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Confirmation of the Fur Operator Site by Insertion of a Synthetic Oligonucleotide into an Operon Fusion Plasmid

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We constructed a synthetic oligonucleotide corresponding to the previously proposed consensus binding site for the Fur protein, a central iron-regulatory protein of *Escherichia coli*. When this oligonucleotide was introduced at the start of transcription of an operon fusion between the *ompF* promoter and the *lacZ* structural gene, β-galactosidase activity became iron regulated. This consensus sequence is sufficient to function as an operator site for the binding of Fur protein in vivo.

Certain strains of *Escherichia coli* produce potent protein toxins that mimic closely the biological activities of the classical Shiga toxin from *Shigella dysenteriae* I (8, 13, 14). These toxins, termed Vero toxins (VT) or Shiga-like toxins (SLT), have been divided into two immunologically distinct types. SLT-I (VT-I) toxins are neutralized by specific anti-Shiga toxin antibody, but SLT-II toxins are not (16). Despite this immunological difference, both toxins have similarly sized A and B subunits, both are encoded by similar temperate bacteriophages, and the genes for the two toxins show weak DNA homology by cross-hybridization (12, 16, 18, 19). We (2) and others (7) have recently determined the nucleotide sequence of the *slt*-IA and *slt*-IB genes of *E. coli* and have suggested that the A and B subunits are transcribed as an operon from a promoter upstream of *slt*-IA.

Several important bacterial toxins, including Shiga toxin from *S. dysenteriae* (5), are repressed in the presence of high concentrations of iron in the growth medium and derepressed when the iron concentration is lowered. We have recently demonstrated similar regulation by iron of SLT-I expression in *E. coli* by using a gene fusion between *slt*-IA and alkaline phosphatase (3). We established that repression of SLT-I expression by iron is mediated by the *fur* locus, a gene in *E. coli* whose product also represses a variety of iron-scavenging systems when sufficient iron is present in the medium (6). Low concentrations of free iron may be an important environmental signal to bacteria that they have entered a mammalian host, and this signal may lead to coordinate expression of a number of virulence determinants. In a previous study (3), we used deletion analysis to localize the iron-regulatory site of the *slt*-I operon to the vicinity of the promoter and suggested that a 21-base-pair dyad symmetric element in this region, which is homologous to similar sequences in the promoters of several other iron-regulated genes in *E. coli*, might represent the operator site for the Fur protein in the presence of iron. DeLorenzo et al. analyzed another iron-regulated gene of *E. coli*, *iucA*, and showed by DNA-footprinting experiments that purified Fur protein (in the presence of heavy metals) protected a region in the vicinity of the *iucA* promoter that contained a dyad symmetric element (4). After comparing the sequence of this region with those of the promoter regions of other iron-regulated genes, they also proposed a Fur protein consensusbinding site which is identical with ours. In the present study, we have made this proposed consensus site as a synthetic oligonucleotide and have inserted it into an operon fusion plasmid. We show that this dyad symmetric element is sufficient to mediate iron regulation by the Fur protein in vivo.

Strains, plasmids, and media. Strain SY327 [F⁻ *araD* Δ(*lac-pro*) *argE*(Am) *rif nalA recA56*] has been described previously (2). Strain SM796 [F⁻ *araD139* Δ(*araABC-leu*)*7697 galE galK* Δ(*lac*)*X74 rpsL thi phoA*Δ*Pvu*II *phoR*] was a generous gift from Dana H. Boyd and Jon Beckwith. Strain SBC796 is strain SM796 with a null mutation of the *fur* locus introduced from strain AB4024 by P1 transduction and verified as previously described (3). Plasmid pRT240, a generous gift of Ronald K. Taylor (17), contains an operon fusion between the promoter of the *ompF* gene and an intact *lacZ* gene; a polylinker nest is located at the end of the *ompF* promoter region, beginning at base +2 of the transcript. Plasmid pRT240 produces approximately 3,300 U of β-galactosidase activity when assayed in an *ompR*⁺ background in LB broth (17). Low-iron and iron-supplemented T media have been previously described (3). Molecular biological techniques were performed as suggested by Maniatis et al. (9) or by the suppliers of individual reagents.

Oligonucleotide synthesis. Oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer, and the products were purified by high-performance liquid chromatography. Two oligonucleotides were made (Fig. 1), one matching the proposed consensus binding site for Fur and the other matching its complement, that when annealed with the first oligonucleotide yielded a double-stranded DNA with *Pst*I and *Bam*HI sticky ends.

Construction of plasmid pSC27 derivatives. Plasmid pSC27.1 was constructed from plasmid pRT240 as outlined in Fig. 1. Equal amounts of the two oligonucleotides (18 pmol) were annealed and ligated overnight with approximately 1 pmol of cesium chloride-purified pRT240 that had been digested to completion with *Bam*HI and digested partially with *Pst*I (6 U of enzyme, 37°C, 15 min). After heat inactivation of the ligase and complete digestion with *Sal*I, DNA was recovered by phenol-chloroform extraction and ethanol precipitation and transformed into competent SY327 cells. Ampicillin-resistant transformants were selected and purified by restreaking on the same medium, and plasmid DNAs were prepared by the method of Birnboim (1). After confirmation of the construction by restriction analysis, the *Eco*RI-*Bam*HI fragments from seven independent isolates were subcloned into M13mp18 (10) and sequenced (15). Plasmid pSC27.1 contained the designed synthetic oligonu-

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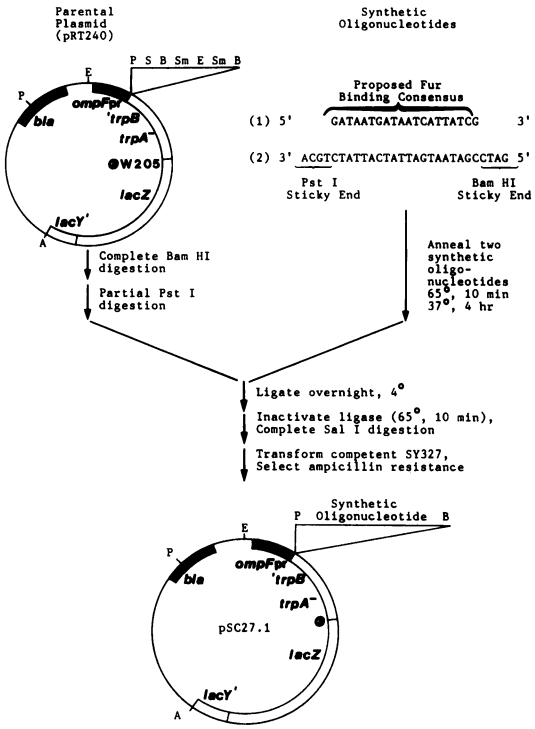

FIG. 1. Construction of plasmid pSC27.1. Plasmid pRT240 contains an operon fusion between the promoter (pr) of *ompF* and an intact *lacZ* gene (17). Restriction sites in plasmids pRT240 and pSC27.1 are abbreviated: E, *Eco*RI; P, *Pst*I; S, *Sal*I; B, *Bam*HI; Sm, *Sma*I; A, *Ava*I.

cleotide in place of the polylinker of pRT240 (Fig. 2). Plasmids pSC27.3 and pSC27.7 (Fig. 2) had spontaneous single-base-pair deletions in the inserted oligonucleotide but were otherwise isogenic with plasmid pSC27.1. Plasmids pRT240 and pSC27.1 were moved into strains SM796 and SBC796 by transformation.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Miller (11), except that cells to be assayed were grown overnight in T medium without or with iron supplementation (10 μM). Additional supplements to T medium included thiamine (10 μg/ml); proline, leucine, and arginine (40 μg/ml); and ampicillin (100 μg/ml). The parental strains SY327, SM796, and SBC796 had less than 1 U of β-galactosidase activity in the absence of plasmids carrying *lacZ*.

Analysis of β-galactosidase assay results. Strains containing the parental plasmid pRT240 showed high levels of β-galactosidase activity without significant regulation by iron (Table 1). In contrast, β-galactosidase activities for SY327(pSC27.1) and SM796(pSC27.1) were nearly twofold lower than for the parental plasmid in the absence of added iron and five- to sevenfold lower at an iron concentration of 10 μM. The lowered β-galactosidase activity even without added iron may reflect Fur binding to the consensus sequence even at the very low iron concentrations present in T medium (≤0.5 μM) (3); nonetheless, this binding is significantly enhanced at higher concentrations of iron. The activities of β-galactosidase made from plasmid pSC27.1 in strain SBC796, an otherwise isogenic *fur*^{0} derivative of SM796, were not dependent on iron concentration.

During construction of plasmid pSC27.1, two spontaneous single-base-pair deletions of the synthetic oligonucleotide arose (Fig. 2). β-Galactosidase activities of SY327 carrying these plasmids (pSC27.3 and pSC27.7) were no longer significantly regulated by iron (Table 1). Thus, single-base-pair deletions in these positions of the synthetic oligonucleotide reverse the ability of iron to repress β-galactosidase activity. The most conserved bases of the proposed consensus sequence for Fur binding are 5′-TCATT-3′, which occur in complementary fashion in each half of the dyad repeat (3, 4). The single-base-pair deletions in the synthetic oligonucleotide occur in the left-hand complement of this sequence and provide direct genetic evidence for the importance of these bases and/or their correct spacing in the formation of a Fur-binding site.

Our data show that the proposed consensus sequence for Fur protein binding is sufficient to mediate iron regulation in vivo and that iron regulation of SLT-I expression in *E. coli* is most likely dependent on the 21-base-pair dyad symmetric element present in the vicinity of the promoter of the *slt*-I operon. We have also shown that plasmid pRT240 is a useful vector for analyzing operator binding sites in vivo. Insertion of an appropriate synthetic oligonucleotide into the polylinker located just downstream of the start site of transcrip-

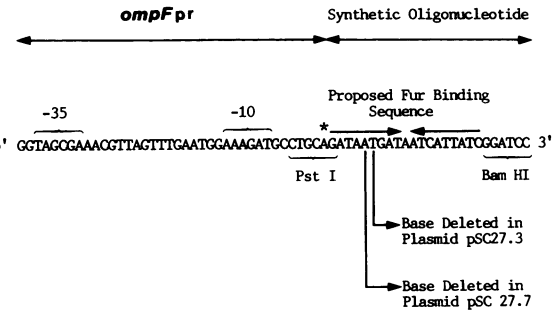

FIG. 2. Nucleotide sequence of the novel fusion joint in plasmid pSC27.1 and its derivatives. Brackets above the sequence delineate the previously defined −35 and −10 regions of the *ompF* promoter (*ompF*pr) (17); *, start site of transcription (base +1) of the parent plasmid. The synthetic oligonucleotide containing the dyad symmetric proposed Fur-binding sequence is inserted in place of the polylinker of pRT240, between the *Pst*I and *Bam*HI restriction sites. Spontaneous single-base-pair deletions in plasmids pSC27.3 and pSC27.7 are indicated.

TABLE 1. β-Galactosidase assays of various strains in low-iron and iron-supplemented T media

Strain	Plasmid carried	Iron added (μM)	β-Galactosidase activity (U)
SY327 (*fur*$^+$)	pRT240	None	4,317
		10	4,174
	pSC27.1	None	2,761
		10	624
	pSC27.3	None	5,193
		10	4,829
	pSC27.7	None	3,916
		10	2,895
SM796 (*fur*$^+$)	pRT240	None	4,467
		10	3,634
	pSC27.1	None	2,575
		10	774
SBC796 (*fur*^{0})	pRT240	None	4,147
		10	3,714
	pSC27.1	None	4,268
		10	3,185

tion of this *lacZ* operon fusion may allow convenient assay of operator function.

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