Functional Genes for Cellobiose Utilization in Natural Isolates of *Escherichia coli*

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Received 17 October 1986/Accepted 7 March 1987

The genes for utilization of cellobiose are normally cryptic in both laboratory strains and natural isolates of *Escherichia coli*. A survey of natural isolates of *E. coli* reveals that functional genes for cellobiose utilization, while rare, are present. The fraction of *E. coli* that utilized cellobiose ranged from <0.01% in human fecal samples to 7% in fecal samples obtained from horses. Samples obtained from sheep, cows, dogs, and pigs contained 0.1 to 0.5% cellobiose-positive *E. coli*. Neither the previously identified *cel* genes nor the *bgl* genes from *E. coli* K-12 were expressed during growth on cellobiose by any of the 14 naturally occurring *Cel*⁺ isolates that were tested. All of the naturally occurring *Cel*⁺ isolates possessed a *cel* operon, but all were deleted for the major portion of the *bgl* operon. The functional *cel*⁺ genes from these natural isolates differed from the mutationally activated *cel*⁺ genes obtained in earlier studies in that (i) the mutationally activated *cel*⁺ genes were temperature sensitive, while the functional genes were not, and (ii) transport of cellobiose was inducible in the strains carrying functional *cel*⁺ genes, while it was expressed constitutively in strains carrying mutationally activated genes.

The cellobiose utilization system of *Escherichia coli* is being used as a model system to investigate the evolutionary basis for the maintenance of cryptic genes in microbial populations. Cryptic genes are phenotypically silent DNA sequences that are not normally expressed during the lifetime of the organism but may be expressed after a mutation, the mobilization of an insertion element, or some other genetic event. The literature provides a variety of examples of activation of cryptic genes by mutations selected in the laboratory (4). Since cryptic genes make no positive contribution to the fitness of the organism, it might be expected that they would eventually be lost due to the accumulation of inactivating mutations. Cryptic genes would therefore be expected to be quite rare. This, however, is not the case. Selection for "novel" functions has revealed the presence of cryptic genes in gram-positive and gram-negative bacteria and even in *Saccharomyces cerevisiae* (10). In a model presented to account for the persistence of cryptic genes, three alternative states of a gene were considered: functional alleles, which are expressed under appropriate circumstances; cryptic alleles, which are not expressed but can revert to a functional state by mutation; and nonfunctional alleles, which are irreversibly inactivated (4). It was suggested that functional alleles are advantageous in one environment, while cryptic and nonfunctional alleles are advantageous in some other environment. Specifically, we suggested that in the normal environment functional alleles would be disadvantageous, whereas in an infrequently encountered alternative environment it would be advantageous to possess a functional allele. Two different analyses of the model indicated that, under these circumstances, fluctuation between these two environments would maintain all three alleles in a population (4, 7).

E. coli do not normally utilize the naturally occurring β -glucoside sugars arbutin, salicin, and cellobiose (1, 12-14).

β -Glucoside-utilizing mutants of *E. coli* K-12 may arise as a consequence of mutations in either the *bgl* operon, in which case the mutants utilize arbutin and salicin (13, 14), or in the *cel* operon, in which case they utilize arbutin, salicin, and cellobiose (2, 5, 6). The *cel* operon is located at 37.8 min on the map of *E. coli* K-12 (5) and encodes a transport protein and a hydrolase, both of which must be active for the organism to utilize β -glucosides (1, 6). The *cel* operon is not expressed in wild-type *E. coli* K-12 strains, but is expressed constitutively in decryptified *cel*⁺ mutants (5, 6). We have cloned the genes of the *cel* operon, and molecular studies of the structure and regulation of this operon are in progress.

A requirement of the model for retention of cryptic genes is that conditions must exist in the normal environment that select against the functional allele (4). For *E. coli* K-12 the presence of both cellobiose and an alternative carbon source such as glycerol in the growth medium strongly selected against *cel*⁺ but not against cryptic (wild-type) alleles (2). We have extended these studies to include natural isolates of *E. coli* (1). None of the 71 natural isolates in the ECOR (*E. coli* Reference) collection (9) was able to utilize any β -glucoside sugars, but 66 of the strains yielded spontaneous β -glucoside-positive mutants, and 45 of these strains yielded cellobiose-positive mutants. Since only five strains failed to yield β -glucoside-positive mutants, only 7% of this collection contained exclusively nonfunctional alleles. A subset of the mutant isolates was tested for synthesis of *cel* and *bgl* mRNA by probing with those specific DNA sequences. Although examples of both *cel*⁺ and *bgl*⁺ mutants of these natural isolates were obtained, several β -glucoside-positive mutants failed to express either of the two identified operons. It was concluded that the population of *E. coli* includes at least four different gene clusters for utilization of β -glucoside sugars and that all of these are cryptic in wild-type strains (1).

The model predicts that all three kinds of alleles, functional, cryptic, and nonfunctional, should be found in natural populations. We lack sufficient information about the mutation rates and fitnesses of these alleles in natural environments to predict the relative frequencies of these alleles;

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TABLE 1. Cel⁺ *E. coli* strains

Strain	Source	Reference
RM30	Camel	8
RM48B	Feral pig	8
WF211A3, WF247A	Horse	This study
GD24, GD31	Cow	This study
GD36, GD47	Dog	This study
GD50, GD55	Sheep	This study
GD84, GD88	Pig	This study
B75, B152, B289, B419	Baboon	12
MK2	Laboratory, <i>E. coli</i> K-12	5
NIM351	Laboratory, Cel ⁺ mutant of ECOR strain 35	2
NIM461	Laboratory, Cel ⁺ mutant of ECOR strain 46	2
NIM481, NIM482	Laboratory, Cel ⁺ mutants of ECOR strain 48	2
NIM701	Laboratory, Cel ⁺ mutant of ECOR strain 7	2

however, the failure to detect any functional cellobiose utilization genes among 71 natural isolates was somewhat surprising. There are a variety of possible explanations for the failure to detect any functional cellobiose utilization genes in the ECOR collection, including (i) functional alleles could be very rare and (ii) the frequency of functional alleles could depend strongly on the host from which the strains were isolated. Routman et al. (12) found that the number of sugars utilized by natural *E. coli* isolates varied strongly with the host from which the isolates were obtained. In particular, the β -glucoside sugar salicin was not utilized by any strains from the ECOR collection, but was utilized by 13% of the isolates from a human-associated tribe or yellow baboons and by 41% of the isolates from a tribe of non-human-associated baboons. While the ECOR collection includes samples from a variety of hosts, the majority (41 strains) were obtained from humans, and most of the animal hosts were in zoos, i.e., human-associated.

The goals of this study were to determine the frequencies of cellobiose-utilizing *E. coli* in fecal samples from a variety of hosts and to compare the properties of these functional cel⁺ genes with those of "decrypted" cel⁺ genes.

MATERIALS AND METHODS

***E. coli* strains.** The strains used in this study are listed in Table 1.

Culture media and conditions. Minimal medium was prepared from a phosphate-buffered mineral salts solution (3) plus a sugar as a carbon source. Plates contained 1.5% agar as a solidifying agent. Glucose and cellobiose were used at a 2 g/liter, and arbutin and salicin were used at 1.1 g/liter.

Molecular techniques. Methods for the preparation of RNA and genomic DNA, isolation and labeling of probe DNA, and both DNA-DNA and RNA-DNA hybridizations have been described (1). The cel-specific probe was made from the 4.2-kilobase (kb) *Hpa*I fragment of plasmid pUF564 (2), and the bgl-specific probes were made from either the 3.5-kb *Hind*III fragment or the 12.0-kb *Eco*RI fragment of plasmid pAR6 (11).

Growth rates. Cultures were grown overnight, with constant aeration, in minimal medium containing a limiting (0.1 g/liter) concentration of glucose. Exhaustion of the glucose limits growth to a density of about 10⁸ cells ml⁻¹. Either

cellobiose or glucose was added to the cultures, and the increase in turbidity was monitored with a Gilford Stasar II spectrophotometer at 600 nm. The growth rates were calculated from the slope of the least-squares fit of ln A₆₀₀ versus time in hours. In all cases the standard error of the growth rate was less than 10% of the reported value.

Sugar transport rates. The rates of glucose and cellobiose transport were determined by measuring the sugar-dependent release of pyruvate from phosphoenolpyruvate at 30°C by permeabilized cells by continuous spectrophotometric measurement of NADH oxidation in a lactate dehydrogenase-coupled reaction as described previously (6). Rates are reported as nanomoles of sugar transported per minute per 10⁹ cells.

Estimation of frequencies of Cel⁺ *E. coli* in fecal samples. Fresh fecal samples were suspended in a small volume of mineral salts buffer, serially diluted, and plated onto MacConkey lactose and cellobiose minimal medium. The total putative *E. coli* count was determined from the number of Lac⁺ colonies on the MacConkey plates. Fifty Lac⁺ colonies were picked to Simmon citrate agar and to EMB lactose agar. Lac⁺ colonies that were citrate negative and produced the typical green sheen characteristic of *E. coli* on EMB lactose agar were considered authentic *E. coli*, and the fraction of authentic *E. coli* was used to correct the total *E. coli* count. Similarly, the total number of cellobiose-positive organisms was determined from the number of colonies on cellobiose minimal medium. Fifty Cel⁺ colonies were tested on MacConkey lactose, EMB lactose, and Simmon citrate agar, and the proportion that were authentic *E. coli* was determined. A few (three to six) Cel⁺ *E. coli* from each host were isolated and tested more thoroughly with the Enterotube system (Roche Diagnostic Systems), which includes 12 biochemical tests for identification of members of the *Enterobacteriaceae*. In only one case was one of these isolates identified as other than *E. coli* (it was *Klebsiella ozaenae* isolated from a pig), and five other isolates from pigs proved to be authentic *E. coli*.

RESULTS

Isolation of Cel⁺ *E. coli*. The proportion of Cel⁺ *E. coli* in fecal samples obtained from humans and from a variety of animals on the University of Connecticut farm was determined. A few confirmed Cel⁺ *E. coli* (Tables 1 and 2) were stored at -70°C for subsequent analysis. Unpaired *t* tests showed that the proportion of Cel⁺ *E. coli* in samples from horses was significantly greater than from any other hosts tested. There were no significant differences among the cow, sheep, pig, and dog samples, but in all of these the proportion of Cel⁺ *E. coli* was significantly greater than in human-derived samples.

Cel⁺ *E. coli* from other collections. Fifty *E. coli* from the Routman et al. (12) collection, including 25 salicin-positive (Sal⁺) and 25 salicin-negative (Sal⁻) isolates, were tested for cellobiose utilization. Four strains were Cel⁺, and these

TABLE 2. Percentage of *E. coli* that were Cel⁺

Source	No. of samples tested	% Cel ⁺ (mean $\pm$ SE)
Human	16	<0.01
Sheep	10	0.16 $\pm$ 0.08
Dog	10	0.15 $\pm$ 0.10
Cow	11	0.51 $\pm$ 0.19
Pig	12	0.15 $\pm$ 0.08
Horse	14	7.0 $\pm$ 0.022

FIG. 1. Hybridization of DNA probes to dot-blots of RNA and DNA. Except as otherwise noted, each sample contained 10 µg of total RNA from cells grown on cellobiose (A and B) or 10 µg of DNA (C and D). The MK2 arbutin sample (A and B only) was taken from cells grown on arbutin, a condition that induces expression of the *bgl* genes. (A and C) Dot-blots were probed with the 4.2-kb *HpaI* fragment of *cel* genes of *E. coli* K-12. (B and D) Dot-blots were probed with the 3.5-kb *HindIII* fragment of the *bgl* operon of *E. coli* K-12. Each panel: (Left to right, top row: MK2, blank, GD24, GD36, GD50, RM30, B158, and WF211A3. Left to right, bottom row: MK2 (1 µg of RNA in panels A and B only), blank, GD31, GD47, GD55, GD88, RM48B, B289, and WF247A. MK2 arbutin is the extreme right in panels A and B.

included a Sal^+ and a Sal^- strain from human-associated baboons and a Sal^+ and a Sal^- strain from non-human-associated baboons (Table 1). The 46 Cel^- strains were streaked onto MacConkey cellobiose medium, and the plates were sealed and incubated for 4 weeks at 30°C. At the end of that time 29 of the strains had generated spontaneous cellobiose-positive mutants, detected as papillae on the surfaces of the colonies and confirmed by isolation and growth on cellobiose minimal medium. Thus, in that collection of *E. coli* strains from baboons, 8% of the isolates carried functional *Cel* alleles, 30% carried nonfunctional alleles, and 58% carried at least one cryptic allele. The frequency of cryptic *cel* genes was thus very similar to the frequency (45%) found in the ECOR collection (1). In addition, two Cel^+ *E. coli*, one from a camel and one from a feral pig, originally from the Roger Milkman collection and provided by D. Dykhuizen, were studied (Table 1). The six strains were confirmed as *E. coli* by the Enterotube test.

Characterization of Cel^+ *E. coli*. *E. coli* K-12 strain MK2 (5) and two Cel^+ isolates from each host were grown on cellobiose. RNA was extracted from each of these strains, dot-blotted, and probed with ^{32}P -labeled *cel* and the *HindIII* fragment of *bgl* DNA sequences (Fig. 1A and B). As a control for expression of *bgl*, RNA was also extracted from strain MK2 growing on arbutin. The *E. coli* K-12 strain MK2 expressed both the *cel* and *bgl* operons. None of the natural Cel^+ isolates expressed either *cel* or *bgl* mRNA. Genomic DNA from each of the strains was also dot-blotted and probed with the same sequences (Fig. 1C and D). The observation that *cel* DNA sequences were detected by hybridization to these probes indicates that the failure to detect mRNA was not the consequence of extreme genetic divergence between the genes of the natural isolates and those of *E. coli* K-12. The failure of the *HindIII* fragment of *bgl* DNA to hybridize to DNA from any of the natural Cel^+ isolates was surprising. To explore the reason for the failure to detect *bgl* DNA with the *bgl* *HindIII* fragment probe, DNA from several strains was digested with restriction endonuclease *ClaI*, subjected to agarose gel electrophoresis,

and transferred to GeneScreen-Plus nylon membranes. Again, the *cel* probe hybridized to *ClaI* fragments from all strains, but the *bgl* *HindIII* fragment probe hybridized only to fragments from the *E. coli* K-12 strain (Fig. 2A and B). The blot shown in Fig. 2B was reprobed with the 12-kb *EcoRI* fragment from plasmid pAR6 (Fig. 2C). That fragment includes the entire *bgl* operon plus the *phoT*, *phoS*, and *glmS* genes that are adjacent to the *bgl* operon (11). Hybridization of this larger probe to DNA from all of the strains confirmed that the failure to detect *bgl* mRNA was not due to low sequence homology with the probe. Instead, it appeared that the region corresponding to the *bglB* and *bglS* genes has been deleted in all of the Cel^+ natural isolates. The sum of the fragment sizes that hybridized to the 12-kb probe was 6.4 kb for strain RM30 and 5.8 kb for strain B289. The size of the deletion in these two strains was therefore at least 5.6 kb. These data indicate that all of the natural isolates are utilizing genes other than *cel* or *bgl* for growth on cellobiose.

Comparison of natural and decryptified cel^+ genes. Because cryptic genes are not contributing to the fitness of the organism, random mutations that occur in cryptic genes are expected to be neutral. Some of those mutations might result in the production of temperature-sensitive gene products when the genes are activated by mutation. In contrast, if functional genes are contributing to the fitness of the organism and are under selection for normal functioning in the mammalian gut, then functional alleles would not be expected to specify temperature-sensitive gene products. The growth rates of six decryptified Cel^+ strains were compared with those of eight functional Cel^+ isolates from nature. To facilitate comparisons among these unrelated strains, the growth rates on cellobiose were normalized to the growth rates on glucose. This normalization is appropriate because cellobiose is phosphorylated during transport (6) and cleaved to yield glucose plus glucose 6-phosphate. The mutationally activated Cel^+ strains, on average, grew only 61% as fast at 37°C as they did at 30°C (Table 3), whereas the naturally functional Cel^+ strains, on average, grew equally as fast at both temperatures. The effect was more dramatic at 42°C

FIG. 2. Hybridization of DNA probes to *Clal* DNA restriction fragments. Each sample contained 5 μ g of total DNA. The filter in panel A was probed with the 4.2-kb *HpaI* fragment of *cel* genes of *E. coli* K-12, that in panel B was probed with the 3.5-kb *HindIII* fragment of the *bgl* operon of *E. coli* K-12, and that in panel C was probed with a 12-kb fragment that includes the *bgl* operon and adjacent genes. Each autoradiogram was cut off 3.5 cm below the position of the wells. Lanes (left to right in each panel): MK2, B289, B152, GD88, GD84, GD31, GD24, WF247A, WF211A3, and RM30.

(the mutationally activated strains failed to grow on cellobiose) while the naturally functional strains grew at about the same rates as they did at 30°C. The mutationally activated *cel*⁺ genes are clearly temperature sensitive, whereas the naturally occurring functional genes are not.

Regulation of *cel*⁺ genes. The decryptified *cel* genes of *E. coli* K-12 are expressed constitutively, as measured by both mRNA hybridizations (1) and cellobiose transport activity (6). Since none of the natural *Cel*⁺ isolates synthesized mRNA that was homologous to either the *cel* or the *bgl* genes of *E. coli* K-12, regulation of gene expression was determined from measurements of cellobiose transport rates. The six natural *Cel*⁺ alleles were all inducible (Table 4). In contrast, the two mutationally activated alleles, from NIM461 and NIM701, were semiconstitutive. Neither of these strains produces either *cel*- or *bgl*-specific mRNA when grown on cellobiose (1).

DISCUSSION

Functional genes for cellobiose utilization were rare in the populations of *E. coli* that we examined. The frequency of *Cel*⁺ isolates ranged from 7% in horses down to <0.01% in humans. The failure to find any *Cel*⁺ isolates in the ECOR collection is explained both by the overall rarity of these genes and by the predominance of human-derived isolates in the ECOR collection.

The observation that none of the natural or wild-type *Cel*⁺ genes corresponded to either the *cel* or *bgl* genes of *E. coli* K-12 is consistent with our earlier suggestion (1) that *E. coli* populations include at least four gene systems for the metabolism of β -glucoside sugars.

The observation that all of the *Cel*⁺ isolates contained a deletion of the *bgl* operon is quite curious. The diversity in restriction patterns among the 11 strains (Fig. 2) shows that

TABLE 3. Growth rates of naturally functional and mutationally activated *Cel*⁺ strains

Strain	Growth rate (h ⁻¹)						Normalized rate on cellobiose ^a		
	Glucose			Cellobiose					
	30°C	37°C	42°C	30°C	37°C	42°C	30°C	37°C	42°C
Mutationally activated									
NIM351	0.41	0.64	0.62	0.17	<0.008	<0.008	0.41	<0.008	<0.008
NIM461	0.33	0.42	0.38	0.26	0.24	<0.008	0.80	0.58	<0.008
NIM481	0.59	0.69	0.52	0.13	0.12	<0.008	0.22	0.18	<0.008
NIM482	0.47	0.66	0.54	0.20	0.21	<0.008	0.43	0.31	<0.008
NIM701	0.66	0.84	0.76	0.44	0.35	<0.008	0.66	0.42	<0.008
MK2	0.43	0.53	0.51	0.21	0.21	<0.008	0.48	0.39	<0.008
Naturally functional									
B75	0.54	0.79	0.73	0.50	0.68	0.71	0.92	0.86	0.98
B158	0.57	0.75	0.62	0.53	0.61	0.58	0.93	0.82	0.94
B289	0.56	0.80	0.75	0.52	0.67	0.64	0.92	0.84	0.85
B419	0.37	0.45	0.39	0.50	0.65	0.57	1.38	1.43	1.48
RM30	0.60	0.6	0.35	0.55	0.70	0.63	0.92	1.17	1.81
RM48B	0.52	0.66	1.14	0.48	0.64	0.90	0.92	0.96	0.79
WF211A3	0.62	0.91	0.92	0.60	0.80	0.83	0.97	0.88	0.90
WF247A	0.61	0.85	0.75	0.53	0.69	0.57	0.86	0.81	0.76

^a Normalized rates are growth rates on cellobiose divided by the growth rates on glucose at the same temperature.

TABLE 4. Sugar transport by natural and mutationally activated *Cel⁺ E. coli*

Strain	Transport rate ^a (nmol/min per 10 ⁹ cells)			
	Glucose ^b		Cellobiose ^b	
	Glucose ^c	Cellobiose ^c	Glucose ^c	Cellobiose ^c
B158	3.5	<0.01	8.6	4.5
B289	11.7	<0.01	5.3	4.9
WF211A3	7.7	<0.01	12.0	11.0
WF247A	5.9	<0.01	12.5	4.3
RM30	5.4	<0.1	8.1	4.0
RM48B	7.8	<0.1	5.1	6.9
NIM461	5.5	0.34	2.1	1.3
NIM701	3.5	0.47	3.4	1.5

^a Means of triplicate assays. In no case was the range more than 15% of the mean value.

^b Carbon sources.

^c Substrates (4 mM).

we are not dealing with repeated isolations of the same strain. We do not yet know whether all of these strains contain the same deletion, which would indicate that they are all descended from the same common ancestor, or whether deletion of *bgl* has occurred repeatedly. Given the diverse origins of these strains, the strong correlation between the deletion of *bgl* and the *Cel⁺* phenotype suggests an interaction between expression of the genes for cellobiose utilization and the presence of the *bgl* operon so that the two cannot coexist for long. More extensive characterization of the *bgl* deletion and a survey for its presence in several collections of natural *E. coli* isolates is in progress.

We originally distinguished three states of alleles: functional, cryptic, and nonfunctional. It now seems reasonable to make a distinction between two categories of functional alleles: those that were functional when the organism was isolated from nature, which we shall call naturally functional, and those that were cryptic when the organism was isolated and were activated by a mutation in the laboratory, which we shall call mutationally activated alleles. This distinction appears to reflect more than just history. Naturally functional alleles seem to be inducible, whereas mutationally activated alleles are constitutive or semi-constitutive. Mutationally activated alleles are temperature sensitive, while naturally functional alleles are not. The temperature sensitivity of mutationally activated alleles is consistent with the notion that silent genes accumulate mutations freely.

These observations are consistent with our model (4, 7) for the retention of cryptic genes. Naturally functional cellobiose utilization alleles are rare, but there is an environment, perhaps the lower intestine of horses, that appears to favor them. We originally envisioned occasional environmental changes that would create temporary conditions favoring functional alleles. It now seems likely that such changes can also be accounted for by migration between different environments (different hosts) which select differently for or against functional *cel* alleles. At the one extreme for *E. coli*, horses appear to favor *cel⁺* alleles, and humans appear to select strongly against them. It is not clear why horses, cows, and sheep, all of which derive energy from cellulose degradation, should differ so much in the frequency of *Cel⁺ E. coli* in their feces.

Together with earlier studies (1, 2), this study provides a picture of an *E. coli* population in which there are several

gene systems for utilization of cellobiose and other β -glucoside sugars. In any one strain, perhaps half of those genes will have been inactivated (rendered nonfunctional), while the others are highly likely to be silent and accumulating mutations that are leading toward extinction of the gene (1). The occasional selection of *Cel⁺* clones as the result of either environmental fluctuation or migration into a favorable environment is expected to generate population bottlenecks and result in periodic selection that repeatedly eliminates much neutral variation. This is consistent with the picture of the structure of *E. coli* populations that has been generated by studies on electrophoretic variation (15).

ACKNOWLEDGMENTS

We gratefully acknowledge the participation of Glenn Doughty in the collection and identification of many of the natural isolates of *E. coli* that are described in this study. We are grateful to Paul W. Betts for his expert technical assistance. We thank Dan Dykhuizen for the gift of several strains.

This work was supported by Public Health Service grants AI-14766 and GM-37110 from the National Institutes of Health.

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