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* 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 terminus.

**INTRODUCTION**

The *wzy* gene (Reeves *et al*., 1996), which encodes the 47,472 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. enterica* *wzy* gene detected homologues in strains of these groups when used as a probe in Southern blotting (Collins & Hackett, 1991), and the same α(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*<sup>D2E1</sup>) (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 α(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*<sup>D3</sup>) which

**Abbreviation:** GST, glutathione-S-transferase.
also gives \( \gamma(1,6) \) 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; Lukomska 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 Kievet 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 Kievet 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 Kievet 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 Kievet 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 a 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 encountered 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. [\( ^{32}P \)]dCTP (\( \sim 1 \times 10^{14} \) Bq mmol\(^{-1} \)) and [\( ^{32}P \)]ATP (\( \sim 1 \times 10^{14} \) 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-FLAG IgG (heavy and light chains)] was purchased from Pierce Laboratories. The secondary antibody for immuno-blotting (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\(\alpha\) (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), pQ5F0 (which contains a promoterless lacZ gene after a HindIII insertion site) (Farinha & Kropinski, 1990), pGEX-2T (Smith & Johnson, 1988) and pUC18 (Norrander 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 MgSO\(_4\) 0.2 mg ml\(^{-1} \), glucose (2 mg ml\(^{-1} \)) and thiamin-HCl.
Bacto tryptone (10 g l\(^{-1}\)) and NaCl (10 g l\(^{-1}\)). 2XYT medium was prepared as 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). Spectinomycin was used at 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\(^{-1}\)).

**Transcription start**

The transgenic expression system was purchased from Amersham Pharmacia. N-terminal protein sequencing was performed at the Bio-technology 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 HindIII 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 strengths.** To assay promoter strengths, PCR fragments, in which various promoters were contained on Spbl–HindIII fragments, were cloned into plasmid pQF50. To amplify the wzy promoter, the primers used were prwzyUP1 (5'-251-GATAGCTgCATGCTATCGGAGGCG-274-3') and prwzyDOWN1 (5'-344-CATCACaAgCTTCC-326-3'). The nucleotides in lower case are substitutions in the sequence (T to G, nt 258; T to A and T to G; C to T, nt 624) in order to create an lac operator (with the lac promoter into pQF50, the primers used were prlacUP (5'-634-GCA-GCTGGatGCAGTTTCCC-612-3') and prlacDOWN (5'-463-TGGTCAaAGCTTCTTTCCTG-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 the sequence (T to G, nt 258; T to A and T to G; nts 336 and 338) to create an Spbl site (prwzyUP1) or a HindIII site (prwzyDOWN1). To insert the lac promoter (with the catabolite-activator-protein binding site and lac operator) into pQF50, the primers used were prlacUP (5'-634-GCA-GCTGGatGCAGTTTCCC-612-3') and prlacDOWN (5'-463-TGGTCAaAGCTTCTTTCCTG-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 Spbl site (prlacUP) or a HindIII site (prlacDOWN).

**General analytical methods.** DNA manipulations followed established methods (Sambrook et al., 1989). Development of Western blots used the Enhanced Chemiluminescence system (Amersham Pharmacia). SPS-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.

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 flag the fusions of Wzy with GST and the FLAG peptide are shown.
strains of E. coli K-12 JM109, carrying these plasmids, were grown in M9 minimal medium to ~5 x 10^6 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-TACTTGAATTCGTCTGTG-37-3) and prwzyDOWN2 (5’-490-GCTATAAGCCTAGCTGTT-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 were used were prGSTUP (5’-249-ACAGTAggTACCCCTATATACA-272-3) and prGSTDOWN (5’-1041-ACAGACAGCATrTGACCCTGCT-CGC-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, the heterologous sequence (gatt tac aaa gat gat gat ) (lane 1), and the annealed primer (prwzyDOWN3) to localize the Wzy transcription startpoint (Fig. 2). The wzy transcription startpoint was shown to be A313 (Figs 1, 2).

**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 transcription startpoint (Fig. 2). The wzy transcription startpoint was shown to be A313 (Figs 1, 2).

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**Fig. 2.** Identification of the wzy transcription startpoint by primer extension, using the oligonucleotide prwzyDOWN3. RNA (40 µg) 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.
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 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 LIISGTPIL (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...
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 (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 ~25 ng per 10^7 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

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**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 µg 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.
per cell, each cell contains \( \sim 30000 \) 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 \( \sim 1 \) 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 \( \sim 6000 \) 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 \( pboA \) 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

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**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 (\( wzy^- \)).
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 polymerase-mediated transcription from a T7 promoter (Hackett, 1991). Previous attempts (Collins & Hackett, 1991) to visualize 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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**REFERENCES**


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