Identification of an EF-Tu protein that is periplasm-associated and processed in *Neisseria gonorrhoeae*

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A 44 kDa protein is a dominant component of periplasmic extracts of *Neisseria gonorrhoeae*. Peptide sequence generated from a cyanogen-bromide-cleaved fragment of this protein indicated sequence homology with elongation factor-Tu (EF-Tu). Polyclonal antiserum was made against the 44 kDa protein purified from periplasm extracts of *N. gonorrhoeae*. The preabsorbed antiserum was immunoblotted against whole-cell lysates on two-dimensional gels. A 44 kDa protein and a smaller 37 kDa protein were recognized by this antiserum. A *N. gonorrhoeae* λ phage DNA library was screened and a clone expressing a 44 kDa protein was identified. The DNA insert in this clone contained several genes homologous to genes contained in the *str* operon of *Escherichia coli*. One ORF product with a calculated molecular mass of 43 kDa was highly homologous to the EF-TuA of *E. coli*. A synthetic peptide antiserum specific for a portion of the C terminus of EF-Tu confirmed that the 37 kDa protein in whole-cell lysates of *N. gonorrhoeae* was a processed form of EF-Tu. Deletion of the *tufA* gene homologue in *N. gonorrhoeae* was attempted but was unsuccessful.

Keywords: *Neisseria gonorrhoeae*, *tufA*, EF-Tu, processing

INTRODUCTION

Elongation factor-Tu (EF-Tu) is a ubiquitous protein involved in protein synthesis and may compose 10% of the total cellular protein of a rapidly dividing *Escherichia coli* bacterium (Van der Meide *et al.*, 1982). EF-Tu has also been described as the dominant protein released in periplasmic extracts of *E. coli* (Jacobson *et al.*, 1976). In Gram-negative bacteria, EF-Tu is typically encoded by two almost identical genes, *tufA* and *tufB*. Therefore, EF-Tu is a generic term which may be applicable to either EF-TuA, EF-TuB, or both. These *tuf* genes have been identified and well characterized in *E. coli* and they differ in only one amino acid (Arai *et al.*, 1980; Jones *et al.*, 1980). During conditions of nutrient limitation, EF-Tu appears to be methylated and cytoplasmically membrane bound (Young & Bernlohr, 1991; Young *et al.*, 1990). Southern analysis has demonstrated that the gonococcus (*Neisseria gonorrhoeae*) has two *tuf* genes (Goldstein *et al.*,

1989); a situation similar to that in *E. coli* (Jaskunas *et al.*, 1975) and *Salmonella typhimurium* (Hughes, 1990).

The 41–45 kDa proteins of *N. gonorrhoeae* have had numerous characteristics described for them (Chen *et al.*, 1984; Hill & Judd, 1988, 1989; Judd *et al.*, 1991; Shafer, 1988; Shafer & Judd, 1991; Shafer & Morse, 1987; Swanson, 1981; Zak *et al.*, 1984). Two closely migrating 44 kDa proteins have been found in periplasm and non-periplasm fractions of *N. gonorrhoeae* (Judd & Porcella, 1993; Porcella & Judd, 1993). In an effort to determine which 44 kDa proteins are responsible for the observed characteristics, we purified a 44 kDa protein from the periplasm of *N. gonorrhoeae*. This protein was cleaved with cyanogen bromide (CNBr) and a peptide fragment was sequenced and found to be highly homologous to EF-Tu (Judd & Porcella, 1993).

In this report we describe the further purification of this periplasm-associated 44 kDa protein and the production of polyclonal antibodies. This antiserum, designated 44 kDa periplasmic antiserum, was preabsorbed with *E. coli* lysates and immunoblotted against whole-cell lysates of *N. gonorrhoeae*. Reactivity of the antisera to a 44 kDa

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protein and a smaller 37 kDa protein implied that the antiserum was sufficiently specific. A gonococcal library was immunoblotted with the polyclonal antiserum and a clone was identified which constitutively produced only one 44 kDa protein. Sequence analysis of the *N. gonorrhoeae* DNA within this clone indicated that it contained several *str* operon-like genes, including a single ORF with a predicted product of molecular mass 44 kDa that was highly homologous to EF-TuA.

EF-Tu has recently been shown to be a primary target in a phage exclusion mechanism where the majority of the molecule is cleaved at a highly conserved site producing a 37 kDa protein (Yu & Snyder, 1994). We used a synthetic, EF-Tu-specific, peptide antiserum previously described (Judd & Porcella, 1993), to probe *N. gonorrhoeae* whole-cell lysates in two-dimensional gels. We found that both the 44 kDa protein and the smaller 37 kDa protein reacted with the antiserum. These results indicate that the 37 kDa protein in *N. gonorrhoeae* is a processed form of EF-Tu.

Analysis of the function of EF-TuA and B in *E. coli* and *S. typhimurium* has indicated that EF-TuA is essential for *E. coli* (Vijgenboom & Bosch, 1987) but not essential for *S. typhimurium* (Hughes, 1990). We attempted to delete the *N. gonorrhoeae tufA* gene via homologous recombination with a selectable, transposon-inactivated gonococcal *tufA*. All mutants obtained were heterodiploid in that they contained an intact genomic copy of *tufA* along with the transposon-inactivated gene in an unknown genomic location.

METHODS

Bacteria. These included *N. gonorrhoeae* strains MS11 (courtesy of John Swanson, Rocky Mountain Laboratory, Hamilton, MT) and FA19 (courtesy of William Shafer, Dept of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA). *E. coli* XL1 was obtained from Stratagene.

Transparent, non-piliated gonococci were grown at 37 °C on clear typing medium as previously described (Swanson, 1982). Cells were passaged daily and large transparent colonies were selected. Cells were harvested at 18 h and washed twice in Dulbecco's PBS (DPBS).

Protein purification. Protocols for whole-cell lysates have been previously described by Judd (1982). Periplasmic fractions of *N. gonorrhoeae* were isolated as previously described (Judd & Porcella, 1993). Five hundred microlitres of solubilized FA19 periplasmic extract was layered onto 9% (v/v) SDS-PAGE preparative gels and electrophoresed. Gels were stained and the 44 kDa protein was excised from gels and purified for antibody production following the methods described by Judd (1988). Protein was quantified using Bio-Rad's BCA kit.

Preparation of antisera. Monospecific, polyclonal rabbit antiserum was generated against a *N. gonorrhoeae* strain FA19 periplasm-associated 44-kDa protein following standardized immunological protocols (Judd, 1991). Preabsorption of the antiserum was performed by first growing *E. coli* strain XL1-Blue (Stratagene) overnight in 100 ml Luria-Bertani (LB) medium. Cells were harvested by centrifugation at 5000 g for 10 min at 4 °C and resuspended in 3 ml 50 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0). The suspension was frozen and thawed three times and sonicated at full power for six

periods of 20 s at 4 °C. DNase (Sigma) was added to the homogenate to a final concentration of 1 unit ml⁻¹ and centrifuged at 12000 g for 10 min at 4 °C. The supernatant was absorbed onto nitrocellulose paper, dried, and subsequently incubated at 10 min intervals in the 44 kDa periplasmic antiserum. The filters were developed according to standard chromogenic Western blot procedures (Maniatis *et al.*, 1982). All *E. coli* cross-reactive antibodies were presumed to be removed from the antisera when nitrocellulose filters no longer showed any reactivity during Western blot development.

Polyclonal antibodies were prepared against a multiple antigenic peptide containing the sequence FRKLLDEGQA as previously described (Judd & Porcella, 1993).

Cloning and DNA sequence analysis. A *N. gonorrhoeae* library, generated by partial digestion of MS11 genomic DNA with the restriction enzyme *EcoRI* and subsequent ligation into the *EcoRI* site in the Lambda Zap II vector (Stratagene), was screened with the 44 kDa periplasmic antiserum following the manufacturer's protocol (Stratagene). Positive clones were plaque purified and rescued as described in the Stratagene protocol. Plasmid DNAs were isolated and purified for sequencing using the Qiagen Midi prep kit (Qiagen). Sequencing of the cloned DNA inserts was performed using forward and reverse primers (Stratagene) and synthetic oligonucleotides made using an ABI oligonucleotide synthesizer at the Murdoch Molecular Biology Facility at the University of Montana and at Rocky Mountain Laboratories in Hamilton. Sequencing reactions were performed using the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). Nucleotide sequencing also was performed using an Applied Biosystems model 373A automated DNA sequencer and PRISM ready reaction DyeDeoxy Terminator cycle sequencing kits according to manufacturer's instructions (Applied Biosystems). Nucleotide and deduced amino acid sequences were analysed and manipulated using the University of Wisconsin Computer Genetics Group version 7.3 (GenBank database release 82.0) (Devereux *et al.*, 1984) and MacVector version 4.1.1 (International Biotechnologies-Kodak) software packages. Potential promoter sites within DNA sequences were searched and assigned similarity scores based upon known *E. coli* promoter sequences using the MACTARGSEARCH algorithm (Mulligan *et al.*, 1984).

Gel electrophoresis. SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970). High-resolution two-dimensional gel electrophoresis was performed as described by O'Farrell (1975) using Bio-Rad's Mini-Protein II 2-D kit.

Shuttle mutagenesis. The shuttle mutagenesis protocol previously described by Seifert *et al.* was used (Seifert & So, 1991; Seifert *et al.*, 1986, 1990) to insertionally inactivate the cloned *tufA* gene. The *N. gonorrhoeae* DNA insert in clone 961 was first digested with *ClaI* and *EcoRI* and a 1.7 kb fragment was gel purified. This 1.7 kb fragment, which contained the entire *tufA* gene as well as *rpsJ*, was cloned into the shuttle mutagenesis vector pHSS6 via *ClaI* and *EcoRI* sites and mutagenized following the guidelines previously described (Seifert *et al.*, 1986, 1990; Seifert & So, 1991). pHSS6 constructs that contained mutagenized *tufA* genes were PCR mapped using primers specific to the DNA 5' of the *tufA* gene (5' GTTGA-AGTGGAACTCCG 3', designated 5'Tuf) and 3' (5' AAG-CTTTCAGGCGGATAC 3', designated 3'Tuf) of the *tufA* gene. Orientation of the chloramphenicol marker relative to the *tufA* gene was determined using the 5'TufA or 3'TufA primers and a primer specific to the minus strand of the chloramphenicol gene (5' TCACCAGCTCACCGTCTTTC 3', designated Cat). A mutagenized *tufA* gene was identified which

contained the inactivated transposon located within the *tufA* gene. The fragment of DNA containing the mutagenized *tufA* gene along with the flanking gonococcal sequences was cut from the vector using the restriction enzyme *NotI* and gel purified. Transformation of *N. gonorrhoeae* was performed with the *NotI* fragment and chloramphenicol-resistant mutants were selected following protocols previously described (Goodman & Scoocca, 1991; Seifert & So, 1991). Long incubation times and close observation of the plates were performed in order to detect extremely slow growing mutants.

PCR and Southern analysis of mutants. PCR was performed using a Perkin-Elmer Cetus Thermocycler and *Taq* polymerase, under reaction conditions previously described (Sambrook *et al.*, 1989). PCR products were gel purified for ligation or Southern blot hybridization procedures using the Qiaex DNA purification kit (Qiagen). PCR mapping of the genomic DNA of the chloramphenicol-resistant mutants, wild-type MS11 genomic DNA (negative control), and the transforming DNA (positive control) was performed using the oligonucleotides 5'Tuf, 3'Tuf and Cat described above. Total genomic DNA was isolated from *N. gonorrhoeae* transformation mutants using a DNA extraction kit (Stratagene) following the procedures recommended by the manufacturer.

Southern blotting and hybridization experiments were performed under high-stringency conditions following the protocol previously described (Cohen, 1990). Specific probes were intrinsically labelled using the Boehringer Mannheim random labelling primer kit.

RESULTS AND DISCUSSION

Two-dimensional isoelectric-focusing SDS-PAGE gels of whole-cell lysates of *N. gonorrhoeae* strain MS11 showed that many proteins (about 11) that differ in pI migrate at a molecular mass of 44 kDa (Fig. 1). This same result has been seen for *N. gonorrhoeae* strain FA19 (data not shown). The fact that so many proteins can be observed to migrate

at this molecular mass may explain why so many diverse functions have been associated with this protein. Immunoblotting of MS11 whole-cell lysates separated by two-dimensional gel electrophoresis with the preabsorbed 44 kDa periplasmic antisera showed that two proteins, with calculated molecular masses of 44 kDa and 37 kDa, were recognized by the antiserum in Fig. 1. This experiment has been repeated with *N. gonorrhoeae* strain FA19 whole-cell lysates with identical results (data not shown).

The 44 kDa periplasmic antiserum was used to probe a *N. gonorrhoeae* lambda Zap II genomic library. Four clones were isolated which reacted with the antiserum. Of the four reactive clones, one slow-growing *E. coli* recombinant clone, designated 961, was selected for further analysis. Of the four clones originally isolated, the 961 clone produced the least amount of 44 kDa protein by immunoblot comparison of the λ plaques, yet it was the only clone which did not die after the second passage following rescue. Fig. 2 (a, b) shows the results of two-dimensional gel analysis and immunoblotting of 961 and host cell XL1 with the 44 kDa periplasmic antiserum. There was minimal cross reactivity with *E. coli* XL1 proteins by the preabsorbed antiserum while a protein from clone 961 with a molecular mass of 44 kDa was observed to react. Interestingly, in Fig. 2(a), a smaller molecular mass protein of approximately 37 kDa was not seen to react with the 44 kDa periplasmic antiserum in clone 961 blots. Based upon the specificity and reactivity of the antiserum, we believe we have cloned the periplasm-associated 44 kDa protein of *N. gonorrhoeae* (Fig. 1).

Induction of the 961 clone with IPTG did not appreciably alter expression of the 44 kDa protein on one-dimensional SDS-PAGE gels (data not shown). This result suggests

Fig. 1. Two-dimensional gel electrophoretic analysis of *N. gonorrhoeae* strain MS11 probed with 44 kDa periplasmic antiserum. Whole-cell lysates of *N. gonorrhoeae* strain MS11 were stained with Coomassie brilliant blue and probed with 44 kDa periplasmic antiserum (Anti-44 kDa periplasmic). The first dimension isoelectric focusing (IEF) direction is shown. Samples were run in the second dimension on a 10% SDS-PAGE gel and either blotted for antibody probing or stained with Coomassie brilliant blue.

Fig. 2. Two-dimensional gel electrophoretic analysis of *E. coli* host strain XL1 and the 961 clone probed with 44 kDa periplasmic antiserum. (a) 961 clone stained with Coomassie brilliant blue (CBB) and probed with 44 kDa periplasmic antisera (Anti-44 kDa periplasmic). (b) *E. coli* host strain XL1 stained with Coomassie brilliant blue and probed with 44 kDa periplasmic antisera (Anti-44 kDa periplasmic). The first dimension isoelectric focusing (IEF) direction is shown. Samples were run in the second dimension on a 9% SDS-PAGE gel and either blotted for antibody probing, or stained with Coomassie brilliant blue.

that the protein is expressed from an endogenous promoter element located in the cloned fragment and that expression was not solely the result of the *lac* promoter in the pSK⁺ vector.

Of note, there are striking differences between the protein profiles for 961 and XL1 (Fig. 2a, b). This disparity in the protein profile may be evidence of a weakly growing *E. coli* recombinant. It is possible that the 44 kDa protein is toxic to *E. coli* and that the 961 clone may contain a truncated insert or a cloning artifact which lowers the expression and/or toxicity of this gene.

Sequence analysis of the 961 clone

The gonococcal DNA fragment contained within the recombinant clone was sequenced in its entirety and this sequence is shown in Fig. 3. The length of the sequenced fragment is 2439 bases. A partial ORF at the 5' end of the DNA insert was found to encode a protein of calculated molecular mass 24.9 kDa. This protein was searched against the databases and found to be most homologous

to *E. coli* EF-G (Blast score 282, probability 4.1×10^{-86}). A Bestfit analysis showed this partial ORF to be 76.7% similar and 65.0% identical to *E. coli* EF-G. Therefore, based upon this high percentage of identity it is most likely that this partially cloned gene is a *N. gonorrhoeae* *fusA* homologue. Immediately downstream of the *fusA* homologue was a potential ribosome-binding site followed by a second ORF which codes for a protein of calculated molecular mass 43 kDa. This protein was searched against the databases and found to be highly homologous to *E. coli* EF-TuA (Blast score 1675, probability 4.7×10^{-228}). A Bestfit analysis of this protein compared to *E. coli* EF-TuA showed 90.6% similarity and 82.9% identity between the two proteins. A Bestfit analysis with *E. coli* EF-TuB showed 90.3% similarity and 82.9% identity. Although it is not possible to differentiate whether or not the *N. gonorrhoeae* EF-Tu protein is an EF-TuA or EF-TuB homologue based upon these percentage identities or similarity scores, we believe that the presence of a *fusA* gene immediately upstream and an *rpsJ* gene (discussed below) immediately down-

Fig. 3. Sequence of the *N. gonorrhoeae* DNA fragment contained within the pSP961 plasmid. (a) The length of the *N. gonorrhoeae* DNA fragment contained within the pSP961 plasmid is 2439 bases. Ribosome-binding sites and the potential promoter element for *tufA* are labelled and underlined as RBS and -10 and -35, respectively. A large stem-loop, potential stop structure for the *str* operon is shown with inverted arrows. The lysine residue believed to be methylated is boxed and the potential cleavage site is shown with an arrow. Primers that were used to make the probe for Southern analysis are shown as line arrows with their respective primer names above. (b) A linkage map of the *fusG*, *tufA* and *rpsJ* genes based upon this PCR data and the sequence data.

stream of the EF-Tu ORF indicates that these genes constitute a portion of the *str* operon of the gonococcus. Therefore, based upon this *str* operon homology, the EF-Tu ORF is most likely encoded by *tufA*. Immediately downstream of the *tufA* homologue was a potential ribosome-binding site followed by a third ORF. This third ORF coded for a protein of calculated molecular mass 11.8 kDa. This protein was searched against the databases and found to be most homologous to *E. coli* S10 (Blast score 365, probability 1.0×10^{-45}). A Bestfit analysis of this ORF compared with *E. coli* S10 showed 82.5%

similarity and 68.9% identity between the two proteins. This high percentage of identity indicates that this ORF is most likely a gonococcal *rpsJ* homologue.

During the double-stranded sequencing of this DNA insert, it was discovered that the putative start codon for the *tufA* gene was an ATA codon. Oligonucleotide primers were made 5' (5' GTTGAAGTGGAACTCCG 3') of the start codon and 3' (5' TTTACCATGGTCA-ACGTG 3') of the start codon and a 424 bp product was amplified by PCR from the chromosome of *N. gonorrhoeae* strain FA19 and MS11. These two PCR products were sequenced with the same PCR primers and it was determined, as shown in Fig. 3, that the start codon for *tufA* was actually ATG for these two strains. It is tempting to speculate that the mutation in the 961 clone may have been selected by enhanced *E. coli* survival, thus producing the ATA codon artifact.

To confirm the genetic linkage of *fusG*, *tufA* and *rpsJ* genes, genomic DNA was isolated from *N. gonorrhoeae* strain MS11 and FA19 and PCR reactions were performed on these templates with primers situated within the 3' end of the *fusG* gene (5' GTTGAAGTGGAACTCCG 3') and a primer located downstream of the *rpsJ* gene (5' CAGCGCATCGGTAGTTT 3'). A PCR product of 1.9 kb, a size consistent with that predicted, was produced (data not shown). A linkage map of the *fusG*, *tufA* and *rpsJ* genes based upon this PCR data and the sequence data is shown in Fig. 3(b).

The *str* operon in *E. coli* is composed of *rpsL*, *rpsG*, *fusA*, and *tufA* (Jinks-Robertson & Nomura, 1987). Downstream of the TAA stop codon for the *rpsJ* gene in the *N. gonorrhoeae* cloned fragment are sequences suggestive of a transcriptional stop signal (Platt, 1986). This stop signal is composed of exact inverted repeats of 14 bp flanking 5 bases of non-homology as shown in Fig. 3. This structure suggests that this is the end of the *str* operon for *N. gonorrhoeae*. The location of the *rpsJ* gene in the gonococcal *str* operon is in contrast to the *str* operon in *E. coli*; the significance of this finding is unclear.

Expression of the *str* operon in *E. coli* is controlled by a promoter in the *rpsL* gene which resides immediately upstream of *rpsG* and *fusA*. In *E. coli*, expression of *tufA* is higher than that of the other genes in the operon. This has been in part explained by the presence of several potential promoter elements in the terminal portion of *fusA* (Zengel *et al.*, 1984). Promoter searches of the terminal region of the gonococcal *fusG* gene and the intergenic region between *fusG* and *tufA* were performed using an algorithm based upon *E. coli* consensus promoter sequences (Mulligan *et al.*, 1984). Only a single potential promoter was identified, upstream of the gonococcal *tufA* gene (Fig. 3), which has a sequence similarity score of 48% denoting a relatively weak promoter.

Processing of the *N. gonorrhoeae* EF-Tu protein

To determine whether the 37 kDa protein which reacted with the 44 kDa periplasmic antiserum was in fact a truncated or a processed form of EF-Tu, antibody

Fig. 4. One- and two-dimensional gel electrophoretic and immunoblot analysis of *N. gonorrhoeae* strain FA19 probed with an EF-Tu-specific antiserum. One- and two-dimensional gel electrophoretic analysis of *N. gonorrhoeae* strain FA19 immunoblotted with the peptide-specific EF-Tu antiserum (FRKLLDEGQA). The blot was subsequently stained with Indian ink. The 44 kDa and 37 kDa proteins reactive with the anti-FRKLLDEGQA antisera and visualized with the India Ink are shown with asterisks.

previously generated against a portion of the gonococcal EF-Tu protein sequence (FRKLLDEGQA) known to be highly conserved amongst many EF-TuA and EF-TuB proteins (Judd & Porcella, 1993) was used in immunoblots of one- and two-dimensional gels of *N. gonorrhoeae* strain FA19 whole-cell lysates. This antiserum reacted with both the 44 kDa and 37 kDa proteins as shown in Fig. 4. The antiserum was immunoblotted against the 961 recombinant clone and only the 44 kDa protein was detected (data not shown). These results suggest that the gonococcal EF-Tu protein is processed or cleaved to a smaller molecular mass form (37 kDa) in *N. gonorrhoeae* but not in the *E. coli* recombinant. Of note, previous use of this antiserum in one-dimensional immunoblots of *N. gonorrhoeae* strain FA19 lysates detected the 37 kDa protein in residue extracts while it failed to detect the 37 kDa protein in whole-cell lysates (Judd & Porcella, 1993). Where a less dilute mixture of the antiserum was used in previous studies a 37 kDa protein was readily detected in whole cell lysates as shown in Fig. 4.

Evidence for a truncated or processed EF-Tu protein was recently described in a phage exclusion system in *E. coli* (Yu & Snyder, 1994). The exclusion mechanism was due to a prophage encoded Lit protease specific for EF-Tu. Cleavage at a very specific and highly conserved sequence produced a 37 kDa EF-Tu fragment which halted protein synthesis as a mechanism of immunity in the infected cell. The conserved site where endogenous cleavage has been demonstrated in *E. coli* (Yu & Snyder, 1994) and predicted for *N. gonorrhoeae* is at position 60 in the EF-TuA protein as shown in Fig. 3. The appearance of a lower molecular mass EF-Tu protein in the gonococcus is the first example

of EF-Tu cleavage in a bacterium other than e14-containing *E. coli* K-12 strains and has interesting implications with regard to possible gonococcal phage exclusion mechanisms, gonococcal prophage elements, or an as yet undefined endogenous self-EF-Tu cleavage system. It is also important to note that no phage capable of infecting the gonococcus has ever been described.

EF-Tu appears to be influenced by nutrient availability and growth rate conditions, being methylated and bound to the cytoplasmic membrane during nutrient-limiting conditions (Young & Bernlohr, 1991; Young *et al.*, 1990). The putative methylation site described for *E. coli* and proposed for *N. gonorrhoeae* is at Lys-57 in the EF-TuA protein sequence as shown in Fig. 3. EF-Tu concentrations, in terms of freely soluble ternary complexes, have been shown to directly affect growth and translation elongation rates (Tubulekas & Hughes, 1993). A computer simulation of the *E. coli* translation system suggests that the concentration of EF-Tu should have a direct cause-and-effect relationship upon the dynamics and accuracy of protein synthesis (Pingoud *et al.*, 1990). Therefore, self-cleavage of EF-Tu, with concomitant modulation of the rate of protein synthesis and/or the possible removal of the methylated portion of EF-Tu may have relevance to gonococcal survival and growth in diverse, nutrient-fluctuating environments.

Attempted deletion of *tufA*

A fragment of DNA containing the *tufA* gene was cut from the cloned insert in 961 using *Cla*I and *Eco*RI enzymes. This fragment was ligated into the pHSS6

Fig. 5. Southern blot analysis of *N. gonorrhoeae* *tufA* mutants. Southern blot analysis was performed with MS11 genomic DNA (MS11), MS11 mutant transformant MS11-Tuf-2 genomic DNA and MS11-Tuf-3 genomic DNA digested with *Hind*III (H), *Hind*III + *Cla*I (HC), or *Cla*I (C).

mutagenesis vector and mutagenized. The fragment containing the insertionally inactivated *tufA* gene was characterized by PCR using the oligos 3'TufA, 5'TufA and Cat. The defective chloramphenicol-containing transposon was located within the *tufA* gene in a 5'-to-3' orientation. The transposon resided approximately 0.6 kb from the 5' end of the *Cla*I/*Eco*RI fragment and 1.1 kb from the 3' end of the fragment. The mutagenized DNA was obtained from the vector by digesting with *Not*I. After gel purification, the linear DNA fragment was transformed into pili-positive MS11 *N. gonorrhoeae* and mutants resistant to chloramphenicol were selected. Twenty-four slow-growing, chloramphenicol-resistant mutants were identified. The plates were rechecked for mutants growing extremely slowly by longer incubation times, but no colonies were detected. From the 24 mutants, two were randomly selected (designated MS11-Tuf-2 and MS11-Tuf-3) and characterized by Southern blot analysis and PCR. The remaining 22 mutants were characterized solely by PCR. Southern blot analysis of the MS11-Tuf-2 and MS11-Tuf-3 mutants is shown in Fig. 5. The double-stranded probe used in the blot was constructed by PCR using primers internal to the *tufA* gene [(5' primer (5' CACTACGCACACGTAGA 3'), 3' primer (5' TTGGTGTGAGGAGTGA 3')]. The locations of these primers are shown in Fig. 3. Southern analysis results in Fig. 5 show that the probe hybridized with both *tufA* and another gene, presumably *tufB* (Goldstein *et al.*, 1989). Based upon the presence of the *Hind*III sites in the cloned fragment, as shown in Fig. 3, we believe that the 1 kb fragment hybridizing in the *Hind*III-restricted lanes is in fact the *tufA* gene. In the lanes marked MS11-Tuf-2 and MS11-Tuf-3 a further hybridizing fragment(s) is present while the wild-type

hybridizing fragments are unchanged. The transposon within the mutated *tufA* allele contains a *Hind*III site that would produce a hybridizing fragment of approximately 2.3 kb. This 2.3 kb hybridizing fragment can be seen in Fig. 5. The *Hind*III fragments seen at approximately 3.8–4.0 kb are probably the result of an unknown *Hind*III site located in the genome adjacent to the mutated allele. If a homologous recombination event had taken place between the transforming DNA and the *tufA* locus a shift or loss of the 1 kb band in the MS11-Tuf-2- and/or MS11-Tuf-3-digested DNA would have been seen in the Southern analysis. Instead, it appears that a duplication has occurred following transformation of MS11 with the mutated *tufA* gene. It is possible that the transposon-mutagenized fragment recombined with the smaller *Cla*I fragment resulting in a lethal event and autolysis. Transformation of a neighbouring cell by this region of the chromosome would result in a duplication of this area in the transformant. This type of result has been described previously (Taha *et al.*, 1988) for the *pilA* gene of *N. gonorrhoeae*. Southern analysis data indicate that the two *tufA* mutants isolated are heterodiploids in that they have gained the chloramphenicol-resistance-conferring fragment while maintaining the integrity of the genomic copies of the *tufA* and *tufB* genes.

PCR analysis using the 3'TufA, 5'TufA and Cat primers was performed on the MS11-Tuf-2 and MS11-Tuf-3 mutants. These data are not shown but are summarized as follows. PCR was first performed using the primers 3'TufA and 5'TufA on the transforming DNA and was shown to produce a fragment of 3 kb; consistent with the size predicted for the transposon-containing fragment. PCR analysis with the same primers performed on the wild-type MS11 DNA produced a 1.5 kb fragment; consistent with the expected size for this portion of the *str* operon. PCR analysis of the wild-type DNA and the transforming DNA with the 5'TufA and Cat primer produced no DNA fragment in the wild-type and a fragment of 2.0 kb (consistent with the location of the transposon) for the transforming DNA. Genomic DNA was isolated from the two mutants and PCR, using the 5'TufA and 3'TufA primers, produced a 1.5 kb fragment for each mutant, thereby indicating that an intact copy of the *tufA* gene still existed. PCR analysis with the 5'TufA and Cat primer produced a fragment of 2 kb in the two mutants indicating that the transforming DNA was located in the chromosome in an unknown location. This PCR analysis confirmed the Southern blot data in that presumably functional copies of the *tufA* gene were still intact while the transforming DNA was present but located in an unknown region of the chromosome.

It is generally believed that the 1% nucleotide difference that exists between the *tufA* and *tufB* genes in *E. coli* and *S. typhimurium* is sufficient to prevent high levels of homologous recombination between the genes. Given this premise and the fact that roughly 1.7 kb of *str* operon-specific DNA flanked the transposon we believed that the odds of recombination occurring at the *tufB* locus were minimal. Given that our primary interest was in the deletion of the *tufA* gene, we decided to characterize the

remaining 22 mutants by PCR analysis alone using the 3'TufA, 5'TufA and Cat primers. Unfortunately, all of the remaining 22 mutants produced PCR profiles identical to those previously described for MS11-Tuf-2 and MS11-Tuf-3. Therefore, we were unable to functionally delete the *tufA* gene of *N. gonorrhoeae*.

The *tufB* gene of *E. coli* has been inactivated by insertion of a Mu phage genome (van de Klundert *et al.*, 1978; Young & Furano, 1981) while the *tufA* gene has been shown to be essential (Vijgenboom & Bosch, 1987). It is possible that in our mutagenesis procedures a potential polar effect upon the *rpsJ* gene may have occurred. Given the unusual location of the *rpsJ* gene in the *N. gonorrhoeae* *str* operon (as compared to the location of this gene in *E. coli*) we do not know whether this gene is essential in this context.

The presence of heterodiploids arising during the mutagenesis of a potentially essential gene such as *tufA* is similar to the phenomenon observed with the *pilA* gene, a gene thought to be essential in *N. gonorrhoeae* (Taha *et al.*, 1988). These results are consistent with the notion that the *tufA* gene of *N. gonorrhoeae* is essential.

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