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# Characterization and Generation of *Escherichia coli* Adenylate Cyclase Deletion Mutants

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***Escherichia coli* Δ*cya-283* is a 75-bp in-frame deletion overlapping the 5′ end of Δ*cya-854*; Δ*cya-201* is a 41-bp frameshift deletion overlapping the 3′ end of Δ*cya-854*. Sequence repeats were found at the boundaries of Δ*cya-283* and Δ*cya-201*, suggesting a mechanism for deletion formation. Recombinant DNA procedures were used to construct a strain in which the total *cya* structural gene in the chromosome was replaced by the kanamycin resistance gene.**

Numerous studies on the physiology of *Escherichia coli* have taken advantage of strains that are incapable of accumulating cyclic AMP (cAMP). Brickman et al. (3) described three spontaneous deletions in the adenylate cyclase gene (*cya*). One of these mutations (Δ*cya-854*) has become the most widely used Δ*cya* background. Recently, Glaser et al. (7) have characterized the Δ*cya-854* mutation as a 200-bp frameshift deletion. This study was designed to characterize the remaining two *cya* deletions described by Brickman et al. and to design a *cya* total deletion strain.

(A preliminary report of some of these findings has been presented as an abstract [18].)

**Characterization of adenylate cyclase deletions.** Adenylate cyclase activity in toluene-treated cells (11) of the parent strain CA8000 (3) was compared with those of cells of the Δ*cya-201* (strain CA8300) and Δ*cya-283* (strain CA8303) deletion strains. Although there was easily detectable activity in the wild-type cells, there was no detectable activity in either of the deletion strains. The lack of adenylate cyclase activity in the deletion strains might be due either to the absence of the protein, resulting from a premature translation termination or instability of the protein product, or, alternatively, to the accumulation of an inactive protein. The analyses described below were performed to evaluate these possibilities.

Polymerase chain reaction (PCR) analysis of Δ*cya-283* compared with that of the wild-type DNA indicates clearly that the deletion is located somewhere between bases 901 and 1320 (Fig. 1A, lanes 4 and 5) and that the size of the deletion is approximately 80 bp. Similarly, PCR analysis of Δ*cya-201* compared with that of the wild-type DNA shows that the deletion is located in the same fragment (Fig. 1B, lanes 4 and 5) and that the deletion size is approximately 50 bp.

A precise analysis of the locations of Δ*cya-283* and Δ*cya-201* was made by sequencing asymmetric PCR products (Fig. 2). Δ*cya-283* was found to span the region from bases 1002 to 1076 (numbering as described in reference 1). This deletion is therefore a 75-bp in-frame deletion and might be expected to produce a truncated gene product. Δ*cya-201* was found to cover the region from bases 1219 to 1259; this corresponds to a 41-bp deletion. Since this deletion is out of frame, it is likely that premature translation termination would result. Inspection of the *cya* sequence (1) indicates that there is a stop codon (TGA) in the shifted reading frame beginning at nucleotide 1332, which might result in a translation product of approximately 22 kDa. The sequence of Δ*cya-854*, which has previously been determined (7), is also shown in Fig. 2. It is of interest that both Δ*cya-283* and Δ*cya-201* overlap the region of Δ*cya-854*. This is consistent with the observations of Brickman et al. (3) that while Δ*cya-283* and Δ*cya-201* could recombine with each other, neither of them could recombine with Δ*cya-854*. It should be noted that the location of Δ*cya-854* suggests that translation might terminate at nucleotide 1332, as does Δ*cya-201*. It has been suggested (7) that translation of Δ*cya-854* might reinitiate at one of the two ATG codons (underscored in Fig. 2) downstream from the deletion. It is conceivable that the second ATG codon might also be a reinitiation site for Δ*cya-201*. Experiments described in the following section support the idea that only the first ATG codon allows a reinitiation.

Sequence repeats were observed in the regions of the termini of Δ*cya-283* (CTGGG) and Δ*cya-201* (GCCGTGCG) (Fig. 2, boxed sequences). Figure 3 outlines a mechanism involving these sequence repeats for the formation of these deletions similar to that previously proposed for the formation of spontaneous deletions in the *lacI* gene (6).

**Expression of *cya* deletions as fusions.** Attempts to hyperexpress the protein products of the Δ*cya-283* and Δ*cya-201* genes under the control of the $\lambda p_L$ promoter (15) were unsuccessful. Therefore, *cya-lacZ* fusions, constructed at the unique *Hpa*I-*Sal*I sites as described previously (5), were placed under the control of the $\lambda p_L$ promoter (Fig. 4A). A *lon* mutant (8) previously transformed with pRK248 (carrying the temperature-sensitive *c*I gene [2]) was transformed with the resultant plasmids, and the transformants were assayed for β-galactosidase activity before (30°C) and after (39°C) heat induction. As expected, there was substantial β-galactosidase activity dependent on heat induction in the wild-type *cya-lacZ* fusion (Fig. 4B). There was also high activity observed in the Δ*cya-283–lacZ* fusion, consistent with the expectation of an in-frame deletion in Δ*cya-283*.

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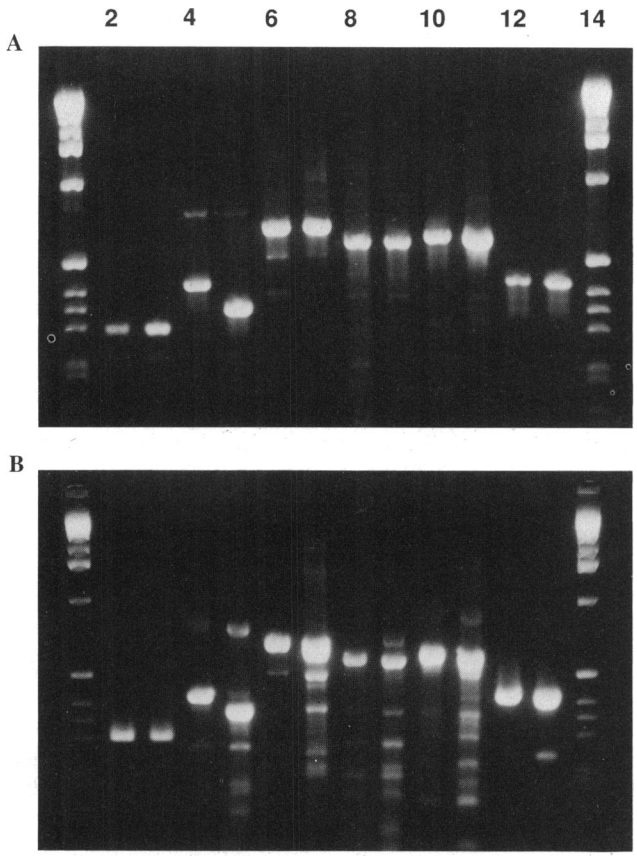


FIG. 1. Location by PCR of Δ*cya-283* (A) and Δ*cya-201* (B) in the *cya* gene. With chromosomal DNA from strain CA8000 (panels A and B, lanes 2, 4, 6, 8, 10, and 12) as a control and strains CA8303 (Δ*cya-283*) (panel A, lanes 3, 5, 7, 9, 11, and 13) and CA8300 (Δ*cya-201*) (panel B, lanes 3, 5, 7, 9, 11, and 13) as templates, the PCR was carried out with a Perkin-Elmer Gene Amp kit. The *cya*-specific primers used in the PCR covered the following regions: bases 652 to 950 (lanes 2 and 3), bases 901 to 1320 (lanes 4 and 5), bases 1282 to 1950 (lanes 6 and 7), bases 1912 to 2499 (lanes 8 and 9), bases 2452 to 3060 (lanes 10 and 11), and bases 2802 to 3230 (lanes 12 and 13). Lanes 1 and 4 contain molecular weight markers (GIBCO BRL, 1-kb ladder). The PCR reaction mixtures were analyzed by agarose gel electrophoresis (3% Nusieve [FMC] plus 1% Seakem [FMC] in 10 mM Tris-borate buffer [pH 8.0] containing 1 mM EDTA).

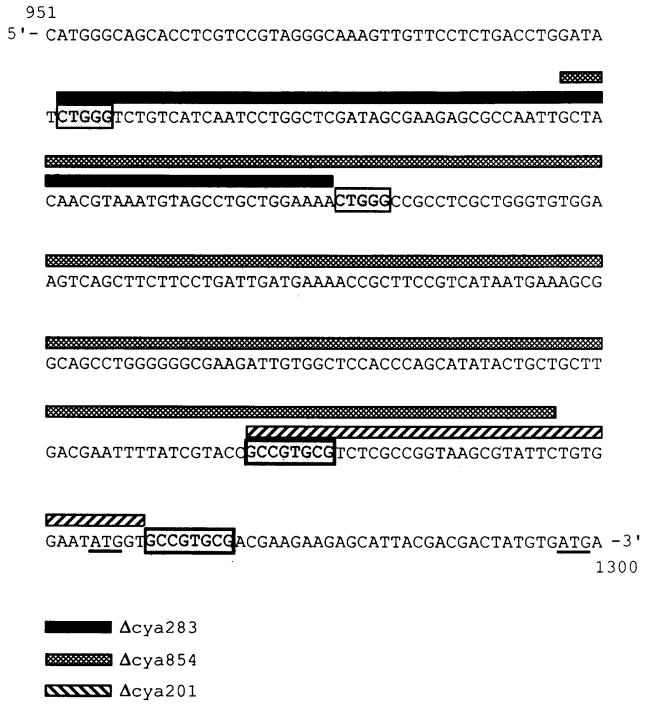


FIG. 2. Nucleotide sequence of the *cya* region showing the localization of Δ*cya-283* and Δ*cya-201*. The boxed sequences show sequence repeats at the endpoints of both of the deletions. Δ*cya-854*, which overlaps the other two deletions, and two ATG codons (underscored) as the possible sites for new translation starts (7) are also shown. The single-stranded PCR products used for sequencing reactions were made by an asymmetric PCR protocol as described previously (12). The chromosomal DNAs of strains CA8303 (Δ*cya-283*) and CA8300 (Δ*cya-201*) were used as templates. DNA was sequenced in both directions. Sequencing reactions with the limiting primer were carried out with Sequenase (U.S. Biochemicals) by the chain termination method of Sanger et al. (17).

There was no observable activity in the Δ*cya-201–lacZ* fusion. The data in Fig. 4B were supported by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic analysis of cell extracts after heat induction. The expected products were observed for the wild-type *cya-lacZ* fusion (175 kDa) and the Δ*cya-283–lacZ* fusion (172 kDa), but no expected 140-kDa product was observed for the Δ*cya-201–lacZ* fusion. The most likely interpretation of these findings is that there is a premature translation termination and no reinitiation at a downstream ATG in the Δ*cya-201–lacZ* fusion (Fig. 2). Because Glaser et al. (7) observed the Δ*cya-854–lacZ* fusion product of 140 kDa, it appears that the reinitiation they observed probably occurs at the first of the two suggested ATG reinitiation codons (Fig. 2).

It had previously been demonstrated by Danchin et al. (5) that the wild-type *cya-lacZ* fusion had not only β-galactosidase activity but also adenylate cyclase activity. Since the data in Fig. 4B showed that the combination of a *lon* mutant background and *lacZ* fusion allowed the expression of a stabilized Δ*cya-283* gene product, it was possible to pose the question of whether the deletion of 25 amino acids characteristic of that deletion affected catalytic activity. Assays for adenylate cyclase activity indicated that the Δ*cya-283–lacZ* fusion is devoid of adenylate cyclase activity. It is therefore reasonable to conclude that the *cya* phenotype of CA8303 is due to a combination of the expression of a protein with no adenylate cyclase activity and the rapid turnover of the protein.

**Construction of total deletion of *cya* structural gene.** Because Δ*cya-854* might synthesize the adenylate cyclase C terminus (7) and because Δ*cya-283* is an in-frame deletion and Δ*cya-201* is only a 41-bp deletion, the construction of a total chromosomal *cya* deletion was undertaken. The kanamycin resistance gene (*Hin*dIII-*Bam*HI fragment) derived from pKC7 (14) was placed into the plasmid pDIA100 (16) to replace most of the *cya* structural gene sequence. The construct was further modified to remove the remaining structural sequences (the product was designated pSAK3). A fragment containing the sequences that border the *cya* structural gene fused to a kanamycin resistance gene was excised from this plasmid and inserted into pMAK705 (10) (a plasmid with a temperature-sensitive replication origin and a chloramphenicol resistance gene) to yield pSAK4. The plasmid was used to make a total deletion in the chromosome of

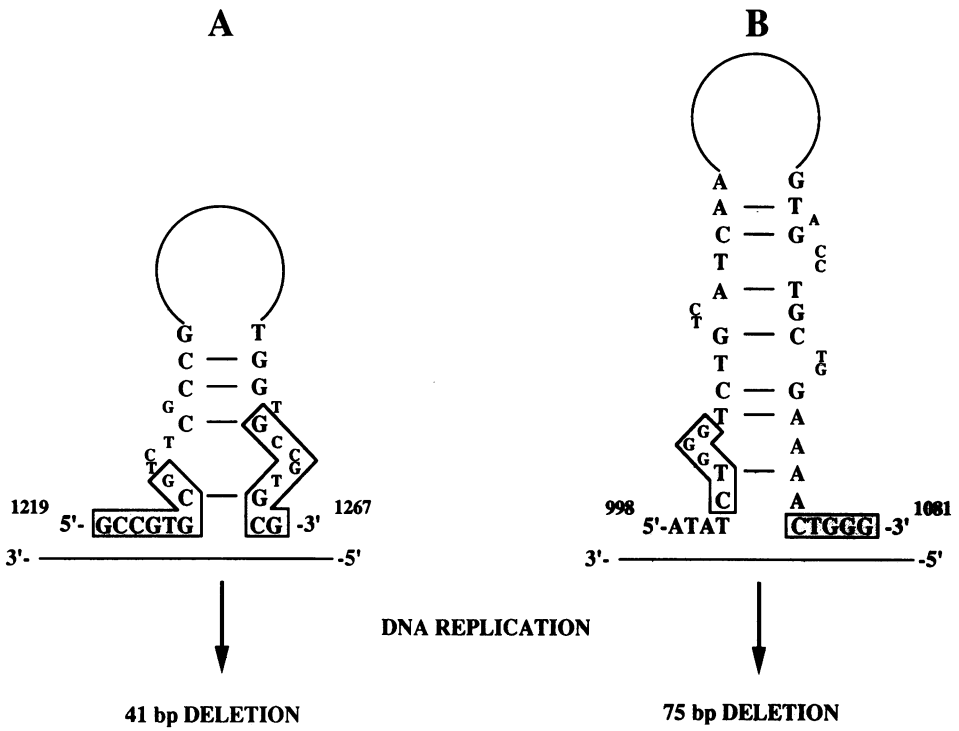


FIG. 3. Proposed model for the looping-out mechanism explaining the involvement of sequence repeats in the formation of spontaneous *cya* deletions. (A) Δ*cya-201*; (B) Δ*cya-283*. Bases in the designated regions are proposed to form loops that juxtapose with another DNA strand to promote, during replication, the indicated deletions. The sequence repeats bordering the deletions are boxed.

*E. coli* MC1061 (4) by the procedure of Hamilton et al. (10). The data in Fig. 5 are the results of an extensive PCR analysis of strain SP793 (presumptive *cya* deletion) compared with that of strain MC1061 (wild-type parent). The results obtained are fully in accord with the expectations if strain SP793 contains a total deletion of the *cya* structural gene. Further evidence that the strain is a chromosomal deletion is that it is sensitive to chloramphenicol (indicating the absence of the modified pMAK705), that it grows at 42°C in the presence of kanamycin (indicating that the kanamycin gene is inserted into the chromosome), and that it is devoid of adenylate cyclase activity.

Growth rates in minimal salts medium (19) supplemented with glucose (0.5%) and with isoleucine, leucine, and valine

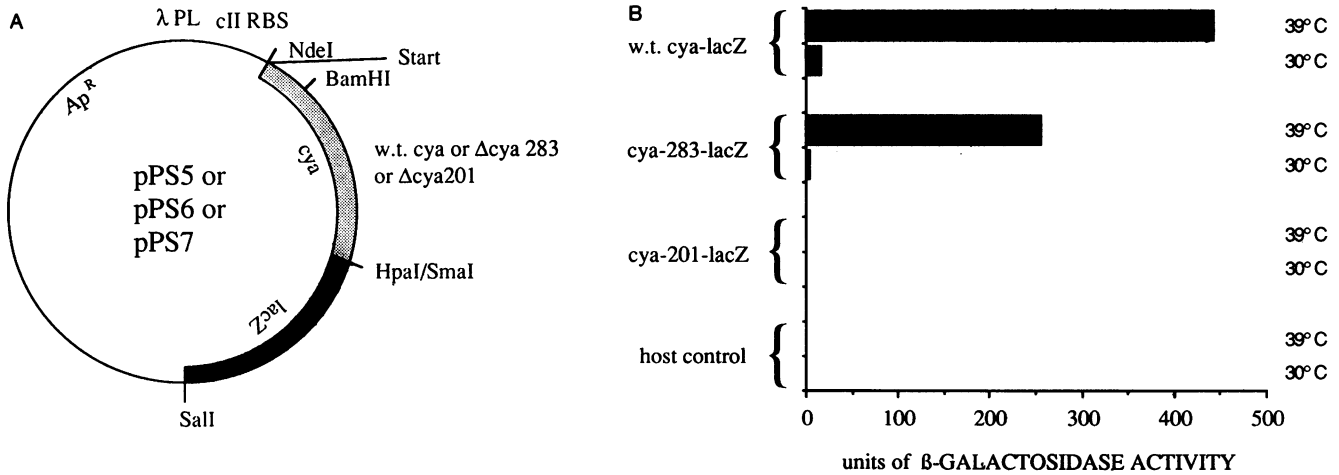


FIG. 4. *cya-lacZ* fusion hyperexpression plasmid vectors. (A) Construction. The *cya HpaI-SalI* restriction fragments of constructs containing the wild-type (w.t.) or mutant *cya* genes were replaced with *SmaI-SalI lacZ* fragments of pMC1871 to make the *cya-lacZ* hyperexpression vectors pPS5 (wild-type *cya-lacZ*), pPS6 (Δ*cya-283–lacZ*), and pPS7 (Δ*cya-201–lacZ*). RBS, ribosome binding site. (B) Expression of β-galactosidase activity in *cya-lacZ* fusions. The cells were grown and heat induced as described previously (15). The cells were harvested by centrifugation at 3,000 × *g* for 10 min, washed in Z buffer (13), and resuspended in the same buffer. The cells were then treated with 2 drops of chloroform and 1 drop of 1% SDS and used to measure β-galactosidase activity, expressed in Miller units (13).

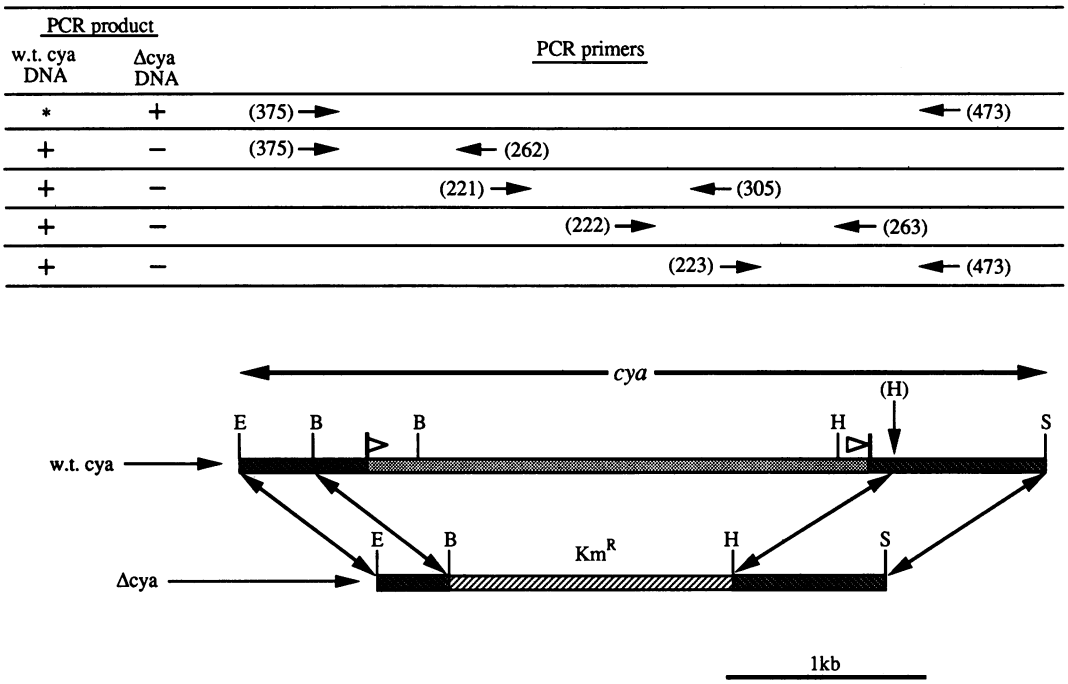


FIG. 5. Demonstration of *cya* (total) deletion in strain SP793 by PCR. PCRs were carried out with boiled cell suspensions (9) (2 min in boiling water bath) of strains MC1061 (wild-type [w.t.] *cya* control) and SP793 (Δ*cya* [total]). Five different sets of primers, covering specific *cya* regions, were as follows: primers 375 and 473, *cya* bases 301 to 3654; primers 375 and 262, *cya* bases 301 to 1320; primers 221 and 305, *cya* bases 1282 to 2499; primers 222 and 263, *cya* bases 1912 to 3230; and primers 223 and 473, *cya* bases 2452 to 3654. Restriction enzymes: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; and (H), newly created *Hin*dIII site. ▷, translation start; ⊳|, translation stop. *, under the conditions of our experimental protocol, it is not possible to generate the expected ~3.3-kb PCR product by using these primers. Electrophoretic analysis of PCR products was carried out with 0.8% agarose in 10 mM Tris-borate buffer (pH 8.0) containing 1 mM EDTA.

(each at 50 μg/ml) in the presence and absence of cAMP (5 mM) were determined for strains SP793 and MC1061 and compared with those for CA8000 (wild-type) and CA8306 (Δ*cya-854*) cultured in minimal salts medium supplemented with glucose and with thiamine (5 μg/ml) and methionine (50 μg/ml). As expected, growth of the two wild-type strains (MC1061 and CA8000) was inhibited by cAMP. But while CA8306 (3) showed the expected growth stimulation by cAMP, growth of SP793 was inhibited by the cyclic nucleotide. In order to test the possibility that the MC1061 strain background or nutritional supplements rather than the creation of the total *cya* deletion was the explanation for the unexpected growth inhibition observed in SP793, the *cya* total deletion from strain SP793 was transduced into the CA8000 strain background by P1 transduction (13). The resultant strain (SP850) was checked for the expected properties by PCR and adenylate cyclase assays. This strain behaved identically to strain CA8306 in showing a cAMP-dependent increase in growth rate. Strain CA8000 had doubling times of 67 min (absence of cAMP) and 79 min (presence of cAMP). Strain CA8306 had doubling times of 115 min (absence of cAMP) and 77 min (presence of cAMP). Strain SP850 had doubling times of 113 min (absence of cAMP) and 84 min (presence of cAMP).

Strain SP850 therefore shows all the properties expected for a total *cya* deletion and is recommended for use as a clean *cya* deletion strain. Strain SP850 has been deposited in the collection of the *E. coli* Genetic Stock Center.

We thank Edith Brickman for providing strains with the *cya-201*, *cya-283*, and *cya-854* deletions and for her very useful advice concerning these deletions. A highly transducing derivative of P1 (P1*c*1.100 *dam rev*6) was kindly provided by Nat Sternberg via Michael Yarmolinsky.

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