Biochemical Genetics of the Cryptic Gene System for Cellobiose Utilization in *Escherichia coli* K12

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ABSTRACT

The cellobiose catabolic system of *Escherichia coli* K12 is being used to study the role of cryptic genes in microbial evolution. Wild-type *E. coli* K12 do not utilize the β-glucoside sugars, arbutin, salicin and cellobiose. A Cel$^+$ (cellobiose utilizing) mutant which grows on cellobiose, arbutin, and salicin was isolated previously from wild-type *E. coli* K12. Biochemical assays indicate that a *cel* structural gene (*celT*) specifies a single transport protein that is a β-glucoside specific enzyme of the phosphoenolpyruvate-dependent phosphotransferase system. The transport protein phosphorylates β-glucosides at the expense of phosphoenolpyruvate. A single phosphoglucosidase, specified by *celH*, hydrolyzes phosphorylated cellobiose, arbutin, and salicin. The genes of the *cel* system are expressed constitutively in the Cel$^+$ mutant, whereas they are not expressed at a detectable level in the wild-type strain. The transport and hydrolase genes are simultaneously silenced or simultaneously expressed and thus constitute an operon. Cel$^+$ strains which fail to utilize one or more β-glucosides express the transport system at a lower level than do Cel$^+$ strains which grow on all three β-glucosides. Other strains inducibly express a gene which specifies transport of arbutin but not the other β-glucosides. The arbutin transport gene, *arbT*, maps outside of the *cel* locus.

CRYPTIC genes are silent genes not normally expressed during the life cycle of an individual organism, but they may be reactivated by various genetic mechanisms. A systematic discussion of cryptic genes is presented in a review by HALL, YOKOYAMA and CALHOUN (1983). Since cryptic genes are not expressed, and thus do not contribute to fitness, it is expected that they would be permanently inactivated by accumulated mutations, and would thus be rare in populations (KOCH 1972; LI 1984). The observation that cryptic genes are commonly found in microorganisms (HALL, YOKOYAMA and CALHOUN 1983) indicates that there is selection for their retention in microbial populations.

It has been proposed that under one set of environmental conditions the expressed allele is more advantageous than the cryptic allele, and under another set of conditions the cryptic allele is favored (HALL, YOKOYAMA and CALHOUN 1983). Alternation of these environments is a sufficient and necessary condition for retention of both the cryptic and functional alleles in the population. Repeated cryptification and decryptification of genes may provide a means of long-term regulation of rarely utilized functions and may account for retention of cryptic genes in the population. We have been investigating a specific cryptic system, the cellobiose utilization (Cel) system of *Escherichia coli* K12 to study the role of cryptic genes in adaptive evolution.

Wild-type *E. coli* K12 does not utilize any β-glucoside sugars as sole carbon and energy sources (SCHAEFLER 1967; SCHAEFLER and MAAS 1967). Mutations in several loci can activate the cryptic *bgl* operon and allow growth on the aryl β-glucosides, arbutin and salicin (DEFEZ and DEFELICE 1981; DINARDO *et al.* 1982; REYNOLDS, FELTON and WRIGHT 1981; SCHAEFLER 1967; SCHAEFLER and SCHENKEIN 1968). Such Bgl$^+$ mutants do not use the dissaccharide, cellobiose, the most common natural β-glucoside (SCHAEFLER 1967). Mutations which activate the *bgl* operon allow inducible expression of two structural genes, *bglC* and *bglB* (PRASAD and SCHAEFLER 1974). The *bglC* gene codes for a transport protein which is a β-glucoside specific enzyme II of the phosphoenolpyruvate dependent phosphotransferase system (FOX and WILSON 1968). The transport protein both transports and phosphorylates β-glucosides. The *bglB* gene specifies a phosphoglucosidase which cleaves phosphorylated arbutin and salicin and a synthetic β-glucoside, *p*-nitrophenyl-β-D-glucoside (PNPG) (PRASAD and SCHAEFLER 1974). Another gene, *bglA*, which maps outside of the *bgl* operon, hydrolyzes phosphorylated arbutin and phosphorylated PNPG, but not phosphorylated salicin. Although the *bglA* gene is expressed constitutively in wild-type *E. coli*, they do not grow on arbutin because they do not transport the substrate (SCHAEFLER and MAAS 1967; PRASAD, YOUNG and SCHAEFLER 1973).

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We have identified a second cryptic system (designated Cel) for β-glucoside utilization in *E. coli* K12 (KRICKER and HALL 1984). The *cel* genes are not expressed in wild-type *E. coli* K12. Activation of the *cel* genes by spontaneous mutation permits utilization of arbutin, salicin, and cellobiose. Mutants that express the *cel* genes also constitutively hydrolyze the synthetic substrate, PNPG. The *cel* genes are located at 37.8 min on the *E. coli* map. We use the notation Cel^+ to refer to the activated, functional allele, and Cel^o to refer to the cryptic (wild-type) allele. The genes of the *bgl* operon are not involved in β-glucoside utilization in Cel^+ strains.

Spontaneous cellobiose negative mutants (designated Cel^-) are easily isolated from Cel^+ strains. Such mutants also become arbutin and salicin negative. From one β-glucoside negative mutant, a series of spontaneous revertants was isolated (KRICKER and HALL 1984). Selection on cellobiose MacConkey medium yielded revertants which grew only on cellobiose. From one such revertant, an arbutin utilizing mutant was selected in a second step, and a salicin utilizing revertant was selected in a third step. Revertants which grow on all three β-glucosides were also selected in only one mutational step on medium containing arbutin or salicin. Additionally, revertants which grow only on arbutin were isolated.

The object of this study was to analyze the genetic and biochemical alterations which gave rise to the variety of phenotypes observed in the series of Cel^+ and Cel^- strains isolated in the earlier study.

MATERIALS AND METHODS

Culture media and growth conditions: Unless otherwise specified, cultures were grown at 37° with aeration. Minimal media consist of mineral salts buffer (HALL and HARTL 1974) containing 0.2% (w/v) of the appropriate sugar as a carbon source except that arbutin and salicin were used at a concentration of 4 mM. When required, amino acids were added to a concentration of 100 μg/ml, purines were added to a concentration of 40 μg/ml, kanamycin was added to a concentration of 75 μg/ml. Solid media contained 1.5% agar.

MacConkey indicator plates contained 1% (w/v) of the indicated sugar and were prepared according to instructions provided by Difco. Colonies unable to ferment the added sugar are white on this medium, whereas fermenting colonies are pink or red.

Transductions: Transductions were performed essentially according to MILLER (1972). Transductions were mediated by bacteriophage P1 *cm,clr100* (ROSNER 1972). Bacteriophage P1 *cm,clr100* lysates and lysogens were prepared according to MILLER (1972).

Transformations: Preparation of competent cells and transformations with plasmid DNA were performed according to the method of MANIATIS, FRITSCH and SAMBROOK (1982).

Transport assays: The 50-ml cultures were grown in minimal medium containing carbon sources as specified in results. When plasmid-bearing strains were assayed, growth medium was supplemented with kanamycin. Cultures were grown to an A_{600} of 0.3–0.4, harvested by centrifugation, washed once with minimal salts buffer, and resuspended in 1.5 ml of 120 mM sodium-potassium phosphate buffer (pH 7.0) with 5 mM $MgCl_2$. The A_{600} of the concentrated cell suspensions were determined in a Gilford spectrophotometer. Cell suspensions (1.0 ml) were permeabilized by the method of IMAI and HALL (1981), and stored on ice until assayed. The cellobiose-dependent release of pyruvate from phosphoenolpyruvate (PEP) was assayed at 30° by continuous spectrophotometric measurement of NADH oxidation at 340 nm in a lactate dehydrogenase coupled reaction. Reaction mixtures (0.45 ml) consisted of 60 μmol of sodium-potassium phosphate buffer (pH 7.0), 2.5 μmol $MgCl_2$, 5 μmol PEP, 0.25 μmol NADH, nonlimiting amounts of lactate dehydrogenase and 10 μl of benzene treated cells. Mixtures were allowed to equilibrate for 5–6 min, and the reactions were initiated by addition of 50 μl of 40 mM β-glucoside. When transport activity was measured using a mixture of two sugars, reactions (0.4 ml) were initiated with 50 μl each of 50 mM sugars.

A control to correct for NADH oxidase activities contained all of the reaction components except that succinate was substituted for β-glucoside. The activity in the presence of succinate was subtracted from the β-glucoside dependent activity to calculate the corrected β-glucoside transport activity. Assays were performed in triplicate and the mean and 95% confidence interval for the three determinations were computed. One unit is equal to the oxidation of 1 nmol of NADH per minute, and corresponds to the transport and phosphorylation of 1 nmol of substrate per minute.

Kinetic analysis of transport enzymes: Cultures were grown in minimal medium with the appropriate carbon source at 30°. Growth conditions and permeabilization of cells were the same as for transport assays. Initial velocities were determined as above for transport assays except that the following concentration ranges of β-glucosides were used: arbutin, 0.02–5 mM; salicin, 0.1–2.5 mM; cellobiose, 0.01–2.5 mM. The apparent K_m and V_{max} were calculated by a computer program that weighs velocities according to the hypothesis that each velocity has a standard error proportional to its true value (CORNISH-BOWDEN, 1976).

***In vivo* phospho-β-glucoside hydrolase assays:** Phospho-β-glucoside hydrolase activities in intact cells were determined from end-point assays performed by modifications of the methods of SCHAEFLER (1967) and SCHAEFLER and MAAS (1967). Hydrolysis of salicin was measured by determination of the liberated aglycone. Cleavage of salicin was measured by the saligenin reaction at 509 nm (SCHAEFLER 1967; SCHAELFER and MAAS 1967). Activities are expressed as nmol of aglycone liberated per minute per A_{600}.

Extinction coefficients for the aglycone was determined from a standard curve of the absorbances of solutions of aglycone at the appropriate concentrations. Triplicate assays were performed for duplicate cultures and the activities for both cultures were pooled to calculate the mean and standard errors.

Plate assays for determination of constitutive PNPG hydrolase activity: PNPG hydrolase phenotypes were detected by plate assay (PRASAD and SCHAEFLER 1974). Colonies grown on glucose minimal medium were overlaid with 2.5 mM PNPG in minimal buffer. Constitutive colonies turn a bright yellow color in 10–15 min. Inducible and negative colonies remain white.

Hydrolase assays in cell-free extracts: Extracts were prepared from 10 ml of cells resuspended in 100 μl of the desired buffer in a microfuge tube. After addition of 1 μl of 10 mg/ml lysozyme, the cell suspension was incubated on ice for 10 minutes. The suspension was then alternately

frozen on powdered dry ice and thawed in ice water five times. The lysed cells were centrifuged to remove cell debris, and the supernatant was assayed for hydrolase activity. Large extracts were prepared by growing 1 L of cells on glucose minimal medium, concentrating the culture to 10 ml in appropriate buffer, and passing the cell suspension through a French pressure cell at 10,00 psi. Concentration of protein in the extracts was determined by the BRADFORD (1976) dye binding assay. Hydrolysis of arbutin phosphate and PNPG phosphate was determined by measuring the aglycone at 400 and 410 nm, respectively (SCHAEFLER 1967; SCHAEFLER and MAAS 1967).

Synthesis of phosphorylated β-glucosides: Phosphorylated derivatives of cellobiose, salicin and PNPG were prepared using permeabilized cells of a strain which is positive for transport of all three β-glucosides, but is hydrolase negative (MK120/pUF 572). A 1-L culture of MK120/pUF 572 was grown to an A_{600} of 1.0 in glucose minimal medium. The cells were washed twice in minimal salts buffer and resuspended in 15.5 ml of 100 mM KPO_4 buffer (pH 7.0) containing 5 mM $MgCl_2$. The resuspended cells were made permeable by the method of IMAI and HALL (1981). Cell suspensions were stored on ice until immediately before use. Suspensions were assayed for PEP dependent phosphotransferase activity on cellobiose. Suspensions which had 20–30 units/A_{600} were used to prepare phosphorylated β-glucosides.

Phosphorylated cellobiose and salicin were prepared in reaction mixtures (5 ml) containing 400 μmol KPO_4 (pH 7.0), 25 μmol $MgCl_2$, 100 μmol PEP and 125 μmol salicin or cellobiose. Reactions were initiated by adding 5 ml of permeabilized cells with a total PEP-phosphotransferase activity on cellobiose of 3.5 μmol/min. Reactions were incubated for 1½ hr at 30°. Reactions were terminated by boiling for 5 min and cell debris was removed by centrifugation.

Phosphorylated PNPG was prepared in a reaction mixture (5 ml) containing 350 μmol KPO_4 buffer, 25 μmol $MgCl_2$, 50 μmol PEP and 62.5 μmol PNPG.

Reactions were initiated by adding 5 ml of permeabilized cells. Permeabilized cells were prepared from cultures grown in glucose minimal medium to A_{600} of 0.6 and concentrated 50 fold in KPO_4 buffer. Reaction mixtures were incubated for 45 min at 30°. Reactions were treated as for preparation of phosphorylated cellobiose and salicin.

The concentration of phosphorylated β-glucosides in the final preparation was determined by assaying with a crude extract of *Klebsiella pneumoniae* strain PRL-R3 (PALMER and ANDERSON, 1971) containing phosphoglucosidase activity for all three substrates. In the absence of ATP these extracts can not phosphorylate β-glucosides, thus the concentration of phosphorylated β-glucosides can be determined from the stoichiometric production of glucose-6-phosphate or aglycone by the phosphoglucosidase reaction.

Preparation of extracts of *K. pneumoniae* strain PRL-R3: A 1-L culture of *K. pneumoniae* strain PRL-R3 was grown to late log phase at 37° in 0.2% cellobiose minimal medium as described in HALL (1979), except that NaCl was used at 20 g/liter. Cells were washed once with minimal salts buffer, harvested by centrifugation, ground in a prechilled mortar and pestle with two times the wet cell weight of alumina, resuspended in 10 ml of ice cold 20 mM Tris-HCl (pH 7.6), and centrifuged at 10,000 × *g* for 10 min at 4°. Nucleic acids were precipitated by adjusting the supernatant to 30 mg/ml streptomycin sulfate and the resulting precipitate was removed by centrifugation at 10,000 × *g* for 10 min at 4°. The supernatant was stored in the refrigerator at 4°.

Measurement of phosphorylated cellobiose or salicin: The assay was based on the spectrophotometric measurement at 30° of $NADP^+$ reduction in a phospho-β-glucosidase, glucose-6-phosphate dehydrogenase linked reaction (PALMER and ANDERSON, 1972a,b). Reaction mixtures (0.5 ml) contained 33.3 μmol glycylglycine buffer (pH 7.5), 3.33 μmol $MgCl_2$, 0.33 μmol of $NADP^+$, 5 μl of cellobiose phosphate or salicin phosphate solution, nonlimiting amounts of glucose-6-phosphate dehydrogenase and 20 μl of extract.

The reactions were allowed to proceed until the absorbance at 340 nm remained constant. The concentrations of the phosphorylated substrates were calculated from the total change in absorbance at 340 nm. As a control, the hydrolysis of the unphosphorylated substrate in the absence of ATP was measured by the same procedure.

The concentration of PNPG phosphate cannot be determined by this assay as the formation of *p*-nitrophenol interferes with the assay. The concentration of PNPG phosphate was determined using the phospho-β-glucosidase reaction of *K. pneumoniae* PRL-R3 extracts by the production of *p*-nitrophenol measured at 410 nm in an end point assay (SCHAEFLER 1967). Reactions (480 μl) contained 33.3 μmol glycylglycine (pH 7.5), 3.3 μmol $MgCl_2$, and twofold dilutions of the PNPG phosphate preparation. Reactions were initiated by addition of 20 μl of *K. pneumoniae* extract. Controls were reactions containing nonphosphorylated PNPG.

Determination of PEP in β-glucoside phosphate reactions: The concentration of PEP was measured by the spectrophotometric determination of NADH oxidation at 340 nm in a lactate dehydrogenase, pyruvate kinase linked reaction. Reactions (0.49 ml) contained 50 μmol KPO_4 buffer (pH 7.6), 1.5 μmol ADP, 5 μmol $MgCl_2$, 0.125 μmol NADH, and excess amounts of lactate dehydrogenase and pyruvate kinase. Reactions were initiated by adding 10 μl of an appropriate dilution of the β-glucoside phosphate solution. The reactions were allowed to proceed until the absorbance at 340 nm remained constant. PEP concentrations were calculated from the total change in absorbance at 340 nm. Controls were reactions containing buffer instead of β-glucoside phosphate solutions.

RESULTS

A series of revertants which utilize one or more β-glucosides are derivatives of one β-glucoside negative mutant, MK912. Strain MK912 and its revertants are described in a previous communication (KRICKER and HALL 1984). Genotypes and phenotypes of these strains are shown in Table 1. The pedigree of these strains is shown in Figure 1.

A new cryptic locus for arbutin utilization in *E. coli* K12: The Cel^+ strain, MK91, its β-glucoside negative mutant, MK912, and various revertants derived from MK912 were analyzed genetically to determine if the β-glucoside utilization genes in those strains mapped at the *cel* locus. In bacteriophage P1 transduction experiments, the *cel* locus in Cel^+ strains such as MK91 cotransduces with the *aroD* locus at a frequency of about 0.3 (KRICKER and HALL 1984).

Strain MK912 is a β-glucoside negative mutant of MK91. The location of the β-glucoside negative mutation in strain MK912 was determined by cotransduction with *aroD* (Table 2). When strain MK912 was

TABLE 1

***Escherichia coli* strains[a]**

Strains	Relevant genotype and phenotype	Source
GMS343	*F⁻aroD6 argE3 lacY1 galK2 man-4 mtl-1 rpsL700 tsx-29? supE44?* λ⁻	Coli Genetics Stock Center
JF201	*lacΔ(×74)bgl-phoΔ(201)ara gyrA thi*	HALL, BETTS and KRICKER (1986)
JF201R	*lacΔ(×74)bgl-phoΔ(201) ara gyrA thi recA srlC: :Tn10* *recA* transductant of JF201	HALL, BETTS and KRICKER (1986)
JF201T	*lacΔ(×74)bgl-phoΔ(201) ara gyrA thi tna: :Tn10* *tna: :Tn10* transductant of JF201	KRICKER and HALL (1984)
MK79	*F⁻ rpsL ara leu purE trp his argG metA or B thi malA xyl mtl gal lacZΔ(W4680)lacY bgl-phoΔ(201) celR° arbT°*	KRICKER and HALL (1984)
MK91	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR1⁺ᴴ arbT°* Arb⁺ Sal⁺ Cello⁺	KRICKER and HALL (1984)
MK120	*arg met thi his xyl mtl rpsL bglA⁻ bglB⁻ bglR⁺ bglSᶜ srlC: :Tn10* Arb⁻ Sal⁻	HALL, BETTS and KRICKER (1986)
MK912	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR2⁻ arbT°* Arb⁻ Sal⁻ Cello⁻ Spontaneous β-glucoside negative mutant of MK91 selected on cellobiose	KRICKER and HALL (1984)
MK912R	*recA* transductant of MK912	
MK3432	*F⁻ aroD6 argE3 lacY1 galK2 man-4 mtl-1 rpsL700 tsx-29? bgl-phoΔ(201) tna: :Tn10* *Δbgl* transductant of GMS343 (donor JF201T)	This study
MK3439	*F⁻ aroD6 argE3 lacY1 galK2 man-4 mtl-1 rpsL700 tsx-29? bgl-phoΔ(201) tna: :Tn10 celR⁺* Arb⁺ Sal⁺ Cello⁺ Spontaneous Cel⁺ mutant of MK3432	This study
MK9123	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR3⁺ᴸ arbT°* Arb⁻ Sal⁻ Cello⁺ Spontaneous revertant of MK912 selected on cellobiose	KRICKER and HALL (1984)
MK91236	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR5⁺ᴴ arbT°* Arb⁺ Sal⁺ Cello⁺ Spontaneous revertant of MK912 selected on arbutin	KRICKER and HALL (1984)
MK91243	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR2⁻ arbT1⁺* Arb⁺ Sal⁻ Cello⁻ Spontaneous revertant of MK912 selected on arbutin	KRICKER and HALL (1984)
MK912301	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR3⁺ᴸ arbT2⁺* Str Trp⁻ His⁻ Arg⁻ Met⁻ Ara⁻ Leu⁻ Lac⁻ Phoᶜ Arb⁺ Sal⁻ Cello⁺ Spontaneous revertant of MK9123 selected on arbutin	KRICKER and HALL (1984)
MK9123011	*F⁻ rpsL trp his argG metA or B ara leu lacY lacZΔ(W4680) bgl-phoΔ(201) celR8⁺ᴴ arbT2⁺* Arb⁺ Sal⁺ Cello⁺ Spontaneous revertant of MK912301 selected on salicin	KRICKER and HALL (1984)

[a] Abbreviations: Arb = arbutin, Sal = salicin, Cello = cellobiose. Genotypes:
Cryptic wild-type β-glucoside alleles: *bglR° celR° arbT°*
Active mutant β-glucoside alleles: *bglR⁺ celR⁺ arbT⁺*
celR allele expressed at a low level: *celR⁺ᴸ*
celR allele expressed at a high level: *celR⁺ᴴ*
Recryptified mutant *celR* allele: *celR⁻*

used as a donor to transduce an *aroD* Cel⁺ recipient strain, MK3439 to *aroD⁺*, the cotransduction frequency between *aroD* and the β-glucoside negative mutation was 0.32, indicating that the mutation pre-

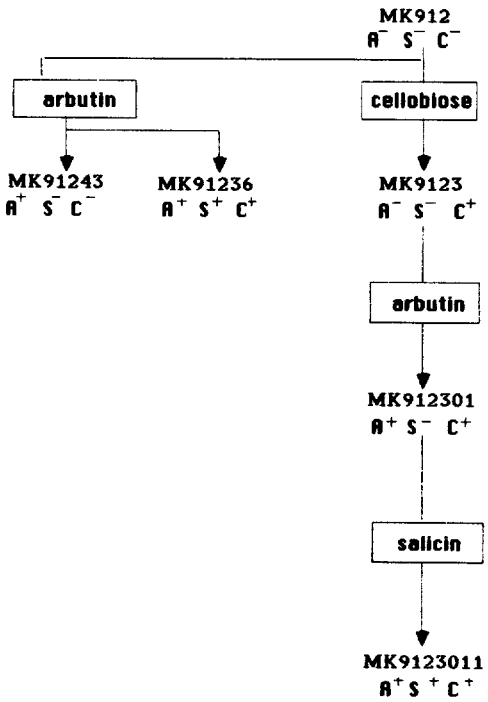

FIGURE 1.—Pedigree for spontaneous β-glucoside positive revertants. Sugars used for selection are enclosed in boxes. Phenotypes are shown below strain names. A = arbutin, S = salicin, C = cellobiose. In each case the strain shown is representative of a class containing multiple independent isolates.

venting utilization of β-glucosides in MK912 maps at the *cel* locus.

Strain MK91236 is a β-glucoside positive revertant of MK912 which grows on cellobiose, arbutin and salicin. When strain MK91236 was used as donor to transduce an *aroD*$^-$ Cel$^-$ recipient to Cel$^+$, the cotransduction frequency between the β-glucoside positive markers and the *aroD* locus was consistent with a mutation at the *cel* locus. No segregation of phenotypes for utilization of arbutin, salicin, or cellobiose was observed in any of these transductions. MK9123, a revertant of MK912, grows only on cellobiose and not on arbutin or salicin. The Cel$^+$ marker in this strain also maps at the *cel* locus.

Strain MK91243 is a revertant of MK912 which grows only on arbutin. The arbutin utilization locus in MK91243 did not cotransduce at all with *aroD*. Failure of the arbutin positive marker in MK91243 to cotransduce with *aroD* indicates that arbutin utilization in that revertant arose as a consequence of an independent mutation outside of the *cel* locus. Therefore there must be another cryptic gene for arbutin utilization, independent of the *cel* locus, which can be activated in *E. coli*. The new gene for arbutin utilization is designated *arbT*. We give evidence below that the *arbT* locus specifies a transport enzyme specific for arbutin.

MK912301, which grows on cellobiose and arbutin, but not on salicin, was derived from MK9123 by selection on arbutin. The cellobiose positive marker from MK912301 mapped at the *cel* locus (Table 2). None of the cellobiose positive transductants were arbutin positive, indicating that the arbutin positive marker of MK912301 also lies outside of the *cel* locus, presumably at *arbT*. The revertant, MK9123011 grows on cellobiose, arbutin, and salicin, and was derived from MK912301 by selection on salicin (Figure 1). All of the β-glucoside positive markers of MK9123011 map at the *cel* locus, indicating that 23011 has a second mutation at the *cel* locus, allowing

TABLE 2

Mapping of β-glucoside markers in mutants and revertants

Donor	Recipient	Recombinant classes (no. of recombinants)		Cotransduction frequency[a]
MK912 C⁻ A⁻ S⁻	MK3439 C⁺ A⁺ S⁺	C⁻ A⁻ S⁻ (40)[b]	C⁺ A⁺ S⁺ (85)	C⁻ A⁻ S⁻ 0.32
MK91 C⁺ A⁺ S⁺	MK3432 C⁻ A⁻ S⁻	C⁺ A⁺ S⁺ (16)	C⁻ A⁻ S⁻ (34)	C⁺ A⁺ S⁺ 0.32
MK91236 C⁺ A⁺ S⁺	MK3432 C⁻ A⁻ S⁻	C⁺ A⁺ S⁺ (38)	C⁻ A⁻ S⁻ (62)	C⁺ A⁺ S⁺ 0.38
MK91243 C⁻ A⁺ S⁻	MK3432 C⁻ A⁻ S⁻		C⁻ A⁻ S⁻ (100)	C⁻ A⁺ S⁻ 0.00
MK912301 C⁺ A⁺ S⁻	MK3432 C⁻ A⁻ S⁻	C⁺ A⁻ S⁻ (67)	C⁻ A⁻ S⁻ (133)	C⁺ A⁻ S⁻ 0.34
MK9123 C⁺ A⁻ S⁻	MK3432 C⁻ A⁻ S⁻	C⁺ A⁻ S⁻ (31)	C⁻ A⁻ S⁻ (69)	C⁺ A⁻ S⁻ 0.31
MK9123011 C⁺ A⁺ S⁺	MK3432 C⁻ A⁻ S⁻	C⁺ A⁺ S⁺ (12)	C⁻ A⁻ S⁻ (29)	C⁺ A⁺ S⁺ 0.29

Note: The selected marker in all transductions was Aro$^+$. Both recipients are genotypically *aroD*. C = cellobiose, A = arbutin and S = salicin.

[a] The cotransduction frequency between *aroD* and the donor locus.

[b] The number of transductants of each class is in parentheses. All classes of transductants obtained are shown in each case.

TABLE 3

β-Glucoside transport activities of wild-type and mutant *E. coli*

Strain	Grown on:	Transport activity[a] on: Cellobiose	Arbutin	Salicin
MK79	Glycerol + β-glucosides[b]	0.33 ± 1.30*	0.27 ± 0.65*	0.37 ± 0.87*
MK91	Glycerol	7.9 ± 2.0	2.8 ± 0.81	3.8 ± 1.6
	Cellobiose	12.6 ± 1.9	5.9 ± 0.81	6.5 ± 0.67
	Arbutin	12.0 ± 2.2	6.2 ± 1.5	7.9 ± 1.1
	Salicin	9.2 ± 0.47	3.8 ± 0.55	5.9 ± 0.81
MK912	Glycerol + β-glucosides[b]	0.91 ± 0.44*	0.75 ± 0.42*	0.34 ± 0.48*
MK9123	Glycerol	2.7 ± 0.70	0.68 ± 0.69*	0.26 ± 0.30*
MK912301	Glycerol	1.8 ± 0.17	1.0 ± 0.24	0.34 ± 0.52*
	Cellobiose	2.7 ± 0.72	0.71 ± 0.23*	0.34 ± 1.7*
	Arbutin	2.3 ± 0.23	5.9 ± 0.67	1.4 ± 0.89
MK9123011	Glycerol	5.3 ± 0.46	2.4 ± 0.35	2.6 ± 0.43*
MK91243	Glycerol	−0.002 ± 1.0*	3.7 ± 0.84	−0.15 ± 1.0*
	Arbutin	−0.33 ± 0.67*	15.6 ± 1.2	−0.63 ± 0.20*
MK91236	Glycerol	4.9 ± 1.1	2.1 ± 0.60	3.5 ± 0.59

* Activity was not significantly different from zero.
[a] Units/A_{600} ± 95% confidence interval.
[b] Cellobiose, arbutin and salicin were present at a concentration of 4 mM in glycerol minimal medium.

utilization of arbutin and salicin as well as cellobiose.

Transport of β-glucosides by the PEP-dependent phosphotransferase system: The *cel* genes specify three functions, utilization of arbutin, salicin and cellobiose. To determine the mechanism of transport of these sugars, Cel$^+$ strains and wild-type strains were assayed for transport activity on arbutin, salicin and cellobiose. The assay detects the activity of sugar-specific enzymes of the phosphoenolpyruvate dependent transport system (PTS). The assay monitors the conversion of PEP to pyruvate in the presence of sugars which are transported by PTSs. Transport activities are given in Table 3.

When a wild-type strain such as MK79 was assayed, the conversion of PEP to pyruvate was not stimulated by β-glucosides. When the Δbgl Cel$^+$ strain MK91 was assayed, the conversion of PEP to pyruvate was stimulated by cellobiose, arbutin, and salicin, indicating that these β-glucosides are transported and phosphorylated by the PTS system.

Synthesis of phosphorylated β-glucosides by the *cel* transport system was confirmed by detecting cellobiose phosphate, salicin phosphate, or PNPG phosphate in reaction mixtures (Table 4).

Nearly one mole of β-glucoside phosphate was formed for each mole of PEP consumed in the reaction, further indicating that the β-glucosides were phosphorylated at the expense of PEP.

Analysis of β-glucoside transport functions in mutants and revertants: The failure of the β-glucoside negative mutant, MK912, and its derivatives to utilize specific β-glucosides could result from inability to transport the sugars, failure to hydrolyze the sugars, or both functions could be inactive. Representative strains were therefore assayed for transport activity on arbutin, salicin, and cellobiose. The Δ*bgl*Cel$^+$ strain, MK91, transported all three β-glucosides at rates significantly higher than wild-type strains when the cultures were grown on glycerol. When strain MK91 was grown on cellobiose, arbutin, or salicin, transport activity was increased less than two fold, indicating that the *cel* transport system is expressed constitutively (Table 3).

The transport activities of strain MK912, the β-glucoside negative mutant of MK91, were not significantly different than the activities of wild-type strains on the three β-glucoside sugars. Transport activity could not be induced in MK912 by growing cultures in the presence of cellobiose, arbutin, or salicin. Thus the transport functions have been recryptified in the β-glucoside negative mutant.

Strain MK9123 is a cellobiose positive, salicin negative, arbutin negative, revertant of strain MK912. Strain MK9123 transported only cellobiose and did not transport arbutin or salicin at detectable rates (Table 3). Strain MK912301 is a cellobiose positive, arbutin positive derivative of MK9123. The cellobiose transport activity of strain MK912301 was similar to that of strain MK9123 and was expressed constitutively (Table 3). The arbutin transport activity of

TABLE 4

Product identification for synthesis of β-glucoside phosphates by the *cel* transport system

Reactants (μmoles)				Products (μmoles)	
Salicin	Cellobiose	PNPG	PEP	β-Glucoside phosphates	PEP consumed
125			100	91	98
	125		100	96	99
		62	50	27	30

TABLE 5

Apparent K_m and V_{max} for β-glucosides

Strain		Cellobiose	Arbutin	Salicin
MK91	K_m[a]:	108	549	652
	V_{max}[b]:	17	7.8	11
MK912301	K_m:	123	517[c]	
	V_{max}:	5.4	18	
MK9123	K_m:	84		
	V_{max}:	2.9		

[a] K_m: μM.
[b] V_{max}: units/A_{600}.
[c] Cultures induced by growth on arbutin.

TABLE 6

Transport activities using mixed β-glucosides

Strain	Substrate[a]	Activity[e]
MK91[b]	Glucose	11.1 ± 0.27
	Cellobiose	12.9 ± 1.0
	Arbutin	4.6 ± 0.72
	Salicin	8.4 ± 2.5
	Glucose + cellobiose	22.9 ± 1.8
	Arbutin + cellobiose	13.7 ± 1.1
	Salicin + cellobiose	13.6 ± 0.57
	Salicin + arbutin	6.4 ± 0.47
MK912301[c]	Cellobiose	2.2 ± 0.56
	Arbutin	3.1 ± 0.99
	Arbutin + cellobiose	6.6 ± 0.43
MK912301[d]	Cellobiose	4.2 ± 0.54
	Arbutin	16.9 ± 0.73
	Arbutin + cellobiose	22.9 ± 1.5

[a] Cellobiose, arbutin and salicin were at concentrations of 5 mM; glucose was at a concentration of 110 mM.
[b] A single culture was grown on glucose at 37°.
[c] A single culture was grown on glucose at 30°.
[d] A single culture was grown on arbutin at 30°.
[e] Activities are reported as units/A_{600} ± 95% confidence interval.

strain MK912301 was inducible by growing cultures on arbutin (Table 3). Cellobiose or salicin transport activity was not induced to any extent by growth on arbutin. Cellobiose did not induce arbutin transport activity. Strain MK91243, a revertant of strain MK912, grows only on arbutin and transports only arbutin. The arbutin transport activity of MK91243 was increased by about fivefold by growth on arbutin (Table 3). The new gene for arbutin utilization, *arbT*, which is active in both strains MK91243 and MK912301, thus specifies an inducible transport system.

Detection of two separate transport systems in MK912301: The arbutin positive phenotype in revertants MK912301 and MK91243 resulted from independent mutations, outside of the *cel* locus which activated an inducible transport system. Strain MK912301 grew on cellobiose in addition to arbutin. Strain MK912301 should contain separate transport systems, one at the *cel* locus and one at *arbT*.

Two PTS systems working simultaneously should convert PEP to pyruvate at a maximum rate equal to the sum of the maximum rates of either system working alone. Separate transport systems for cellobiose and arbutin should therefore yield a transport activity on a mixture of cellobiose and arbutin equal to the sum of the transport activities on cellobiose alone and on arbutin alone, provided that the substrate concentrations employed are in the V_{max} range for both systems. The kinetic constants for these transport systems are given in Table 5.

Excess concentrations of the β-glucosides (five to ten times the value of the apparent K_m) were used to determine transport activities. As shown in Table 6, the transport activities for MK912301 on cellobiose and arbutin are additive, indicating that strain MK912301 has separate transport genes for cellobiose and arbutin.

The *cel* genes specify a single transport protein for cellobiose, arbutin and salicin: To determine the number of β-glucoside specific transport systems specified by the *cel* locus, strain MK91 was assayed on mixtures of β-glucosides. The values for apparent K_m and V_{max} for MK91 are shown in Table 5. When cellobiose and arbutin were mixed, or cellobiose and salicin were mixed, the rates were non-additive and equalled the rate on cellobiose (Table 6). Similarly, when arbutin and salicin were mixed, the transport activities were non-additive.

As a control, glucose and cellobiose were mixed and the transport activity was determined. Glucose is expected to be transported by separate PEP-dependent sugar specific enzymes unrelated to β-glucoside specific transport proteins of the *cel* system. Excess concentrations of cellobiose and glucose were used in the assay. As shown in Table 6, activity toward a mixture of glucose and cellobiose was additive, demonstrating that the assay could identify separate transport systems where such exist. These data indicate that one structural *cel* gene specifies a single protein for transport of cellobiose, arbutin, and salicin.

Strain MK9123 transports cellobiose at a significantly lower rate than that of the Cel$^+$ strain MK91

or of revertants which grow on all three substrates such as strains MK91236 and MK9123011 (Table 3). For all strains, transport activity toward salicin and arbutin is two- to threefold less than transport activity toward cellobiose. Consistent with this, arbutin and salicin transport activity of MK9123 is also about twofold lower than its activity toward cellobiose. Thus, transport of arbutin and salicin in MK9123 occurs at rates not significantly different than in wild-type strains, and those rates are apparently too low to support growth on those substrates.

We suggest that the ability of strain MK9123 to transport cellobiose, but not arbutin and salicin, results from the decreased expression of one gene specifying a single transport system for cellobiose, arbutin, and salicin. When the gene is expressed at levels yielding cellobiose transport activities similar to those of MK91236 or MK91, the transport activity towards arbutin and salicin is high enough to allow growth on those substrates.

A single *cel* hydrolase gene is expressed constitutively: Cloning of the *cel* genes was described previously (HALL, BETTS and KRICKER 1986). The *cel* genes were located by insertional inactivation with the transposable element $\gamma\delta$. A number of inactivated plasmids were isolated which expressed transport functions, but had lost hydrolase functions. These plasmids contained insertions of $\gamma\delta$ throughout a 1.9-kb region. All of the plasmids fail to hydrolyze arbutin, salicin and cellobiose, indicating that the hydrolase functions for those substrates cannot be separated and that the 1.9-kb region specifies a single hydrolase enzyme (HALL, BETTS and KRICKER, 1986).

To determine if the hydrolase gene was expressed constitutively, the salicin hydrolase activity of strain MK91 was measured by an *in vivo* assay. When MK91 was grown on salicin, the salicin hydrolase activity was 1.5 ± 0.3 units/A_{600}. The salicin hydrolase activity for glycerol grown cells was 2.6 ± 0.6 units/A_{600}, indicating that the hydrolase gene is expressed constitutively.

Coordinated expression of transport and hydrolase genes: Mutants and revertants which failed to grow on one or more β-glucosides, did not transport those β-glucosides. To determine if the hydrolase functions were expressed independently of the transport functions plasmid pUF 572, which carries a functional *cel* transport gene, but is hydrolase negative (HALLS, BETTS and KRICKER 1986), was introduced into the β-glucoside negative strain MK912R and into several revertant strains that fail to transport one or more substrates. The plasmid-bearing hosts transported all three β-glucosides at rates comparable to those of the Cel$^+$ strain MK91. If chromosomally specified hydrolase functions are active in the hosts, the plasmid bearing strains should therefore grow on the β-glucoside sugars.

Fifteen transformants of each host were tested for β-glucoside positive phenotypes on arbutin, salicin and cellobiose MacConkey medium, and were screened for constitutive hydrolysis of PNPG by plate assay. All of the plasmid bearing strains grew on arbutin and hydrolyzed PNPG constitutively. These functions probably resulted from constitutive activity of the *bglA* gene product. Neither strains MK912R/pUF 572 nor MK91243/pUF 572 utilized cellobiose or salicin, indicating that the *cel* hydrolase gene is not expressed in strains that fail to express the chromosomal *cel* transport gene. Strains MK9123/pUF 572 and MK912301/pUF 572 grew on salicin, indicating that the host strains do synthesize salicin hydrolase activity.

Only MK9123 and MK912301 express the chromosomal *cel* transport functions, and only those hosts hydrolyzed salicin when high level transport activity was provided by the plasmid pUF 572. This indicates that expression of the *cel* hydrolase gene requires *cis* activation of the *cel* transport gene.

Low level expression of the Cel transport system does not cause sensitivity to β-glucosides: Cellobiose and salicin inhibit the growth rate of some Cel$^+$ strains on glycerol, while wild-type Cel$^-$ strains are not inhibited. Inhibition is thought to result from accumulation of phosphorylated β-glucosides by the transport system, and low hydrolase activity compared to transport activity may account for the sensitivity of Cel$^+$ mutants (HALL, BETTS and KRICKER 1986).

To determine if strain MK91, its β-glucoside negative derivative strain, MK912, and revertants of strain MK912 were sensitive to β-glucosides, cultures were grown in glycerol minimal medium, and an aliquot from each culture was spotted onto glycerol minimal plates and glycerol minimal plates containing either arbutin, salicin, or cellobiose as previously described (HALL, BETTS and KRICKER 1986). Strains MK91, MK91236, and MK9123011, which grew on all three β-glucosides, were severely inhibited by cellobiose, and strains MK91 and MK91236 were severely inhibited by salicin. None of the strains were sensitive to arbutin. Strains MK912 and MK91243 do not transport either cellobiose or salicin and were not inhibited by those substrates. Strains MK9123 and MK912301, which express the *cel* transport system at a lower level than do Cel$^+$ strains MK91 and MK91236, were not inhibited by any of the three β-glucosides. This suggests that mutations that allow low level expression of the *cel* transport system do not confer sensitivity to β-glucosides.

CONCLUSIONS

Our observations indicate that cellobiose, arbutin and salicin are transported and phosphorylated by a single β-glucoside specific enzyme of the PEP-dependent PTS in Cel$^+$ strains. The transport mechanism is

thus the same as that of the *bgl* specified transport system. We designate the gene specifying this transport enzyme as *celT*.

Transport studies indicate that there are three cryptic arbutin transport systems in *E. coli* K12: An inducible transport system for arbutin, salicin and PNPG, specified by *bglC*; a constitutive system for arbutin, salicin, PNPG and cellobiose, specified by *celT*, and an inducible transport system for arbutin encoded by *arbT* at an unknown location.

We suggest that *arbT* is a cryptic gene for arbutin transport which has been activated by mutation. It is unlikely that the arbutin transport function was acquired by alterations in an existing pathway for two reasons. Spontaneous *arbT* mutants were obtained in a single mutational step from β-glucoside negative mutants. It is unlikely that another pathway would not only acquire in a single mutational step the ability to regulate the transport system by arbutin, but would then also specifically transport arbutin and not the other β-glucosides. Additionally, arbutin-utilizing Salmonella mutants have been obtained in a second step from cellobiose positive strains (SCHAEFLER and MALAMY, 1969), paralleling our *E. coli arbT* revertant, MK912301. These observations suggest that the *arbT* gene of *E. coli* did not evolve from an existing transport gene, but is a homologue of the arbutin-utilization gene activated in Salmonella. We do not know if an arbutin hydrolase gene is associated with *arbT*, since all of our $arbT^+$ mutants also contain a wild-type *bglA* gene.

Insertional inactivation of the *cel* genes carried by the Cel^+ plasmid, pUF564, indicates that a single gene, *celH*, specifies a single hydrolase for arbutin, salicin, and cellobiose (HALL, BETTS and KRICKER, 1986). Our results suggest that mutations which inactivate expression of the transport gene also inactivate expression of the hydrolase gene. The chromosomal *cel* hydrolase gene is expressed only when the *cel* transport system is expressed in *cis* to the hydrolase gene. Simultaneous expression of the transport and hydrolase functions indicates that *celT* and *celH* comprise a single genetic unit, the *cel* operon.

Mutations in *celR* activate the *cel* operon: The cellobiose, arbutin and salicin transport and hydrolase functions were acquired in a single mutational step (KRICKER and HALL 1984). Analysis of a series of mutants showed that both transport and hydrolase functions can be lost by a single mutational step and can be regained at a frequency consistent with activation at a single locus (KRICKER and HALL 1984). These mutations thus define a locus, *celR*, where mutations occur which alternately activate or cryptify the *cel* structural genes. The *celR* site can be assigned four states: (1) $celR^o$ indicates the wild-type allele; (2) $celR^{+H}$ is assigned to strains which grow on cellobiose, arbutin and salicin and express the *cel* operon at a high level as measured by transport assays; (3) $celR^{+L}$ is assigned to strains which express the *cel* genes at a low level and therefore grow only on cellobiose; (4) $celR^-$ is reserved for those strains which have lost the Cel^+ phenotype and no longer express either *celH* or *celT*. Although there is no phenotypic distinction between $celR^o$ and $celR^-$, we distinguish them based upon their genetic histories in order to emphasize that we do not know if the allelic states are identical. We do not know if these four states are different states of a promoter for the *cel* structural genes. These designations are convenient descriptions of differences in expression of *cel* structural genes as indicated by growth characteristics and enzyme assays. To illustrate the different allelic states of *celR*, the relevant genotypes of various β-glucoside negative and positive strains are given in Table 7.

TABLE 7

Genotypes and phenotypes of representative strains[a]

Strain	Phenotype	Genotype
MK79	A^- S^- C^-	$celR^o$ $arbT^o$
MK91	A^+ S^+ C^+	$celR1^{+H}$ $arbT^o$
MK912	A^- S^- C^-	$celR2^-$ $arbT^o$
MK91243	A^+ S^- C^-	$celR2^-$ $arbT^+$
MK9123	A^- S^- C^+	$celR3^{+L}$ $arbT^o$
MK912301	A^+ S^- C^+	$celR3^{+L}$ $arbT^+$
MK9123011	A^+ S^+ C^+	$celR4^{+H}$ $arbT^+$
MK91236	A^+ S^+ C^+	$celR5^{+H}$ $arbT^o$

[a] A = arbutin, S = salicin, C = cellobiose; "+" indicates utilization of the sugar; "−" indicates no utilization of the sugar.

It can be argued that all of these states could be explained on the basis of a series of nonsense mutations and reversions in *celT*. Were this the case then $celR^{+L}$ and $celR^{+H}$ would actually be the results of different amino acid replacements in *celT*, and the transport proteins would be expected to differ not only in activities but in kinetic properties as well. Contrary to this expectation, the transport systems from $celR^{+L}$ and $celR^{+H}$ strains exhibit indistinguishable K_m values for cellobiose. We therefore conclude that these different alleles do, in fact, represent different regulatory states.

Alternating environments cryptify and decryptify the *cel* genes: The observation that Cel^+ strains, but not wild-type or Cel^- strains, are inhibited by β-glucosides when they are growing on glycerol suggested that in a mixed carbon resource environment Cel^+ strains are at a selective disadvantage compared to cryptic strains (HALL, BETTS and KRICKER 1986). From a Cel^+ strain which grew on arbutin, salicin and cellobiose, β-glucoside negative mutants were selected on rich medium containing cellobiose (KRICKER and HALL 1984), further indicating that Cel^+ strains are less fit than cryptic strains in a mixed resource environment which includes β-glucosides.

From a β-glucoside negative mutant we selected cellobiose positive revertants on the same rich medium that was used to select the β-glucoside negative mutant itself (KRICKER and HALL 1984). These revertants probably arose when carbon sources other than cellobiose were depleted from the medium. In environments where cellobiose is the primary carbon source Cel$^+$ strains would have a selective advantage. These *celR*$^{+L}$ revertants expressed the *cel* transport functions at a lower level than did the original Cel$^+$ ancestor and were no longer sensitive to inhibition by β-glucosides. They may therefore have had a selective advantage over mutants that expressed the *cel* genes at a high level (*celR*$^{+H}$) in that particular mixed resource environment.

When we selected cellobiose positive revertants on cellobiose minimal medium, the majority of them also grew on arbutin and salicin (KRICKER and HALL 1984) and were thus *celR*$^{+H}$. The observation that *celR*$^{+H}$ strains are selected on cellobiose minimal medium, but not on rich medium containing cellobiose, further suggests that *celR*$^{+L}$ strains are at a selective advantage in a mixed resource environment.

The observation that *celR*$^{+L}$ strains are not inhibited by β-glucosides indicates that the ability to utilize cellobiose does not automatically carry with it the penalty of being at a disadvantage in a mixed resource environment. These observations thus suggest a fitness hierarchy that changes in different environments. Our understanding of the physiology of the *cel* system, as a result of this study, will permit us to make and test specific predictions about the relative fitnesses of these alleles in specific single resource and mixed resource environments.

We have shown by isolation of β-glucoside negative mutants and Cel$^+$ revertants that these allelic states are interchangeable by mutation. We have also shown that the level of expression of the *cel* genes can be modulated by mutation, and that the class of mutations selected depends upon the composition of the selection medium, *i.e.*, the environment. Mutation could be an important adaptive regulatory mechanism, not only to activate or silence genes, but to modulate the level of expression. This type of regulation would be more suitable than would physiological mechanisms, such as induction by the substrate, when the substrates themselves are toxic to the cell, as has been shown for cellobiose when it is metabolized by the *cel* operon (HALL, BETTS and KRICKER 1986).

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