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RESEARCH NOTE

Mutational analysis of *b*-glucoside utilization in *Klebsiella aerogenes*: evidence for the presence of multiple genetic systems

TIRUMALAI R. RAGHUNAND[1,2] and S. MAHADEVAN[1]*

[1]*Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560 012, India*
[2]*Present address: Center for TB Research, Johns Hopkins University School of Medicine, 1503 E Jefferson Street, Baltimore, MD 21231-1001, USA*

Introduction

Escherichia coli possesses multiple systems for uptake and utilization of the *b*-glucoside sugars salicin, arbutin and cellobiose. However, these genes are not expressed in the wild-type organism. Activating mutations at three different loci, the *bgl* operon, the *cel* operon, and the *asc* system, enable *E. coli* to utilize these sugars (see Mukerji and Mahadevan 1997 for a review). Of the three, the *bgl* operon, involved in uptake and degradation of the aromatic *b*-glucosides arbutin and salicin, is best characterized (Prasad and Schaefler 1974; Reynolds *et al.* 1981; Mahadevan *et al.* 1987; Schnetz and Rak 1987).

The *cel* operon, located at 37.8 min on the genetic map, upon mutation, allows utilization of cellobiose (Kricker and Hall 1984; Parker and Hall 1990a,b). The operon can be activated by insertion of IS*1*, IS*2* or IS*5* into a region 72–180 bp upstream from the transcription start site, by an unknown mechanism that does not involve provision of a promoter sequence. It can also be activated by base substitutions in the gene for the putative repressor *celD* that alter the repressor so that it can recognize cellobiose, arbutin and salicin as inducers. The induction in phospho-*b*-glucosidase activity in response to cellobiose ranges from 70-fold to 100-fold depending on the activating allele. Recent studies have shown that the *cel* genes allow utilization of the disaccharide chitobiose in the absence of any mutations (Kehyani and Roseman 1997; Plumbridge and Pellagrini 2004), and the operon has been renamed the *chbBCARFG* operon. The *chbBCA* genes code for the IIB, IIC and IIA domains of the PTS permease respectively, *chbR* encodes a repressor/activator, and *chbF* a phospho-chitobiase. On the basis of comparison of the nucleotide sequences of the *chb* genes in the wild type and Cel^+ strains, it has been suggested that the Cel^+ conversion is the result of multiple mutational events in the structural and regulatory genes (Kehyani and Roseman 1997).

The *asc* operon (Parker and Hall 1988) specifies a transport system and a hydrolase that acts on arbutin and salicin, and to a lesser extent on cellobiose. It encodes three genes *ascG*, *ascF* and *ascB*. AscG has been identified as a repressor, based on its similarity to the GalR repressor and the presence of a DNA-binding helix–turn–helix motif. Activation of the operon is mediated by disruption of *ascG* by IS*186*, which leads to semiconstitutive expression of the operon. AscF resembles the PTS enzyme II for *b*-glucosides, such as BglF. AscB is the phospho-*b*-glucosidase, which can cleave salicin, cellobiose and arbutin and shows 70.5% sequence similarity to BglB (Hall and Xu 1992). Three sequential mutations are required before the *asc* operon allows utilization of the three sugars. The wild-type strain can mutate first to Arb^+, followed by Sal^+ and then Cel^+ (Parker and Hall 1988).

Erwinia chrysanthemi is the only other member of the enteric bacteria for which more than one *b*-glucoside utilization system has been identified. The *clb* genes responsible for cellobiose metabolism in *E. chrysanthemi* were found to confer on *E. coli* the ability to utilize cellobiose as well as salicin and arbutin (Barras *et al.* 1984). The sequence of these genes have still not been reported. Subsequently the *arb* genes responsible for the utilization of salicin and arbutin were isolated, and sequence analysis indicated that these genes comprised an operon with a high degree of similarity to the *E. coli bgl* operon (el Hassouni *et al.* 1992).

Members of the genus *Klebsiella* possess the ability to utilize the aromatic *b*-glucoside sugars salicin, arbutin

*For correspondence. E-mail: mahi@mrdg.iisc.ernet.in.

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and cellobiose. A cellobiose utilization system cloned from *K. oxytoca* allowed metabolism of all the three *b*-glucoside sugars by *E. coli* transformed with this system. The system, named the *casRAB* operon, showed a high degree of similarity to the *E. coli bgl* genes at the protein level (Lai *et al.* 1997). We have recently described the characterization of the homologue of the *E. coli bgl* genes in a sewage isolate of *Klebsiella aerogenes* (Raghunand and Mahadevan 2003). The objective of the present work is to examine the systems involved in mediating the utilization of the *b*-glucoside sugars in *K aerogenes* at the genetic level and to determine if, as in *E. coli* multiple systems exist for utilization of these sugars.

Materials and methods

Chemical reagents and growth media

All chemical reagents used in this study were obtained from Sigma Chemical Company, USA, and Invitrogen – Life Technologies, USA. Bacteriological growth media were from HiMedia Laboratories, India.

Phospho-b-glucosidase assays

(i) Assays using p-nitrophenyl-b-D-glucoside (pNPG): Enzyme assays were carried out essentially as described in Schaefler (1967), with minor modifications. Cells were grown in M9 minimal medium with 0.4% succinate as carbon source. At mid-log phase, cells in 1 ml of culture were pelleted by centrifugation, washed, and resuspended in 0.9 ml of 0.075 M phosphate buffer (pH 7.5) containing 1 mM $MgSO_4$. The reaction was started by adding 0.1 ml of 20 mM pNPG, and the suspension was incubated for 10 min at 37°C. The reaction was stopped by adding 0.5 ml of 2 M Na_2CO_3, and the amount of *p*-nitrophenol estimated by measuring absorbance at 410 nm. Absorbance at 600 nm was used to normalize values. Enzyme activity was determined using the formula:

$$\text{Activity units} = \frac{A_{410} \times 10^3}{A_{600} \times 10 \times v \times t},$$

where v is the volume of concentrated cells used in the assay, and t the time of incubation.

(ii) Saligenin assay: Estimation of BglB specific activity was carried out using a procedure similar to that described by Prasad and Schaefler (1974). Cells were grown to mid-log phase in M9 minimal medium with 0.4% succinate as carbon source. One millilitre of cells were harvested, washed in 0.8% saline, and resuspended in 0.1 ml saline. Salicin (0.1 ml of 4% solution) was added and the mixture incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 2 M Na_2CO_3. Production of saligenin by cleavage of phosphosalicin was detected by addition of 0.5 ml of 0.6% 4-amino-antipyrine followed by 0.25 ml of $K_3Fe(CN)_6$ after 15 min at room temperature. A positive reaction, indicated by appearance of a red colour, was quantified by measuring absorbance at 509 nm. Absorbance at 600 nm was used to normalize values. Enzyme activity was determined using the formula:

$$\text{Activity units} = \frac{A_{509} \times 10^3}{A_{600} \times 10 \times v \times t},$$

where v is the volume of concentrated cells used in the assay, and t the time of incubation.

In both the assays described above, the fold induction (I/U) was estimated by comparing the units of enzyme activity in cells grown in uninduced (U) and induced (I) conditions.

EMS mutagenesis

Mutagenesis using ethyl methanesulphonate (EMS) was carried out by the procedure detailed in Miller (1992). *K. aerogenes* AN292 cells were grown in 30 ml Luria broth (LB) to mid-log phase, harvested, washed and resuspended in 15 ml of minimal A buffer (1× A per litre: 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate·$2H_2O$). The resuspended cells were allowed to grow with shaking at 37°C and 2 ml samples were withdrawn and treated with 0.03 ml EMS for 0, 15, 30, 45 and 60 min. Following treatment, the cells were washed twice with minimal A buffer and resuspended in 2 ml of the same buffer. Viable counts corresponding to each time point were determined by dilution plating of cells on LB agar plates.

Results

Phospho-b-glucosidase activity in K. aerogenes AN292 is inducible by b-glucosides

The basal level of salicin-specific phospho-*b*-glucosidase activity in *K. aerogenes*, as assessed by the saligenin assay, was observed to be 18-fold higher than that in *E. coli* RV^+ (Raghunand and Mahadevan 2003). More significantly, this activity was induced fourfold, an observation consistent with what was described as semiconstitutive enzyme activity in the *Klebsiella–Aerobacter* group of organisms (Schaefler and Malamy 1969). Induction of phospho-*b*-glucosidase activity in members of the *Klebsiella* genus in response to cellobiose was estimated using the pNPG assay. A 4-fold to 6-fold induction was observed (figure 1).

These results still do not shed light on whether there are independent systems for utilization of the aryl *b*-glucosides salicin and arbutin, and the aliphatic cellobiose, or all are metabolized via the same system. This point becomes even more relevant in light of the proper-

ties of the *casRAB* genes from *K. oxytoca* (Lai *et al.* 1997). To clarify this issue, an attempt was made to generate mutants of *K. aerogenes* that are unable to utilize salicin (Sal^-). The cellobiose phenotype of such mutants would be a pointer to the nature of the systems involved.

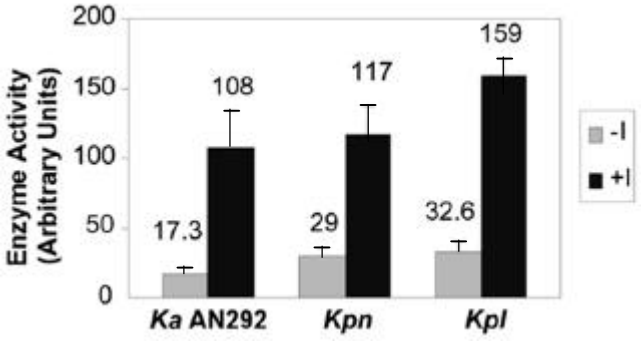

Figure 1. Induction of phospho-*b*-glucosidase activity in response to cellobiose. Results of pNPG assays carried out with *K. aerogenes* AN292 (*Ka*), *K. pneumoniae* (*Kpn*), and *K. planticola* (*Kpl*) grown in minimal succinate medium in the presence (+l) and absence (–l) of 1% cellobiose.

K. aerogenes has at least two independent systems for utilization of the aromatic b-glucosides and cellobiose

No Sal^- mutants could be obtained either by plating 10^4 bacteria to isolate spontaneously arising mutants or by following mutagenesis with EMS, suggesting that the mutation frequency was lower than 10^{-4}. To enrich for Bgl^- cells, *K. aerogenes* AN292 was mutagenized with EMS and these cells were treated with 200 mM phenyl-*b*-D-glucoside (p-glu), a toxic analogue of salicin. As depicted in figure 2a, this compound is specifically cytotoxic to Bgl^+ cells, since no significant cytotoxic effect was observed in *E. coli* RV, which is phenotypically Bgl^-. Nine mutants were obtained following the above treatment and plating cells on MacConkey agar media supplemented with salicin, and their phenotypes characterized (table 1). As the conditions under which the Sal^- mutants were obtained do not permit their growth, it is unlikely that

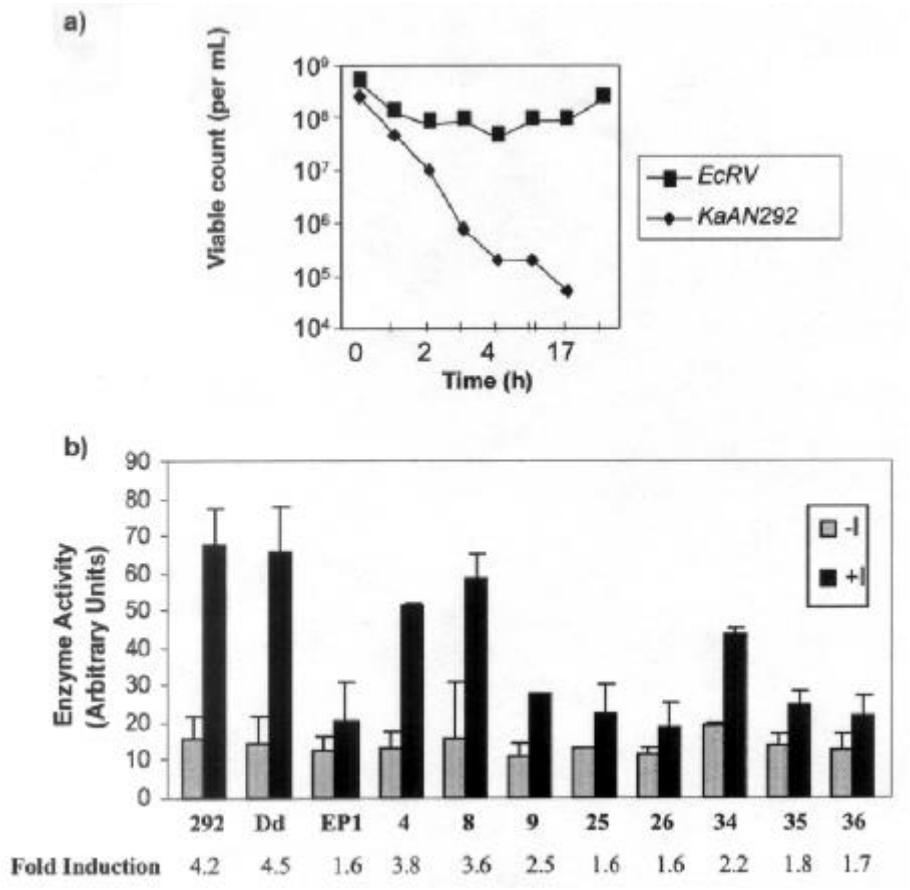

Figure 2. Isolation and properties of Sal^- mutants of *K. aerogenes* AN292. (a) Contrasting effects of 200 mM phenyl-*b*-D-glucoside (p-glu) on the growth of *K. aerogenes* AN292 (*Ka* AN292) and *E. coli* RV (*Ec* RV). (b) Saligenin assays carried out on *K. aerogenes* AN292 (292), *K. aerogenes* Dd (Dd), and all the mutants generated in this study. Cells grown in minimal succinate medium in presence (+ l) and absence (– l) of 7 mM salicin.

Table 1. Bacterial strains used in this study.

Strain	Genotype or description	Source/reference
Klebsiella strains:		
*Ka*AN292	*K. aerogenes* (sewage isolate) Amp^r, Kan^r	(Raghunand and Mahadevan 2003)
KaDd	*K. aerogenes* (lab strain) Amp^r	This work
Kpn	*K. pneumoniae* (lab strain) Amp^r, Kan^r, Tet^r, Cam^r	This work
Kpl	*K. planticola* (lab strain)	This work
EP1	*K. aerogenes* AN292 Sal^- Arb^- mutant	This work
EP4	–do–	This work
EP8	–do–	This work
EP9	–do–	This work
EP25	–do–	This work
EP26	–do–	This work
EP34	–do–	This work
EP35	–do-	This work
EP36	–do-	This work
E. coli strains (all K-12 derivatives)		
RV	F^- Δ*lac*X74 *thi bglR*° $bglG^+$ $bglF^+$ $bglB^+$ (Bgl^-)	Wright A.
RV^+	F^- Δ*lac*X74 *thi bglR*::IS*1* $bglG^+$ $bglF^+$ $bglB^+$ (Bgl^+)	Wright A.

these are siblings and are likely to be the result of independent mutations. All the Sal^- mutants also showed an Arb^- phenotype, but retained the ability to utilize cellobiose. Two significant conclusions can be drawn from these observations:

(a) *K. aerogenes* has at least two independent *b*-glucoside utilization systems. One for the aromatic sugars salicin and arbutin, and the other for utilization of cellobiose.
(b) The system that encodes the components for metabolism of cellobiose does not have the ability to cleave the aryl-*b*-glucosides.

The mutations that lead to a Sal^- phenotype possibly lie in a regulatory gene

The location of the mutation by introduction of the *E. coli bgl* genes carried on plasmids was unsuccessful because the *Klebsiella* strain was resistant to most standard antibiotics. To surmount the inability to localize the mutations using genetic complementation, a biochemical analysis of the mutants was carried out (figure 2b). Salicin-specific phospho-*b*-glucosidase activity of the mutants was estimated under induced and uninduced conditions, and as expected most of the mutants showed lower levels of induction than wild-type cells. Significantly, however, the basal level of activity in all the mutants was similar to that in wild-type cells, suggesting that the mutation did not affect the activity of the phopho-*b*-glucosidase per se. The mutations therefore could lie in a regulatory gene, involved in mediating induction by the aryl-*b*-glucosides.

Discussion

E. coli invests substantially in maintaining three major *b*-glucoside systems. All these systems are cryptic under laboratory conditions but it is believed that these could be transiently activated in its natural environment leading to increased fitness under certain conditions (Hall *et al.* 1983; Li 1984). The various systems have overlapping substrate specificities although the efficiencies with which these substrates are utilized vary in each system. The overlapping specificities are believed to be physiologically relevant but they might well be relics of the evolutionary process itself.

Members of the genus *Klebsiella* showed a higher basal level of phospho-*b*-glucosidase activity over *E. coli*. In their natural environment that includes sewage, humus, soil and plants, these organisms possibly encounter a variety of *b*-glucosides that are predominantly of plant origin. The elevated level of enzyme activity would allow them to use these sugars as carbon sources to generate energy for cellular metabolic processes. In contrast, *E. coli*, which shows a strong association with the mammalian intestine, is unlikely to come across sugars of this kind. In addition, *b*-glucosidase activity in *K. aerogenes* is inducible by both the aryl-*b*-glucosides and cellobiose, which presumably allow upregulation of specific genes involved in the metabolism of these different classes of sugars when they are present in the milieu.

All the Sal^- mutants generated in this study were found to be Arb^- but Cel^+, indicating the presence of at least two independent systems for utilization of these sugars. The metabolism of the aryl-*b*-glucosides is likely to be mediated primarily by the homologue of the *E. coli bgl*

operon recently identified (Raghunand and Mahadevan 2003), although the role of a putative *asc* homologue cannot be discounted. In *E. coli*, activation of the *cel* genes allows utilization of salicin and arbutin as well, but this does not seem to be true for *Klebsiella*. *K. aerogenes* therefore seems to have put to use homologues of the *E. coli bgl* and *cel* operons to exclusively metabolize these two different classes of sugars; the overlap in substrate specificities, if any, is not likely to be significant. In this respect, *K aerogenes* differs from *K. oxytoca*, which has a single genetic system for catabolism of aryl-*b*-glucosides as well as cellobiose (Lai *et al.* 1997).

Analysis of phospho-*b*-glucosidase activity of the mutants showed that these mutants are unable to undergo induction by salicin. A basal level of activity that is similar to the activity in wild-type cells implied that the mutations did not lie in the enzyme that cleaves the substrate. This observation is puzzling since the most obvious class of mutations that would lead to a Sal^- phenotype would be those in the phospho-*b*-glucosidase itself. Perhaps the probability of such mutations arising is low, and they may be isolated on screening a larger number of mutants. Since the mutants were also Arb^-, the mutations could possibly lie in the *K. aerogenes* homologue of *bglF*. In *E. coli*, BglF is the PTS permease and is also involved in mediating induction in response to these sugars. Mutations in *bglF* abolish induction and lead to a $Sal^- Arb^-$ phenotype (Mahadevan *et al.* 1987). This is because *bglF* mutants are unable to accumulate the phosphorylated form of the *b*-glucoside that is the substrate for the phospho-*b*-glucosidase and no enzyme activity would be detected.

A TBLASTN analysis carried out with protein sequences of the *E. coli asc* and *cel* operons and the *bglA* gene as queries on the yet unannotated *K. pneumoniae* genome sequence database (http://genome.wustl.edu/projects/bacterial/kpneumoniaee) did reveal potential matches (data not shown). The *cel* and *asc* genes are present in an operon-like organization, as reported in *E. coli*. The functional status of these genes has to be confirmed in the case of all members of the *Klebsiella* genus.

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