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NOTES

Cross-Species Complementation of the Indispensable *Escherichia coli era* Gene Highlights Amino Acid Regions Essential for Activity

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Era is an essential GTP binding protein in *Escherichia coli*. Two homologs of this protein, Sgp from *Streptococcus mutans* and Era from *Coxiella burnetii*, can substitute for the essential function of Era in *E. coli*. Site-specific and randomly generated Era mutants which may indicate regions of the protein that are of functional importance are described.

The *era* (*Escherichia coli ras*-like) gene encodes an essential 34-kDa GTP binding protein (1, 6, 9, 15). It occurs at 55.5 min on the *E. coli* chromosome as the second gene of the tripartite *rnc* operon, which consists of *rnc* (encoding RNase III [5, 8, 15]) upstream and *recO* downstream of *era* (10, 15). Similar to other GTPases (3), Era contains conserved amino acid motifs which facilitate GTP binding and hydrolysis (5, 9). Era is autophosphorylated at specific serine and threonine residues (14). However, despite various biochemical and genetic analyses, the function of Era in the cell remains elusive.

Recent work with *Streptococcus mutans* (a gram-positive bacterium responsible for causing human dental caries) has identified a gene, *sgp* (16), that encodes a protein in which 44.3% of the amino acids are identical to Era (Fig. 1). This gene appears to be part of an operon expressing a diacylglycerol kinase homolog and may be essential for growth (16). Another gram-positive bacterium, *Coxiella burnetii*, the causative agent of Q fever, has also been found to contain an Era homolog with 50.5% similarity to the *E. coli* Era (GenBank accession no. L27436). Like *E. coli*, *C. burnetii* appears to contain the *era* gene within an intact *rnc* operon.

Previous attempts to identify indispensable amino acid positions within the Era protein have been hampered by lack of information regarding functionally important regions outside the nucleotide-binding domain. The discovery of the Era homologs described above has made it possible to identify potentially important residues on the basis of conservation between these proteins. In the present work, we investigate whether non-*E. coli era*-like genes can substitute for the essential function of *era* within the *E. coli* context and describe several mutations of Era that were generated either by localized random PCR mutagenesis or by site-directed mutagenesis based on sequence comparison with the other Era homologs.

Sgp from *S. mutans* can supply essential Era function in *E. coli*. To determine whether Sgp from *S. mutans* can functionally replace Era in *E. coli*, plasmids pAC19era and pMCL21PBBsp expressing Era and Sgp, respectively, were used. The host vector pAC19E (13) served as a control. pAC19E contains the *lac* promoter-operator and the *lacZ* gene with multiple cloning sites from pUC19, the ColE1-compatible origin p15A, and the chloramphenicol resistance gene from pACYC184 (New England Biolabs). The *era* gene from pJR302 (9) was introduced into pAC19E to obtain pAC19era. Plasmid pMCL21PBBsp contains the *sgp* coding region under control of a *lac* promoter, in a vector carrying the p15A origin and chloramphenicol resistance marker (also derived from pACYC184).

E. coli CL213(pXC001) [K-12 *era-1*::*kan recA56 ara* Δ(*lac-proAB*) *thi* ϕ*80dlacZ*ΔM15 F′ (*proAB-lacI*^{q})] (7) was used to screen for functional complementation. This strain is viable at low temperatures (30°C) because of the presence of the helper plasmid pXC001, which carries a wild-type copy of the *era* gene, confers ampicillin resistance, and has a pSC101 origin of replication that is temperature sensitive for replication. CL213 (pXC001), transformed with plasmids pAC19era, pMCL21 PBBsp, and pAC19E, was plated onto L broth agar plates containing chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml) and incubated at either 30 or 42°C. Growth of the CL213 strain at 42°C was rescued by the *sgp* gene with 100% efficiency when compared with that obtained with wild-type *era*. Transformants grown at 42°C had lost the parent *era*$^+$ plasmid and were found to be Amp^s (data not shown).

Similar results were obtained by complementing with the *C. burnetii* gene homologous to *era* (data not shown), indicating that both of the *era* homologs could supply the essential *era* function in *E. coli*.

Generation of mutations by site-specific PCR mutagenesis. On the basis of amino acid sequence alignment (Fig. 1) between Era and the two homologous proteins, four mutations, K32A (a K-to-A mutation at position 32), Q41A, W278A, and

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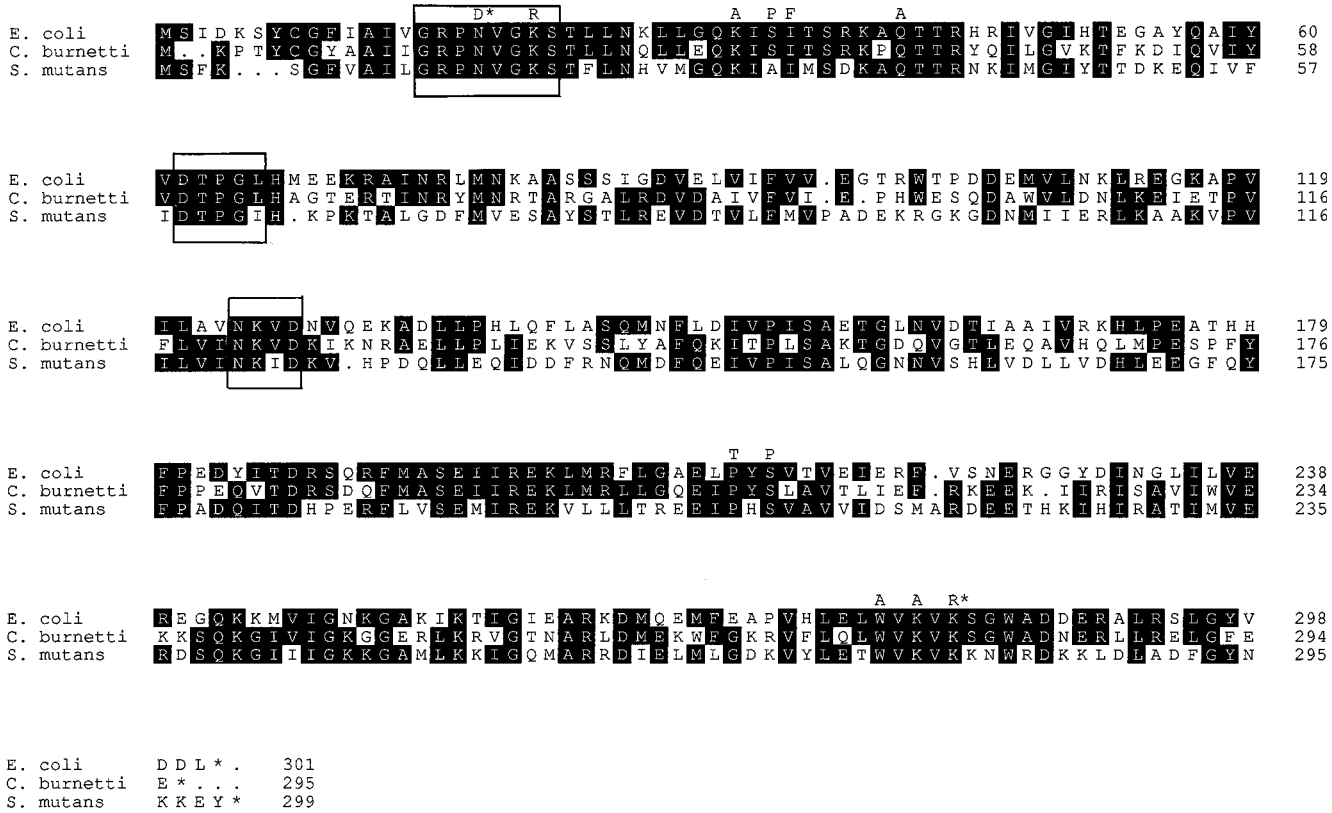

FIG. 1. Amino acid sequence alignment of Era from *E. coli* and its homologs from *S. mutans* and *C. burnetii*. Regions of identity are highlighted in black. Regions conserved in all GTP binding proteins are boxed. Amino acids shown above the sequences indicate the alterations found in the mutant proteins described in the text. The changes in the double mutant identified by random mutagenesis are indicated by an asterisk following the amino acid.

K280A, were constructed by site-specific PCR mutagenesis (2) in pJR302. Positions 32 and 41 are conserved residues within the predicted effector domain, while positions 278 and 280 are located within a highly conserved region near the carboxy terminus. Following confirmation of the desired nucleotide sequence alteration by DNA sequencing, complementation analysis was carried out with CL213(pIE-CAT:SB). In this case, the helper plasmid pIE-CAT:SB is similar to pXC001 except that it has a chloramphenicol resistance marker instead of an ampicillin resistance marker (7).

Sequence comparison (Fig. 1) of the three Era homologs shows that positions 278 and 280 are located in a region encompassing 11 consecutive identical residues shared between *C. burnetii* Era and *E. coli* Era and are nearly invariant in all three proteins. This particularly high degree of similarity may indicate conservation of a functionally important domain. However, when either W278A or K280A was introduced into CL213(pIE-CAT:SB) and the strain was plated on L broth plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml), we found that both mutations retained the ability to complement growth at 42°C. The same result was observed for mutations K32A and Q41A. All transformants grown at 42°C were found to be Cm^s (data not shown). One possible explanation for this could be that, although these residues are essential, the change to alanine is not sufficient to cause a detectable loss of function.

Isolation of Era mutants by localized random PCR mutagenesis. Since none of the mutations in the highly conserved regions described above seriously affected Era function, we next attempted to generate defective Era mutations by random PCR mutagenesis. For this purpose, the plasmid pCLKS+ ERA was constructed by subcloning the *era* gene from pJR302 into the pBluescriptKS$^+$ vector (Stratagene) containing a ColE1 origin and an ampicillin resistance marker. With pCLKS+ ERA as the template, a 1.1-kb sequence containing *era* was amplified by 25 cycles of PCR under standard reaction conditions with *Taq* DNA polymerase (Gene-Amp DNA Amplification reagent kit; Perkin-Elmer Cetus, Norwalk, Conn.). To enhance misincorporation activity during PCRs, dITP was added to final concentrations ranging from 0 to 400 μM. A library of PCR-mutagenized *era* genes in pBluescriptKS$^+$ was generated in this manner and introduced into *E. coli* CL213 (pIE-CAT:SB) to screen for nonfunctional mutations. Of a total of 1,146 transformants screened, 25 failed to rescue growth at 42°C. The plasmids encoding these nonfunctional mutations were introduced into SB221 (11), a wild-type *E. coli* strain, to examine protein expression. Seven candidates demonstrated inducible expression of Era, while the rest were apparently promoter site or nonsense mutations and did not express the protein. Of the seven mutations, one was found to express a truncated protein and was aborted. The remaining six candidates were sequenced, and five were found to contain point mutations while the sixth carried a double mutation. The alterations found were K21R, S34P, I35F, P211T, S213P, and the double mutation N18D, K282R (Fig. 1). Interestingly, all of the mutations occurred at residues that were absolutely conserved among the three Era proteins.

Previous work (14) that identified a tryptic peptide ISITSR from residues 33 to 38 as the site of autophosphorylation demonstrated that mutations T36A and S37A, at potential

phosphorylation sites, were by themselves insufficient to block function whereas the double mutant (T36A, S37A) became nonfunctional. Interestingly, however, the mutations S34P and I35F, although carrying only a single residue change, resulted in total loss of function. Serine at position 34 may therefore also be a potential phosphorylation site. The role of isoleucine 35 remains to be determined, although it seems quite clear that it is important for function and is highly conserved.

Sequence comparison of yeast ras proteins RAS1 and RAS2 (12) with each other and with mammalian H-ras shows that homology is predominantly restricted to the amino-terminal 180 amino acids, with little or no homology in the carboxy-terminal domain. On the other hand, sequence comparison of *E. coli* Era with the *C. burnetii* homolog shows 49.4% identity in the amino-terminal half of the protein (up to and including residue 180 of *E. coli* Era), which contains the regions conserved in all GTP binding proteins (shown in Fig. 1), and 52% identity in the carboxy-terminal domain (the last 121 residues of *E. coli* Era). The *E. coli* Era shows 43.3% identity with Sgp of *S. mutans* in the amino-terminal domain and 43% identity in the carboxy-terminal domain. Thus, homology between these three bacterial Eras appears to extend throughout the protein, much like the situation with the more highly conserved GTPases, such as translocation factors (4), which are functionally identical in all species. The conservation of sequence in the carboxy-terminal domain and the ability to complement in *E. coli* suggest that despite the large phylogenetic distance that separates gram-negative bacteria from gram-positive bacteria, Era responds to the same signal in all of these bacteria and is therefore interchangeable among these organisms. Identification of residues within this region that are critical for function is therefore important for gaining insights into the physiological function of Era. Mutations P211T and S213P have identified residues in the carboxy-terminal domain that were hitherto unknown to be important for function. Generation of more mutations within this region would provide a useful tool for investigating the role of Era in the cell.

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