Cloning and characterization of the *Neisseria gonorrhoeae* MS11 *folC* gene

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**Abstract** The gene coding for folylpoly-(γ)-glutamate synthetase (FPGS)-dihydrofolate synthetase (DHFS) of *Neisseria gonorrhoeae* (*Ngo*) has been cloned by functional complementation of an *Escherichia coli* *folC* mutant (SF4). The sequence encodes a 224-residue protein of 46.4 kDa. It shows 46% identity to the *E. coli* FPGS-DHFS and 29% identity to the FPGS of *Lactobacillus casei*. Sequence comparisons between the three genes reveal regions of high homology, including ATP binding sites required for bifunctionality, all of which may be important for FPGS activity. In contrast to *L. casei* FPGS, the *E. coli* and *Ngo* enzymes share some additional regions which may be essential for DHFS activity. The products of *Ngo* *folC* and flanking genes were monitored by T7 promoter expression. Interestingly, deletion of the upstream *folI* gene, which encodes a 16.5 kDa protein, abolishes the capacity of *folC* to complement *E. coli* SF4 to the wild-type phenotype. The ability to complement can be restored by *folI* provided in trans. Unlike *folC* mutants, gonococcal *folI* mutants are viable and display no apparent phenotype. Thus, in contrast to *E. coli*, *Ngo* *folC* is expressed at a sufficiently high level from its own promoter, in the absence of *Foll*. This study provides the first insights into the genetic complexity of one-carbon metabolism in *Ngo*.

**Key words** Gonococcus · Folic acid · Dihydrofolate synthetase · Folylpolyglutamate synthetase · One-carbon metabolism

**Introduction**

The addition of *p*-aminobenzoic acid to 2-amino-4-oxo-6-hydroxymethyl-7,8 dihydropteridine pyrophosphate produces dihydropteroate (Brown et al. 1961; Weisman and Braun 1964). This is the substrate for the ATP-dependent attachment of L-glutamate to form dihydrofolate, a reaction catalysed by dihydrofolate synthetase (DHFS; Griffin and Brown 1964). The reduced form of dihydrofolate, tetrahydrofolate (THF), can accept, in a readily reversible reaction, a CH$_2$OH group from serine to give rise to glycine plus a one-carbon fragment (hydroxymethyl-THF). Reaction of glycine with THF yields a second CH$_2$OH-THF (and CO$_2$ plus NH$_3$). The CH$_2$OH-THF derived from serine or glycine is used in a variety of biosynthetic reactions. It can be isomerized to 5,10 methylene-THF, reduced to 5-methyl-THF, or oxidized to 5,10 methenyl-THF and its isomers 1-, 5- or 10-formyl-THF. At these various levels of oxidation it provides the formyl group of formyl-Met-tRNA or for the closure of the two rings in purines, the methyl group of methionine, thymine, and ketopantoate, a precursor of panthotenate and acetyl-CoA (Brown and Williamson 1987).

Nearly all of the intracellular folate coenzymes in bacteria as well as in mammalian cells occur as polyglutamate derivatives containing glutamate residues in γ-peptide linkage (Griffin and Brown 1964; Masurekar and Brown 1975) (for *Escherichia coli* additional z-peptide linkages have been shown; Ferone et al. 1986a, b). These folylpolyglutamates are preferentially retained by bacteria and mammalian tissues helping to concentrate folates intracellularly (Shane and Stokstad 1975; Taylor and Hanna 1977). The various oxidation levels of THF vary in glutamate chain configuration and length, and therefore represent different coenzymes for the enzymes of one-carbon metabolism which recognize and favour a specific polyglutamate tail (McGuire and Coward 1984). Glutamate chain
lengths have been suggested to be regulated by the physiological state of a cell, thereby indirectly influencing the one-carbon flux through the various folate-dependent reactions (Kisliuk 1981; Cichowicz et al. 1981; Shane et al. 1983).

The enzyme folic poly-(r)-glutamate synthetase (FPGS), which catalyzes the ATP-dependent attachment of glutamates to pteroylmonoglutamates has been purified from Corynebacterium spp. (Shane 1980a, b), Lactobacillus casei (Bognar and Shane 1983), and E. coli (Bognar et al. 1985), as well as from a variety of mammalian sources (Cichowicz and Shane 1987; McGuire et al. 1980). Whereas mammalian cells and L. casei are not able to produce folate and require exogenous folate for growth (Bognar and Shane 1986; Garrow et al. 1992), Corynebacterium (Shane 1980b) and E. coli (Bognar et al. 1985) have been shown to possess an additional DHFS activity responsible for folate synthesis de novo. DHFS and FPGS activities have been shown to copurify and are mediated by a bifunctional enzyme encoded in E. coli by folC (Bognar et al. 1985; Ferone et al. 1983; Ferone and Warkow 1983). The DHFS activity has been proposed to be essential (Pyne and Bognar 1992), whereas the loss of FPGS activity may only result in methionine auxotrophy (Ferone et al. 1983). For folC in E. coli, differential loss of one of the two activities could not be observed in viable mutants, suggesting that a single site is required for both activities (Ferone et al. 1983). One viable mutant, SF4 (Bognar et al. 1985; Ferone et al. 1983), contains a point mutation causing an alanine-to-threonine amino acid change at codon 309 (Keshavjee et al. 1991) which leads to methionine auxotrophy in the absence of vitamin B12 due to a decrease in intracellular folates to 10–30% of the wild-type level (Ferone et al. 1983). To date, the folC genes of L. casei (Toy and Bognar 1990), E. coli (Bognar et al. 1985; Bognar et al. 1987), and humans (Garow et al. 1992) have been cloned and characterized. Homologous genes were found by sequence comparisons in Bacillus subtilis (Margolis et al. 1993), Streptococcus pneumoniae and Saccharomyces cerevisiae (Lacks et al. 1995), all of which await further characterization. In this report, we describe the cloning, sequencing and expression in wild-type E. coli and its mutant SF4 of the FPGS-DHFS gene, folC, of Neisseria gonorrhoeae (Ngo). We also describe an upstream gene, folI, which is involved in folC regulation.

Materials and methods

Strains and plasmids

Commensal and pathogenic Neisseria strains are from our laboratory collection. Most experiments described in this work were performed with Ngo strain MS11, N200 (PilE variant A). Gonococci were grown on GC agar base with vitamin supplement (Becton Dickinson), at 37°C in 5% CO2 and passaged daily. E. coli E131 (recA sCh616) was used for cloning and transposon mutagenesis (Haas et al. 1993). E. coli auxotrophic mutant SF4 (F' folC strA recA srlc::TnlO) kindly provided by Barry Shane, was used for folC complementation analysis. E. coli strains were grown in LB medium supplemented with ampicillin (30 µg/ml, 25 µg/ml for complementation), tetracycline (10 µg/ml) or chloramphenicol (30 µg/ml). SF4 was grown on Vogel-Bonner minimal medium (Vogel and Bonner 1956) supplemented with methionine (50 µg/ml) and glycine (50 µg/ml). Unsupplemented medium solidified with 1.5% agar was used for complementation analysis.

Plasmid constructions

The selected plasmid library clone pMF50 was constructed as described (Kahrs et al. 1994). Plasmid pMF47 contains the NsiI-PesII fragment cloned into the PstI/EcoRV sites of pBluescript II KS−. The transcriptional orientation of the structural gene folC is towards the lac promoter and it can be expressed from the T7 promoter. Plasmid pMF70 contains the same fragment as pMF47 under control of the lac promoter on the low-copy-number plasmid pWSK29 (Wang and Kushner 1991). The fragment was cut out of pMF47 with BamHI + HindIII and ligated to the corresponding sites of pWSK29. pMF46 contains the NsiI-BamHI fragment of pMF50 cloned into pBluescript II KS− PstI/BamHI; folC and the downstream gene can be expressed from the T7 promoter. pMF42 contains the downstream gene and its promoter, which is located within the folC gene. The EcoRI fragment of pMF50 was cloned into pBluescript II SK+ (EcoRI) to give pMF66. pMF66 contains the EcoRI fragment of the inducer gene folI under control of the lac promoter; pMF71 contains the same fragment of the reversed orientation, under the control of the T7 promoter. pMF68 is a pACYC184-based derivative (Chang and Cohen 1978) containing folI on the BamHI-HindIII fragment of pMF66 and was used for in-trans induction of folI on pMF47.

DNA manipulations

All standard cloning and DNA analysis procedures were performed according to Sambrook et al. (1989). Plasmid DNA was purified from E. coli by the alkaline lysis procedure. E. coli cells for electroporation were prepared according to the recommended protocol for the Gene Pulser apparatus (BioRad). Transformation of naturally competent Ngo was performed as described before (Gibbs et al. 1989; Rudel et al. 1992). Transformants were selected on chloramphenicol (10 µg/ml) and verified by Southern blot analysis. Southern blotting and hybridizations with DNA fragments as probes were performed using the ECL labelling and detection system according to the manufacturer’s protocol (Amersham). For hybridization 0.5 M NaCl was used; washing was done using 0.5% SSC; 6 M urea and 0.4% SDS at 42°C. Chromosomal DNA of Ngo was isolated by a protocol adapted from that described by Sakari et al. (1994) for N. meningitidis. Sequencing was performed according to the diodeoxyribonucleotide chain termination method (Sanger et al. 1977) using a Prism Ready Reaction Dye Cycle Sequencing Kit (Applied Biosystems) with fluorescence-labelled M13 and RP1 primers on an Applied Biosystems 373A automated sequencer. Plasmid pMF50 with various TnMax5 inserts was digested with BamHI and used as template. These plasmids carried mobile M13 and RP1 primers on the TnMax5 mini-transposon which was inserted at different locations within folI, folC or the gene located downstream. An average of 430 nucleotides could be sequenced upstream and downstream of each TnMax5 insertion, resulting in overlapping sequence on both strands. Additional sequence data were obtained from the subclones in pBluescript II, sequenced from their M13 and RP1 primer-binding sites.