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In Vivo Expression of the β-Glucoside (*bgl*) Operon of *Escherichia coli* Occurs in Mouse Liver

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An *Escherichia coli* DNA fragment was identified that contained part of the β-glucoside (*bgl*) operon. This fragment was identified because it contained a promoter that was responsible for the expression of a reporter gene, the chloramphenicol acetyltransferase gene, in a mouse liver during bacterial infection but not when a bacterial clone was grown in vitro. This fragment contained a promoter and a rho-independent transcription terminator which were flanked by the 3′ end of *bglG* and the 5′ end of *bglF*. Reverse transcription-PCR confirmed that *cat*-specific mRNA was produced in infected mouse liver but not in vitro. mRNA encoding the positive regulator of the *bgl* operon, *bglG*, also was detected in mouse liver infected with an *E. coli* strain. These results demonstrated that expression of the *bgl* operon occurs in infected mouse liver and suggests a unique role for this operon in vivo.

The β-glucoside (*bgl*) operon of *Escherichia coli* encodes enzymes required for catabolism of aromatic β-glucosides. In *E. coli* K-12 the operon is cryptic, and wild-type cells cannot metabolize β-glucoside sugars, such as arbutin or salicin. The operon consists of three genes that map at 83.8 min on the *E. coli* chromosome (Fig. 1) (5). The three genes, *bglG*, *bglF*, and *bglB*, encode all the functions necessary for uptake and degradation of β-glucoside as well as regulation of the operon (23), while *bglR* is a *cis*-acting regulatory sequence. The *bgl* operon of *E. coli* K-12 has been extensively studied because, while the operon is cryptic, *cis*-active mutations have been identified that activate it. These activating mutations result from the insertion of IS*1* or IS*5* elements or various other point mutations within *bglR* (18–20). Mutations in another gene, *bglJ*, located at 99 min on the *E. coli* chromosome, also have been shown to activate the *bgl* operon (9). In addition, enhanced transcription from the *bgl* promoter P1 due to mutations in the genes encoding DNA gyrase (*gyrA* and *gyrB*) and histone-like nucleoprotein (*hns*) have been reported (7, 11).

The functions of the various *bgl* genes have been determined. *bglR* is a *cis*-acting regulatory element located upstream of *bglG*. *bglG* encodes a positive regulator of the operon, BglG, which can be phosphorylated. In the dephosphorylated state BglG has antiterminator activity and prevents early transcription termination (1, 12, 21). Two rho-independent transcriptional terminators, t1 and t2, are located upstream of the *bglG* and *bglF* genes, respectively (14, 23). BglG can serve as an antiterminator for both terminator sequences. *bglF* encodes a β-glucoside-specific membrane-bound transport protein which is part of the phosphoenolpyruvate-sugar phosphotransferase system (22). BglF also senses the presence of β-glucosides and, in the absence of β-glucoside, is a negative regulator of the *bgl* operon (15). In the absence of β-glucosides, BglF phosphorylates BglG, thus inhibiting the antiterminator activity of BglG (1–3). The last gene of the operon, *bglB*, encodes a phospho-β-glucosidase that hydrolyzes phosphorylated β-glucosides.

The *bgl* operon is actively kept silent due to negative effects exerted by DNA structural elements near the promoter region (16, 24, 25). Expression of the *bgl* operon containing an activating mutation requires a β-glucoside inducer and is dependent on cyclic AMP and catabolite-activating protein (CAP) (17, 18). A CAP binding site is present upstream of *bglG*. In the presence of cyclic AMP and CAP, transcription initiates constitutively at the promoter proximal to *bglG*, but in the absence of β-glucosides it terminates at the rho-independent terminator t1. The *cis*-acting mutations in *bglR* increase transcription initiation, and in the presence of inducer β-glucosides, the negative effect of BglF is relieved, permitting expression of all three genes (1, 2, 21).

In this communication we provide evidence that the wild-type *bgl* operon is induced in mouse liver and is not linked to *cis*-acting mutations in the *bglR* locus. We have employed in vivo expression technology (IVET) to identify genes important in the pathogenesis of *E. coli*-induced septicemia. This technology allows the detection and identification of genes that are expressed in vivo during infection but not under standard laboratory conditions. The approach was employed to identify novel "silent" genes that may contribute to survival of the bacteria in the animal and to the development of disease. During the screening process, *bglF* was identified as a gene expressed in vivo. Our results suggest that the wild-type *bgl* operon is derepressed in the microenvironment of the liver.

E. coli i484, which is the focus of this study, was isolated from a patient with septicemia. Strain i484 has a serotype of O25:H autoagglutinating. A streptomycin-resistant mutant of i484 was selected by plating on Luria-Bertani (LB) agar containing streptomycin (30 μg/ml). To identify in vivo-induced genes, a modified plasmid-based IVET strategy was devised which used the promoter selection vector pKK232-8 (Pharmacia Biotech) (6). Plasmid pKK232-8 carries a promoterless chloramphenicol acetyltransferase (*cat*) gene cassette that serves as a reporter. Upstream of the *cat* cassette are transcriptional terminators and translational stop codons in each reading frame, followed by a multiple cloning site. A genomic library of *E. coli* i484 DNA was prepared by insertion of genomic fragments into the multiple cloning site of pKK232-8. This allowed the selection of active promoters based on the expression of *cat*. Total DNA was isolated from strain i484 by using cetyltrimethylammonium bromide (4) and was partially

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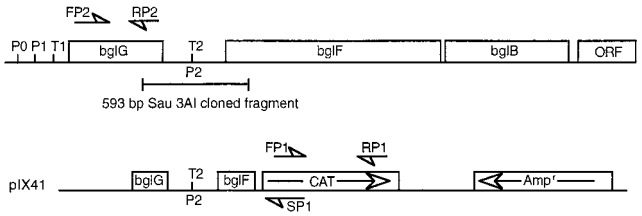

FIG. 1. The *bgl* operon showing all three genes required for regulated uptake and utilization of β-glucosides. The identified promoters are designated P0, P1, and P2, and the rho-independent transcription terminators are designated T1 and T2. Two primers, FP2 and RP2, were used to identify *bglG* mRNA by RT-PCR. In plasmid pIX41 the *bgl* fragment that contains the 5′ end of *bglG*, promoter P2, terminator t2, and the 3′ end of *bglF* is cloned upstream of the promoterless *cat* gene. Primers designated FP1 and RP1 were used for RT-PCR identification of the *cat* gene. A reverse primer, SP1, was used for sequencing. ORF, open reading frame.

digested with the restriction endonuclease *Sau*3AI. Fragments (0.5 to 2.0 kb) were collected from a sucrose density gradient. The pooled DNA fragments were precipitated with ethanol, collected, and dried. The fragments were ligated into the *Bam*HI site within the multiple cloning site upstream of the *cat* cassette. Recombinant plasmids were introduced into *E. coli* i484 by electroporation and plated on LB agar containing ampicillin (50 μg/ml).

A chloramphenicol-treated mouse infection model was developed to select the bacterial clones that expressed the *cat* gene in vivo. Mice (CD-1) (Harlan Sprague Dawley, Indianapolis, Ind.) were inoculated intraperitoneally with 10^8 cells from the library and immediately after challenge were treated with chloramphenicol (500 μg). Chloramphenicol was administered intraperitoneally six times at 4-h intervals. Thirty minutes after treatment, the concentration of chloramphenicol in the livers of the mice was 80 μg/ml, as measured by an agar diffusion assay. The concentration dropped to 25 μg/ml after 1 h. Two days postchallenge the mice were euthanized by CO_2, and bacterial cells were recovered from infected mouse livers by plating on LB containing streptomycin (30 μg/ml) and ampicillin (100 μg/ml); 1.3×10^6 bacterial cells were recovered per gram of liver. Bacterial clones were replicated on LB agar containing chloramphenicol (20 μg/ml), and those clones sensitive to chloramphenicol in vitro were pooled. Eighty-two percent of the clones were sensitive to chloramphenicol in vitro. Subsequently, 10^8 in vitro chloramphenicol-sensitive cells were used to challenge a second group of chloramphenicol-treated mice. Once again bacterial cells sensitive to chloramphenicol in vitro were recovered from the mouse livers. After the second challenge the percentage of in vitro chloramphenicol-sensitive clones was 99.9%. These clones were presumed to be resistant to chloramphenicol in vivo. Random clones were selected, and limited sequencing of purified plasmids isolated from these clones was performed. The oligonucleotide primer SP1 (Fig. 1) (5′-GAAAATCTCGTCGAAGCTC-3′), which is specific for the 5′ end of *cat*, was used to sequence DNA flanking *cat*. Two clones, i673 and i678, were found to contain similar DNA fragments, and sequencing revealed a sequence located in the intracistronic region between *bglG* and *bglF*. This 593-bp *Sau*3AI fragment, nucleotide positions 1319 to 1911 (23), contained the 3′ end of *bglG*, promoter P2, the rho-independent transcription termination sequence t2, and the 5′ end of *bglF* (Fig. 1). Both clones were confirmed to be sensitive to chloramphenicol in vitro by replating on LB agar containing streptomycin and LB agar containing chloramphenicol, reaffirming that the cloned promoter was not active in vitro.

Even though these clones did not express *cat* in vitro, we wanted to confirm that the *bgl* operon had not been permanently activated by growth in vivo due to alteration of important *cis*-active elements. Therefore, the wild-type *E. coli* strain i484 and the two in vivo-induced clones, i673 and i678, were grown on *Bgl* indicator plates. Indicator plates were prepared by adding 0.5% filter-sterilized salicin to a liter of sterile minimal medium followed by a 10-ml solution of 2% bromothymol blue, 50% ethanol, and 0.2 N NaOH. Ampicillin (100 μg/ml) was added to the medium if needed. All the strains tested exhibited a *bgl*-negative phenotype. This indicated that the chromosomal copy of the *bgl* operon was not activated by *cis* mutations after growth in vivo and that the regulation of the *cat* gene was under the control of a promoter in the cloned fragments (presumably P2). In addition, data obtained by employing reverse transcription (RT)-PCR also support this conclusion (see below).

Because we were concerned that the clones selected in vivo might arise for reasons not related to production of Cat that allowed the organism to overcome the inhibitory effects of systemic chloramphenicol, an RT-PCR-based assay was used to determine the presence of *cat*-specific mRNA in vivo. Total RNA was isolated from the liver of a mouse infected with i673 and from i673 cells grown in LB medium. As a control, total RNA was also isolated from the liver of a mouse challenged with *E. coli* i484 containing pKK232-8 but with nothing inserted in the multiple cloning site. Two primers were designed for PCR with *cat* gene sequences: a reverse primer, 5′-TTCT GCCGACATGGAAGCCATCAC-3′ (RP1), and a forward primer, 5′-CCTATAACCAGACCGTTCAGCTGG-3′ (FP1), that could be used to transcribe and amplify a 516-bp fragment of *cat* mRNA (Fig. 1). Using primer RP1, we performed reverse transcription at 66°C for 20 min with *Tth* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.), followed by PCR amplification. The conditions of the first cycle included a denaturation step at 95°C for 2 min, annealing at 60°C for 1 min, and polymerization at 72°C for 1.5 min; this was followed by another 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and polymerization at 72°C for 1.5 min. At the end of the 36 cycles, the reaction mixtures were left at 72°C for 5.5 min. Ten microliters of each sample was subjected to agarose gel electrophoresis (Fig. 2). A product of 516 bp was detected when RNA was prepared from the liver of a mouse infected with i673 (Fig. 2, lane 3), whereas no RT-PCR products were detected in RNA isolated from mouse liver infected

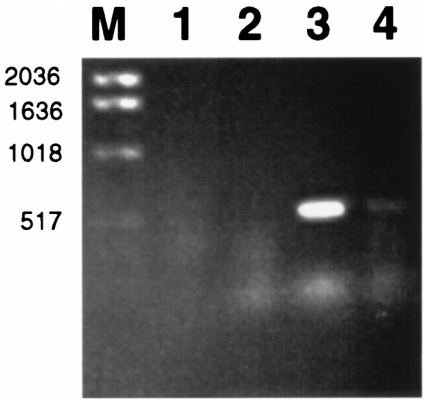

FIG. 2. Identification of *cat* mRNA by RT-PCR. RT-PCR was performed with RNA isolated from mouse liver infected with i484 carrying pKK232-8 (lane 1), in vitro-grown i673 (lane 2), mouse liver infected with i673 (lane 3), and RNase-treated RNA from mouse liver infected with i673 (lane 4). Lane M, molecular weight marker.

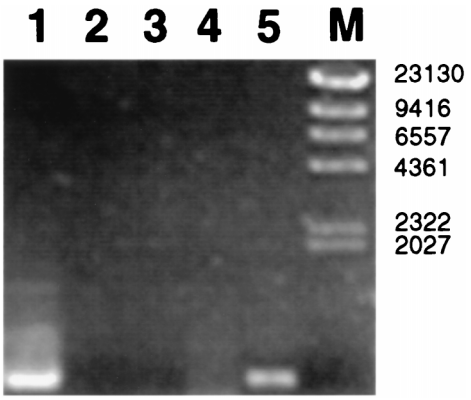

FIG. 3. RT-PCR amplification of *bglG* mRNA. Lane 1, PCR-amplified *bglG*, using total DNA from strain i484; lane 2, RT-PCR with RNA isolated from uninfected mouse liver; lane 3, RT-PCR with RNA isolated from in vitro-grown strain i673; lane 4, PCR amplification of RNase-treated total RNA from infected mouse liver; lane 5, RT-PCR with total RNA from infected mouse liver; lane M, molecular weight marker.

with i484 carrying pKK232-8 (Fig. 2, lane 1) or from in vitro-grown *E. coli* i673 (Fig. 2, lane 2). To eliminate the possibility that the 516-bp fragment was amplified from contaminating plasmid DNA, a second RT-PCR experiment was performed. Prior to RT-PCR amplification the RNA was subjected to RNase digestion. The RNase was boiled for 20 min prior to use to eliminate any contamination by DNase. After RNase digestion, *cat*-specific amplification did not occur (Fig. 2, lane 4).

The DNA fragments cloned in pIX41 and pIX46 (strains i673 and i678, respectively) contain the promoter P2, which is required for transcription of *bglF*. However, for that to occur, the antiterminator activity of the dephosphorylated BglG is required. Moreover, the 5′ end of the *bglG* gene is absent in pIX41 and pIX46. Therefore, we hypothesized that *bglG* was supplied in these clones from the wild-type genomic copy and that growth in vivo promoted *bglG* expression. To determine whether *bglG* was derepressed in vivo, two PCR primers were designed that could be used to detect *bglG* mRNA. The reverse primer 5′-GCGGGTAATTTTGTTTTACTGC-3′ (RP2) and the forward primer 5′-CAACAGCGGGAAAAAGTCG-3′ (FP2), were used for reverse transcription and amplification of a 657-bp internal fragment of *bglG*. As shown in Fig. 3, a DNA fragment of 657 bp was detected when RNA prepared from infected mouse liver was used (Fig. 3, lane 5) while no RT-PCR amplification was observed when RNA prepared from in vitro-grown i673 and RNA from uninfected mouse liver were used (Fig. 3, lanes 2 and 3). As before, to eliminate the possibility that the amplification product was due to contaminating DNA in the RNA preparation from infected mouse liver, RNase-treated total RNA was used for PCR amplification. The subsequent PCR amplification did not yield any DNA fragments (Fig. 3, lane 4). To confirm that the RT-PCR-amplified product contained *bglG*, it was sequenced; as expected, the sequence of the PCR product contained the *bglG* sequence. These results indicate *bglG*-specific mRNA is produced in vivo, and they are consistent with our hypothesis that the *bgl* operon is derepressed when grown in infected mouse liver.

Our results demonstrate that the induction of the *bgl* operon can occur in a specific in vivo habitat. This expression is not linked to activation of the cryptic *bgl* operon and does not result in constitutive expression. Expression occurs only in vivo, and the subsequent regrowth in vitro results in the repression of this operon. This result was confirmed by using RT-PCR, where *cat*-specific mRNA driven by the weak promoter P2 of *bglF* was detectable in the livers of mice infected with strain i673 (which contains pIX41) but was not produced when cells were grown in vitro.

Because the DNA fragment in pIX41 contained the 3′ end of *bglG*, a weak promoter, P2, located within or just downstream of the terminator t2 (21), and the 5′ end of *bglF* followed by *cat*, and because the expression of *bglF*, and therefore *cat*, requires antiterminator activities associated with BglG, it was assumed that BglG was supplied in *trans* from the chromosomal copy of the entire operon. Thus, BglG could act as an antiterminator for t2 located in the chromosome and the cloned DNA fragment. If this hypothesis was correct, *bglG*-specific mRNA should be produced by cells grown in mice but not by those grown in vitro. By using RT-PCR, this was found to be the case.

The purpose of this investigation was to identify *E. coli* genes that were exclusively expressed during the course of disease. Such an analysis will begin to describe the repertoire of genes expressed in the hostile in vivo environment and through this process define the adaptive ability of *E. coli*. The discovery that the cryptic *bgl* operon was exclusively induced in a murine infection model raises interesting questions about the regulation of this phenotypically silent operon and the importance of these genes in infection. A number of mutations that activate the *bgl* operon have been identified either in *bglR* or in other genes, like *hns*, *gyrA*, and *gyrB*. More recently the products of two genes, *bglJ* and *leuO*, have been identified as positive *trans* activators of the *bgl* operon (9, 26). However, an increased expression of these *trans* activators is required for activation of the *bgl* operon. We hypothesize that complex host-pathogen interactions in infected mouse liver may derepress certain bacterial genes that could directly relieve the silencing effect of the *bgl* operon or may in turn increase the expression of the *trans* activators, *bglJ* and *leuO*, leading to induction of the *bgl* operon. Growth condition-dependent induction of another cryptic operon, *cel*, has been reported (8). The growth of thermotolerant and mesothermophilic mutants of *E. coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* at 48 and 54°C results in the induction of the *cel* operon. While the *bgl* operon generally is not expressed except under specific activating conditions, a previous report demonstrated that it can be induced by growth under anaerobic conditions (13). In that study, expression was increased 22-fold but remained substantially below the levels achieved after activation. The results presented here confirm that other growth conditions (i.e., growth in mice) also induce this operon. We have not been able to quantify the degree of induction and therefore do not know whether expression in vivo achieves the levels obtained by growth under anaerobic conditions or activating conditions. The growth of bacteria in mammalian hosts also presents *E. coli* with unique stresses. These environmental changes could lead to the induction of a variety of genes during the disease process, including *bgl*. The importance of the *bgl* operon in the survival of the pathogen during infection in mouse liver is not clear. We have begun to ask whether *bgl* is required in vivo. Mice have been challenged with a mutant of i484 containing a deletion in *bglF* (P2) (unpublished results). No significant differences in mortality were observed compared to that of mice infected by the wild-type strain. However, in these experiments the challenge dose was quite high and more subtle effects on virulence might not have been detected. Since 90% of the natural isolates of *E. coli* carry the *bgl* operon (10), this cryptic metabolic system probably confers an advantage under specific growth conditions.

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