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Transcriptional activation by MarA, SoxS and Rob of two *tolC* promoters using one binding site: a complex promoter configuration for *tolC* in *Escherichia coli*

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Summary

The *Escherichia coli tolC* encodes a major outer membrane protein with multiple functions in export (*e. g.*, diverse xenobiotics, hemolysin) and as an attachment site for phage and colicins. *tolC* is regulated in part by MarA, SoxS and Rob, three paralogous transcriptional activators which bind a sequence called the *marbox* and which activate multiple antibiotic and superoxide resistance functions. Two previously identified *tolC* promoters, *p*1 and *p*2, are not regulated by MarA, SoxS or Rob but *p*2 is activated by EvgAS and PhoPQ which also regulate other functions. Using transcriptional fusions and primer extension assays, we show here that *tolC* has two additional strong overlapping promoters, *p*3 and *p*4, which are downstream of *p*1, *p*2 and the *marbox* and are activated by MarA, SoxS and Rob. *p*3 and *p*4 are configured so that a single *marbox* suffices to activate transcription from both promoters. At the *p3* promoter, the *marbox* is separated by 20 bp from the −10 hexamer for RNA polymerase but at the *p4* promoter, the same *marbox* is separated by 30 bp from the −10 hexamer. The multiple *tolC* promoters may allow the cell to respond to diverse environments by coordinating *tolC* transcription with other appropriate functions.

Keywords

gene regulation; outer membrane protein; transcriptional start sites; efflux pumps; antibiotic resistance

Introduction

TolC is an outer membrane protein (Koronakis *et al.*, 2004) with diverse functions including efflux of multiple antibiotics, colicin uptake, phage adsorption and export of hemolysin and enterobactin. A major function of TolC is to interact with certain inner membrane transporters (*e.g.*, AcrB) and periplasmic membrane fusion proteins (*e.g.*, AcrA) to form a tripartite structure which makes it possible for substrates to be pumped directly out of the cell. At least eight such pairs of partners of TolC have been reported (Keseler *et al*., 2005). Basal levels of *tolC* expression are critical in providing wild-type *E. coli* with intrinsic resistance to many xenobiotics including clinically important antibiotics and bile salts; higher levels of expression are found in certain multidrug resistant mutants (Piddock, 2006).

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TolC-mediated efflux also seems to play a role in pathogenesis. Interestingly, recent evidence suggests that non-efflux functions of TolC may be involved in pathogenesis (Baucheron *et al.*, 2005; Buckley *et al.*, 2006; Piddock, 2006; Imuta *et al.*, 2008; Virlogeux-Payant *et al.*, 2008). Thus, TolC plays important roles in xenobiotic resistance and virulence.

tolC is a member of the *marA*/*soxS*/*rob* regulon (Fralick, 1996; Aono *et al.*, 1998) which includes over 40 genes that promote resistance to multiple antibiotics, numerous other xenobiotics and to superoxides (Martin and Rosner, 2003; for comprehensive reviews, see articles in White *et al.*, 2005). These genes are transcriptionally activated by three paralogous proteins, MarA, SoxS and Rob. *marA* and *soxS* expression can be increased by treating cells with salicylate (Cohen *et al.*, 1993) and paraquat (Demple, 1996), respectively, and the activity of Rob can be increased post-translationally by treatment with 2,2′-dipyridyl, bile salts or decanoate (Rosenberg *et al.*, 2003; Rosner *et al.*, 2002).

MarA, SoxS and Rob bind as monomers to an asymmetrical and degenerate 19 bp DNA sequence known as the *marbox* (Martin *et al.*, 1999; Wood *et al.*, 1999). Because of *marbox* degeneracy, there are about 10,000 such binding sites per genome (Griffith *et al.*, 2002; Martin *et al.*, 2002). From an analysis of the promoters that are activated by these proteins, it is clear that the orientation of the *marbox* and its distance from the −10 hexamer of the promoter are critical in determining whether MarA, SoxS and Rob can activate the promoter.

Three functional promoter configurations have been found (Martin *et al.*, 1999; Wood *et al.*, 1999). At Class II promoters (*e. g.*, *fumC*), the *marbox* is separated by ~20 bp from the −10 hexamer, overlaps the −35 hexamer and is in the “forward” orientation. At Class I promoters, the *marbox* is separated by ~39 (*e. g.*, *marRAB*) or 50 bp (*e. g.*, *acrAB*) from the −10 hexamer and is in the “backward” orientation. At a subset of the Class I promoters, Class I* (*e. g.*, *zwf*), the *marbox* is separated by ~30 bp from the −10 hexamer but can be functional in either orientation.

Eguchi and coworkers have described two *tolC* promoters, *p*1 and *p*2 (Eguchi *et al.*, 2003). The *p*2 promoter is activated by the joint action of the two-component systems EvgAS and PhoPQ but the regulation of the *p*1 promoter is not known. It has been assumed that the *tolC* promoter is activated by MarA, SoxS and Rob via a *marbox* identified only by its activity in chimeric promoters (Martin *et al.*, 1999). However, the *tolC* transcription start sites (TSS) for *p*1 and *p*2 (Eguchi *et al.*, 2003), were inconsistent with that interpretation because they lie upstream of the *marbox* (Fig. 1, top). On reinvestigation, we found two new TSSs for *tolC* (corresponding to promoters *p*3 and *p*4) that lie downstream of the *marbox* and are activated by MarA, SoxS and Rob. The single *tolC marbox* is uniquely configured so that it can be used for activation of both promoters.

Results and Discussion

tolC transcription start sites

Transcription of *tolC* is activated by MarA, SoxS and Rob (Aono *et al.*, 1998) but the relevant *tolC* promoter has not yet been defined. Two transcription start sites (TSS) corresponding to two promoters had been reported for *tolC* by nuclease S1 digestion (Eguchi *et al.*, 2003): a principal site, P1 (position 3176026, Fig. 1) and a secondary site, P2 (position 3176034), whose promoter, *p2*, is activated by EvgA and PhoPQ. Activation by the monomeric proteins MarA, SoxS and Rob requires that they bind to a *marbox* located in a particular orientation upstream of the TSS. However, a sequence (positions 3176034 to 3176052) that fits the 19 bp consensus *marbox* sequence and that has been found to have *marbox* activity in chimeras (Martin *et al.*, 1999) lies downstream of both P1 and P2. Since

no activator-responsive regulon *marbox* has yet been found downstream of its associated TSS (Martin and Rosner, 2002), we considered it unlikely that promoter *p1* or *p2* could be activated by this *marbox*.

To locate the *tolC* promoter(s) stimulated by MarA, SoxS and Rob and to identify the *marbox* required for *tolC* activation, three *tolC::lacZ* fusions (Fig. 1) were constructed in the bacteriophage λRS45 vector (Simons *et al.*, 1987) and analyzed in single copy lysogens. Fusion **B**, commencing only 19 bp upstream of the putative *marbox* and thus lacking the −35 and −10 signals for *p1* and *p2* (Eguchi *et al.*, 2003), had 50% of the basal activity of fusion **A** (200 vs 400 MU) which begins 43 bp farther upstream than fusion **B** and contains those promoters. Both fusions terminate at position 3176145, which is 91 bp downstream of the *marbox*, and both were significantly activated by agents known to activate *marA* and *soxS* transcription or Rob function (salicylate, paraquat and 2,2′-dipyridyl, respectively). Fusion **C** which lacks promoters *p1* and *p2* and 11 bp of the 19 bp *marbox* had 30% of the basal activity of fusion **A** (120 vs 400 MU) and was not activated by these agents. We conclude that the *tolC* promoter(s) activated by MarA, SoxS and Rob must lie downstream of *p1* and *p2*.

To identify the *tolC* promoters activated by MarA and SoxS, primer extension analyses were carried out with total cellular RNA isolated from strains M3710 and M3944. These *ΔmarRAB, rob::kan* strains carry plasmid pRGM9818 (pMarA) and pJLR70 (pSoxS) (Martin *et al.*, 2000) which express *marA* and *soxS*, respectively, from a *plac* promoter but also carry an F' $lacI^q$ so that only in the presence of IPTG is MarA or SoxS made. To analyze activation of *tolC* by Rob, a different approach was used since *in vivo* Rob protein has to be post-translationally activated for it to be a potent transcriptional activator (Rosner *et al.*, 2002; Rosenberg *et al.*, 2003). To achieve this, we compared the RNA from two strains, M4110 and M4113, treated with a Rob activator, 2,2′-dipyridyl (DIP), for 1 hr during exponential growth. Both strains have *Δmar sox::cat rob::kan* null mutations but M4110 carries the vector pTA108 whereas M4113 carries pTA108:*rob* which expresses *rob* from a *lac* promoter. Assays of β-galactosidase due to the expression of *micF::lacZ* fusions in strains M3710 and M3944 and *inaA::lacZ* fusions in strains M4110 and M4113 showed 19-, 16- and 61-fold increases of fusion expression due to the upregulation of MarA, SoxS and Rob, respectively.

In the absence of IPTG (*marA* and *soxS* repressed) or Rob (M4110), six extension products were apparent (Fig. 2, top): at the adjacent G and T residues, positions 3176096 and 3176097, respectively; at two A residues at positions 3176084 and 3176087 (apparent on longer exposure); at a residue upstream near position 3176021; and at one near the top of the gel. The first four were greatly enhanced on overexpression of MarA and SoxS (growth in IPTG) or on activation of Rob in strain M4113 by DIP whereas the latter two products were diminished. (Overexpression of MarA and SoxS, and activation of Rob resulted in the appearance of two weak bands at positions 317616 and 317617 which we interpret as premature terminations of the reverse transcription assay or as mRNA degradation products.)

Corresponding to these four TSSs are two potential promoters. A potential extended −10 signal (TGnaATAAT) is spaced 6 nt upstream of the TSSs labeled P4 and another potential extended −10 signal (cGnTAgAAT) is spaced 7 nt upstream of the stronger P3 TSSs (Fig. 2, bottom). Extended −10 signals are frequently observed for promoters that have very poor −35 signals (Keilty and Rosenberg, 1987; Schultzaberger *et al.*, 2007), as is the case for these promoters. We conclude that *tolC* has two overlapping promoters, *p3* and *p4* (Fig. 2): *p3* has its *marbox* in the Class II configuration (Martin *et al.*, 1999;Wood *et al.*, 1999) separated by 20 bp from the −10 signal with TSSs 5 and 8 nt downstream of the −10 signal

(positions 3176084 and 3176087); *p4* has the Class I* configuration with the *marbox* separated by 30 bp from the −10 signal with TSSs 7 and 8 nt downstream of the −10 signal (positions 3176096 and 3176097). That one *marbox* can activate transcription from two different promoters is unique among the known *marA/soxS/rob* regulon promoters.

Similar results were obtained in strains in which SoxS was overproduced or Rob was activated by treatment with 2,2′-dipyridyl. Quantitation of the products indicated that MarA and Rob utilized *p*3 and *p*4 to about equal extents whereas SoxS utilized *p*4 to a greater extent than *p*3 (approximately 2-fold).Under the experimental conditions employed, we did not expect to observe transcripts starting at P2 because they require high levels of EvgA. We should, however, have seen P1 at position 3176026 (Eguchi *et al.*, 2003) but instead observed an apparent TSS 5 nt upstream at position 3176021. Since the only apparent 10 signal in this region, and the one suggested by Eguchi *et al.* (2003), TTAAAT, is 6 nt distant from the TSS we observed but unusually distant (11 nt) from the TSS they reported, we suspect that our P1 TSS assignment is correct.

The multiplicity of *tolC* promoters may reflect the need to adapt the expression of *tolC* to different environments. The role of TolC in the efflux of xenobiotics has been well studied. Recently, a role in adherence to vertebrate cells has been reported for the TolC of *Salmonella enterica* serovar Typhimurium (Buckley *et al.*, 2006; Virlogeux-Payant *et al.*, 2008). Since *acrB* mutants were not similarly defective in adherence, this may suggest that the outer membrane TolC surface itself has important functions in colonization. It might be beneficial to regulate these different functions differently. Promoter *p*2 appears to be activated by phosphorylated EvgA (in a strain with a mutant evgS) and PhoPQ together (Eguchi *et al.*, 2003). Concomitant with upregulation of *tolC*, EvgA upregulated several multidrug resistance genes (*acrAB*, *emrKY*, *mdfA* and *yhiUV*) and showed increased antibiotic resistance that was fully dependent on TolC but only partially dependent on AcrA. The environmental signal for EvgAS is not known but PhoPQ is activated by low Mg++ concentrations such as exists in the phagosome (Groisman, 2001).

The four *tolC* promoters respond to at least five different environmental signals. The *p*1 promoter is not responsive to EvgAS, PhoPQ, MarA, SoxS or Rob but seems to have significant constitutive expression under laboratory conditions (Eguchi *et al.*, 2003; and Figs. 1 and 2). The *p*2 promoter is responsive to EvgAS and PhoPQ but not the others. As shown here, *p*3 and *p*4 are activated by MarA, SoxS and Rob, with each activator being regulated by different environmental signals. The nesting of the *p*3 and *p*4 promoters with a single activator binding site might facilitate even further levels of control as intimated by the different relative extents to which *p*3 and *p*4 are utilized by these activators. These three activators also upregulate *acrAB* and other genes which are critical for increased multidrug and superoxide resistances whereas EvgAS and PhoPQ regulate different sets of genes. Thus, *tolC* has multiple transcriptional regulatory elements which respond to different environments and possibly tailor the response to the particular TolC function (efflux, protein transport, adherence) that is adaptive.

Experimental procedures

Bacterial strains and plasmids

All strains are derivatives of *Escherichia coli* K-12. *tolC::lacZ* transcriptional fusions (see Fig. 1) were made using phage λRS45 as described (Simons *et al.*, 1987). Strain GC4468 (*Δlac*) was infected with the phage, and lysogens with a single-copy prophage were used for β-galactosidase assays. Strains M3710 and M3944 are *Δmar rob::kan micF::lacZ F' lacI*^{q} carrying plasmid pRGM9818 or pJLR70 in which *marA* or *soxS*, respectively, are under the control of the *tac* promoter (Martin *et al.*, 2000). Strains M4110 and M4113 are *Δmar*

rob::kan sox::cat inaA::lacZ. Strain M4110 carries plasmid pTA108 (vector) and M4113 carries plasmid pTA108:*rob* in which *rob* is constitutively expressed from the *lac* promoter (Rosner *et al*., 2002).

Culture media and chemicals

LB (Lennox) media contained per liter: 10 gm Bacto-tryptone (DIFCO, Detroit, MI), 5 gm Bacto-yeast extract and 5 gm NaCl, pH adjusted to 7.5 with NaOH. Chemicals were purchased from Sigma Chemicals, St. Louis, MO

β-galactosidase assays

Bacteria were grown overnight in LB medium at 32°C, diluted 1000-fold in fresh medium and grown to an A_{600} of about 0.15 unless otherwise indicated. To test the transcriptional activation of *tolC::lacZ* fusions by MarA, SoxS or Rob, cells were grown to an A_{600} of about 0.15, diluted into an equal volume of LB medium containing sodium salicylate, paraquat or 2,2′-dipyridyl (final concentrations of 5mM, 50 μM and 5 mM, respectively) and aerated for 1 hr at 32°C. β-galactosidase was then measured using the CHCl3-SDS method of Miller (1972). All assays presented were performed at least twice in duplicate and the standard errors of the mean were <± 5%.

Primer extension analysis

Strains with wild-type *tolC* were grown in LB medium at 32°C to exponential phase (A_{600} = 0.4). Strains M3710 (pMarA) and M3944 (pSoxS) were then diluted two-fold into LB without (control) or with IPTG (0.5 mM final concentration) for 1 hr to induce plasmid-borne *marA* or *soxS* expression, respectively. Strains M4110 (pTA108 vector control) and M4113 (pTA108:Rob) were diluted into LB with 2,2′-dipyridyl (5 mM final concentration) for 1 hr to activate Rob. Total cellular RNA was isolated by using the TRIzol Reagent (Invitrogen, Carlsbad, CA). Primer extension assays (Zhang *et al.*, 1998) were performed by incubating 20 μg of total RNA and 1 pmole of 5′-end ^{32}P-labeled primer AZ#1006 (5′-CTC AGG CCG ATA AGA ATG G) at 65°C for 5 min, followed by incubation with 10 U of AMV Reverse Transcriptase (Life Sciences, Inc,, St. Petersburg, FL), 5 U of RNase Inhibitor (Invitrogen) in 1X RT buffer (Life Sciences, Inc.) in a 15-μl reaction volume at 42°C for 1 hour. The reactions were terminated by adding 7.5 μl Stop/Loading buffer and cDNA products were separated on an 8% polyacylamide gel containing 8 M urea in 1X TBE buffer at 70 watts for 70 min. To generate a sequence ladder, a *tolC*-specific PCR fragment was generated by using primers AZ#1007 (5′-CGC CGC ACC TCA TGA CTC AT) and AZ#1008 (5′-TGT GGC AGT AAT GGA CTG CG), and DNA sequencing reactions were carried out with AZ#1006 (above) and SequiTherm EXCEL™ II DNA sequencing Kit (Epicentre, Madison, WI). Bands on gels were quantitated using a Typhoon Trio Variable Mode Imager (GE Healthcare Life Sciences, Piscataway, NJ).

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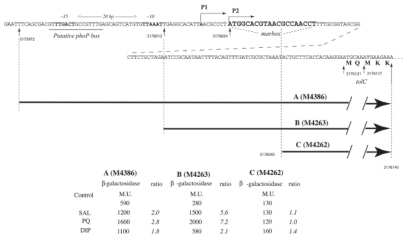

	A (M4386) β-galactosidase	ratio	B (M4263) β -galactosidase	ratio	C (M4262) β -galactosidase	ratio
Control	M.U. 580		M.U. 280		M.U. 130	
SAL	1200	*2.0*	1500	*5.6*	130	*1.1*
PQ	1600	*2.8*	2000	*7.2*	120	*1.0*
DIP	1100	*1.8*	580	*2.1*	160	*1.4*

Fig. 1.

The sequence of the promoter region upstream of the structural gene for *tolC* is indicated with P1 and P2 transcription start sites (TSSs) and the putative −35, −10 and *phoP* box sites as suggested by Eguchi *et al.* (2003), and the presumptive *marbox* (Martin *et al.*, 1999). Seven digit numbers refer to positions on the *E. coli* chromosome (Blattner *et al.*, 1997). Below are indicated the fragments of this region incorporated into various *promoter::lacZ* fusions with strain numbers in parentheses and the β-galactosidase activities (Miller Units) obtained for these strains after treatment with 5 mM Na salicylate (SAL), 50 μM paraquat (PQ) or 5 mM 2,2′-dipyridyl (DIP) for 1 hr at 32°C. The ratio is the activity of the treated cells divided by that of the untreated cells.

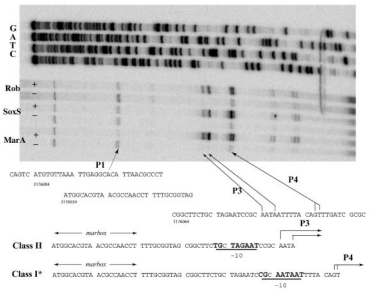

Fig. 2.

Mapping of *tolC* transcription start sites. Above: Primer extension analysis was carried out using total RNA isolated from the cells of strain M3710 (pMarA) or M3944 (pSoxS) grown for 1 hr during exponential phase without (−) or with (+) IPTG treatment to overexpress MarA and SoxS, respectively, as described in Materials and Methods. For activation by Rob, the RNA from M4110 (−) and M4113 (+) treated with 2,2′-dipyridyl for 1 hr during exponential phase was used. Sequencing ladders are also shown. The TSSs P3 and P4 from MarA-, SoxS- and Rob-dependent promoters (*p3* and *p4*) are indicated by arrows as is a new position suggested for P1, 5 nt upstream of that previously suggested (Eguchi *et al.*, 2003). Below: two alignments of the *marbox* with respect to promoters *p3* and *p4*; their TSSs (P3 and P4); and potential extended −10 signals are underlined.