Identification of O-antigen polymerase transcription and translation start signals and visualization of the protein in *Salmonella enterica* serovar Typhimurium

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The *wzy/rfc* gene, encoding the O-antigen polymerase, of *Salmonella enterica* serovar Typhimurium has been previously cloned and sequenced. In the present work, the *wzy* transcriptional startpoint was initially identified by primer extension. Next, *wzy* promoter strength in *Escherichia coli* K-12 was measured, and was found to be greater than that of the induced *lac* promoter. To define the Wzy translational startpoint, DNA including the *wzy* promoter and the putative first five residues of the Wzy protein was fused to the N-terminus of glutathione-S-transferase, and the fusion protein purified by affinity chromatography. N-terminal amino acid sequencing yielded the Wzy translational startpoint. Next, the Wzy protein was C-terminally tagged with the FLAG peptide, and immunoblotting of an *S. typhimurium* strain expressing a low-copy *wzy-FLAG* gene (five copies per cell) localized the intact Wzy protein in the cytoplasmic membrane of *S. typhimurium* cells. The Wzy protein was not well-expressed from a multi-copy *wzy-FLAG*⁺ plasmid in *S. typhimurium*, or in *E. coli* K-12.

Keywords: Wzy, Rfc, lipopolysaccharide, semi-rough

INTRODUCTION

The *wzy* gene (Reeves *et al.*, 1996), which encodes the 47472 Da Wzy protein, was previously cloned and sequenced, from *Salmonella enterica* serovar Typhimurium (henceforth termed *S. typhimurium*) (Collins & Hackett, 1991). The Wzy protein is the O-antigen polymerase. Thus, *wzy* mutants of *S. typhimurium* have a semi-rough phenotype, in which individual lipid A molecules carry at most a single O-antigen unit. If Wzy is synthesized, the typical O-antigen ladder of wild-type smooth *S. typhimurium* is seen (Collins & Hackett, 1991). The *wzy* gene of *S. typhimurium* maps in the 35·6 centisome region on the chromosome (Naide *et al.*, 1965), at a site remote from the *rfb* gene cluster.

The *wzy* genes of several other enterobacteria have since been cloned and sequenced. Genes analysed in this manner have been identified as *wzy* genes either on the basis of homology with other *wzy* genes, or because the

genes complement mutations that result in semi-rough phenotypes. The *wzy* genes of various *S. enterica* serovars have received most attention. It is likely that a close homologue of the *S. typhimurium wzy* gene is active in *S. enterica* strains of groups A and D1, as the *S. typhimurium wzy* gene detected homologues in strains of these groups when used as a probe in Southern blotting (Collins & Hackett, 1991), and the same $\alpha(1,2)$ linkage is present between O-antigen units of strains of groups B (*typhimurium*), A and D1 (Mäkelä, 1965; Nurminen *et al.*, 1971). A group C2 strain of *S. enterica* also carries a gene with detectable homology to *S. typhimurium wzy* within a block of group C2-unique genes in the main *rfb* gene cluster (Brown *et al.*, 1992). A different *wzy* gene (termed *wzy*^{D2E1}) (Xiang *et al.*, 1994), located in the main *rfb* gene cluster, has been identified in *S. enterica* strains of groups D2 and E1 [which have the same $\alpha(1,6)$ O-antigen linkage], partly on the basis of the fact that this is the only gene common to the *rfb* clusters of strains of these groups to which no *rfb* function has been assigned (Xiang *et al.*, 1994). However, a *wzy* gene of *S. enterica* group D3 (*wzy*^{D3}) which

Abbreviation: GST, glutathione-S-transferase.

also gives $\alpha(1,6)$ linkage has no detectable sequence similarity to *wzy*^{D2E1} (Curd *et al.*, 1998).

The *wzy* genes of some smooth strains of *Escherichia coli* have been identified either by detectable homology to *S. typhimurium wzy* (Yao & Valvano, 1994) or by the complementation of mutations in *rfb* regions (Batchelor *et al.*, 1991; Liu & Reeves, 1994; Lukomski *et al.*, 1996). Complementation analysis has also indicated that the *wzy* genes of *Shigella dysenteriae* (Klena & Schnaitman, 1993), *Shigella flexneri* (Morona *et al.*, 1994), *Yersinia enterocolitica* (Zhang *et al.*, 1996) and *Pseudomonas aeruginosa* (Coyne & Goldberg, 1995; De Kievit *et al.*, 1995) are located in their respective *rfb* gene clusters.

Putative Wzy proteins share certain structural features. First, the putative proteins have multiple putative membrane-spanning domains (Collins & Hackett, 1991; De Kievit *et al.*, 1995; Morona *et al.*, 1994; Xiang *et al.*, 1994; Zhang *et al.*, 1996). The GC content of *wzy* genes is generally lower than that of the bulk DNA of the bacterium in which the genes are located (Collins & Hackett, 1991; De Kievit *et al.*, 1995). Analysis of codon usage in *wzy* genes indicates that 'rare' codons (Grosjean & Fiers, 1982) are overrepresented, compared to codon usage by other genes sequenced from bacteria containing *wzy* genes (Collins & Hackett, 1991; De Kievit *et al.*, 1995; Morona *et al.*, 1994).

Previously (Collins & Hackett, 1991), it was not possible to view labelled Wzy protein in minicells of an *E. coli* K-12 strain containing a high-copy-number *wzy*⁺ plasmid, nor was Wzy detectable when the *wzy* gene was under the control of the T7 promoter in a high copy-number plasmid, in an *E. coli* strain expressing the bacteriophage T7 RNA polymerase. Likewise, expression of the *S. flexneri* Wzy protein could not be detected in *E. coli* K-12 under conditions where the products of adjacent cloned genes were visible after protein labelling (Morona *et al.*, 1994). It has been suggested (Collins & Hackett, 1991) that the high usage of rare codons in the *wzy* gene of *S. typhimurium* may greatly limit Wzy production in *E. coli*, and the concentration of rare codons in the N-terminal region of the *S. flexneri rfc* gene has been proposed to exert an attenuating effect on Wzy translation (Morona *et al.*, 1994). Indeed, when 2–3 rare codons in the N-terminal region of the *S. flexneri wzy* gene were mutated to synonymous major codons, and the mutated gene then placed under the control of the T7 promoter and the Shine–Dalgarno sequence from gene 10 of phage T7, a low level of Wzy was detected in whole *E. coli* K-12 cells following induction of the T7 polymerase (provided on a separate plasmid) (Daniels *et al.*, 1998).

Unlike other *wzy* genes, that of *S. typhimurium* is not located in the *rfb* gene cluster, but at a distant site. The *S. flexneri wzy* gene does not have its own promoter, being part of the *rfb* operon. Earlier, no attempt was made to determine (other than by sequence inspection) the transcriptional start point of the *S. typhimurium wzy* gene, and the possibility that *wzy* might be transcribed by readthrough from a plasmid promoter

was not formally excluded. Also, because of the difficulties experienced in obtaining Wzy expression, it was not possible to determine (by N-terminal analysis of purified protein) the translational start point, or to raise antibody against purified Wzy for use in defining the subcellular localization of the protein. Pulse-chase experiments have shown that polymerization of O-specific lipopolysaccharide chains takes place at the periplasmic face of the inner membrane (McGrath & Osborn, 1991), and it is thus likely that Wzy might be located in the cytoplasmic-membrane fraction. Also, Daniels *et al.* (1998) constructed (*S. flexneri*) Wzy–PhoA fusion proteins, and found that fusion proteins with up to 167 aa of Wzy were located in the 'membrane fraction'. The fusion points of expressive *lacZ* and *phoA* fusions with *S. flexneri wzy* further indicated that the Wzy protein was located in the cytoplasmic membrane, with 12 transmembrane segments (Daniels *et al.*, 1998). It remains true, however, that a full-length Wzy protein has never been visualized, in either whole cells or the cytoplasmic-membrane fraction. The topics of *S. typhimurium wzy* transcriptional and Wzy translational start signals, the subcellular localization of Wzy and Wzy quantification are addressed in this paper.

METHODS

Materials. Enzymes active on DNA were obtained from either Gibco-BRL or Boehringer Mannheim and were used as directed by the suppliers. IPTG was purchased from Amersham Pharmacia. [α -³²P]dCTP ($\sim 1.1 \times 10^{14}$ Bq mmol⁻¹) and [γ -³²P]ATP ($\sim 1.1 \times 10^{14}$ Bq mmol⁻¹) were from Amersham Pharmacia. Oligodeoxynucleotide primers were synthesized by Gibco-BRL. Rabbit anti-FLAG peptide antibodies were the product of Santa Cruz Biotechnology. The secondary antibody for ELISA [phosphatase-labelled goat anti-rabbit IgG (heavy and light chains)] was purchased from Pierce Laboratories. The secondary antibody for immunoblotting (donkey anti-rabbit IgG, conjugated with horseradish peroxidase) was from Amersham Pharmacia. As an ELISA standard, bacterial alkaline phosphatase, C-terminally tagged with the FLAG peptide, was obtained from Eastman Kodak. ELISA plates were purchased from Corning.

Strains and vectors. *E. coli* K-12 DH5 α (Hanahan, 1983) was the host for recombinant plasmids. Promoter strengths were assessed in the background of *E. coli* K-12 JM109 (Yanisch-Perron *et al.*, 1985). *E. coli* BL21(DE3) pLysS (Phillips *et al.*, 1984) was the host for expression of a fusion protein in which part of Wzy was attached to glutathione-S-transferase (GST) (Phillips *et al.*, 1984). *S. typhimurium* J357 (Cerin & Hackett, 1989) was used to modify plasmid DNA before transformation into other *S. typhimurium* strains. *S. typhimurium* C5 (wild-type) and *S. typhimurium* LV386 (*wzy*::IS10) have been described previously (Collins & Hackett, 1991). The low-copy plasmids pCL1920 (Lerner & Inouye, 1990), pQF50 (which contains a promoterless *lacZ* gene after a *Hind*III insertion site) (Farinha & Kropinski, 1990), pGEX-2T (Smith & Johnson, 1988) and pUC18 (Norrandar *et al.*, 1983), were used. Plasmid pADE206 (*wzy*⁺) has been described previously (Collins & Hackett, 1991).

Growth media. Minimal M9 medium was prepared as described by Miller (1972) and supplemented prior to use with MgSO₄ (0.2 mg ml⁻¹), glucose (2 mg ml⁻¹) and thiamin-HCl

Fig. 1. Constructions in the N- and C-terminal regions of the *wzy* gene of *S. typhimurium*, and features of interest in these regions. The nucleotide numbers refer to GenBank submission M60066. The *wzy* promoter region, the *Wzy* translational start, the *Wzy* C-terminus, and the junctions of fusions of *Wzy* with *GST* and the *FLAG* peptide are shown.

(50 µg ml⁻¹). Luria-Bertani (LB) medium was prepared with Bacto tryptone (10 g l⁻¹), Bacto yeast extract (5 g l⁻¹) and NaCl (10 g l⁻¹). 2XYT medium has been described previously (Collins & Hackett, 1991). Liquid media were solidified, when required, by the addition of 1.5% Oxoid bacteriological agar. Bacterial growth was at 37 °C unless specified otherwise. Ampicillin was added to broth and solid medium at a final concentration of 50 µg ml⁻¹. Spectinomycin was used at 50 µg ml⁻¹ (*E. coli*) or 500 µg ml⁻¹ (*S. typhimurium*).

Cell fractionation. Cultures (40 ml) were grown in 2XYT for 3 h. Cells were harvested by centrifugation at 5000 g. The pellet, after washing in 30 mM Tris/HCl, pH 8.1, was resuspended in 0.8 ml 20% (w/v) sucrose in 30 mM Tris/HCl, pH 8.1. Lysozyme (10 mg ml⁻¹ in 0.1 M EDTA) was added to a final concentration of 0.4 mg ml⁻¹. The cells were frozen in an ethanol/dry ice bath for 30 min and then thawed at room temperature. Twelve millilitres of 3 mM EDTA was added and cells were lysed by sonication (Branson sonicator) until the cell lysate became clear (usually 4 × 25 s bursts). Unlysed cells were removed by centrifugation at 5000 g. The lysate was separated into cytoplasmic and total membrane fractions by centrifugation at 200000 g for 1 h. The resulting pellet (total membrane) was dissolved in PBS (0.3 ml), followed by the addition of sodium lauryl sarcosine (Sarkosyl) in 7 mM EDTA, to 1%. The mixture was incubated at 37 °C for 30 min and then centrifuged at 200000 g for 2 h. The supernatant (Sarkosyl-soluble fraction) is the cytoplasmic-membrane fraction, while the pellet is the outer-membrane fraction. The pellet was washed with 10 mM Tris/HCl, pH 7.4 and recentrifuged at 200000 g for 2 h. The final pellet was then dissolved in PBS.

General analytical methods. DNA manipulations followed established methods (Sambrook *et al.*, 1989). Development of Western blots used the Enhanced Chemiluminescence system (Amersham Pharmacia). SDS-PAGE was usually performed on 5% (stacking gel)–12% (separating gel) polyacrylamide, and proteins were stained with 0.25% Coomassie brilliant blue G250. The gel loading buffer contained 2% SDS and 100 mM DTT (Sambrook *et al.*, 1989). Lipopolysaccharide-specific silver staining of SDS-PAGE gels was performed as described by Hitchcock & Brown (1983). The ELISA method

has been published by Ausubel *et al.* (1993). The *GST* Gene Fusion System was purchased from Amersham Pharmacia. N-terminal protein sequencing was performed at the Biotechnology Laboratory/NAPS Unit, University of British Columbia, Canada.

Accessing the *S. typhimurium wzy* sequence. When the *wzy* sequence is mentioned below, the numbers shown are the nucleotide numbers from GenBank submission M60066, where nt 1 is the first nucleotide in the 1750 bp *Hind*III fragment carrying the *wzy* gene of *S. typhimurium*. Fig. 1 shows the *wzy* promoter region, the translational start, the C-terminus, and the junctions of fusions of *Wzy* with *GST* and the *FLAG* peptide (see below).

Testing of promoter strength. To assay promoter strengths, PCR fragments, in which various promoters were contained on *Sph*I–*Hind*III fragments, were cloned into plasmid pQF50. To amplify the *wzy* promoter, the primers used were *prwzy*UP1 (5′-251-GATAGCTgCATGCTATCGGAGGCG-274-3′) and *prwzy*DOWN1 (5′-344-CATCACAAGCTTCCCGATA-326-3′). The nucleotides in lower case are substitutions in the sequence (T to G, nt 258; T to A and T to G; nts 336 and 338) to create an *Sph*I site (*prwzy*UP1) or a *Hind*III site (*prwzy*DOWN1). To insert the *lac* promoter (with the catabolite-activator-protein binding site and *lac* operator) into pQF50, the primers used were *prlac*UP (5′-634-GCA-GCTGGCAtGcCAGGTTTCCC-612-3′) and *prlac*DOWN (5′-463-TGGTCAaAGCTtTTTCTGTG-483-3′), where the numbers refer to the sequence of pUC18 (GenBank accession no. L09136) (Norlander *et al.*, 1983). The nucleotides in lower case are substitutions in this sequence (C to T, nt 624 and A to C, nt 622; T to A, nt 469 and G to T, nt 474) in order to create an *Sph*I site (*prlac*UP) or a *Hind*III site (*prlac*DOWN). To insert the *tac* promoter into pQF50, the primers used to amplify DNA from plasmid pGEX-2T were *prtac*UP (5′-65-GGCTGTGCAgGcCGTAAATCAC-86-3′) and *prtac*DOWN (5′-262-GACATGAAgctTGTTTCTGTG-243-3′) where the numbers refer to the GenBank sequence U13850 (Smith & Johnson, 1988). The bases in lower case are substitutions in the sequence (G to T, nt 74 and T to C, nt 76; TAC to GCT, nts 254–252) in order to create an *Sph*I site (*prtac*UP) or a *Hind*III site (*prtac*DOWN). Transformed

strains of *E. coli* K-12 JM109, carrying these plasmids, were grown in M9 minimal medium to $\sim 5 \times 10^8$ cells ml⁻¹. Cells were pelleted, resuspended in Z-buffer (0.1–0.5 ml) (Miller, 1972) and lysed with 2 drops chloroform and 1 drop 0.1% SDS. The lysates were assayed for β -galactosidase activity as expressed in Miller units (Miller, 1972).

Fusion of part of *wzy* with the *gst* gene. To determine the translation startpoint of the Rfc protein, a portion of the *wzy* gene, including the *wzy* promoter and the DNA encoding the first 5 residues of the putative Wzy protein, was fused to the N terminus of the *gst* gene. First, an *EcoRI*–*KpnI* fragment, containing the required *wzy* DNA, was cloned into pUC18 and the resulting plasmid then received a *KpnI*–*HindIII* fragment containing the required *gst* DNA, to make plasmid pUST013. The primers used to amplify the *wzy* DNA were *prwzyUP2* (5′-39-TACTTGgAATTCTGTGTG-57-3′) and *prwzyDOWN2* (5′-490-GCATAAgGtAccGTATGAAATT-AT-467-3′). The nucleotides in lower case are substitutions in this sequence (T to G, nt 45; T to G, nt 484, C to T, nt 482 and AT to CC, nts 480–479) to create an *EcoRI* site (*prwzyUP2*) or a *KpnI* site (*prwzyDOWN2*). To amplify the *gst*-containing DNA from plasmid pGEX-2T, primers used were *prGSTUP* (5′-249-ACAGTAgtAccTCCCCTATACTA-272-3′) and *prGSTDOWN* (5′-1041-ACAGACAAGCTtTGACCGTCT-CCG-1018-3′), where the numbers refer to the GenBank sequence U13850 (Smith & Johnson, 1988). The bases in lower case are substitutions in this sequence (TTC–TG to GGT–CC, nts 255–260 and G to T, nt 1030) in order to create a *KpnI* site (*prGSTUP*) or a *HindIII* site (*prGSTDOWN*). As a result of this construction, the first 5 putative residues of Wzy were fused, via 2 residues encoded by the *KpnI* site, to the second residue (after M) of GST (Fig. 1).

Primer extension. To determine the transcription startpoint of the *wzy* gene, the primers used in the primer extension experiment were *prwzyDOWN3* (5′-483-GCAATGTATGA-AATTATAAGCATATAGAGC-454-3′), *prwzyDOWN4* (5′-374-TTCTGAATCTGGTAGTCTATAATCATTACTC-344-3′) and *prwzyDOWN5* (5′-405-GGTGTGGTACGGA-AAATCTTCTGGCAAGAT-376-3′). These primers were 5′ end-labelled with [γ -³²P]ATP using T4 polynucleotide kinase (Gibco BRL) and purified from a polyacrylamide gel. Total RNA was isolated, from strains grown in LB, using a Stratagene Micro RNA Isolation Kit. RNA (40 μ g) was mixed with 0.8 pmol purified labelled primer, and the mixture precipitated with NaCl and ethanol (Sambrook *et al.*, 1989). Reverse transcription was performed using SUPERScript RNaseH⁻ reverse transcriptase (Gibco BRL), and the annealing and subsequent extension reactions were performed as described in the SUPERScript RNaseH⁻ reverse transcriptase protocol, except that actinomycin D and RNasin ribonuclease inhibitor (Promega) were added to the reaction mixture at 50 μ g ml⁻¹ and 2 U μ l⁻¹, respectively. The reaction was stopped by heating at 70 °C for 15 min. RNase treatment, followed by ethanol precipitation, was performed as described by Sambrook *et al.* (1989). The samples were loaded next to a set of sequencing reactions, with pADE206 as template, which used the same primer. The sequencing reaction was performed using a T7 Sequencing Kit (Amersham Pharmacia).

Insertion of the FLAG tag at the C-terminus of *wzy*. The FLAG tag (DYKDDDDK) (Hopp *et al.*, 1998) was created at the C-terminus of Wzy by insertion of a heterologous sequence between the penultimate and ultimate residue of Wzy (Fig. 1). PCR primers used were *prwzyUP3* (5′-gattacaagatgatgat-1679-AAATAAGTTATACGGCGGCAATGCCGCCGTT-1709-3′) and *PrwzyDOWN6* (5′-atcatcatcttgaatc-1678-ATTGTTTCTTAGTAAACGAATCTTATATGC-

1648-3′). The heterologous sequence (gat tac aaa gat gat gat gat) in the primer *prwzyUP3* encodes DYKDDDD, while the final K of the FLAG tag is provided by the ultimate codon (AAA) of the native *wzy* gene. A sequence complementary to this segment is in the primer *prwzyDOWN6*. Two PCR reactions were performed, using pADE206 as template. The first reaction used Universal primer (5′-GTAAAACGACG-GCCAGT-3′, from the Amersham Pharmacia T7 sequencing kit) and *prwzyDOWN6*, and the other reaction used *prwzyUP3* and a primer 5′-TCACACAGGAAACAGCT-ATGAC-3′, which is a modification of the Reverse sequencing primer (Amersham Pharmacia). The two PCR products were mixed and annealed (at the complementing sequences in the primers *prwzyUP3* and *prwzyDOWN6*). The annealed product was used as a template for a final PCR reaction using the Universal and modified Reverse primers. The final product was digested with *HindIII* and cloned into pUC18 to give pUST011, or into pCL1920 to give pUST012.

RESULTS

The transcriptional startpoint of the *wzy* gene

Cellular RNA from *S. typhimurium* LV386 (*wzy*) and the complemented multi-copy *wzy*⁺ strain *S. typhimurium* LV386/pADE206, was subjected to primer extension from *prwzyDOWN3* to localize the *wzy* transcriptional startpoint (Fig. 2). The *wzy* transcriptional startpoint was shown to be A313 (Figs 1, 2).

Fig. 2. Identification of the *wzy* transcription startpoint by primer extension, using the oligonucleotide *prwzyDOWN3*. RNA (40 μ g) from *S. typhimurium* LV386 (*wzy*) (lane 1), and the complemented *wzy*⁺ strain *S. typhimurium* LV386/pADE206 (lane 2) was annealed with the *wzy*-complementary primer *prwzyDOWN3*, and reverse transcription followed (see Methods). A sequencing reaction, using the same primer, was also performed. The letters on the right show DNA sequence, and the bold A at position 313 shows the *wzy* transcription startpoint.

Fig. 3. Purification of the Wzy-GST fusion protein. The *wzy* promoter and the putative first 5 residues of Wzy were fused to the second residue of GST. The fusion protein was purified by affinity chromatography using agarose-bound glutathione and subsequent glutathione elution (Smith & Johnson, 1988). Lane 1, purified Wzy-GST (50 µg); lane 2, molecular-mass markers (sizes, in kDa, are shown on the right).

Primer extension from two other primers, *prwzy*DOWN4 and *prwzy*DOWN5, confirmed this (data not shown). This indicated that the *S. typhimurium wzy* gene does indeed have its own promoter as previously suggested (Collins & Hackett, 1991), and allowed the -35 and -10 promoter regions to be positively identified (Fig. 1). Both the -10 signal (TAATAT, nts 301-306) and the -35 signal (TAG-ATA, nts 278-283) have 4/6 nucleotides of the canonical sequences (TATAAT and TTGACA, respectively) (Hawley & McClure, 1983; Rosenberg & Court, 1979).

To measure the strength of the *wzy* promoter, the *wzy* promoter region, and control *lac* and *tac* promoters, were inserted upstream of the promoterless *lacZ* gene of plasmid pQF50, and the transcriptional efficiency of the *LacZ* gene product measured (see Methods). The strength of the *wzy* promoter was 73.0-99.9 Miller units in two tests: it was thus stronger than the induced *lac* promoter (30.6-38.0 Miller units) but much weaker than the *tac* promoter (665-689 Miller units).

The translational startpoint of Wzy

Initial attempts to purify the Wzy protein, from *E. coli* K-12 or *S. typhimurium* cells with the *wzy* gene in low- or multi-copy plasmids, were unsuccessful, possibly due to the fact that the protein is membrane associated (see below). A different approach to determination of the N-terminal amino acid sequence of Wzy was therefore taken. Routinely, all or part of foreign proteins may be fused to the C-terminus of GST without affecting the ability of GST to bind glutathione, so that the fusion

Fig. 4. Lipopolysaccharides of *S. typhimurium* strains on an SDS-PAGE gel, with lipopolysaccharide-specific silver staining. Each lane received material from 10^7 cells grown in 2XYT. Lane 1, *S. typhimurium* C5 (wild-type); lane 2, *S. typhimurium* LV386 (*wzy*); lane 3, *S. typhimurium* LV386/pUST010 (low-copy *wzy*⁺; the *wzy*⁺ *Hind*III fragment was inserted into pCL1920); lane 4, *S. typhimurium* LV386/pUST011 (multi-copy *wzy*-FLAG); lane 5, *S. typhimurium* LV386/pUST012 (low-copy *wzy*-FLAG).

proteins may be purified by affinity chromatography. It was thought that it might be possible to fuse a small portion of a foreign protein to the N terminus of GST, also without affecting the glutathione-binding capacity of GST. Accordingly, the entire *wzy* promoter and a sequence containing the first 5 putative residues of Wzy was fused to the second residue (thus, the starting M was omitted) of GST (see Methods) (Fig. 1), and a fusion protein sought by affinity chromatography (Fig. 3). This approach was successful. From an 800 ml culture of *E. coli* BL21(λDE3) pLysS/pUST013, a total of 3.6 mg Wzy-GST fusion protein was obtained. A portion of this protein was subjected to N-terminal amino acid sequencing and the first 10 residues were LIISGTSPIL (Fig. 1). This identifies ATG461-463 as the Wzy start codon (Fig. 1), as previously suggested (Collins & Hackett, 1991). The sequence AAAGG450-454 (Fig. 1) may, as indicated previously (Collins & Hackett, 1991), serve as part of a ribosome-binding site upstream of the Wzy start codon.

Visualization of the Wzy protein in cells of *S. typhimurium*

Initially, it was planned to purify the Wzy protein so that anti-Wzy antibody might be raised. Fusion proteins with all or part of Wzy linked to the C-terminus of GST were uniformly unstable, and were obtained in only small and variable yields. Accordingly, the FLAG tag was inserted at the C-terminus of Wzy (see Methods) (Fig. 1). This insertion did not obviously affect Wzy function (Fig. 4), as the typical O-antigen ladder was obtained when the *wzy* strain, *S. typhimurium* LV386, was complemented with the *wzy*-FLAG gene, resident in either multi- or low-copy plasmids.

Next, FLAG-tagged Wzy protein, expressed in the *S. typhimurium* LV386-based strains containing either the multi- or low-copy *wzy*-FLAG plasmids, was sought by

Fig. 5. Detection of the Wzy protein by immunoblotting. For panels (a) and (c) (10^8 cells per track), whole cells, grown in LB, were solubilized and analysed by SDS-PAGE. Panel (a) was developed with Coomassie blue and panel (c) by immunoblotting, using antibodies against the FLAG tag (1:500 dilution; $0.2 \mu\text{g protein ml}^{-1}$) as the primary antibody, and the secondary antibody at a 1:1000 dilution. Lanes 1, *S. typhimurium* C5 (*wzy*⁺); lanes 2, *S. typhimurium* LV386 (*wzy*); lanes 3, *S. typhimurium* LV386/pUST011 (multi-copy *wzy*-FLAG); lanes 4, *S. typhimurium* LV386/pUST012 (low-copy *wzy*-FLAG). For panels (b) and (d), whole cells of *S. typhimurium* LV386/pUST012 (low-copy *wzy*-FLAG), grown in LB broth, were fractionated (material from 10^7 cells per track) before SDS-PAGE. Lanes 1, cytoplasmic fraction; lanes 2, whole membrane; lanes 3, cytoplasmic membrane; lanes 4, outer membrane. Panel (b) was developed with Coomassie blue, and panel (d) by immunoblotting, using antibodies against the FLAG tag as the primary antibody. The positions of molecular mass markers, with sizes, are shown on the left. The small arrow (left) marks the position of a cytoplasmic protein, present in all strains, which cross-reacts with the anti-FLAG antibodies. The large arrow (right) shows the FLAG-tagged Wzy protein in whole cells, whole membrane, and the cytoplasmic membrane of *S. typhimurium* LV386/pUST012.

immunoblotting (Fig. 5). No genuine FLAG signal was obtained on immunoblots of whole cells of either *S. typhimurium* C5 or *S. typhimurium* LV386 (neither strain expresses a FLAG peptide), although a protein cross-reactive with the anti-FLAG antibodies was present in all strains examined (and is shown, below, to be located in the cytoplasm of *S. typhimurium* LV386/pUST012). Surprisingly, a genuine FLAG signal was also absent upon immunoblots of whole cells of *S. typhimurium* LV386/pUST011 (which carries multi-copy *wzy*-FLAG), even though such cells clearly expressed active Wzy protein (Fig. 4). The low level of Wzy expression (below the sensitivity level of the immunoblot) in this strain will be discussed later. When whole cells of *S. typhimurium* LV386/pUST012 (which has low-copy *wzy*-FLAG) were examined, however, FLAG-tagged Wzy protein was visible (Fig. 5c, track 4), and cell fractionation localized the protein to the cytoplasmic membrane (Fig. 5d, track 3).

In an effort to quantify the Wzy protein in *S. typhimurium* LV386/pUST012, whole cells of this strain, and of control *S. typhimurium* LV386, were disrupted by boiling in gel-loading buffer, and then used to coat wells of a 96-well tray. An ELISA assay, using a commercial pure FLAG-tagged protein (similarly boiled) as standard, followed (Fig. 6). The Wzy protein level in the (low-copy *wzy*) strain of *S. typhimurium* LV386/pUST012 was $\sim 25 \text{ ng per } 10^7 \text{ cells}$ (after correction for cross-reaction between the anti-FLAG antibodies and a cytoplasmic protein present in both control and test strains). This calculation assumes that the efficiency of binding to the plastic tray of the FLAG-tagged Wzy protein from solubilized whole cells, was the same as that of the pure FLAG-tagged protein standard, and that the FLAG tag does not affect (inhibit?) the natural degradation rate of the unmodified Wzy protein. If these two conditions are met, it may be calculated that when the *wzy* gene is present at 5 copies

Fig. 6. Quantification of the Wzy protein by ELISA. (a) FLAG-tagged standard protein solubilized in gel-loading buffer was used to coat duplicate wells of a 96-well tray at 5, 10, 50 and 100 ng protein per well, and an ELISA assay (using the commercial anti-FLAG antibodies at a dilution of 1/200) followed. The A_{450} development over 20 min was monitored (and was linear for all standards during this time). The mean absorbancies, with the ranges, are shown. (b) Whole cells of *S. typhimurium* LV386 and *S. typhimurium* LV386/pUST012, grown in either 2XYT broth (thick lines) or M9 broth (thin lines) were solubilized in gel-loading buffer and the material from 10^7 cells used to coat duplicate wells of a 96-well tray; the A_{450} development over 20 min is shown (means and ranges). The control (*S. typhimurium* LV386) A_{450} levels at 20 min were subtracted from those of *S. typhimurium* LV386/pUST012, and the figures obtained then interpolated on the standard curve of (a), to yield estimates of the amount of Wzy-FLAG protein per 10^7 cells. T, test (wzy^+); C, control (wzy^-).

per cell, each cell contains ~ 30000 copies of the Wzy protein, which would thus comprise $\sim 1.4\%$ of total cellular protein (Neidhardt & Umbarger, 1996). As the Wzy protein is a cytoplasmic-membrane protein, it is relevant to mention that this would equate to ~ 1 molecule of Wzy protein for every 700 molecules of membrane phospholipid (Neidhardt & Umbarger, 1996). If it is assumed that Wzy production from a single *wzy* gene copy would be one-fifth of the amount seen from the low copy-number *wzy*⁺ plasmid, then Wzy would be present, in wild-type *S. typhimurium*, at ~ 6000 copies per cell, comprising $\sim 0.3\%$ of total protein: one Wzy molecule for each 3500 molecules of phospholipid.

DISCUSSION

Subcellular localization of Wzy

Pulse-chase experiments have previously shown that polymerization of O-specific lipopolysaccharide chains takes place at the periplasmic face of the inner membrane (McGrath & Osborn, 1991), and it was thus always likely that Wzy might be located in the cytoplasmic-membrane fraction. Daniels *et al.* (1998) located a (*S. flexneri*) Wzy-PhoA fusion with 167 aa of Wzy in a 'membrane fraction'. It was not possible to view fusions

with longer Wzy segments, and it was suggested that this was due to the increasing number of rare codons in the longer *wzy* segments of such fusions, with consequent poor fusion-protein expression. Also, the locations of expressive *lacZ* and *phoA* fusions in the *wzy* gene were indicative of a cytoplasmic-membrane location for Wzy, with 12 transmembrane segments (Daniels *et al.*, 1998).

The results in this paper are in full agreement with these data. In this work, the full-length *S. typhimurium* Wzy protein is shown to be located, by immunoblotting of an attached FLAG tag, in the cytoplasmic membrane.

Transcription of *wzy*

When an extract of (wild-type) *S. typhimurium* C5, which contains a single copy of the *wzy* gene, was used for primer extension, no labelled product was detectable on a gel such as that shown in Fig. 2 (data not shown). The sensitivity of this method thus requires that the *wzy* gene be present in a multi-copy state. Transcription of the *wzy* gene from a single startpoint in the multi-copy *wzy*⁺ plasmid, pADE206, was noted in *S. typhimurium* (Fig. 2), and this transcript was clearly translated into functional Wzy protein (Collins & Hackett, 1991). It is reasonable to assume that the transcription startpoint of *wzy* in pADE206 is the same as that used in wild-type

S. typhimurium. It was further shown that the *wzy* gene has its own strong promoter.

Level of translation of Wzy

Previous attempts (Collins & Hackett, 1991) to detect Wzy translation used systems (maxicells, minicells, T7-polymerase-mediated transcription from a T7 promoter) requiring that Wzy be translated in *E. coli* K-12; as suggested (Collins & Hackett, 1991), it seemed possible that such expression might be suboptimal, as the Wzy protein has no obvious function in *E. coli* K-12. This suggestion may have merit. Thus, when pUST012 (which allows good levels of Wzy protein expression in a *S. typhimurium* background; Fig. 6) was transformed into various *E. coli* K-12 strains and Wzy protein sought, after SDS-PAGE of lysed whole cells, by immunoblotting with anti-FLAG antibodies, no Wzy protein was detected (data not shown). The absence of obvious Wzy in *E. coli* K-12 is not due to poor expression of the *wzy* promoter in *E. coli* K-12. It may be that the lack of function for Wzy in *E. coli* K-12 causes the protein to be targeted by the protein-degradation systems of the cell, resulting in a steady-state level of Wzy which is very low or zero.

It was also suggested previously (Collins & Hackett, 1991) that the presence of many attenuating codons in *wzy* might cause Wzy to be translated at a low level. With the multi-copy *wzy*⁺ plasmid, pUST011, in *S. typhimurium*, no Wzy protein was seen, and it is possible that the simultaneous initiation of many Wzy protein molecules, with consequent high demand for attenuating tRNA species, results in completion of only a few Wzy protein molecules, so that Wzy protein levels are below the detection level of the immunoblot (Fig. 5) but still adequate to complement the chromosomal *wzy* mutation (Fig. 4). Daniels *et al.* (1998) placed the *S. flexneri* *wzy* gene downstream of the T7 promoter and the Shine-Dalgarno sequence from gene 10 of phage T7, induced the T7 polymerase, and found that expression of labelled Wzy increased (from undetectable to weak) when 2–3 rare codons in the N-terminal region of *wzy* were mutated to major synonyms. This is the most direct evidence that translation attenuation by rare codons is a controlling factor in Wzy production.

If only a few Wzy molecules are being synthesized at any time (from the low-copy *wzy*⁺ plasmid pUST012), the protein completion rate may be much higher than seen when *wzy* is present in a high copy-number plasmid (Fig. 5). This greater Wzy protein completion rate, coupled with possible good stability of the completed protein (embedded, and active, in the cytoplasmic membrane), appears to result in a steady-state level of Wzy equivalent to ~0.3% of bacterial protein/*wzy* copy, in *S. typhimurium*.

The main conclusions of this study are that the *wzy* transcriptional startpoint has been identified at residue A313 and confirms that the *S. typhimurium* *wzy* gene has its own promoter, with its –10 and –35 sequences,

as predicted previously (Collins & Hackett, 1991). The *wzy* promoter strength was assessed and lies between that of the *lac* and *tac* promoters. N-terminal amino acid sequencing of a Wzy–GST fusion protein confirmed the translation start point which agreed with sequence prediction. Using FLAG as an antibody-reactive tag, a Wzy–FLAG protein was visualized by SDS-PAGE and was localized to the inner membrane. The level of Wzy in wild-type *S. typhimurium* was estimated to be ~6000 copies/cell by ELISA analysis.

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