Use of *cir-lac* Operon Fusions to Study Transcriptional Regulation of the Colicin Ia Receptor in *Escherichia coli* K-12

PATRICIA L. WORSHAM AND JORDAN KONISKY*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

We describe *cir-lac* operon fusions constructed by using phage Mu *d*(Ap^r *lac*). Expression of β -galactosidase in these fusion strains is analogous to known regulatory properties of *cir* gene expression. It is concluded that the observed regulation by iron of the *cir* gene is under transcriptional control.

The biosynthesis of enterochelin and the production of the outer membrane receptor specific for this phenolate siderophore are coordinately regulated by the amount of intracellular iron (2, 9, 10, 12, 15, 16). In addition, the levels of two other outer membrane polypeptides seem to be similarly regulated: an 83,000-dalton protein with no known function and the 74,000-dalton *cir* gene product, which functions as the receptor for colicins Ia and Ib (2, 9-12, 15). It is known that the colicin I receptor is not required for ferric enterochelin transport (19). It seems likely that this protein serves as a receptor for an, as yet, undetermined siderophore.

Currently the enumeration of colicin I receptors involves either a determination of ¹²⁵I-colicin I adsorption to cells or analysis of outer membrane polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Unfortunately, these assays are not convenient for the kinds of studies that will be required to define the mechanism whereby receptor production is regulated by iron. Recently, Casadaban and Cohen (4, 5) have developed an operon fusion technique whereby it is possible to study the regulation of genes by coupling the expression of the *lacZ* gene product to the promoter of the gene in question. Bacteriophage Mu *d*1(Ap^r *lac*) allows construction of such fusions in a single step. This defective phage carries the structural genes for lactose utilization but does not contain the regulatory regions of this operon. Therefore, expression of β -galactosidase activity requires that these genes be fused to a bacterial promoter. This phage also carries the ampicillin resistance determinant from transposon Tn3, providing a simple method for selection of lysogens. In this communication we report on the use of this method to construct *cir::Mu d*(Ap^r *lac*) mutants in which the *lac* genes are fused to the promoter of the *cir* gene.

Overnight culture samples of strain MC4100 (colicin Ia sensitive; see Table 1 for strain designations) grown in LB (13) were infected with

Mu *d*1(Ap^r *lac*) phage (prepared by heat induction of the double lysogen MAL103) at a multiplicity of infection of approximately 1. Adsorption of the phage was carried out in LB broth containing 10 mM MgSO₄ and 5 mM CaCl₂ for 20 min at 30°C. After 1:10 dilution into LB broth, the culture was grown for 30 min at 30°C to allow expression of ampicillin resistance. After another 1:10 dilution into LB broth, ampicillin (25 μ g/ml; Sigma Chemical Co.) was added, and the cells were grown overnight at 30°C. Portions were then plated at 30°C on 5-bromo-4-chloro-3-indoyl- β -D-galactoside (Bachem Chemicals) (40 μ g/ml) minimal Tris plates (13, 18), with glycerol (0.4%) as the carbon source. Cells carrying operon fusions were characterized by blue-colored colonies after 48 h and were pooled into a mass culture in LB broth. Purified colicin Ia (5 μ g/ml) was added to this pooled culture, and binding was allowed to proceed for 30 min at 30°C. Portions of the culture were then plated on LB agar, and survivors were picked on the following day for further study.

As spontaneous mutation to colicin resistance is not a rare event, survivors of the colicin treatment were screened to determine whether β -galactosidase activity was affected by factors that are known to regulate the amount of the *cir* gene-determined product. The number of colicin Ia receptors is low in cells grown in medium containing high levels of iron, yet elevated in media containing ZnCl₂ (11). Therefore, mutants carrying the presumptive operon fusions were streaked on parallel 5-bromo-4-chloro-3-indoyl- β -D-galactoside minimal plates containing 100 μ M FeCl₃ and 100 μ M ZnCl₂, respectively. After incubation at 30°C for 48 h, isolates displaying high levels of β -galactosidase activity on plates containing zinc but low activity on plates containing high iron were picked and retested for resistance to colicin Ia. All proved to be resistant to the colicin. Two independent isolates were chosen for further study, strains PW400 and PW402. As *tonB* lesions lead to insensitivity

TABLE 1. *Strains*

Strain/ plasmid	Genotype ^a	Source
Strain		
JK1	F ⁻ λ ⁻ <i>rpsL</i>	This laboratory
MC4100	F ⁻ <i>araD139</i> Δ(<i>lacIOPZYA</i>)U169 <i>rpsL</i> <i>thiA</i>	T. Silhavy
MAL103	F ⁻ Mu cts <i>d1</i> (Ap ^r <i>lac</i>) Mu cts Δ(<i>proAB-lacIOPZYA</i>) _{xIII} <i>rpsL</i>	M. Casadaban via J. Gardner
MH221	MC4100 Φ(<i>ompC-lac</i>) <i>malQ7</i>	T. Silhavy
MH513	MC4100 Φ(<i>ompF-lac</i>)	T. Silhavy
pop3105	MC4100 Φ(<i>lamB-lacZ</i>) hybrid 61-4	T. Silhavy
PW400	MC4100 Φ(<i>cir-lac</i>)	This study
PW402	MC4100 Φ(<i>cir-lac</i>)	This study
1830	<i>pro met</i> (pJB4J1)	J. Cronan
PW401	PW400(pJB4J1)	This study ^b
PW414	PW401 <i>fepA</i>	This study
Plasmid		
pJB4J1	Plasmid determining resistance to gentamycin, spectinomycin, streptomycin, and kanamycin/neomycin and carrying a Mu phage with a normal repressor (1)	

^a Φ denotes a fusion, and Δ denotes a deletion.

^b A derivative of PW400 made thermally stable by introduction of plasmid pJB4J1.

to colicin Ia (6), these isolates were tested for their TonB character. Both isolates were found to utilize ferric enterochelin as a source of iron and to be sensitive to colicin B and phage φ80 *vir* and are, therefore, *tonB*⁺. Furthermore, in contrast to *tonB* mutants, which show enhanced levels of colicin Ia receptor due to iron stress (11), strains PW400 and PW402 bound little or no ¹²⁵I-colicin Ia (data not shown). Indeed, colicin Ia resistance due to a defect in colicin adsorption is the classic phenotype defining *cir* mutants (3). As expected for strains carrying a mutant *cir* gene, these isolates were resistant to colicin Ib and insensitive to colicin V (3, 6). Thus, both isolates satisfied every established phenotypic criterion which uniquely defines mutations in *cir*.

To be assured that β-galactosidase activity in the fusion strains was no longer under control of the *lac* regulatory region, β-galactosidase levels were determined with and without added isopropyl-β-D-thiogalactopyranoside (IPTG). IPTG had no effect on enzyme levels in the fusion strains. In contrast, *E. coli* JK1, which carries a normal *lac* operon, was induced 22-fold by IPTG.

Addition of 10 mM citrate to growth medium leads to the induction of a citrate-specific high-affinity iron uptake system in *Escherichia coli* (7). Cells grown in citrate have decreased levels of the *cir* gene product (11). When either *cir-lac* fusion strain was grown in iron-depleted medium, elevated levels of β-galactosidase were found compared with the strains grown in normal medium. Addition of 10 mM citrate to such

low-iron medium resulted in repression of β-galactosidase synthesis in these mutants (Table 2). In contrast, β-galactosidase levels in fusion strains in which *lacZ* expression is determined by the activity of promoters for three distinct outer membrane polypeptides (8, 17) were not affected by citrate. Similarly, citrate did not influence induction of β-galactosidase in the Lac⁺ strain JK1.

Growth of *E. coli* in medium containing the iron-binding protein transferrin leads to an increase in the amount of the three outer membrane proteins that are normally induced under low-iron conditions; one of these is the *cir* gene product (20). To examine the effect of transferrin on expression of *cir*, strains PW400 and 402 were grown in M63 medium containing transferrin (Table 3). Both *cir-lac* fusion strains showed a substantial increase in the level of β-galactosidase when grown in transferrin-containing medium. In contrast, transferrin did not affect β-galactosidase levels in uninduced or induced (IPTG) strain JK1.

We have previously reported that *fepA* mutants have an enhanced level of colicin Ia receptors, but that receptor levels in these mutants can be reduced by addition of ferrichrome or citrate (11). To examine the effect of a *fepA* lesion on β-galactosidase levels in the *cir-lacZ* fusion strains, colicin B-resistant derivatives of strain PW401 were isolated. One isolate, designated PW414, was selected for further study.

Strain PW414 was unable to grow in minimal medium unless the medium was supplemented with FeSO₄ or sodium citrate. It did not utilize

TABLE 2. Effect of citrate on β -galactosidase levels in fusion strains^a

Strain	Medium	β -Galactosidase (U)
PW402	Iron depleted	479 $\pm$ 6
	Iron depleted + citrate	103 $\pm$ 2
PW400	Iron depleted	590 $\pm$ 5
	Iron depleted + citrate	122 $\pm$ 4
MH221	Iron depleted	252 $\pm$ 6
	Iron depleted + citrate	305 $\pm$ 2
pop3105	Iron depleted	98 $\pm$ 1
	Iron depleted + citrate	95 $\pm$ 3
MH513	Iron depleted	836 $\pm$ 11
	Iron depleted + citrate	801 $\pm$ 5
JK1	Iron depleted	17 $\pm$ 2
	Iron depleted + citrate	20 $\pm$ 1
JK1 ^b	Iron depleted + IPTG	1,196 $\pm$ 12
	Iron depleted + IPTG + citrate	1,139 $\pm$ 15

^a Strains were grown at 30°C in M63 medium (13) depleted of iron by the method of Murphy et al. (14). Where indicated, this same medium was supplemented with sodium citrate (10 mM). In this and in subsequent experiments, the medium contained 0.4% glycerol and thiamine as required (1 μ g/ml).

^b IPTG (1 mM) was added 2 h before enzyme levels were determined (13).

exogenously added ferric enterochelin. The mutant was sensitive to phage ϕ 80 and is, therefore, *tonA*⁺ *tonB*⁺. Based on this phenotype, we consider it very likely that the strain carries a lesion in *fepA*. Table 4 shows the effect of the presumptive *fepA* lesion on β -galactosidase levels in the *cir-lac* fusion strain. When grown in M63 minimal medium containing 50 μ M iron, strain PW414 exhibited a ninefold-higher level in β -galactosidase activity when compared with its parental strain PW401. This difference was drastically reduced by addition of citrate to the medium.

These results establish that regulation by iron of the *cir* gene product is under transcriptional control. Although it is likely that *cir* is the structural gene for the 74,000-dalton outer membrane polypeptide identified as the colicin I receptor, this relationship has not been unambiguously established. We cannot rule out the possibility that *cir* encodes a regulatory element specific to expression of the colicin Ia receptor structural gene.

These strains should prove useful in studies of iron metabolism in *E. coli*. In contrast to other genes regulated by iron, such as *fepA*, *fes*, or *ent* (16), the inactivation of *cir* does not affect operation of the ferric enterochelin utilization system (19). Thus, it is possible to use the *cir-lac* system to monitor the iron state of the fusion strains under conditions in which the endogenous iron transport system is intact.

TABLE 3. Effect of transferrin on fusion strains^a

Strain	Medium	β -Galactosidase (U)
PW402	No addition	27 $\pm$ 1
	+ Transferrin	426 $\pm$ 1
PW400	No addition	42 $\pm$ 1
	+ Transferrin	466 $\pm$ 1
JK1	No addition	8 $\pm$ 1
	+ Transferrin	10 $\pm$ 1
	+ IPTG	1,034 $\pm$ 4
	+ Transferrin + IPTG	1,044 $\pm$ 3

^a Strains were grown at 30°C in M63 medium containing no added iron. Where indicated, this medium was supplemented with transferrin (250 μ g/ml; Sigma, human). β -Galactosidase was determined as described in footnote b Table 2.

TABLE 4. Effect of *fepA* on β -galactosidase levels in a *cir-lac* fusion strain

Strain ^a	Medium	β -Galactosidase (U)
PW401 <i>fepA</i> ⁺	No addition	101 $\pm$ 4
	+ Citrate	63 $\pm$ 1
PW414 <i>fepA</i>	No addition	883 $\pm$ 16
	+ Citrate	90 $\pm$ 1

^a Strains were grown in M63 medium containing 50 μ M FeCl₃ and, where indicated, 10 mM sodium citrate. β -Galactosidase was determined as described in footnote b of Table 2.

Owing to the straightforward selection procedures available for isolation of mutations in the lactose utilization genes, the *cir-lac* fusion strains should allow isolation of *cir* regulatory mutants. In the past this has been difficult since there was no suitable selection of *cir* constitutive phenotypes. However, selection in the fusion strains for lactose utilization under conditions of repression by iron should yield mutations in regulatory elements specifically controlling *cir* gene expression, as well as in elements which might be involved in the coordinate regulation of the many genes subject to control by iron.

It has recently been shown that certain plasmids which confer pathogenicity to *E. coli* (20) determine iron sequestration systems. The *cir-lac* fusion strains described in this paper may present an ideal system to screen for such plasmid-mediated systems. The plasmid in question can be introduced into the fusion strain, and β -galactosidase levels can be monitored to determine the response of the organism to iron stress. This approach seems particularly promising since Williams (20) has shown that the presence of the ColV plasmid which specifies iron sequestration activity represses synthesis of the colicin I receptor.

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