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Cloning and Nucleotide Sequence of *bisC*, the Structural Gene for Biotin Sulfoxide Reductase in *Escherichia coli*

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Clones of the *Escherichia coli bisC* locus have been isolated by complementing a *bisC* mutant for growth with *d*-biotin *d*-sulfoxide as a biotin source. The complementation properties of deletions and Tn*5* insertions located the *bisC* gene to a 3.7-kilobase-pair (kbp) segment, 3.3 kbp of which has been sequenced. A single open reading frame of 2,178 bp, capable of encoding a polypeptide of molecular weight 80,905, was found. In vitro transcription of plasmids carrying the wild-type sequence and deletion and insertion mutants showed that BisC complementation correlated perfectly with production of a polypeptide whose measured molecular weight (79,000) does not differ significantly from 80,905.

The *Escherichia coli* protein biotin *d*-sulfoxide (BDS) reductase reduces a spontaneous oxidation product of biotin, BDS, back to biotin (8). Mutants that are unable to synthesize biotin can use BDS as their sole source of biotin (25). The BDS reductase protein, which is encoded by the *bisC* gene, requires the presence of both a small thioredoxinlike protein and a molybdenum cofactor for activity (8, 9). The thioredoxinlike protein has not been well characterized. On the other hand, the genes required for molybdenum cofactor production have been studied intensively. The cofactor is a pterin ring structure which is extremely oxygen labile (6, 19). The production of the pterin cofactor, the addition of molybdenum to this molecule, and the insertion of the molybdopterin into apomolybdoenzymes require the products of eight genes in *E. coli*: *chlA*, *B*, *D*, *E*, *F*, *G*, *M*, and *N* (10, 20, 36, 37). Cells with a mutation in any one of these genes have a pleiotropic phenotype. In addition to being unable to reduce BDS, they cannot utilize nitrate, trimethylamine-*N*-oxide, or dimethyl sulfoxide anaerobically as a terminal electron acceptor (2, 14, 38), they cannot use formate as an electron donor (5, 16), and, as their name implies, they are resistant to chlorate (14, 28, 29). Chlorate reduction appears to be catalyzed by several molybdoenzymes of *E. coli*, and only when all are inactivated do cells become resistant to chlorate (37).

The function of BDS reductase in *E. coli* is unknown. It may serve as a scavenger, allowing the cell to utilize BDS as a biotin source. Another possible role for this protein is to protect the cell from oxidation damage, as do superoxide dismutase and methionine sulfoxide reductase (3, 11, 13). BDS reductase may restore activity to enzymes that have been inactivated by the spontaneous oxidation of the biotin cofactor covalently bound to them.

A recombinant phage containing the *bisC* gene was isolated by M. Martin in our laboratory by selecting from a clone bank clones that allowed strains with a particular *bisC* point mutation to grow on minimal media containing BDS as the sole biotin source. A clone bank of the *E. coli* chromosome from the *bisC*$^+$ *bio* deletion strain KS302 (Table 1) was prepared by inserting a *Sau*3A partial digest of bacterial chromosomal DNA prepared by the method of Redfield and Campbell (30) into a *Bam*HI digest of the λ cloning vector λ1059 (21). *bisC*$^+$ clones were isolated from the bank by lytic complementation of the *bisC32* point mutation found in strain S1187 (Table 1) as follows. A 0.1-ml volume of a stationary culture of S1187 was mixed with the *E. coli* clone bank and plated in minimal A (26) soft agar on glucose minimal A plates containing 4×10^{-3} μg of BDS per ml and 0.007% triphenyltetrazolium chloride as an indicator dye. Feeding plaques were identified by a red halo that was formed by the growth of cells around the plaque due to release of biotin by cells lysed by bacteriophage containing *bisC32*-complementing sequences. Feeding plaques were picked and repurified twice. These phage were tested further for lytic complementation of another *bisC* allele, in strain S1130 (Table 1), which they complemented, and for the complementation of two *chl* gene mutations, *chlG21* and *chlE41*, which they failed to complement. A single positive clone containing an insert of 19.5 kilobase pairs (kbp) was used for all further studies. A partial restriction map of this clone, λbisC, is shown in Fig. 1A. A 5.8-kbp internal *Eco*RI-*Pst*I fragment of λbisC was subcloned into plasmid pUC9 (39). This subclone, pBISC352, complemented all *bisC* alleles tested, including spontaneous temperature-sensitive mutations, nitrosoguanidine-induced mutations, Tn*5* insertions, and Mu insertions.

To define the position of the *bisC* gene on pBISC352 and to determine its orientation, the clone was subjected to deletion and insertion analysis (Fig. 1B and C) Deletions constructed by subcloning different restriction fragments of pBISC352 into pUC9 were tested for complementation of the *bisC32* mutation. This deletion analysis places the gene on a 3.2-kbp *Hin*cII-*Hin*dIII fragment (Fig. 1A). The position of the *bisC* gene was further restricted by insertional mutagenesis with the two transposable elements Tn*5* and Mu dI1681 (7, 12). Cells harboring pBISC352 were infected with the Tn*5*-containing phage λNK467 (Table 1) at a multiplicity of 5 and incubated at 30°C for 20 min to allow Tn*5* to transpose from the phage to the plasmid and the chromosome. Plasmid DNA isolated from these cells by the boiling lysis method of Holmes and Quigley (18) was used to transform strain S1187 to ampicillin and kanamycin resistance by the calcium chloride transformation method (24), thus selecting for plasmids into which Tn*5* had transposed. These transformants were then tested for growth on BDS. Cells that were lysogenic for Mu dI1681 phage (POI1681 [Table 1]) were

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TABLE 1. Bacterial strains, plasmids, and phages used

Strain	Genotype	Origin or reference
S1130	F⁻ *araD139 ΔlacU169 thi rpsL Φ(bioA-lacZ)301 bisC*	4
S1134	F⁻ *araD139 ΔlacU169 thi rpsL Φ(bioA-lacZ)301 chlE41*	4
S1187	F⁺ *bioA24 bisC32* (λ *imm*^{434})	8
S1196	F⁺ *bioA24 chlG21*	Curing of S1177 (8)
S1312	F⁻ *araD139 ΔlacU169 thi rpsL bioA24* (λ *imm*^{434}) *zbh-428*::Tn*10 bisC8*::Mu cts	Transduction to Tc^r of *bisC*::Mu cts mutant of A. del Campillo-Campbell
S1316	F⁻ *araD139 ΔlacU169 thi rpsL bioA24 zbh-428*::Tn*10 bisC9*::Mu cts	Same as for S1312
KS302	HfrH Δ(*gal-bio*)	33
POI1681	*araD139 araB*::Mu cts Δ*lac rpsL* (Mu dI1681 [Km^r] Δ*Bam*HIcts)	12
pUC9	*lac*$^+$ Ap^r	39
pDEPO	Ap^r	27
M13mp18, mp19		40
λ1059		21
λNK467	*c*I857 *b*221 *rex*::Tn*5* *O*am *P*am	N. Kleckner

transformed (24) with pBISC352, and a Mu-transducing lysate was prepared from these cells by inducing the Mu prophage in a logarithmically growing culture at 43°C for 2.5 h. A *bisC*::Mu strain, S1312 (Table 1), was transduced (26) to resistance to ampicillin and kanamycin with this lysate. These transductants, which must harbor pBISC352 into which Mu dI1681 has transposed, were screened for growth on BDS. The positions of these insertions (Fig. 1C) suggest that the *bisC* gene occupies a maximum of 2.8 kbp of the original clone. There is a small region of overlap of complementing and noncomplementing insertions into the *bisC* clone. Since both of the noncomplementing Mu dI1681 insertions are in the same orientation relative to the *bisC* gene but the noncomplementing insertion is in the opposite orientation, this overlap is probably due to polarity effects of the insertion element in one orientation but not the other. This result suggests that these insertions are between the *bisC* promoter and the start of the *bisC* coding region.

The polypeptides produced by the original *bisC* gene clone, pBISC352, were identified both in vivo by maxicell analysis (31) of cells containing the clone (data not shown) and in vitro by transcription and translation (41) of plasmid DNA. These polypeptides were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23) of [^{35}S] methionine (Amersham)-labeled proteins prepared in an S30 extract (Amersham) used according to the instructions of the manufacturer with 3 μg of cesium chloride gradient-purified (24) plasmid DNA. Three polypeptides with molecular weights of 79,000, 34,000, and 22,000 (79K, 34K, and 22K polypeptides) were produced by this clone (Fig. 2). The 79K polypeptide was the sole polypeptide required for the BisC$^+$ phenotype (Fig. 2). In experiments in which the products of the three Tn*5* insertion mutants and the six deletion mutants which fail to complement *bisC32* were produced by in vitro transcription or translation of purified DNA, the 34K and 22K polypeptides were produced but the 79K polypeptide was not (Fig. 2, lanes 3, 5, 8, 9, 10, 11, and 14). More

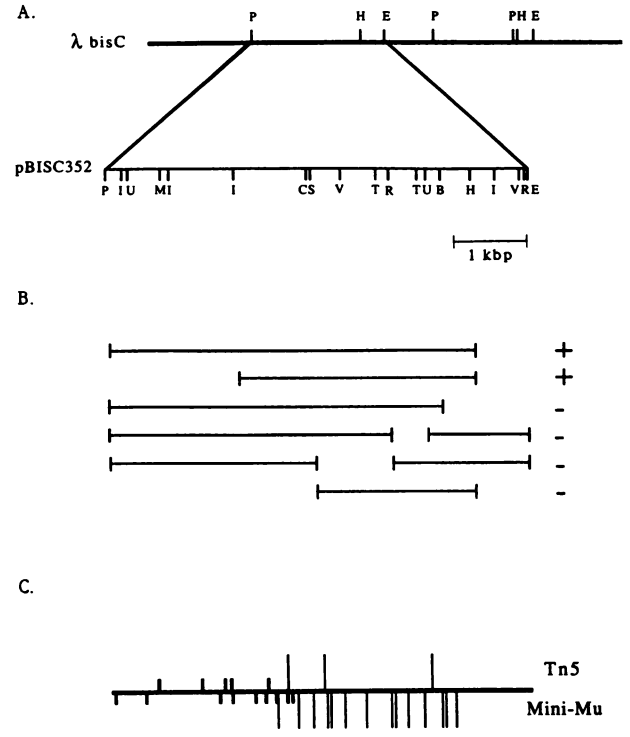

FIG. 1. Location of the *bisC* gene on the *bisC* clone. (A) Restriction maps of the 19.5-kbp bacterial DNA insert in λbisC and of the 5.8-kbp subclone, pBISC352. The restriction enzymes used were *Pst*I (P), *Hin*cII (I), *Pvu*I (U), *Mlu*I (M), *Cla*I (C), *Stu*I (S), *Pvu*II (V), *Bst*EII (T), *Eco*RV (R), *Bgl*II (B), *Hin*dIII (H), and *Eco*RI (E). The 1-kbp scale is for the pBISC352 map and the maps in panels B and C. (B) The fragments of *bisC* DNA present in several subclones of pBISC352 are aligned with the map of pBISC352 in panel A. The abilities of these subclones to complement the *bisC32* allele are shown on the right. Some clones (+) complement the mutant for growth on BDS minimal plates, and other (−) do not. (C) Positions of Tn*5* insertion are indicated above the line, and positions of Mu dI1681 insertions are indicated below the line; the map is aligned with the map of pBISC352 in panel A. Tn*5* insertion clones were tested for the ability to complement the *bisC32* mutation. Mu dI1681 insertion clones were tested for the ability to complement the *bisC8*::Mu mutation. Insertion clones that complement are indicated by a short line; those that do not are indicated by a long line.

important, the 34K polypeptide was not produced by one of the Tn*5* insertion mutants that can complement the *bisC32* mutation (lane 15), and the 22K polypeptide was not produced by a different complementing Tn*5* insertion mutant (lane 4). The 79K polypeptide was produced, however, in these two insertion mutants and in two others that can complement the *bisC32* mutation (lanes 12 and 13).

The fact that our cloned segment complements *bisC* mutations suggests (but does not prove) that the gene responsible for the complementation is really the *bisC* locus. To prove that it is, one of the *bisC* temperature-sensitive structural gene mutations isolated by del Campillo-Campbell and Campbell (8), *bisC117*(Ts) was cloned by using its ability to hybridize to the wild-type clone. The region containing the mutation was mapped to the interval between bp 1180 and 1780 on the wild-type *bisC* sequence map (see below and Fig. 3), which is within the open reading frame proposed here to be the BDS reductase coding region. The DNA sequence of this mutant from bp 1290 to 2050 was deter-

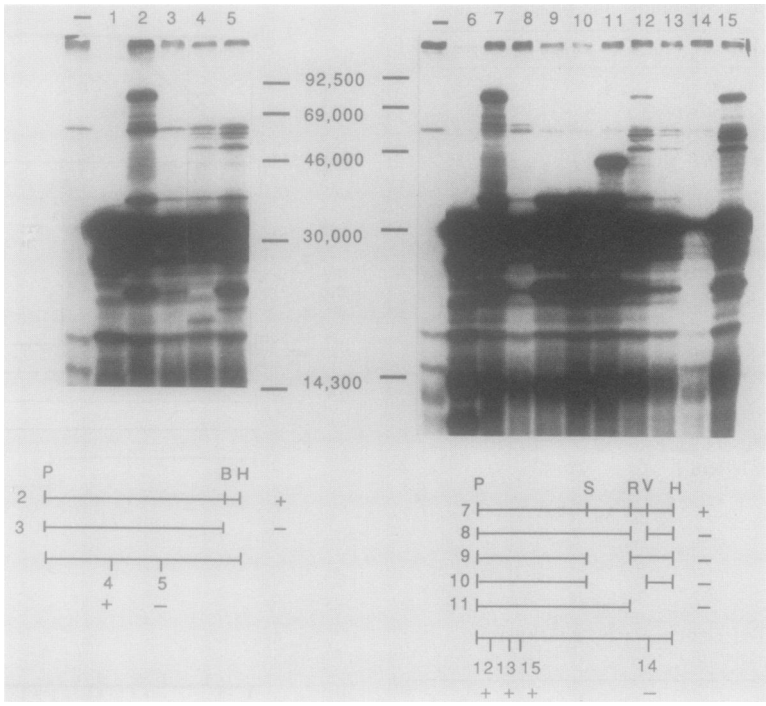

FIG. 2. Analysis of polypeptide products of deletion and Tn*5* insertion mutations in the *bisC* clone expressed in a coupled in vitro transcription-translation system. Polypeptides labeled with [^{35}S]methionine were produced in an S30 extract of *E. coli* (41). These were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiograms of two gels are shown. The *bisC* fragments and insertion mutants tested in this system are indicated below the autoradiograms. Those marked with a plus sign complement the *bisC32* mutation and produce the 79K polypeptide. Those marked with a minus sign do not complement the *bisC32* mutation and do not produce the 79K polypeptide, although several do produce polypeptides of altered sizes. Lanes: —, no DNA; 1, pDEPO (27); 2, 5-kbp *Pst*I-*Hin*dIII *bisC* fragment cloned into pDEPO; 3, 4.6-kbp *Pst*I-*Bgl*II *bisC* fragment cloned into pDEPO; 4 and 5, Tn*5* insertions in plasmid used in lane 2; 6, pUC9 (39); 7, 5-kbp *Pst*I-*Hin*dIII *bisC* fragment cloned into pUC9; 8, *Eco*RV-*Pvu*II internal deletion of plasmid used in lane 7; 9, *Stu*I-*Eco*RV internal deletion of plasmid used in lane 7; 10, *Stu*I-*Pvu*II internal deletion of plasmid used in lane 7; 11, 3.9-kbp *Pst*I-*Eco*RV fragment into pUC9; 12 through 15, Tn*5* insertions in plasmid used in lane 7.

mined by the dideoxy sequencing method, and the sole change from the wild-type sequence was shown to be a G-to-T transversion at bp 1443 (data not shown). This base change results in a change from a methionine to an isoleucine codon in the BDS reductase protein. Additional evidence comes from Southern analysis of a Mu insertion (in S1316 [Table 1]) that has been mapped to the *bisC* locus by genetic means. By using probes representing different regions of the cloned gene, we have mapped the Mu insertion to the 0.3-kbp *Bst*EII-*Eco*RV fragment in the center of the gene (Fig. 1). Sequences on either side of this region are still present in this strain (data not shown). Thus, two known *bisC* mutations map within the region proposed to be the *bisC* coding region.

The sequence of the 3.3 kbp of the 3.7-kbp *Hin*cII-*Eco*RI fragment containing the *bisC* gene was determined by the dideoxy chain termination method (32). M13mp18 and M13mp19 clones of this fragment were subjected to exonuclease III deletions from either end (17). This produced a complete sequence, including every base of 3.3 kbp of the 3.7-kbp fragment, a minimum of two times in each direction. The sequence of this 3.3 kbp is shown in Fig. 3. An open reading frame thought to correspond to the *bisC* coding region and capable of encoding a polypeptide with a predicted molecular weight of 80,905 is also shown in Fig. 3. The three noncomplementing Tn*5* insertions and the six deletion mutants which do not produce the 79K polypeptide in the in vitro transcription-translation system all map to this open reading frame. The sequence places the 5′-to-3′ orientation of the *bisC* gene from left to right on the map of Fig. 1A. An examination of the hydropathicity of the predicted BisC open reading frame by the method of Kyte and Doolittle (22) suggests that the product is a soluble protein, which is consistent with the location of the enzyme in partially purified preparations (9; unpublished observations). The codon usage in the predicted open reading frame (data not shown) is typical of weakly expressed *E. coli* proteins (15). The 700-bp sequence upstream of the *bisC* coding region includes no sequences with appreciable homology to the consensus ribosome-binding site (34) and promoter of *E. coli* (35). Because of this, we have no reason to assume that the major initiation site for translation is at nucleotide 577 (as shown) rather than at the downstream ATG at nucleotide 643. The *bisC* gene appears to be expressed at a low rate in vivo. The *bisC* product, the 79K polypeptide, is expressed in vitro at rates much lower than those of the vector-borne gene product, the β-lactamase polypeptide, and indeed lower than those of the other two polypeptides produced by the cloned DNA (Fig. 2). This low level of expression may explain the difficulty in identifying sequences that match consensus sequences important for gene expression in *E. coli*.

The sequences of two other molybdoenzymes from *E. coli* show some similarities to that of *bisC* (1).

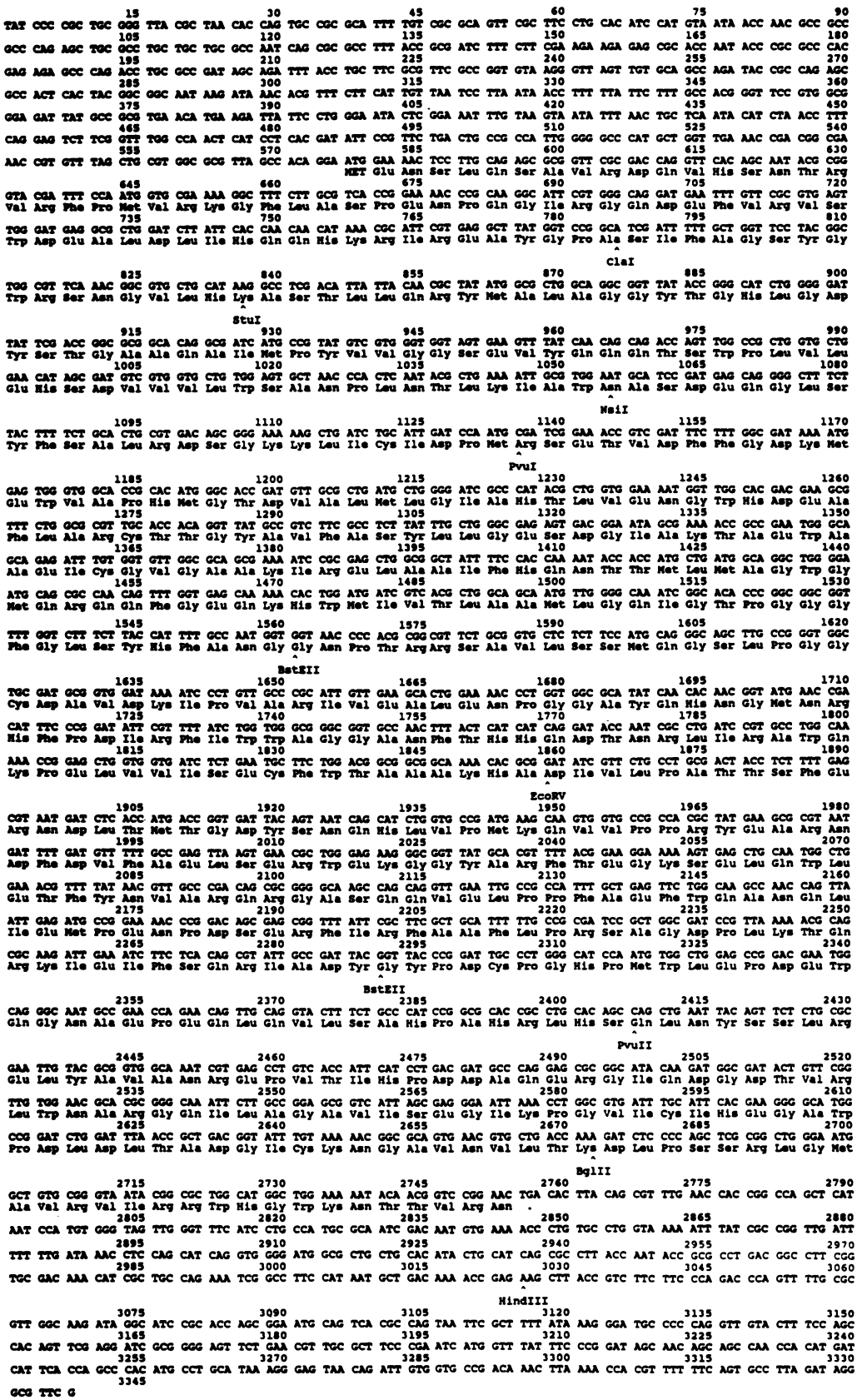

FIG. 3. Sequence of the *bisC* gene. The sequence of 3,336 bp within the 3.5-kbp *Hin*cII fragment containing the *bisC* gene is shown. The position and sequence of an open reading frame capable of encoding a polypeptide of 80,905 molecular weight are also shown.

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