Construction of *cys:lac* Gene Fusions in *Escherichia coli* and Their Use in the Isolation of Constitutive *cysB^c* Mutants

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Operon fusions of the *lacZ* gene to two different genes of the cysteine regulon controlled by the *cysB* regulatory protein were isolated. The fusion strains were used for selection of *cysB* constitutive mutants. Three *cysB^c* alleles have been characterized and cloned into multicopy plasmids.

INTRODUCTION

The *cysB* gene of *Salmonella typhimurium* or *Escherichia coli* codes for a regulatory protein (Mascarenhas & Yudkin, 1980; Baptist *et al.*, 1982) required for expression of all other genes of the cysteine regulon except *cysE* (Jones-Mortimer, 1968*a, b*; Kredich, 1971) and *cysG* (Ostrowski & Hulanicka, 1979). The *cysB* alleles from both organisms have been cloned (Jagura-Burdzy & Kredich, 1983), sequenced (Ostrowski *et al.*, 1987) and shown to code for a 36 kDa polypeptide with 95% protein sequence homology. Maximum expression of the cysteine regulon requires, in addition to a functional *cysB* gene product, an internal inducer – *O*-acetyl-L-serine (the *cysE* gene product) – and sulphur limitation, because cysteine represses the cysteine biosynthetic activities (Jones-Mortimer 1968*b*; Pasternak *et al.*, 1965; Kredich, 1971). Most known *cysB* mutations result in cysteine auxotrophy. In *S. typhimurium* several *cysB* alleles have been found that cause constitutive expression of the cysteine biosynthetic pathway irrespective of *O*-acetylserine and sulphur source. These alleles, designated *cysB^c*, confer a Cys⁺ phenotype together with resistance to 1,2,4-triazole and selenate (Kredich, 1971; Borum & Monty, 1976; Sledziewska & Hulanicka, 1978). So far no *cysB^c* mutants of *E. coli* have been described and there has not been an easy way of systematically isolating regulatory *cys* mutations in this organism.

We employed the technique of Casadaban & Cohen (1979) to isolate strains in which genes of the *lac* operon were fused to structural *cys* genes and thereby placed under the control of the regulatory elements for the cysteine regulon: sulphur source and the *cysB* gene product. In this report we describe the construction of *cys:lac* operon fusions under *cysB* gene control and the use of these fusion strains for selection of *cysB^c* mutants in *E. coli*. The *cysB^c* alleles were characterized and cloned into multicopy plasmids.

METHODS

Bacterial strains and bacteriophages. All strains used were derivatives of *Escherichia coli* K12. Their characteristics are described in Table 1. MuCtsd1(Ap^r *lac*) phage (Casadaban & Cohen, 1979) was prepared from the lysogenic strain Mal103. λ p1(209) was obtained from M. Casadaban. P1vir and T4 phages were used for transductions performed according to Miller (1972) and Wilson *et al.* (1979), respectively.

Abbreviations: DES, diethyl sulphate; OASS, *O*-acetylserine sulphydrylase; SR, sulphite reductase.

Table 1. *Escherichia coli* strains and plasmids

Strain	Genotype	Reference or source
Mal103	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla ara::Mud1(Ap^r lac)</i> (Mucts)	M. J. Casadaban
JA199	<i>trpE5 leu-6 thi r⁻m_k⁺</i>	Stock collection
NK1	<i>trpE5 leu-6 thi r⁻m_k⁺ cysB</i>	N. M. Kredich
EC1119	<i>trpE5 leu-6 thi r⁻m_k⁺ cysA</i>	N. M. Kredich
EC1177	<i>leu-6 thi r⁻m_k⁺ cysB</i>	Phage grown on JA199 × NK1
EC1250	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla trp-3</i>	Jagura-Burdzy & Hulanicka (1981)
EC2250	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla trp-3</i> <i>cys329::Mud1(Ap lac)</i>	This work
EC2251	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla trp-3</i> <i>cys342::Mud1(Ap lac)</i>	This work
EC2256	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla trp-3 cys329::lac imm^λ</i>	This work*
EC2257	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla trp-3 cys342::lac imm^λ</i>	This work*
EC2266	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys329::lac imm^λ cysB</i>	Phage grown on EC1177 × EC2256
EC2267	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys342::lac imm^λ cysB</i>	Phage grown on EC1177 × EC2257
EC2270	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys329::lac imm^λ</i>	Phage grown on EC1177 × EC2256
EC2271	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys342::lac imm^λ</i>	Phage grown on EC1177 × EC2257
EC2276	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys329::lac imm^λ</i> <i>cysB^c302</i>	This work
EC2277	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys329::lac imm^λ</i> <i>cysB^c303</i>	This work
EC2278	F ⁻ <i>araD139 ΔlacU169 rpsL thi fla cys342::lac imm^λ</i> <i>cysB^c301</i>	This work
EC2279	<i>leu-6 thi r⁻m_k⁺ cysB^c302</i>	Phage grown on EC2276 × NK1
EC2280	<i>leu-6 thi r⁻m_k⁺ cysB^c303</i>	Phage grown on EC2277 × NK1
EC2281	<i>leu-6 thi r⁻m_k⁺ cysB^c301</i>	Phage grown on EC2278 × NK1
Plasmids		
pGBK5	<i>cysJIH</i>	G. Jagura-Burdzy (pers. comm.)
pDHK14	<i>cysA cysM</i>	Hulanicka <i>et al.</i> (1986)
pDHK16	<i>cysB</i>	Jagura-Burdzy & Hulanicka (1981)
pMAH1	<i>cysB^c301</i>	This work
pMAH2	<i>cysB^c302</i>	This work
pMAH3	<i>cysB^c303</i>	This work

* Strains derived from *cys::Mud(Ap lac)* in which λ p1(209) has been lysogenized and the intervening Mu has subsequently been deleted.

Media and growth conditions. Sulphate-free medium (SF) was the minimal medium C, in which the sulphur source was 0.1 mM-L-cystine or 1 mM-L-djenkolic acid. Glucose was added to 0.5%, amino acids to 0.2 mM, and ampicillin and tetracycline to 50 and 25 mg l⁻¹, respectively, when required. All solid media contained 1.5% (w/v) agar. LB liquid medium or agar plates (Miller, 1972) were used as rich media. Lactose solid medium contained 1% (w/v) lactose as sole carbon source. Bacterial cultures were incubated with aeration at 37 °C.

Enzyme assays. β -Galactosidase (EC 3.2.1.23) was assayed in toluenized cells with *o*-nitrophenyl β -D-galactopyranoside as substrate (Miller, 1972). *O*-Acetylserine sulphydrylase (OASS; EC 4.2.99.8) was determined as described by Becker *et al.* (1969). Sulphite reductase (SR, EC 1.8.1.2) was assayed by the method of deVito & Dreyfuss (1964) and sulphate permease according to Karbonowska *et al.* (1977). Protein was determined by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

Isolation of *cys::lac* fusion strains. Preparation of Mud1(Ap lac) phage lysate and conditions of infection of *E. coli* were as described by Casadaban & Cohen (1979). Conversion of Mud1(Ap lac) lysogens to λ p1(209) lysogens was done as described by Komeda & Iino (1979) and Gowrishankar & Pittard (1982).

Selection of *cysB* constitutive mutants. Portions (0.2 ml) of overnight cultures of the *cys::lac* fusion strains were spread on lactose plates supplemented with 0.1 mM-L-cystine, and a 3MM Whatman paper disc saturated with 20 μ l DES was placed in the centre of each plate. After overnight incubation at 37 °C, fast-growing colonies were picked up, grown on L-cystine or L-djenkolate and tested for OASS activity in toluenized cells.

Cloning techniques. DNA of plasmids pBR322 or pBR325 was prepared by the alkaline lysis method and purified by CsCl gradient centrifugation (Maniatis *et al.*, 1982). Chromosomal DNA was prepared according to Silhavy *et al.* (1984). Vector and chromosomal DNA were separately digested with *Eco*RI restriction endonuclease, mixed in a ratio of 1:5 (w/w) and ligated with T4 DNA ligase at 14 °C overnight. Transformation of *E. coli* was done by the method of Kushner (1978).

Table 2. Activities of β -galactosidase in *cys* : :*lac* fusion strains

The results are means of three to five experiments; the deviations from these means were less than 7%. RI, repression index: the ratio of the enzyme activity of bacteria grown on L-djenkolic acid to that of bacteria cultured on L-cystine. CT, L-cystine (0.1 mM); Dj, L-djenkolic acid (1 mM).

Strain	Pertinent genotype	β -Galactosidase (Miller units)		RI
		CT	Dj	
EC2250	<i>cys329</i> : :Mud1(Ap <i>lac</i>)	26	234	9.0
EC2251	<i>cys342</i> : :Mud1(Ap <i>lac</i>)	35	347	9.9
EC2256	<i>cys329</i> : : <i>lac imm</i> ^h	26	276	10.6
EC2257	<i>cys342</i> : : <i>lac imm</i> ^h	27	294	10.9

Table 3. Activities of SR and OASS in fusion strains

Enzyme activities of the control strain grown on L-djenkolic acid as sulphur source were taken as 100%; these values were 12 nmol S²⁻ min⁻¹ (mg protein)⁻¹ for SR and 7.5 units (mg protein)⁻¹ for OASS.

Strain	Pertinent genotype	Enzyme activity (% of control)	
		SR	OASS
EC1250	<i>cys</i> ⁺	100	100
EC2250	<i>cys329</i> : :Mud1(Ap <i>lac</i>)	96	108
EC2251	<i>cys342</i> : :Mud1(Ap <i>lac</i>)	2	96
EC2256	<i>cys329</i> : : <i>lac imm</i> ^h	98	102
EC2257	<i>cys342</i> : : <i>lac imm</i> ^h	4	94

RESULTS

Isolation and characterization of *cys* : :*lac* fusion strains

Strain EC1250 (Δ lac) was mutagenized with Mud1(Ap *lac*) phage and lysogens containing random insertions of Mud1 were selected at 30 °C as ampicillin-resistant colonies on LB agar plates supplemented with 0.1 mM-L-cystine. Of about 10⁴ replica-plated lysogens, 160 had acquired new auxotrophic mutations, and 26 of these were cysteine auxotrophs. Ten of the cysteine-requiring Mud1(Ap *lac*) lysogens expressed β -galactosidase at high levels and were able to utilize lactose as sole carbon source. Two fusion strains (EC2250 and EC2251) were chosen for further study. In these strains β -galactosidase expression was regulated by sulphur source in the growth medium (derepressed on L-djenkolate and repressed on L-cystine: Table 2) and the *lac* genes were fused to two different regulatory regions of the cysteine genes (see below). Because Mud1(Ap *lac*) fusions tend to undergo rearrangements (Mud prophage is still capable of further transpositions) we stabilized these *cys* : :Mud1(Ap *lac*) fusion strains by converting them to λ p1(209) lysogens free from intervening Mu and *bla* sequences. The resulting strains, EC2256 and EC2257, showed the same dependence of β -galactosidase activity on sulphur source as the original *cys* : :Mud1(Ap *lac*) strains (Table 2).

The *cys* : :*lac* fusions were characterized by enzyme assays and genetic mapping. The fusion *cys342* : :*lac* (strain EC2251 and its λ -lysogenic derivative, EC2257) has been identified as a fusion of *lac* genes to the regulatory region of the *cysJIH* operon. The *cysJI* genes (59 unit on the *E. coli* chromosome) code for two polypeptides of SR. The following data support this location of the *cys342* : :*lac* fusion: (a) lack of SR activity in crude extracts of strains EC2251 and EC2257 (Table 3); (b) complementation of cysteine auxotrophy by plasmid pGBK5 carrying the *cysJIH* region of the *S. typhimurium* chromosome; and (c) 10% linkage of the *cys* mutation with the *srl* marker (58 unit on the *E. coli* chromosome) in T-4-mediated transduction (data for b and c not shown).

The *cys329* : :*lac* fusion strains (EC2250 and the corresponding λ -lysogen, EC2256) had wild-type SR and OASS activities (Table 3). The complementation of cysteine auxotrophy by

Table 4. *Activity of sulphate permease*

Strain	Pertinent genotype	Sulphate permease [pmol SO ₄ ²⁻ min ⁻¹ ml ⁻¹ (OD ₆₅₀ unit) ⁻¹]
EC1250	<i>cys</i> ⁺	690
EC1119	<i>cysA</i>	0.0
EC2256	<i>cys329::lac</i>	4.8

Table 5. *Effect of cysB on expression of cys::lac fusions*

All strains were Trp⁺ transductants from a cross with strain EC1177 (*cysB*) as a donor. Because the recipient strains were cysteine auxotrophs the *cysB* transductants were identified as those with significantly reduced OASS activity (data not shown). The deviations from mean values (three to five experiments) were less than 7%.

Strain	Pertinent genotype	β -Galactosidase (Miller units)	
		CT*	Dj*
EC2270	<i>cys329::lac</i>	38	386
EC2266	<i>cys329::lac cysB</i>	1	3
EC2271	<i>cys342::lac</i>	50	423
EC2267	<i>cys342::lac cysB</i>	2	3

* See Table 2.

plasmid pDHK14 carrying *cysA* and *cysM* genes of *S. typhimurium*, and the lack of sulphate permease activity in strain EC2256 (Table 4) allowed us to localize the *cys329::lac* fusion in the *cysA* region.

Selection of cysB regulatory mutants

The rationale for the use of *cys::lac* fusion strains to select *cysB* regulatory mutants required β -galactosidase expression to be controlled by the *cysB* gene product. In order to prove that these fusion strains fulfilled this requirement the *cysB* mutant allele from strain EC1177 was introduced into fusion strains by P1*vir*-mediated transduction. Assay of β -galactosidase activity showed an almost complete lack of *lacZ* gene expression in *cysB* transductants (Table 5).

The common phenotype of strains with *cys::lac* fusions under *cysB* control was the ability to grow on lactose as sole carbon source when L-djenkolic acid but not L-cystine was provided as sulphur source. This feature was exploited to search for *cysB* constitutive mutants among clones expressing the Lac⁺ phenotype in the presence of cystine. Fusions with two different regulatory regions of the *cys* structural genes (strains EC2270 and EC2271, Trp⁺ derivatives of the parental strains EC2256 and EC2257) were used for selection of Lac⁺ clones resistant to cystine repression after DES mutagenesis. Fast-growing colonies were found on lactose plates supplemented with L-cystine at a frequency of 10⁻⁶–10⁻⁷. Twenty-four stable cystine-resistant Lac⁺ clones were screened for OASS activity in toluenized cells grown in the presence of L-cystine or L-djenkolate. In three of the mutant strains examined (EC2276, EC2277 and EC2278) the OASS levels were almost the same for cultures grown on both sulphur sources (data not shown). In order to check if constitutive levels of OASS resulted from mutation in the *cysB* gene (linked to the *trp* operon) P1 lysates of strains EC2276, EC2277 and EC2278 were used for transduction of a *cysB* strain (NK1) to tryptophan prototrophy. About 70% of the transductants were Trp⁺Cys⁺.

The activities of SR and OASS in these transductants (strains EC2279, EC2280 and EC2281) are presented in Table 6. The activities of both enzymes were nearly the same for cultures grown on L-cystine or L-djenkolate, with a repression index of 1–1.7, in contrast to that of 8–10 for the wild-type strain.

Table 6. Activities of SR and OASS in *cysB^c* strains

The results are presented as percentages of the SR and OASS activities of the wild-type strain grown on L-djenkolic acid; these values were 110 nmol S²⁻ min⁻¹ (mg protein)⁻¹ for SR and 6.5 units (mg protein)⁻¹ for OASS. ND, Not determined; other abbreviations as in Table 2.

Strain	Pertinent genotype	SR (% of control)			OASS (% of control)		
		CT	Dj	RI	CT	Dj	RI
JA199	Wild-type	12	100	8.3	9.6	100	10.4
EC2279	<i>cysB^c302</i>	93	120	1.3	75	102	1.4
EC2280	<i>cysB^c303</i>	45	72	1.6	53	84	1.6
EC2281	<i>cysB^c301</i>	101	109	1.1	109	127	1.2
NK1	<i>cysB</i>	ND	3		ND	5	
NK1/pMAH1	<i>cysB^c301</i>	83	117	1.4	93	140	1.5
NK1/pMAH2	<i>cysB^c302</i>	78	133	1.7	85	120	1.4
NK1/pMAH3	<i>cysB^c303</i>	44	79	1.8	54	107	2.0

Cloning of *cysB^c* mutant alleles

The wild-type *cysB* allele was originally cloned in a 5 kb *EcoRI* restriction fragment of the *E. coli* chromosome (plasmid pDHK16). Therefore, we used *EcoRI* for shot-gun cloning of the newly isolated *cysB^c* alleles into a multicopy plasmid. Genomic DNA of strains EC2279 (*cysB^c302*), EC2280 (*cysB^c303*) and EC2281 (*cysB^c301*) was digested with *EcoRI* and ligated with *EcoRI*-cleaved DNA of plasmids pBR322 or pBR325. The ligation mixtures were used to transform the *E. coli cysB* strain NK1 to cysteine prototrophy. A few Cys⁺ transformants were found; these were analysed by agarose gel electrophoresis for the presence of plasmids containing a 5 kb *EcoRI* chromosomal insert.

The following plasmids were isolated: pMAH1, consisting of the pBR325 vector and a 5 kb *EcoRI* fragment of EC2281 (*cysB^c301*) chromosomal DNA; and pMAH2 and pMAH3, consisting of the pBR322 vector and 5 kb *EcoRI* chromosomal inserts of EC2279 (*cysB^c302*) and EC2280 (*cysB^c303*) DNA, respectively. The identity of the chromosomal inserts of the pMAH plasmids with that of plasmid pDHK16 was shown by double *EcoRI/SalI* restriction analysis. The introduction of plasmids containing each of the cloned *cysB^c* alleles into the *cysB* strain NK1 resulted in derepressed levels of cysteine biosynthetic enzymes regardless of the sulphur source in the medium (Table 6); similar results have been obtained with another *E. coli cysB* strain (EC1258) and with an *S. typhimurium cysB* strain (TK3213) bearing these plasmids (data not shown).

DISCUSSION

Fusions of the *E. coli lacZ* gene to regions controlling other genes have become one of the most powerful tools for studying the regulation of gene expression (Silhavy & Beckwith, 1985). Application of the defective phage Mud1(Ap *lac*) constructed by Casadaban & Cohen (1979) allows simple *in vivo* isolation of *lac* operon fusions to almost any gene of interest. In such fusion strains the easily measurable β -galactosidase activity reflects the level of transcription from the promoter to which the *lac* operon is fused.

One of the many uses of the *lac* fusion technique is employment of fusion strains to isolate new classes of regulatory mutants. We used the procedure of Casadaban & Cohen (1979) to generate fusions linking *lac* genes to *cys* gene promoters controlled by sulphur source and the *cysB* regulatory protein. Two strains with *lac* genes fused to the *cysA* or *cysJIIH* regulatory regions (EC2250 and EC2251 and their λ -lysogenic derivatives, EC2256 and EC2257, respectively) were characterized and chosen for selection of *cysB* constitutive mutants in *E. coli*. Expression of the *lacZ* gene in both fusion strains required a functional *cysB* gene product and sulphur limitation; the strains were therefore able to utilize lactose as sole carbon source when djenkolate but not cystine was provided as sulphur source.

We reasoned that secondary mutations enabling these fusion strains to escape cystine repression might belong to two categories: (1) *cysB*^c mutations, and (2) mutations in regulatory regions of *cys* genes fused to *lacZ*. Mutants able to use lactose as sole carbon source in the presence of cystine arose with relatively high frequency (10^{-6} – 10^{-7}) both spontaneously and after DES mutagenesis of *cysJI::lac* and *cysA::lac* fusion strains. Among 16 clones resistant to cystine repression analysed, two mutants (EC2276 and EC2277) derived from the *cysA::lac* strain and one mutant (EC2278) derived from the *cysJI::lac* strain expressed OASS at constitutive levels (independently of sulphur limitation). The mutations resulting in constitutive expression of cysteine enzymes in these strains were mapped in the *cysB* region. The transfer of each of the *cysB*^c alleles to a *cysB* strain by P1 transduction resulted in cysteine prototrophy, constitutive levels of two enzymes representative of the cysteine biosynthetic pathway, SR and OASS, and also in resistance to 1,2,4-triazole and selenate. The features of the *cysB*^c alleles isolated were similar to those of previously described *cysB*^c mutations in *S. typhimurium* (Kredich, 1971; Borum & Monty, 1976; Sledziewska & Hulanicka, 1978).

As the first step toward detailed characterization of *E. coli cysB*^c alleles by sequence analysis we cloned them into multicopy plasmids. The introduction of plasmids carrying cloned *cysB*^c alleles into a *cysB* strain resulted in derepressed levels of cysteine biosynthetic enzymes similar to those in strains with a single copy of the respective allele.

The use of strains with *lac* genes fused to structural *cys* genes controlled by *cysB* offers an easy way to isolate a large number of both *cysB*^c mutations and mutations in the regulatory regions of *cys* genes that change their response to regulatory effectors of the cysteine regulon. A similar approach has been used to isolate *malT*^c mutants (Debarbouille *et al.*, 1978). Fusions of *lacZ* to the *malPQ* operon were constructed. The basal level of β -galactosidase in these strains was too low to allow growth on lactose, and only colonies with *malT*^c mutations could use lactose as sole carbon source.

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