Biochemical and molecular analyses of the C-terminal domain of Era GTPase from *Streptococcus pneumoniae*

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Era, an essential GTPase, is present in many bacteria and *Mycoplasma* spp. and appears to play a major role in the cell cycle and other cellular processes. To further understand its function, an *era* gene from *Streptococcus pneumoniae* was identified and cloned, and a mutant *era* gene with a deletion of 68 codons from its 3'-terminus was constructed. The truncated Era protein was then purified and characterized, and the ability of the truncated *era* gene to complement an *Escherichia coli* mutant strain defective in Era production was examined. Like the full-length Era protein, the truncated Era protein was able to bind and hydrolyse GTP, but its binding activity was increased twofold and its hydrolytic activity was reduced sevenfold when compared with those of the full-length Era protein. Unlike the full-length Era protein, the truncated Era protein lost its ability to bind to the *E. coli* cytoplasmic membrane. The full-length *era* gene was able to complement the *E. coli* mutant deficient in Era production when carried on pACYC184, while the truncated *era* gene failed to do so when carried on pACYC184, pBR322 or pUC18. The cellular amounts of the truncated Era and the full-length Era proteins in *E. coli* and *S. pneumoniae*, respectively, were then determined by Western blot analysis. In addition, the minimal amount of the *S. pneumoniae* Era protein needed for complementation of the *E. coli* mutant was also measured. Taken together, these results suggest that the C-terminus of the Era protein might be responsible for the binding of the protein to the cytoplasmic membrane and be essential for function.

Keywords: Era, membrane binding, GTPase, growth phase regulation, *Streptococcus pneumoniae*

INTRODUCTION

The *era* gene was first identified in *Escherichia coli*, and was thought to encode a RAS-like protein (Ahnn *et al.*, 1986). Later, detailed sequence analysis suggested that Era is not a RAS-like protein (Chen *et al.*, 1990). Recently, a human homologue of Era was reported that is distinct from RAS proteins (Britton *et al.*, 1998). Nevertheless, *era* is present in Gram-positive and Gram-

negative bacteria, and *Mycoplasma* spp. (Ahnn *et al.*, 1986; Fleischmann *et al.*, 1995; Fraser *et al.*, 1995; Kawabata *et al.*, 1997; Zuber *et al.*, 1990, 1997), and is essential for bacterial growth (Gollop & March, 1991b; Inada *et al.*, 1989; March *et al.*, 1988; Sato *et al.*, 1998; Takiff *et al.*, 1989, 1992). Era proteins are highly conserved among different bacteria and appear to share the same function as evidenced by the fact that *era* genes from quite diverse bacterial species are able to complement *E. coli* mutants (Pillutla *et al.*, 1995; Zuber *et al.*, 1990, 1997).

The bacterial Era proteins are a unique class of GTPases that bind GTP and GDP and hydrolyse GTP to GDP (March, 1992). The GTP binding and hydrolysis ac-

Abbreviations: Amp, ampicillin; Cam, chloramphenicol; GST, glutathione S-transferase; Tet, tetracycline.

The GenBank accession number for the nucleotide sequence of the *Streptococcus pneumoniae era* gene is AF07281.

tivities of Era appear to be essential for its function since mutations in the *era* gene of *E. coli* that reduce either or both activities are lethal (Inada *et al.*, 1989; Lerner *et al.*, 1992, 1995; Lerner & March, 1991). Point mutations in the C-terminal domain of the *E. coli* Era protein were isolated and these mutant *era* genes failed to complement *E. coli era* mutants (Inada *et al.*, 1989; Lerner *et al.*, 1995; Pillutla *et al.*, 1995). However, the biochemical basis for their failure to complement the *E. coli era* mutants remains unknown as biochemical characterization of these mutant Era proteins was not described (Lerner *et al.*, 1995; Pillutla *et al.*, 1995).

Genetic studies showed that depletion of the cellular amount of Era inhibited the growth of *E. coli* cells and caused the cells to elongate (Britton *et al.*, 1997, 1998; Gollop & March, 1991b; March *et al.*, 1988). Also, the viability of these cells was drastically reduced upon repression of Era production (Gollop & March, 1991b; Lerner & March, 1991; March *et al.*, 1988). Under these conditions, cell constriction or septum formation was not observed (Gollop & March, 1991b), suggesting that Era may play a role in cell cycle control. More recently, genetic studies also demonstrated that a mutation in the *E. coli era* gene suppressed temperature-sensitive mutations in the genes encoding chromosome replication and partitioning enzymes such as *dnaB*, *dnaG*, *gyrB*, *mukB*, *parB* and *parC*, but not those of the cell division genes *ftsA*, *ftsI* and *ftsZ* (Britton *et al.*, 1997, 1998). Some of the *era* mutant cells were found to contain two or four segregated nucleoids (Britton *et al.*, 1997, 1998). These studies suggest that Era plays a role in cell cycle progression at some point, probably after chromosome partitioning but before cytokinesis (Britton *et al.*, 1998).

Immunoelectron microscopic studies localized the *E. coli* Era protein on the internal side of the cytoplasmic membrane and also revealed that the Era protein exists in patches on the membrane (Gollop & March, 1991a). Later, biochemical studies confirmed that the *E. coli* Era protein is associated with the cytoplasmic membrane (Lin *et al.*, 1994). This association was GTP- or GDP-dependent and could be disrupted by salt treatment (Lin *et al.*, 1994). These studies led to the suggestions that potential binding sites exist on the membrane for the Era protein (Lin *et al.*, 1994). However, the significance of this membrane binding activity of Era and the part of Era that is responsible for this association remain unknown.

In this study, we report the biochemical and molecular characterizations of the full-length Era and C-terminally truncated Era (Δ Era) proteins from *Streptococcus pneumoniae*. The results of this study show that the Δ Era protein exhibited a reduced GTP-hydrolysis activity and an increased GTP-binding activity, and failed to bind to the *E. coli* cytoplasmic membrane. The truncated *era* (Δ era) gene was unable to complement an *E. coli* mutant defective in Era production (Takiff *et al.*, 1989, 1992). Together, these results suggest that the C-terminus of the Era protein is essential for the *in vivo* function of the protein.

METHODS

Materials. Alkaline-phosphatase-conjugated goat anti-rabbit monoclonal IgG, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, GTP, GDP, ampicillin (Amp), tetracycline (Tet), chloramphenicol (Cam), IPTG and BSA were purchased from Sigma. Prestained molecular mass standards for SDS-PAGE (myosin, 208 kDa; β -galactosidase, 116 kDa; BSA, 83 kDa; ovalbumin, 48.7 kDa; carbonic anhydrase, 33.4 kDa; soybean trypsin inhibitor, 28.2 kDa; lysozyme, 20.7 kDa; and aprotinin, 7.6 kDa) were obtained from Bio-Rad Laboratories. Chelating Sepharose Fast Flow resin and Glutathione Sepharose resin were obtained from Pharmacia LKB Biotechnology. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

Bacterial strains and culture conditions. The *E. coli* strains and plasmids used in this study are described in Table 1. *S. pneumoniae* (hex) R6 is an unencapsulated derivative of the Rockefeller University strain R36A that was kindly provided by Dr A. Tomasz (Rockefeller University).

Luria-Bertani (LB) medium capsules were purchased from Bio101. Brain Heart Infusion medium was purchased from Difco. Amp (100 μ g ml⁻¹), Cam (45 μ g ml⁻¹), Tet (15 μ g ml⁻¹) and IPTG were added to media as indicated in each experiment.

Cloning of the *era* gene from *S. pneumoniae* (hex) R6. To clone the *S. pneumoniae era* gene, we first searched our M13 *S. pneumoniae* sequence database (Baltz *et al.*, 1998; Rosteck *et al.*, 1997) using BLAST (Altschul *et al.*, 1990). The *era* gene was located; however, only a partial sequence was available. By comparing the *S. pneumoniae* DNA sequence to that of the *E. coli era* gene (Ahnn *et al.*, 1986; Chen *et al.*, 1990) we found that the available sequence was missing both the 5'- and 3'-terminal regions of the gene. An internal 500 bp fragment from the *S. pneumoniae era* gene was amplified using the primers 5' CGCGGATCCTCTAGATTCGCGAAGTGGA-CACTGTTC 3' and 5' CGCGGATCCAAACGTTCTGGA-TGATCTGTG 3', and used as a probe for Southern blot analysis of *S. pneumoniae* chromosomal DNA (Sambrook *et al.*, 1989). The amplified *era* probe hybridized to two *Clal* fragments with approximate sizes of 1.0 and 3.0 kb (data not shown). To obtain the missing 3'- and 5'-terminal sequences of the *era* gene, we employed inverse PCR technology using *Clal*-digested *S. pneumoniae* genomic DNA as template (DNA was diluted to $\approx 1 \mu$ g ml⁻¹) (Innis *et al.*, 1990). Primers were designed based on both sides of the sequences of the internal *Clal* site. To amplify the 1.0 kb *Clal* fragment that contained the missing 5'-terminal sequence of the *era* gene and the 3.0 kb *Clal* fragment that contained the missing 3' end of the gene, we used the following primers: 5' AAAGAACAGTGTCCTACTCGC 3' and 5' AAGGGGACGATATGATTATC 3'; and 5' GACAGACAAGGTTTCACATCCG 3' and 5' AACCTTCATCCAGATTTTTCAC 3', respectively. The PCR reaction mixtures contained ≈ 10 ng *Clal*-digested and circularized *S. pneumoniae* chromosomal DNA, 10 μ l 10 $\times$ PCR buffer containing MgCl₂ (Boehringer Mannheim), 8 μ l dNTPs (2.5 mM), 1 μ l of each primer (50 pmol), 2.5 U *Taq* DNA polymerase (Boehringer Mannheim) and 78.5 μ l H₂O (Innis *et al.*, 1990). The following PCR amplification conditions were employed: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and polymerization at 72 °C for 45 s for the 1 kb fragment and 90 s for the 3 kb fragment for a total of 25 cycles. For each *Clal* fragment amplified, five PCR reaction products were combined and sequenced directly to derive a consensus sequence. PCR primers were then designed based on the consensus sequence and used to amplify the *era* gene for cloning into *E.*

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype	Source
Strains		
<i>E. coli</i> BL21(DE3) pLysS	F ⁻ <i>dcm ompT hsdS</i> (<i>r</i> _B ⁻ <i>m</i> _B ⁻) <i>gal λ</i> (DE3) (pLysS, Cam ^r)	Stratagene
<i>E. coli</i> XL2-Blue	<i>rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F' <i>proAB lacI</i> ^a <i>ΔM15</i>) <i>Tn10</i> (Tet ^r)	Stratagene
<i>E. coli</i> HT120	W3110 <i>rnc-40::ΔTn10</i> (A40) (Tet ^r)	Takiff <i>et al.</i> (1989, 1992)
LY41	<i>E. coli</i> XL2-Blue/pLY41 (GST- <i>era</i> ⁺ , Amp ^r)	This study
LY42	<i>E. coli</i> BL21(DE3)pLysS/pLY42 (<i>era</i> ⁺ , Amp ^r)	This study
LY52	<i>E. coli</i> XL2-Blue/pLY52 [GST- <i>Δera</i> ⁺ (deleted <i>era</i>), Amp ^r]	This study
LY54	<i>E. coli</i> BL21 (DE3)pLysS/pLY54 (<i>Δera</i> ⁺ , Amp ^r)	This study
LY56	<i>E. coli</i> BL21(DE3)pLysS/pLY56 (His- <i>Δera</i> ⁺ , Amp ^r)	This study
Plasmids		
pSPN- <i>era</i>	<i>era</i> was cloned into pCZA342 at <i>Bam</i> HI site	This study
pLY41	<i>era</i> was cloned into pGEX-2T at <i>Nde</i> I/ <i>Bam</i> HI sites (GST- <i>era</i> ⁺ , Amp ^r)	This study
pLY42	<i>era</i> was cloned into pET-11a at <i>Nde</i> I/ <i>Bam</i> HI sites (<i>era</i> ⁺ , Amp ^r)	This study
pLY44	<i>era</i> was cloned into pET-16b at <i>Nde</i> I/ <i>Bam</i> HI sites (His- <i>era</i> ⁺ , Amp ^r)	This study
pLY52	The deleted <i>era</i> gene (<i>Δera</i>) was cloned into pGEX-2T at <i>Nde</i> I/ <i>Bam</i> HI sites (GST- <i>Δera</i> ⁺ , Amp ^r)	This study
pLY54	<i>Δera</i> was cloned into pET-11a (<i>Δera</i> ⁺ , Amp ^r)	This study
pLY56	<i>Δera</i> was cloned into pET-15b at <i>Nde</i> I- <i>Bam</i> HI sites (His- <i>Δera</i> ⁺ , Amp ^r)	This study
pLY58	<i>Δera</i> was cloned into pACYC184 at <i>Bam</i> HI site and transcribed from the opposite direction of the Tet resistance gene (Cam ^r)	This study
pLY60	<i>Δera</i> was cloned into pACYC184 at <i>Bam</i> HI site and transcribed from the promoter of the Tet resistance gene (Cam ^r)	This study
pLY62	<i>era</i> was cloned into pACYC184 at <i>Bam</i> HI site and transcribed from the promoter of the Tet resistance gene (Cam ^r)	This study
pLY64	<i>era</i> was cloned into pACYC184 at <i>Bam</i> HI site and transcribed from the opposite direction of the Tet resistance gene (Cam ^r)	This study
pLY75	<i>Δera</i> was cloned into pUC18 at <i>Bam</i> HI site and transcribed from P _{<i>lac</i>} (Amp ^r)	This study
pLY76	<i>Δera</i> was cloned into pBR322 at <i>Bam</i> HI site and transcribed from the promoter of the Tet resistance gene (Amp ^r)	This study
pCZA342	Apramycin resistance	P. Matsushima (pers. comm.)
pET-11a	Amp ^r	Novagen
pET-15b	Amp ^r	Novagen
pET-16b	Amp ^r	Novagen
pGEX-2T	Amp ^r	Pharmacia
pACYC184	Cam ^r Tet ^r	New England BioLabs
pBR322	Amp ^r Tet ^r	New England BioLabs
pUC18	Amp ^r	New England BioLabs

coli expression systems. The 5' PCR primer (5' CGCGG-ATCCTCTAGATTTCGCGAAGTGGACACTGTTC 3') was designed at the ATG start codon of *era* and contains *Bam*HI and *Nde*I sites for cloning purposes. The 3' PCR primer (5' CGCGGATCCAAACGTTCTGGATGATCTGTG 3') was designed at the stop codon of *era* and contains a *Bam*HI site after the stop codon. Using these primers, *era* was PCR-amplified from *S. pneumoniae* under the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and polymerization at 72 °C for 30 s for 25 cycles. Five PCR reaction products were combined and a portion of the pooled PCR products was digested with *Bam*HI. The *Bam*HI-digested

PCR fragment was cloned into pCZA342, a low-copy-number plasmid (Baltz *et al.*, 1997), previously digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase. *era* from several pCZA342 clones was sequenced and a clone containing the consensus *era* gene sequence (pSPN-*era*) was used for constructing expression systems and complementation plasmids. pSPN-*era* was digested with *Nde*I and *Bam*HI. The *Nde*I-*Bam*HI DNA fragment containing *era* was subcloned into pET-11a and pET-16b (Novagen). The resulting constructs were designated pLY42 and pLY44, respectively (Table 1). pSPN-*era* was also digested with *Bam*HI, and the *Bam*HI fragment of *era* was subcloned into

pGEX-2T and pACYC184. The resulting constructs were designated pLY52, and pLY62 and pLY64, respectively (Table 1).

To generate a 3'-terminal deletion mutant of *era*, a 3' PCR primer was designed and used for PCR amplification. The 3' PCR primer contains DNA sequence complementary to codons 207–213 of the *era* gene and a *Bam*HI site. A stop codon was also included (5' CGCGGATCCTCAAACCTA-CTGCTACAGAATGCGG 3'). The 5' PCR primer was the same one described for cloning the full-length *era* gene into expression vectors. PCR-amplified products were pooled, sequenced and cloned into pCZA342 as described above. The truncated *era* (Δ era) gene was subsequently cloned into pET-11a, pET-15b and pGEX-2T as described above and the resulting constructs were designated pLY54, pLY56 and pLY52, respectively (Table 1). The Δ era gene was also cloned into pACYC184, pBR322 and pUC18 at their *Bam*HI sites, and the resulting constructs were designated pLY58, pLY60, pLY76 and pLY75, respectively (Table 1).

Enzyme assay and kinetics. For determination of K_m and V_{max} values of Era proteins, reaction mixtures (300 μ l each) containing 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$ and 1.17 μ M GST-Era or GST- Δ Era (GST-truncated Era) were incubated at 23 °C. To initiate the reactions, GTP was added at concentrations of 15–2000 μ M. Aliquots (100 μ l each) were removed after incubations for 0.0 and 30 min, and reactions were stopped by adding 5 μ l 1 M HCl. Then, 50 μ l of each aliquot was injected onto a 4.4 $\times$ 250 mm ODS-AQ HPLC column (YMC) and separated under isocratic conditions (79 mM potassium phosphate, pH 6.0, 4 mM tetrabutylammonium hydrogen sulfate, 21% methanol). Amounts of GDP produced were quantified by comparing its peak areas with those of GDP standards. Since GDP is bound to the protein when purified, the total amount of GDP produced after 30 min incubation was calculated by subtracting GDP released at the 0.0 min incubation.

The k_d values of Era proteins were also determined. Briefly, Era proteins were mixed with various amounts of [3H]GTP, and the [3H]GTP bound to Era proteins was captured using MANP NOB filter plate (Millipore) and quantified by scintillation counting.

Purification of Era proteins. *E. coli* cells of LY41 [pLY41 (GST-Era)], LY42 [pLY42 (Era)], LY52 [pLY52 (GST- Δ Era)] and LY56 [pLY56 (His- Δ Era)] were first grown overnight with vigorous shaking at 30 °C in LB medium supplemented with 100 μ g Amp ml⁻¹. The overnight cultures (4%) were inoculated into 1.25 l each of fresh LB medium containing Amp. The cultures were induced with 0.8 mM IPTG at OD₆₀₀ 0.5–0.6 for 3 h at 30 °C. Cells were harvested by centrifugation at 4000 g for 10 min, and washed with 20 mM Tris/HCl, pH 8.0, and 5 mM $MgCl_2$ (buffer A).

For purification of GST- Δ Era, cells of LY52 were resuspended in 20 mM Tris/HCl, pH 7.5, 140 mM NaCl and 5 mM $MgCl_2$ (buffer B). The resulting suspension was disrupted by passing twice through a 138 MPa French press cell (Aminco Laboratories) and centrifuged at 180000 g for 45 min. The supernatant fraction collected was loaded onto a 10 ml Glutathione Sepharose column. The column was washed with 60 ml buffer B and eluted with 10 mM glutathione in buffer B. All fractions were subjected to SDS-PAGE analysis (Laemmli, 1970), and those containing GST- Δ Era were collected and dialysed using dialysis tubing (molecular mass cut-off, 25 kDa; Sigma) in 4 l buffer A at 4 °C overnight.

For purification of GST-Era, cells of LY41 were disrupted and

the resulting suspension was centrifuged as described above. GST-Era was purified as described for GST- Δ Era.

For purification of His- Δ Era, cells of LY56 were resuspended in 20 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$ and 0.5 M NaCl (buffer C) (Novagen). The resulting suspension was disrupted and centrifuged as described above. The supernatant fraction collected was loaded onto a 10 ml chelating Sepharose column charged with 50 mM $NiCl_2$. The column was washed with 60 ml buffer C and then 60 mM imidazole in buffer C, and eluted with 0.5 M imidazole in buffer C. Fractions containing His- Δ Era were collected and dialysed as described above except that a 6000–8000 kDa molecular mass cut-off tubing was used.

For purification of the full-length native Era protein, cells of LY42 were disrupted and the resulting suspension was centrifuged as described above. The supernatant fraction collected was loaded onto a Source Q (30 μ m diameter) column (2.6 by 10 cm) that was previously equilibrated with buffer A. The column was washed with 250 ml buffer A and eluted with a linear gradient of 0–1000 mM KCl in buffer A. The presence of Era in fractions was monitored by SDS-PAGE (Laemmli, 1970). The fractions containing Era were pooled and dialysed as described above against 4 l (no change) 20 mM potassium phosphate, pH 7.5. The dialysed enzyme preparation was applied to a Macro-Prep ceramic hydroxyapatite type I (80 μ m diameter) column (2.6 by 10 cm) that was equilibrated with 20 mM potassium phosphate, pH 7.5. The column was washed with 250 ml of the buffer and eluted with a linear gradient of 20–700 mM potassium phosphate, pH 7.5. The fractions containing Era were pooled and dialysed as described above in buffer A and loaded onto a Heparin (Bio-Rad) column (2.6 by 10 cm) that was equilibrated with buffer A. The column was washed with 250 ml buffer A and eluted with a linear gradient of 0–1000 mM KCl in buffer A. The fractions containing Era were pooled and dialysed as described above in buffer A. The Era protein purified was divided into aliquots and stored as described above.

Antibody preparation and quantitative Western blot analysis of Era in *S. pneumoniae*. Polyclonal antibodies against SDS-denatured full-length Era protein were prepared (Harlan Bioproducts for Science). Purified Era was denatured by SDS and subjected to SDS-PAGE (10% gel; Bio-Rad) (Laemmli, 1970). Protein bands were visualized by incubating the gel in a solution containing 0.5 M KCl and 50 mM potassium phosphate (pH 7.2) and cut out (Zhao & Winkler, 1995). Each protein band, which contained approximately 100 μ g Era, was injected into two New Zealand White rabbits.

To compare the expression levels of Era in *S. pneumoniae* and *E. coli*, *E. coli* strains were grown in LB medium as described above and *S. pneumoniae* was grown as described below. A frozen stock of *S. pneumoniae* was inoculated into Brain Heart Infusion broth (Difco) at dilutions of 10⁻⁶ to 10⁻⁸. The cultures were grown at 37 °C overnight without shaking and harvested at OD₆₀₀ 0.4. The cells were washed with 20 mM potassium phosphate, pH 7.2, resuspended in buffer A and 1 M NaCl, disrupted and centrifuged as described above. Supernatant fractions were collected, denatured by SDS (Laemmli, 1970), heated at 100 °C for 2 min, and subjected to Western blot analysis, which was carried out essentially as described by Zhao & Winkler (1995) with the following modifications. Transfer was done in 12 mM Tris/HCl, 96 mM glycine and 20% methanol (Novex) at room temperature (40 V, constant) for 2 h using a Blot Module (Novex). Membranes were blocked in 1 $\times$ PBS (BRL) containing 5% dry milk at 4 °C overnight, incubated with primary antibodies

(1/500 dilutions) and secondary antibodies (1/2000) for 2–3 h at room temperature, and washed three times with $1 \times$ PBS. Protein concentrations were determined using a Bradford assay kit (Bio-Rad) with BSA as a standard (Bradford, 1976).

Determination of association of Era proteins with the membrane. *E. coli* cells of LY42 and LY54 were first grown in LB medium containing Amp ($100 \mu\text{g ml}^{-1}$) overnight at 35°C and the overnight cultures (3%) were inoculated into 300 ml LB medium supplemented with Amp. The cultures were grown for 1 h at 33°C and then induced with 1 mM IPTG for 2 h. The cultures were collected and washed as described above. The cells were resuspended in 8 ml buffer A and disrupted as described above. The resulting crude extracts were centrifuged at $12000 g$ for 8 min to remove unbroken cells and potential inclusion bodies. The supernatant fractions collected (4 ml each) were mixed with 1 ml buffer A or 1 ml buffer A containing 5 M NaCl (final, 1 M) and centrifuged at $140000 g$ for 30 min. The supernatant fractions, designated cytoplasmic fractions, were collected, denatured with SDS, and subjected to SDS-PAGE analysis as described above. The resulting pellets, designated membrane fractions, were washed twice with buffer A, resuspended in buffer A, and subjected to SDS-PAGE analysis as described above.

S. pneumoniae was grown and harvested at OD_{660} 0.4 as described above. Cells were resuspended in 12 ml buffer A, and disrupted as described above. The resulting crude extracts (4 ml each) were mixed with 1 ml buffer A or 1 ml buffer A containing 5 M NaCl (final, 1 M NaCl), incubated on ice for 40 min, and centrifuged at $180000 g$ for 45 min. Supernatant fractions were collected, denatured by SDS, and subjected to SDS-PAGE analysis as described above. The presence of the Era protein was analysed by Western blotting as described above.

RESULTS

Identification, cloning and expression of the *S. pneumoniae* era gene

To clone the *S. pneumoniae* era gene, we first searched our sequence database (Baltz *et al.*, 1998; Rosteck *et al.*, 1997) using the *E. coli* era sequence as a query in the BLAST program and found that the era gene was not completely sequenced. Both 5'- and 3'-terminal sequences for era were missing. We then recovered the missing sequences of the gene by inverse PCR and determined its entire sequence. The *S. pneumoniae* era gene, 900 bp long, encodes a protein consisting of 299 amino acids with a predicted molecular mass of 33810 Da. The *S. pneumoniae* Era protein shares 40, 45 and 95% identities with those of *E. coli*, *Haemophilus influenzae* and *Streptococcus mutans*, respectively (Ahnn *et al.*, 1986; Chen *et al.*, 1990; Fleischmann *et al.*, 1995; Yamashita *et al.*, 1993). The *S. pneumoniae* Era protein contains all conserved motifs for GTP binding at the N-terminal part of the protein and also all conserved motifs of boxes A–D identified by Zuber *et al.* (1997), which are located at the C-terminus of the protein (Zuber *et al.*, 1997). The *S. pneumoniae* era gene complements an *E. coli* mutant strain defective in Era production (see below). We also constructed a truncated era (Δ era) gene by removing 68 codons from its 3'-terminus. As a result, the Δ Era protein does not contain

Fig. 1. SDS-PAGE analysis of the association of the *S. pneumoniae* Era and Δ Era proteins with the *E. coli* cytoplasmic membrane. Cells were grown, collected and disrupted as described in Methods. The resulting crude extracts were centrifuged at $12000 g$ for 8 min to remove unbroken cells and potential inclusion bodies. The supernatant fractions collected were mixed with buffer A (20 mM Tris/HCl, pH 8.0, 5 mM MgCl_2) or buffer A plus 1 M NaCl, and centrifuged (Methods). The supernatant fractions collected, designated cytoplasmic preparations, were denatured and subjected to SDS-PAGE analysis (Methods). The pellet fractions collected, designated membrane preparations, were washed twice with and resuspended in buffer A, and denatured and subjected to SDS-PAGE analysis (Methods). Lanes: 1, prestained molecular mass markers (from top to bottom: myosin, 208 kDa; β -galactosidase, 116 kDa; BSA, 83 kDa; ovalbumin, 48.7 kDa; carbonic anhydrase, 33.4 kDa; soybean trypsin inhibitor, 28.2 kDa; lysozyme, 20.7 kDa; and aprotinin, 7.6 kDa); 2 and 3 (6 μl each), the cytoplasmic preparation of LY42 (era-pET-11a) which was treated with buffer A and 1 M NaCl in buffer A, respectively; 4 and 5 (6 μl each), the membrane preparation of LY42 which was treated with buffer A and 1 M NaCl in buffer A, respectively; 6 and 7 (6 μl each), the cytoplasmic preparation of LY54 (Δ era-pET-11a) which was treated with buffer A and 1 M NaCl in buffer A, respectively; 8 and 9 (6 μl each), the membrane preparation of LY54 which was treated with buffer A and 1 M NaCl in buffer A, respectively.

the last two conserved motifs of boxes C–D (Zuber *et al.*, 1997). The full-length and Δ era genes were cloned into *E. coli* expression vectors and expressed in native form, in GST-fusion form or in His-fusion form (Table 1).

Purification and characterization of Δ Era proteins

To understand the role of the C-terminal portion of Era, we purified GST- Δ Era and His- Δ Era to homogeneity (Fig. 1; and data not shown). Our first attempt to purify Δ Era from LY54 using the three-step column chromatography procedure (Methods) failed to yield a homogeneous protein preparation ($\approx 50\%$ pure) due to its relatively low level of expression in *E. coli* (Fig. 1). Therefore, we characterized the purified GST- Δ Era and His- Δ Era proteins for their ability to utilize GTP under the conditions described (Methods). Like the full-length native Era proteins (Ahnn *et al.*, 1986; Chen *et al.*, 1990; Lerner *et al.*, 1992; March *et al.*, 1988; Wu *et al.*, 1995), GST- Δ Era and His- Δ Era proteins were able to bind and hydrolyse GTP. The apparent K_m value determined for

Table 2. Complementation of *E. coli* HT120 by *era* and Δ *era*

E. coli HT120 was transformed with different plasmids and selected on LB plates containing appropriate antibiotics. The LB plates were incubated at 30 °C for 24 h and examined for growth. + + +, Large colonies; +, small colonies; –, no growth.

Complementing plasmid	Orientation of <i>era</i> and Δ <i>era</i> *	Growth of transformants	
		+ Tet	– Tet
pLY62 (<i>era</i> -pACYC184)	The same as P _{tet}	+ + +	+ + +
pLY64(<i>era</i> -pACYC184)	Opposite to P _{tet} , but the same as P _{cam}	+ + +	+
pLY60 (Δ <i>era</i> -pACYC184)	The same as P _{tet}	+ + +	–
pLY58 (Δ <i>era</i> -pACYC184)	Opposite to P _{tet} , but the same as P _{cam}	+ + +	–
pLY76 (Δ <i>era</i> -pBR322)	The same as P _{tet}	+ + +	–
pLY75 (Δ <i>era</i> -pUC18)†	The same as P _{lac}	+ + +	–
pLY41 (<i>era</i> -pGEX-2T)	The same as P _{lac}	+ + +	+ + +
pACYC184		+ + +	–
pBR322		+ + +	–
pGEX-2T		+ + +	–
pUC18†		+ + +	–

* P_{tet}, the Tet resistance gene promoter of pACYC184 and pBR322; P_{cam}, the Cam resistance gene promoter of pACYC184; and P_{lac}, the *lac* promoter of pUC18.

† The *E. coli* HT120 transformants carrying pLY75 and pUC18 were selected on LB plates containing 0.0, 0.2 or 1.5 mM IPTG, incubated, and examined as described in Methods.

GST- Δ Era was 232 μ M and the apparent $V_{\max}$ value obtained for the enzyme was 28 mmol min⁻¹ mol⁻¹. Similar results were obtained for His- Δ Era (data not shown). The k_d value for GST- Δ Era was determined to be 2.1 μ M. The kinetic parameters (K_m , $V_{\max}$ and k_d) determined for the full-length Era were 430 μ M, 200 mmol min⁻¹ mol⁻¹ and 4.2 μ M, respectively. Therefore, the removal of the C-terminal part of Era resulted in a twofold increase in its affinity for GTP and a sevenfold decrease in its GTP-hydrolysis activity.

Association of Era proteins with the *E. coli* and *S. pneumoniae* cytoplasmic membranes

The Era proteins of *E. coli* and *S. mutans* have been shown to be associated with the cytoplasmic membrane (Gollop & March, 1991a; Lin *et al.*, 1994; Wu *et al.*, 1995). To examine whether the *S. pneumoniae* Era protein is also associated with the cytoplasmic membrane, we performed Western blot analysis of the crude extracts of *S. pneumoniae*. We found that there was about twofold more Era protein detected in the crude extract that was treated with 1 M NaCl than in the untreated sample (data not shown). This finding suggests that like other Era proteins, the *S. pneumoniae* Era protein is also associated with the cytoplasmic membrane and this association can be disrupted by salt treatment.

We also showed that the full-length native Era protein of *S. pneumoniae*, when expressed in *E. coli*, was clearly

associated with the cytoplasmic membrane (Fig. 1, lane 4), and that this association can be disrupted by salt treatment (Fig. 1, lane 5). Consistent with this finding, there was twofold more Era protein detected in the cytoplasmic preparation that was treated with 1 M NaCl than in the untreated sample (Fig. 1, lanes 2 and 3). Similarly, the full-length GST-Era (pLY41) and His-Era (pLY44) proteins were also found to be associated with the *E. coli* cytoplasmic membrane when expressed in *E. coli* (data not shown).

We also analysed the truncated Era (Δ Era) proteins for their possible association with the *E. coli* cytoplasmic membrane. However, we found no evidence that the Δ Era proteins were associated with the *E. coli* cytoplasmic membrane when expressed directly (pLY54) or in a GST-fusion (pLY52) or His-fusion (pLY56) form in *E. coli*, and the salt treatment of the crude extracts of LY52, LY54 and LY56 had no effect at all on its possible association with the cytoplasmic membrane (Fig. 1, lanes 6–9; and data not shown). Thus, the removal of the C-terminal part of Era resulted in the failure of the Δ Era protein to associate with the cytoplasmic membrane.

Complementation of an *E. coli* mutant strain defective in Era production

To further analyse the function of the C-terminal part of Era *in vivo*, we tested whether the full-length *era* and Δ *era* genes complement the *E. coli* mutant strain

Fig. 2. Immunodetection of cellular amounts of the Era and Δ Era proteins produced in *S. pneumoniae* and *E. coli* HT120, respectively, with antibodies prepared against the *S. pneumoniae* Era protein. *S. pneumoniae* was grown, collected and disrupted as described in Methods. *E. coli* HT120 harbouring pUC18 or pLY75 was grown in LB and induced with 1.5 mM IPTG upon inoculation with overnight cultures. Cells were collected and disrupted as described in Methods. The resulting crude extracts were centrifuged, and supernatant fractions were collected and denatured as described in Methods. (a) Lanes: 1–4, 2, 4, 6 and 8 ng purified full-length Era protein, respectively; 5–7, 10, 15 and 20 μ g of the supernatant fraction of *S. pneumoniae* R6, respectively; 8–10, 10, 15 and 20 μ g of the supernatant fraction of *E. coli* HT120(pLY64) (*era*-pACYC184, opposite to P_{tet}), respectively. (b) Lanes: 1 and 2, 3.75 and 7.5 μ g of the supernatant fraction of *E. coli* HT120(pLY75) (Δ Era-pUC18), respectively; 3 and 4, 60 and 80 ng purified His- Δ Era protein, respectively.

defective in Era production, HT120 (Takiff *et al.*, 1989, 1992). HT120 contains a mini-Tn10 insertion upstream of the *rnc* gene which forms part of an operon with its downstream gene, *era* (Takiff *et al.*, 1989, 1992). The mini-Tn10 carries two divergent Tet-inducible promoters (Takiff *et al.*, 1989, 1992). In the absence of Tet, HT120 could not grow on LB plates because the mini-Tn10 insertion is downstream polar to *era*, which in turn blocks the transcription of the *era* gene. Thus, growth of HT120 is Tet-dependent. Like *era* genes from

several other organisms (Pillutla *et al.*, 1995; Zuber *et al.*, 1990, 1997), the full-length *era* gene of *S. pneumoniae* complemented the *E. coli* mutant HT120 when cloned into pACYC184 (pLY62 and pLY64) in either orientation with respect to the direction of transcription of the Tet resistance gene because the HT120 transformants were able to grow on LB plates without supplementation of Tet (Table 2). When HT120 was transformed with pLY62 (e.g. *era* was cloned into pACYC184 in the same orientation as the transcription of the plasmid Tet resistance gene), the resulting transformants grew equally well on LB plates supplemented with or without Tet (Table 2), showing that pLY62 was able to fully complement HT120. Interestingly, when HT120 was transformed with pLY64 (e.g. *era* was cloned into pACYC184 in the opposite orientation with respect to the transcription of the Tet resistance gene, but in the same orientation as the transcription of the distant Cam resistance gene), the resulting transformants grew very slowly on LB plates without the supplementation of Tet (Table 2). The colony size of these transformants [HT120(pLY64)] was only 1/4–1/3 of those growing on LB plates supplemented with Tet after 24 h incubation at 30 °C (Table 2). This observation suggests that the amount of Era protein expressed from pLY64 is limiting to the cell growth. Therefore, the amount of Era produced under these conditions was probably close to the minimal level of Era that is required for complementation of the *E. coli* mutant HT120. Our Western blot analysis showed that the minimal level of Era required for growth is 2.6 ng per 10 μ g or 0.26 ng per μ g total cellular protein (Fig. 2a, lane 8).

When the *era* gene was cloned into pGEX-2T (pLY41), the resulting GST-*era* fusion gene also complemented the *E. coli* HT120 mutant (Table 2), even in the absence of IPTG, suggesting that the GST-Era fusion protein was expressed and functional in the cell.

Unlike the full-length *era* gene, the Δ era gene of *S. pneumoniae* was unable to complement the *E. coli* mutant HT120 when carried on pACYC184, pBR322 or pUC18 (Table 2). The failure of the Δ era gene to complement the *E. coli* mutant suggested two possibilities. First, the C-terminal part of the Era protein is essential for function. Second, the Δ era gene was not expressed or expressed at a very low level. To test these possibilities, we carried out Western blot analysis of the *E. coli* mutant HT120 carrying the Δ era gene on the plasmids. The expression levels of the Δ era gene were barely detectable in HT120 harbouring pLY58 and pLY60 (Δ era-pACYC184) and pLY76 (Δ era-pBR322) (data not shown). Thus, the Δ era gene was poorly expressed when carried on these plasmids. However, when HT120 was transformed with pLY75 (Δ era-pUC18) and induced with 1.5 mM IPTG, the amount of Δ Era produced in HT120 was 48 ng per 3.75 μ g or 12.8 ng per μ g total cellular protein (Fig. 2b, lane 1). This is 20-fold higher than that of Era in *S. pneumoniae* since the amount of Era present in *S. pneumoniae* was estimated to be 6.3 ng per 10 μ g or 0.63 ng per μ g total

cellular protein (Fig. 2a, lane 5). Thus, the failure of the Δ era gene to complement the *E. coli* mutant deficient in Era production was not due to its poor expression; rather it suggests that the C-terminal domain of the Era protein is probably essential for function.

DISCUSSION

In this study, we found that removal of the C-terminal portion of the *S. pneumoniae* Era protein resulted in a twofold increase in its GTP-binding activity, a sevenfold decrease in its GTP-hydrolysis activity, and the loss of its ability to associate with the *E. coli* cytoplasmic membrane. In addition, the removal of the 3'-terminal part of the era gene also led to the inability of the Δ era gene to complement the *E. coli* mutant strain defective in Era production. The failure of the Δ era gene to complement the *E. coli* mutant HT120 was not due to its poor expression or insoluble protein formation. When the *E. coli* mutant HT120 was transformed with pLY75 (Δ era-pUC18) and induced with 0.2 and 1.5 mM IPTG, the amounts of the Era protein produced were estimated to be 6.8 (data not shown) and 12.5 ng per μ g total protein, respectively, which are at least 10- and 20-fold higher than that of the Era protein present in *S. pneumoniae*. Since the molecular mass of the Δ Era protein is 26 kDa, which is 24% smaller than that of the full-length Era protein, the molar concentrations of Δ Era produced in *E. coli* HT120 should be 13- and 26-fold higher than that of Era in *S. pneumoniae*. The amounts of the Era protein detected in HT120(pLY75) represent all soluble protein retained in the crude extract after centrifugation at 180 000 g for 45 min. We also established that the minimal amount of the *S. pneumoniae* Era needed for complementation of the *E. coli* mutant HT120 was 0.26 ng per μ g total cellular protein, which is twofold lower than that of Era present in *S. pneumoniae*. The minimal amount of Era determined was probably an overestimate since the antibodies prepared against the *S. pneumoniae* Era protein appear able to recognize the *E. coli* Era protein (Fig. 2b, lanes 1 and 2). Therefore, the amounts of Δ Era produced in HT120(pLY75) are at least 26- and 52-fold higher than the minimal amount of Era needed for complementation. Since the Δ Era protein exhibits a sevenfold reduction in its GTP-hydrolysis activity but a twofold increase in its GTP-binding activity, the amount of the Δ Era protein produced in HT120(pLY75) should make up the total activity that is needed for complementation. Clearly, these results suggest that the removal of the C-terminus of the Era protein renders the Era protein unable to function *in vivo*. Therefore, the C-terminal domain of the Era protein is essential for the function of the protein.

Several studies have suggested that the C-terminal domain of Era proteins might be important for function. However, in many cases a complete study was not presented. Point mutations in the C-terminal domain of the *E. coli* Era protein were isolated and these mutant era genes failed to complement an *E. coli* era mutant

(Pillutla *et al.*, 1995), but biochemical characterization of these mutant Era proteins was not given (Pillutla *et al.*, 1995). A temperature-sensitive mutation in the GTPase domain of a truncated *E. coli* Era protein has been reported and the biochemical characterization of this mutant Era protein was also described (Inada *et al.*, 1989). This era mutant, however, requires the C-terminal deletion of the Era protein for its temperature sensitivity (Inada *et al.*, 1989). Cold-sensitive point mutations in the C-terminal domain of the *E. coli* Era protein have also been reported, but biochemical characterization of these mutant proteins was not presented (Lerner *et al.*, 1995). Thus, our study represents the first complete biochemical and genetic analyses of the C-terminal domain of an Era protein.

The era gene was first discovered in *E. coli* by the similarity of its derived amino acid sequence to the RAS proteins of *Saccharomyces cerevisiae* (Ahnn *et al.*, 1986). Later, further detailed sequence analysis of the RAS proteins and the *E. coli* Era protein did not appear to support the contention that the *E. coli* Era protein is a RAS-like protein (Chen *et al.*, 1990). Recently, a human homologue of Era was reported which exhibits 30% identity to the *E. coli* Era protein (Britton *et al.*, 1998). The human homologue is distinct from RAS proteins (Britton *et al.*, 1998). Nevertheless, some of the nucleotide-binding motifs of the yeast RAS, human Ras, human Era and bacterial Era proteins seem to be conserved during evolution (Ahnn *et al.*, 1986; Bourne *et al.*, 1991; Britton *et al.*, 1998; Chen *et al.*, 1990; March, 1992; Pillutla *et al.*, 1995; Zuber *et al.*, 1990, 1997), but the C-terminal part of the Era proteins (from residues 201 to the end) appears to represent a domain that is unique to and conserved among the bacterial Era proteins (Pillutla *et al.*, 1995; Zuber *et al.*, 1990, 1997).

Unlike RAS proteins that are attached to the cytoplasmic membrane through acylation of palmitic acid (Barbacid, 1987), the bacterial Era proteins are found to be associated with the cytoplasmic membrane (Gollop & March, 1991a; Lin *et al.*, 1994; Wu *et al.*, 1995). The significance of this membrane association has not yet been assessed. Our studies show that the Δ Era protein of *S. pneumoniae* can no longer bind to the cytoplasmic membrane and also the Δ era gene is unable to complement the *E. coli* mutant deficient in Era. These observations suggest that the C-terminal domain of the Era protein might be responsible for its attachment to the membrane, and this attachment (or association) of the Era protein to the membrane appears to be essential for the biological function of the protein. Thus, this study supports the hypothesis that Era is involved in a GTPase-receptor-coupled membrane-signalling pathway (Lin *et al.*, 1994).

The minimal amount of *S. pneumoniae* Era protein required for complementation of the *E. coli* mutant HT120 is 0.26 ng per μ g or 0.026% of total protein. The cellular amount of Era protein in *S. pneumoniae* is about 0.63 ng per μ g or 0.063% of total protein. The *E. coli* Era protein was estimated to be 10 ng per 1.5×10^8 cells or

$\approx 0.022\%$ of total protein since the mean soluble protein of an *E. coli* K-12 cell was determined to be $3.3 \times 10^{-7} \mu\text{g}$ (Gollop & March, 1991a; Zhao & Winkler, 1995). Thus, the cellular amounts of Era proteins are similar in *E. coli* and *S. pneumoniae*. These results have several implications. First, the amount of Era protein produced *in vivo* is probably very close to what is needed for cell growth, and may be tightly modulated. Second, the Era protein of *S. pneumoniae* appears able to function very efficiently in *E. coli* because overexpression of the protein was not required. Finally, all Era proteins examined seem to share the same function regardless of their phylogenetic histories.

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REFERENCES

- Ahnn, J., March, P. E., Takiff, H. E. & Inouye, M. (1986). A GTP-binding protein of *Escherichia coli* has homology to yeast RAS proteins. *Proc Natl Acad Sci USA* **83**, 8849–8853.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Baltz, R. H., Matsushima, P., Peery, R. B. & 10 other authors (1997). Use of genomics and insertional mutagenesis to identify targets for antibiotic development in *Streptococcus pneumoniae*. Abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy (Ontario, Canada), 391(S-46).
- Baltz, R. H., Norris, F. H., Matsushima, P. & 13 other authors (1998). DNA sequence sampling of the *Streptococcus pneumoniae* genome to identify novel targets for antibiotic development. *Microb Drug Resist* **4**, 1–9.
- Barbacid, M. (1987). Ras genes. *Annu Rev Biochem* **56**, 779–827.
- Bourne, H. R., Sanders, D. A. & McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349**, 117–127.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Britton, R. A., Powell, B. S., Court, D. L. & Lupski, J. R. (1997). Characterization of mutations affecting the *Escherichia coli* essential GTPase Era that suppress two temperature-sensitive *dnaG* alleles. *J Bacteriol* **179**, 4575–4582.
- Britton, R. A., Powell, B. S., Dasgupta, S., Sun, Q., Margolin, W., Lupski, J. R. & Court, D. L. (1998). Cell cycle arrest in Era GTPase mutants: a potential growth rate-regulated checkpoint in *Escherichia coli*. *Mol Microbiol* **27**, 739–750.
- Chen, S.-M., Takiff, H. E., Barber, A. H., Dubois, G. C., Bardwell, J. C. A. & Court, D. L. (1990). Expression and characterization of RNaseIII and Era proteins: products of the *rnc* operon of *Escherichia coli*. *J Biol Chem* **265**, 2888–2895.
- Fleischmann, R. D., Adams, M. D., White, O. & 37 other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.
- Fraser, M., Gocayne, J. D., White, O. & 26 other authors (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397–403.
- Gollop, N. & March, P. E. (1991a). Localization of the membrane binding sites of Era in *Escherichia coli*. *Res Microbiol* **142**, 301–307.
- Gollop, N. & March, P. E. (1991b). A GTP-binding protein (Era) has an essential role in growth rate and cell cycle control in *Escherichia coli*. *J Bacteriol* **173**, 2265–2270.
- Inada, T., Kawakami, K., Chen, S., Takiff, H. E., Court, D. L. & Nakamura, Y. (1989). Temperature-sensitive lethal mutant of Era, a G protein in *Escherichia coli*. *J Bacteriol* **171**, 5017–5024.
- Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (1990). *PCR Protocols: a Guide to Methods and Applications*. New York: Academic Press.
- Kawabata, S., Terao, Y., Andoh, T. & Hamada, S. (1997). Nucleotide sequence and molecular characterization of a gene encoding GTP-binding protein from *Streptococcus gordonii*. *FEMS Microbiol Lett* **156**, 211–216.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lerner, C. G. & March, P. E. (1991). Pleiotropic changes resulting from depletion of Era, an essential GTP-binding protein in *Escherichia coli*. *Mol Microbiol* **5**, 951–957.
- Lerner, C. G., Sood, P., Ahnn, J. & Inouye, M. (1992). Cold-sensitive growth and decreased GTP-hydrolytic activity from substitution of Pro17 for Val in Era, an essential *Escherichia coli* GTPase. *FEMS Microbiol Lett* **95**, 137–142.
- Lerner, C. G., Gulati, P. S. & Inouye, M. (1995). Cold-sensitive conditional mutations in Era, an essential *Escherichia coli* GTPase, isolated by localized random polymerase chain reaction mutagenesis. *FEMS Microbiol Lett* **126**, 291–298.
- Lin, Y. P., Sharer, J. D. & March, P. E. (1994). GTPase-dependent signaling in bacteria: characterization of a membrane-binding site for Era in *Escherichia coli*. *J Bacteriol* **176**, 44–49.
- March, P. E. (1992). Membrane associated GTPases in bacteria. *Mol Microbiol* **6**, 1253–1257.
- March, P. E., Lerner, C. G., Ahnn, J., Cui, X. & Inouye, M. (1988). The *Escherichia coli* Ras-like protein (Era) has GTPase activity and is essential for cell growth. *Oncogene* **2**, 539–544.
- Pillutla, C. R., Sharer, J. D., Gulati, P. S., Wu, E., Yamashita, Y., Lerner, C. G., Inouye, M. & March, P. E. (1995). Cross-species complementation of the indispensable *Escherichia coli* era gene highlights amino acid regions essential for activity. *J Bacteriol* **177**, 2194–2196.
- Rosteck, P. R., Jr, Norris, F. H., Matsushima, P. & 13 other authors (1997). *Streptococcus pneumoniae* genome sequence sampling. In *Developments in Industrial Microbiology*, pp. 13–20. Edited by R. H. Baltz, G. D. Hageman & P. L. Skatrud. Washington, DC: Society for Industrial Microbiology.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sato, T., Wu, J. & Kuramitsu, H. (1998). The *sgp* gene modulates stress responses of *Streptococcus mutans*: utilization of an antisense RNA strategy to investigate essential gene functions. *FEMS Microbiol Lett* **159**, 241–245.
- Takiff, H. E., Chen, S.-M. & Court, D. L. (1989). Genetic analysis of the *rnc* operon of *Escherichia coli*. *J Bacteriol* **171**, 2581–2590.
- Takiff, H. E., Baker, T., Copeland, T., Chen, S.-M. & Court, D. L. (1992). Locating essential *Escherichia coli* genes by using mini-Tn10 transposon: the *pdxJ* operon. *J Bacteriol* **174**, 1544–1553.

Wu, J., Cho, M.-I. & Kuramitsu, H. K. (1995). Expression, purification, and characterization of a novel G protein, SGP, from *Streptococcus mutans*. *Infect Immun* **63**, 2516–2521.

Yamashita, Y., Takehara, T. & Hiramitsu, H. K. (1993). Molecular characterization of a *Streptococcus mutans* mutant altered in environmental stress responses. *J Bacteriol* **175**, 6220–6228.

Zhao, G. & Winkler, M. E. (1995). Kinetic limitation and cellular amount of pyridoxine (pyridoxamine) 5'-phosphate oxidase of *Escherichia coli* K-12. *J Bacteriol* **177**, 883–891.

Zuber, M., Hoover, T. A., Powell, B. S. & Court, D. L. (1990).

Analysis of the *rnc* locus of *Coxiella burnetii*. *Mol Microbiol* **14**, 291–300.

Zuber, M., Hoover, T. A., Dertzbaugh, M. T. & Court, D. L. (1997). A *Francisella tularensis* DNA clone complements *Escherichia coli* defective for the production of Era, an essential Ras-like GTP-binding protein. *Gene* **189**, 31–34.

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