-$	ilv D$^+$β-gl$^-$ 1545 (66.2%)	ilv D$^+$β-gl$^+$sal$^-$ 468 (20.2%) a	ilv D$^+$β-gl$^+$ 321 (13.6%) b
	RECIPIENT	ilv D$^-$β - gl$^-$			
II	DONOR	ilv D$^+$β - gl$^-$	ilv D$^+$β-gl$^-$ 521 (35.2%) c	ilv D$^+$β-gl$^+$sal$^-$ 963 (64%)	ilv$^+$β-gl$^+$ 12 (0.08%) d
	RECIPIENT	ilv D$^-$β - gl$^+$sal$^-$			
III	DONOR	ilv D$^+$ met E$^+$β-gl$^+$sal$^-$	ilv D$^+$met E$^+$β-gl$^-$ 1379 (99.5%)	ilv D$^+$met E$^+$β-gl$^+$sal$^-$ 0	ilv D$^+$met E$^+$β-gl$^+$ 7 (0.05%) e
	RECIPIENT	ilv D$^-$ met E$^-$β-gl$^-$			

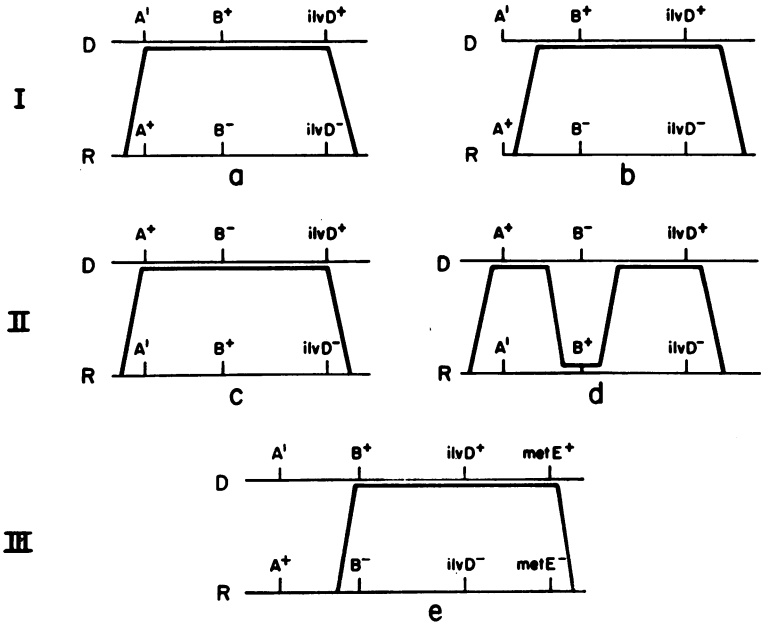

FIG. 4. *Cotransduction of β-gl loci with ilvD and metE as selected markers. (I) K-12 ilvD$^+$ β-gl$^+$ sal$^-$ × AB1450 ilvD$^-$ β-gl$^-$. (II) K-12 ilvD$^+$ β-gl$^-$ × AB1450 ilvD$^-$ β-gl$^+$ sal$^-$. (III) K-12 ilvD$^+$ metE$^+$ β-gl$^+$ sal$^-$ × AB2071 ilvD$^-$ metE$^-$ β-gl$^-$.*

A tentative map of the analyzed *E. coli* chromosome region has the following order of loci . . . *mtl, pyrE, (tna), β-glA, β-glC, β-glB, ile, ilvA, B, C, D, E, metE*. . . .

DISCUSSION

The genetic analysis of the *β-gl*$^-$ strains and their *β-gl*$^+$, *β-gl*$^+$ *sal*$^-$, and *β-gl*$^+$ *c* mutants reveals the existence of at least three mutational sites, possibly representing three distinct loci associated with the fermentation of aryl β-glucosides. We refer to these as: *β-glA*, *β-glB*, and *β-glC*.

The *β-glA* locus, at whose level the *β-gl*$^+$ → *β-gl*$^+$ *sal*$^-$ mutation occurs, appears to be the structural gene of an aryl β-glucoside-splitting enzyme whose *β-glA*$^+$ allele is present in *β-gl*$^+$ and *β-gl*$^-$ cells. This is indicated by the preservation of the inducible β-glucoside permease in *β-gl*$^+$ *sal*$^-$ mutants and the high frequency of *β-gl*$^+$ recombinants in *β-gl*$^+$ *sal*$^-$ × *β-gl*$^-$ crosses. It is, however, not clear whether the *β-glA* gene is the structural gene for an aryl β-glucoside-splitting enzyme with broad specificity or of a specific salicin-splitting enzyme, the enzyme(s) splitting

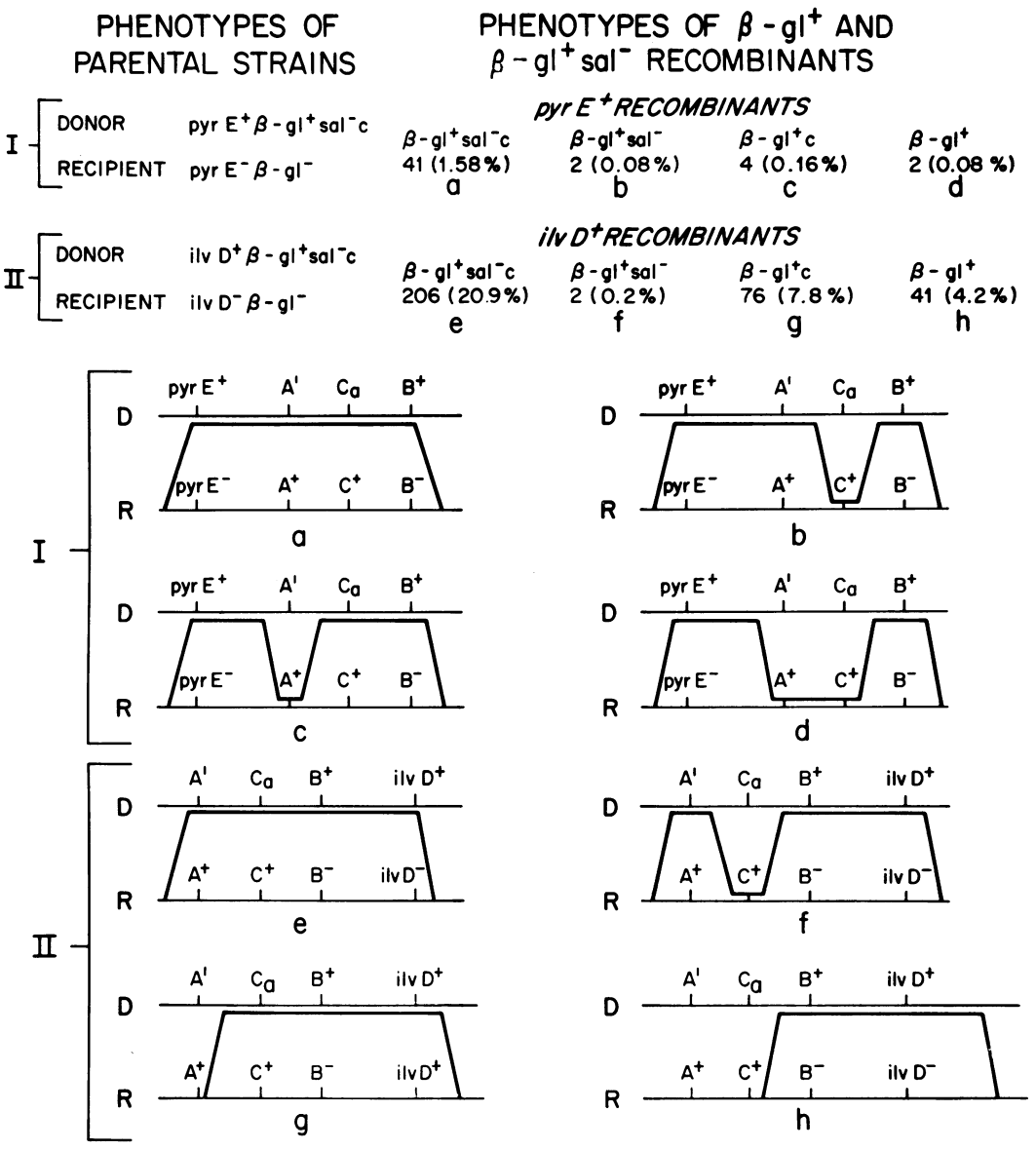

FIG. 5. *Cotransduction of constitutive splitting of β-glucosides with pyrE and ilvD as selected markers. (I) K-12 pyrE+ β-gl+ sal- c1 × AT1243 pyrE- β-gl-. (II) K-12 ilvD+ β-gl+ sal- c1 × AB1450 ilvD- β-gl-. β-Glucoside-fermenting recombinants were reisolated twice and grown in patch form on plates with medium A with 0.5% succinate, 0.1% yeast extract, and the required growth factors, and with medium LB; the plates were then sprayed with p-nitrophenyl β-glucoside.*

other aryl β-glucosides being determined by another gene(s) of still unknown location.

The multiple enzyme hypothesis presumes that all structual genes of the enzymes involved become phenotypically expressed as a result of the $\beta\text{-}gl^- \rightarrow \beta\text{-}gl^+$ mutation, that their inducers and substrates are taken up by the same permease, and that all enzymes together with the β-glucoside permease belong to the same regulon. Multiple β-glucosidases have been described in fungi, yeasts, and higher plants (1, 3, 5, 7; P. R. Mahadevan, Thesis, Princeton Univ., Princeton, N.J.). Indications for the existence of multiple enzymes in the *E. coli* system are the higher temperature sensitivity of the splitting of salicin and the very similar K_m value for the splitting of

p-nitrophenyl β-glucoside in *β-gl*$^+$ and *β-gl*$^+$ *sal*$^-$ strains. The lower specific activity in splitting *p*-nitrophenyl β-glucoside in most *β-gl*$^+$ *sal*$^-$ strains analyzed could result from interactions of a still unknown nature between the structural genes of the two aryl β-glucoside-splitting enzymes (2, 4). The isolation of mutants which split salicin but not other aryl β-glucosides would be an important argument in favor of the multiple enzyme hypothesis. So far the search for such mutants has been unsuccessful.

If there is a single enzyme with broad specificity which splits salicin as well as other aryl β-glucosides, then the *β-gl*$^+ \rightarrow$ *β-gl sal*$^-$ mutation probably determines a change in the enzyme at a site, or sites, different from the active site. Although there is no direct evidence for the existence of a single enzyme with a broad specificity, certain similarities are indicative of such a possibility, e.g., the same degree of inactivation of the splitting of *p*-nitrophenyl β-glucoside and salicin by sonic treatment and freezing and thawing, a parallel increase of activity in the 20 to 35% ammonium sulfate fraction, the same cofactor requirements, and competitive inhibition by salicin and *p*-nitrophenyl β-glucoside (Table 2 and S. Schaefler, *unpublished data*). Further purification of the enzyme is needed to clarify this situation.

The *β-glB* locus, at whose level the *β-gl*$^- \rightarrow$ *β-gl*$^+$ mutation occurs, is either the structural gene for the β-glucoside permease or a regulatory gene. The existing data do not permit a clear-cut differentiation between these two possibilities. If the *β-gl*$^+$ mutation is a regulatory mutation, it could involve the type characterized by the $i^s \rightarrow i^+$ change in the lactose system (13) or a change of the type $C^- \rightarrow C^+$, as described for the L-arabinose system (2). The pleiotropic *β-glC* locus could be either of regulator or of operator type. If the mutation in the *β-glB* site involves a change in regulation, it is possible that both the *B* and *C* sites are located in the same cistron. To disinguish between these theoretical alternatives, further genetic analysis is required, especially complementation tests for dominance and *cis-trans* relationships. Such studies are in progress.

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