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Identification of *greA* Encoding a Transcriptional Elongation Factor as a Member of the *carA-orf-carB-greA* Operon in *Pseudomonas aeruginosa* PAO1

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A homolog of the transcriptional elongation factor, GreA, was identified in *Pseudomonas aeruginosa* PAO1. The deduced amino acid sequence for GreA from this organism exhibits 65.2% identity to its counterpart in *Escherichia coli* K-12. The nucleotide sequence of *greA* from *P. aeruginosa* overlaps by four bases the 3′ terminus of *carB* which encodes the large subunit of carbamoylphosphate synthetase. S1 nuclease experiments showed that level of the *greA* transcript is elevated approximately 10-fold under conditions of pyrimidine limitation, consistent with the conclusion that transcription is initiated from the previously identified pyrimidine-sensitive promoter upstream of the *carA-orf-carB-greA* operon. Transcriptional fusion experiments showed the presence of an additional weak promoter within the *carB* sequence. A *greA* insertional mutant of *Pseudomonas aerugionsa* was constructed by gene replacement. The mutant derivative grew well in rich medium but did not grow in minimal medium supplemented by arginine and nucleosides. The *greA* phenotype was suppressed by secondary mutations at a relatively high rate, consistent with the notion of an important physiological role for GreA.

Recent reports by Borukhov et al. (2, 3) demonstrated that the transcription elongation factors GreA and GreB of *Escherichia coli* induce the endonucleolytic cleavage that occurs at the 3′ ends of arrested ternary transcription complexes. This cleavage reaction, which was first observed by Surratt et al. (24), permits the resumption of elongation from the newly generated 3′ end if nucleotides are present. It now appears that the RNA polymerase of *E. coli* possesses an intrinsic transcript cleavage activity which is enhanced by the Gre proteins (16).

We have previously reported that the *carA-orf-carB* operon, encoding carbamoylphosphate synthetase of *Pseudomonas aeruginosa*, is transcribed from a single promoter upstream of *carA* and that this promoter is controlled by pyrimidines and arginine (10). In the present paper, we report the finding that the coding sequence of a homolog of *greA* of *E. coli* (20) overlaps the 3′ terminus of *carB*. The results presented here indicate that this *greA* homolog is expressed as part of a larger transcript, *carA-orf-carB-greA*, as well as from a weak promoter within the *carB* coding sequence. In the present paper, we also report the construction and the phenotype of a derivative of *P. aeruginosa* in which *greA* is inactivated by gene replacement.

Nucleotide sequence of *greA* and downstream region. Following determination of the nucleotide sequence (13, 18) of *carB* and its flanking downstream region on pKA1 (Fig. 1), a *carAB* clone of *P. aeruginosa* PAO1 (10), analysis revealed the presence of an open reading frame (ORF) that overlapped by four bases the coding sequence of *carB*. A data bank search showed that this ORF is highly homologous to the GreA protein of *E. coli* (20). Since the chromosomal insert on pKA1 contained only part of the ORF sequence, the complete nucleotide sequence of this ORF and its flanking regions (Fig. 2) was then determined from another construct, pKA96, which has an additional 1.2 kb. A comparison of the derived amino acid sequence of this ORF with the GreA proteins from other organisms is shown in Fig. 3; it exhibits 65.2% identity and 76.6% similarity to GreA of *E. coli*. Further, the derived sequence consists of 158 amino acid residues, as does GreA of *E. coli* (20).

The *greA* homolog of *P. aeruginosa* is preceded by a putative ribosome binding site (AGGA) complementary to the 3′ end of the 16S rRNA of *P. aeruginosa*, 3′-AUUCCUCU (6). A potential RNA stem-loop structure with very high free energy ($\Delta G = -46.2$ kcal/mol) was found immediately after the UAA stop codon of *greA* (Fig. 2). The DNA sequences found downstream of *greA* in both *E. coli* (GenBank accession no. U18997) and *P. aeruginosa* (Fig. 2) have similar features. In both cases, an ORF transcribed in the opposite direction is found following *greA*. The deduced amino acid sequences for the two ORFs in *P. aeruginosa* (104 residues) and *E. coli* (97 residues) share 43.3% identity and 65% similarity. Furthermore, in both organisms, these ORFs are followed by *ftsJ*, which is transcribed in the same direction as *greA*. The *ftsJ* gene of *P. aeruginosa* on pKA96 is truncated by the cloning site; the deduced partial sequence for FtsJ of *P. aeruginosa* exhibits 53.8% identity and 68% similarity with its counterpart in *E. coli*. Aside from these similarities, it is important to note that *greA* of *E. coli* is considered a single-gene operon (20, 21). Furthermore, its chromosomal location in this organism (21) is about 69 min away from that of the *carAB* operon.

Regulation of *greA* transcripts. Earlier work from this laboratory (10) established that the *carA-orf-carB* operon is transcribed from a single promoter upstream of *carA* and that this promoter is derepressed nine- and twofold by the limitation of pyrimidines and arginine, respectively. The overlap of the nucleotide sequences of *greA* and *carB* as well as the absence of a rho-independent terminator structure following *carB* suggested that *greA* might be also transcribed as part of this large transcript.

Quantitative S1 experiments (8) were carried out with RNA extracted from cultures (12) grown under conditions of pyrimidine limitation or excess. For pyrimidine limitation conditions, RNA samples were isolated from a pyrimidine-auxotrophic mutant, PAO483 (*pyrE70*) (9), grown in citrate-containing minimal medium (22) supplemented with 0.1 mM cytosine. Under these conditions, the specific growth rate is one-third of

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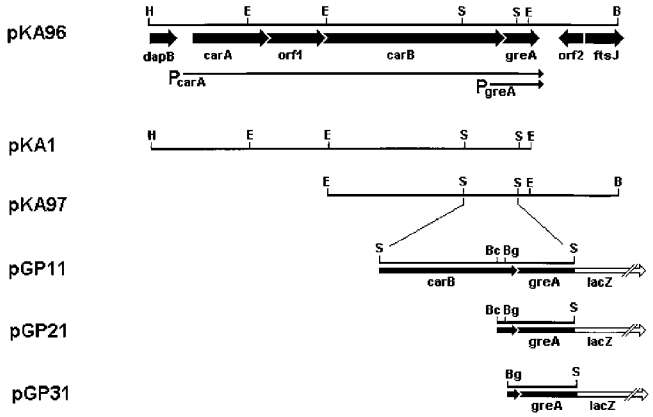

FIG. 1. Schematic representation of the *carA-orf-carB-greA* operon and the construction of *greA*::*lacZ* promoter fusions. Depicted are restriction sites used to form the different plasmids as described in the text. Two different transcripts for *greA* expression are indicated by arrows following P_{carA} and P_{greA}. H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; S, *Sal*I; Bc, *Bcl*I.

that with excess pyrimidine (10). For conditions of pyrimidine excess, the medium was supplemented with 1 mM uracil.

For preparation of the probe, the *Bcl*I/*Sal*I fragment of pGP21 (Fig. 1) was cloned into *Bam*HI and *Sal*I sites of M13mp18 (25), and the single-stranded DNA produced by this construct was used as a template for primer extension from a ^{32}P-labeled oligonucleotide with a sequence complementary to nucleotides (nt) 324 to 344 of Fig. 2. The extended single-stranded product was isolated by agarose gel electrophoresis after endonuclease *Eco*RI digestion. The resulting probe has a sequence complementary to nt 7 to 344 (Fig. 2) plus the sequence derived from the multiple cloning site of the vector at the 3′ end. The results (Fig. 4) show a major cluster of consecutive bands, with the most distal site corresponding to the 5′-terminal nucleotide of the single-stranded probe used (nt 7 [Fig. 2]). Pyrimidine limitation results in significant elevation (approximately 10-fold) of the level of the protected consecutive bands. These results are consistent with the conclusion that this transcript is initiated at the previously identified pyrimidine-sensitive promoter (10). No transcript corresponding specifically to *greA* was detected in these experiments.

While no *greA*-specific transcript was detected in the S1 experiments, studies with *lacZ* fusions in *P. aeruginosa* indicated the possible presence of an independent weak promoter upstream of *greA*. Three *greA*::*lacZ* transcriptional fusions (pGP11, pGP21, and pGP31 [Fig. 1]) were constructed by employing the low-copy-number plasmid pQF50 (5) and were introduced into the pyrimidine-auxotrophic derivative of *P. aeruginosa* (PAO483) by transformation. Screening of the transformants on Luria-Bertani (LB) plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) indicated that both pGP11 and pGP21, which carry 938 and 187 nt of the 3′-terminal region for *carB*, exhibit promoter activity. In contrast, pGP31, which has a shorter segment of the 3′-terminal region of *carB*, does not exhibit promoter function. Measurements of β-galactosidase activity (14) in *P. aeruginosa* carrying either pGP11 or pGP21 showed that the level of activity from both constructs was relatively low (about 100 Miller units on average). This level was not affected by pyrimidine availability in citrate-containing minimal medium or by growth in LB medium. Due to the constitutively low expression from this putative promoter, we were not able to assign a corresponding transcriptional start site from several weak signals observed in S1 experiments after longer exposure time (data not shown).

Construction and characterization of a *greA* insertion mutant. A 1.6-kb gentamicin resistance cassette (GmΩ) was isolated from plasmid pGMΩ1 (19) by agarose gel electrophoresis and cloned into the *Eco*RI site within *greA* on plasmid pKA97 (Fig. 1). The *greA*::GmΩ region on the resulting plasmid was released by digestion with *Kpn*I and subcloned into the *Kpn*I site of a conjugation vector, pRTP1-M. This vector was derived from pRTP1 (23) by ligation of the multiple cloning site of pUC19 into the *Eco*RI and *Hin*dIII sites of pRTP1. The resulting gene replacement plasmid was mobilized into a spontaneous streptomycin-resistant *P. aeruginosa* strain (PAO1-Sm) by biparental plate mating with *E. coli* SM10 as described by Gambello and Iglewski (7). Following incubation at 37°C for

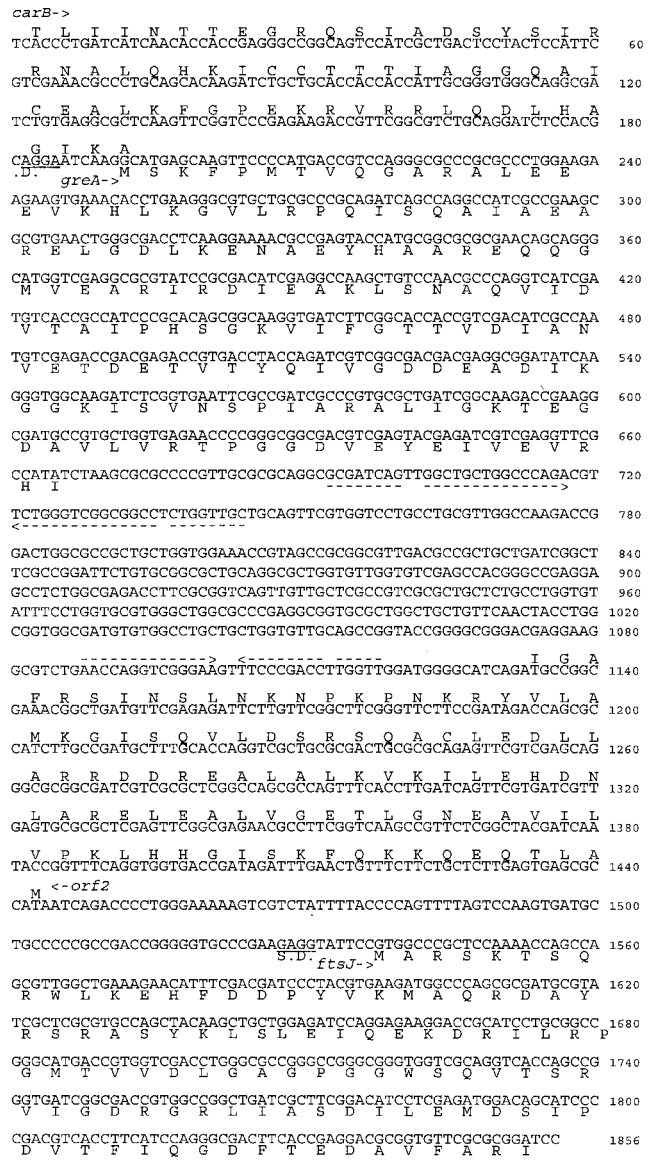

FIG. 2. Nucleotide sequence of the *greA* gene and flanking regions from *P. aeruginosa* PAO1. The deduced amino acid sequences are indicated by one-letter symbols. Also shown is the Shine-Dalgarno (S.D.) sequence for *greA* and *ftsJ*. Converging arrows define regions of dyad symmetry at the end of *greA* and *orf2* genes and may form stem-loop structures within the transcribed RNA.

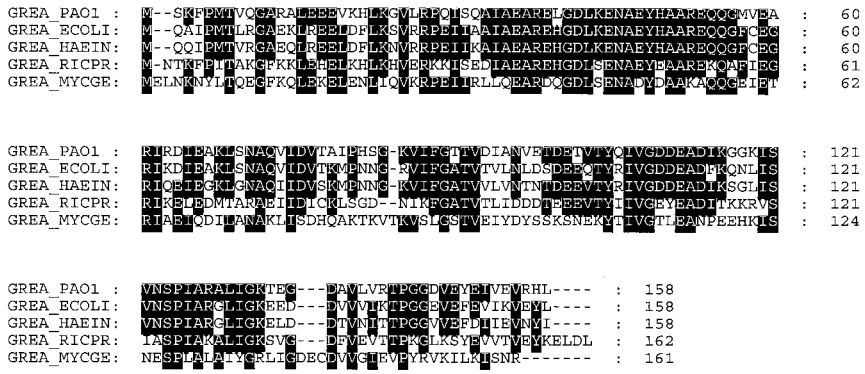

FIG. 3. Comparison of the derived amino acid sequences for *greA* from *P. aeruginosa* (PAO1), *E. coli* (ECOLI), *Haemophilus influenzae* (HAEIN), *Rickettsia prowazekii* (RICPR), and *Mycoplasma genitalium* (MYCGE). Identical residues are shaded.

16 h, transconjugants were selected on LB plates supplemented with gentamicin (250 μg/ml) and streptomycin (500 μg/ml) to select for those organisms in which *greA* had been replaced by double-crossover events. Transconjugants were obtained with a frequency comparable to that for null mutations introduced by the same protocol in this laboratory in other genes of *P. aeruginosa* ($\sim 2 \times 10^{-8}$/donor). The occurrence of gene replacement in one such transconjugant (PAO-G1) was confirmed by Southern blot analysis. The hybridization pattern (Fig. 5) shows a shift from 1.2 kb for the wild type to 2.8 kb for PAO-G1, confirming that *greA* in this isolate was replaced with the GmΩ-marked insertion.

The growth behavior of strain PAO-G1 and that of other similar isolates were examined on various media. Isolates carrying the inactivated *greA* gene grew well on LB plates but did not grow on citrate-containing minimal medium plates. They were also not able to grow in citrate-containing minimal medium supplemented with 1 mM uracil and hypoxanthine as sources for pyrimidines and purines, respectively. When approximately 10^6 cells of strain PAO-G1 were spread on a citrate-containing minimal medium plate, there were about 10 to 30 colonies on the plate after overnight incubation at 37°C. These prototrophic colonies were still resistant to both streptomycin and gentamicin, indicating that the prototrophic phenotype is the result of secondary mutations that suppressed the *greA* phenotype of PAO-G1. The observed rate of suppressor mutations in the *greA* derivative (1×10^{-5} to 3×10^{-5}) is significantly higher than that for the rate of spontaneous streptomycin resistance observed with the isogenic strain carrying the functional copy of *greA* (10^{-8} to 10^{-9}).

Recent reports from other laboratories have suggested two possible functions for *greA*, namely, preventing the accumulation of arrested transcription complexes on the DNA (2, 15, 16) and increasing transcriptional fidelity by removal of misincorporated nucleotides (1, 4, 16). These proposed roles for GreA provide a possible explanation for the inability of the *greA* derivative to grow in minimal medium; these effects could result in the reduced activity of essential rate-limiting biosynthetic enzymes that are not required in rich medium. While mutations in *greA* of *E. coli* have been reported (16, 21), whether such mutants can grow in minimal medium has not been indicated. Recently, Orlova et al. (16) reported that a *greA greB* double-mutation strain of *E. coli* fails to grow at a high temperature (43°C) in complex medium, whereas neither single mutant shows this effect. No temperature sensitivity was observed in this work for the *greA* insertion of *P. aeruginosa*.

While no experiments on regulation of the *greA* operon of *E. coli* have been reported, analysis of its nucleotide sequence led Sparkowski and Das (20) to suggest that this operon is controlled by an attenuation-type mechanism. The results reported here for *P. aeruginosa* show that *greA* in this organism is expressed primarily as part of a larger operon, *carA-orf-carB-greA*. We have previously shown (10) that pyrimidine control of the single promoter upstream of *carA* in this organism is mediated by an attenuation-type mechanism (11, 17). The higher level of *greA* expression under conditions of pyrimidine limitation reported here might be of physiological significance. When intracellular pyrimidine concentrations are low, the elongating RNA polymerase has a higher probability of being arrested on DNA templates with high pyrimidine content, such as those present in the leader sequences for operons controlled by pyrimidine-sensitive attenuation mechanisms (11). A higher

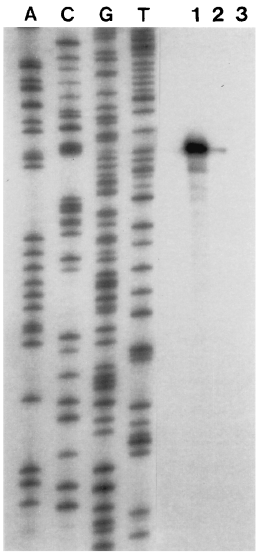

FIG. 4. S1 nuclease experiments with a pyrimidine-auxotrophic derivative of *P. aeruginosa*. A single-stranded DNA probe with sequence complementarity to nt 7 to 344 in Fig. 2 was labeled at the 5′ end with ^{32}P as described in the text. After hybridization to cellular RNA, the hybridization mixture was treated with S1 nuclease and was analyzed on a 6% sequencing gel. The dideoxy sequence ladder was derived by using the primer that was used to generate the probe for the S1 nuclease experiments. Lane 1, PAO483 grown in the presence of 0.1 mM cytosine (limiting pyrimidine); lane 2, PAO483 grown in the presence of 1 mM uracil (excess pyrimidine); lane 3, yeast tRNA (negative control). The same amount of RNA was used in these experiments.

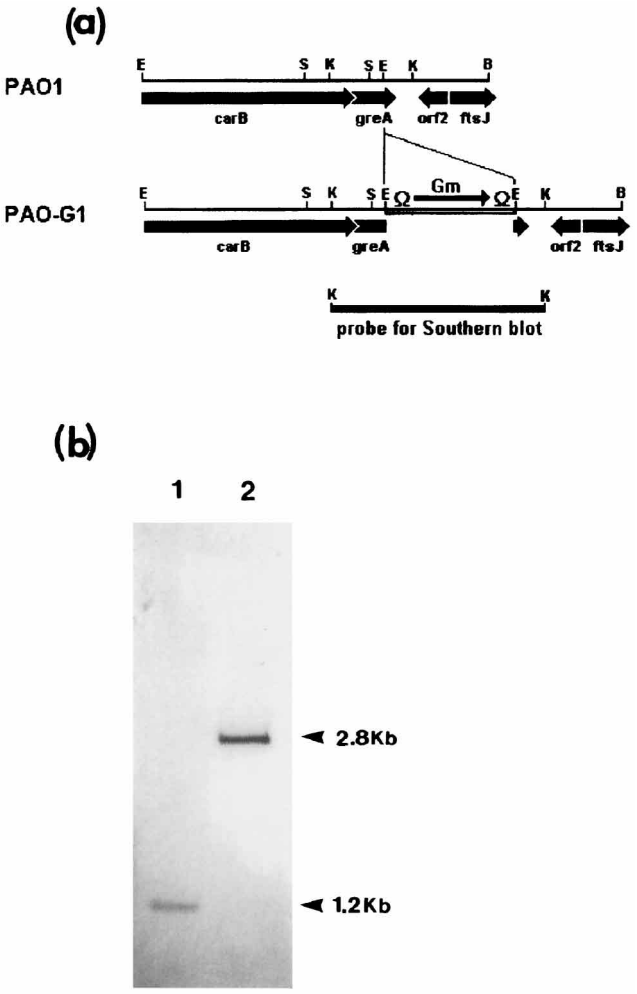

FIG. 5. (a) Schematic representation of gene arrangement on the chromosomes of *P. aeruginosa* PAO1 and PAO-G1. Filled arrows indicate the coding region of genes in this region. A gentamicin cassette with omega loops was inserted to inactivate the *greA* gene in PAO-G1 as described in the text. Also indicated is the probe for Southern hybridization. (b) Southern hybridization analysis of the *greA* locus in *P. aeruginosa* strains. Chromosomal DNAs were digested with *Kpn*I, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and probed with the non-radioactivity-labeled 2.8-kb fragment shown in panel a. Lane 1, PAO1 (wild type); lane 2, PAO-G1 (*greA*).

level of GreA under these conditions could be helpful in keeping arrested transcription complexes to a minimum.

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