Control of hexuronate metabolism in *Escherichia coli* by the two interdependent regulators, ExuR and UxuR: derepression by heterodimer formation

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Two homologous proteins, UxuR and ExuR, were previously predicted to repress synthesis of enzymes required for hexuronic acid metabolism, but little is known about the relative roles of these proteins in gene regulation. We confirmed the previous report that UxuR is essential for rapid growth with D-glucuronate as the primary source of carbon and energy. In contrast, an exuR mutant grew more rapidly on D-glucuronate than the parent. Transcription of *exuR* is initiated at a σ70–dependent promoter predicted in silico. Purified ExuR bound to the *exuR* regulatory region in the presence, but not in the absence, of D-glucuronate. Apparently weaker UxuR binding in the presence of glucuronate was also detected, and its addition decreased ExuR binding by forming ExuR–UxuR heterodimers. Glucuronate induced *exuR* transcription in the parental strain, but not in the *exuR* mutant. No evidence was obtained for cAMP-dependent regulation of *exuR* by the catabolite repressor protein (CRP). A previous study reported that the divergent *yjjM* and *yjjN* genes, essential for L-galactonate metabolism, are repressed by UxuR. We showed that ExuR binds to the *yjjM-yjjN* regulatory region, and that the binding is also glucuronate-dependent. As for the *exuR* promoter, UxuR appeared to decrease ExuR binding. ExuR is required for glucuronate induction of *yjjM* and *yjjN*, and CRP is required for their transcription. The combined data established that UxuR and ExuR fulfil contrasting roles in regulating hexuronic acid metabolism and indicate that ExuR can function as a transcription activator, possibly by inactivating the repressor function of UxuR by heterodimer formation.

INTRODUCTION

Hexuronic acids are relatively abundant in natural environments, including the bodies of warm-blooded animals. Mounting evidence suggests that their catabolism is important for the colonization and motility of *Escherichia coli* (Chang *et al*., 2004; Fabich *et al*., 2008; Peekhaus & Conway, 1998; Sweeney *et al*., 1996). They are metabolized by the Ashwell pathway, which generates intermediates that are converted to pyruvate via the Entner–Doudoroff pathway. Regulation of hexuronic acid and uronamide metabolism in *E. coli* and related bacteria has been predicted to be complex, involving three global regulators, Catabolite Repressor Protein (CRP), Fumarate and Nitrate Reductase regulatory protein (FNR), and Histone-like Nucleoid-Structuring protein (H-NS), as well as two more specific repressors, UxuR and ExuR (De Wulf *et al*., 2002; Rodionov *et al*., 2000; Shulami *et al*., 1999; Tan *et al*., 2001). The amino acid sequences of UxuR and ExuR are 45.5% identical. They are members of the Gluconate Repressor protein (GntR) family of transcription factors in which the amino terminal helix-turn-helix domain interacts with promoter DNA, and the C-terminal domains are required for ligand binding and oligomerization.

Previous studies have established that the *uxuR* gene is autoregulated by UxuR (Ritzenhaler & Mata-Gilsinger, 1982). Other genes in the UxuR regulon include *gntP*, encoding a fructuronate transporter, *uxuAB* and *uidABC*, encoding enzymes (*uxuA*, *uxuB* and *uidA*) and transporters (*uidB* and *uidC*) involved in hexuronate metabolism, as well as the *yjjN* and *yjjM* genes encoding L-galactonate oxidoreductase and another putative transcription regulator of the GntR family, respectively (Bates Utz *et al*., 2004; Suvorova *et al*., 2011).

In contrast to UxuR, relatively little is known about ExuR. It was originally proposed that ExuR primarily controls
expression of genes for galacturonate metabolism, but UxuR regulates glucuronate metabolism (Medina-Rivera et al., 2011; Rodionov et al., 2000). However, it was soon realized that this had been an oversimplification as evidence of cross-regulation by both UxuR and ExuR was subsequently reported (Rodionov et al., 2000). It was shown that the glucuronate transporter protein, ExuT, and the first enzyme required for glucuronate conversion to fructuronate, UxaC, are members of the ExuR regulon (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1981; Mata-Gilsinger & Ritzenhalter, 1983; Nemoz et al., 1976). Fructuronate, which specifically prevents DNA-binding by UxuR (Bates Utz et al., 2004), is then metabolized by products of UxuR-repressed genes such as UxuB and UxuA.

The repressor function for ExuR has been confirmed only for exuT, encoding a hexuronate transporter, uxaB, and the uxaCA operon involved in galacturonate metabolism (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1981; Mata-Gilsinger & Ritzenhalter, 1983; Nemoz et al., 1976; Portaliert et al., 1980). Bioinformatic analysis of the regulatory regions of genes known to be regulated by UxuR and ExuR led us to propose that they share similar target DNA-binding sites: atATGGTaaACCAattt for UxuR; and aAAgTgGTaTgA-cAAcTtt for ExuR (Suvorova et al., 2011). As these consensus sequences are not identical, UxuR and ExuR might be expected to bind individual promoters with different affinities. The starting point for the current study was, therefore, based upon this background information and the assumption that both ExuR and UxuR are ligand-responsive transcription repressors, as summarized in Fig. 1. Using three approaches, we have investigated whether there is cross-regulation of genes required for hexuronic acid metabolism by both transcription factors. First, we compared the effects of uxaR and exuR deletions on growth in the presence of glucose or glucuronate. The results of this experiment indicated that UxuR, and not ExuR, is required for rapid growth in the presence of glucuronate. The second aim was to determine whether exuR expression, like uxaR expression, is autoregulated, regulated by UxuR, or regulated by cAMP-CRP. Our final aim was to test the effect of ExuR on the transcription of genes that are repressed by UxuR.

**METHODS**

**Strains, plasmids and growth conditions.** Strains and plasmids used in this study are listed in Table S1, available on the Online Supplementary Material. Oligonucleotide primers are listed in Table S2. The exuR and uxaR genes were disrupted in *E. coli* BW25113 using recombineering technique (Datsenko & Wanner, 2000). The gene::kan mutations were transferred by P1-transduction into *E. coli* K-12 MG1655 and into its Δlac derivative, *E. coli* M182. Strain M182 was transformed with *PexuR*:lacZ, *PygM*:lacZ or *PygN*:lacZ fusion plasmids constructed on the basis of pBR224 (Islam et al., 2012). Purified transformants were grown in minimal salts (MS) medium supplemented with 5 % (v/v) LB, 0.2 % (v/v) of the appropriate carbon source (d-glucose or d-galactonate) and 35 μg ml⁻¹ tetracycline. Cultures were grown aero- bically at 37 °C under constant shaking. Cells were harvested after 4.5 h of growth (mid-log phase with OD~0.2–0.4) and used to measure β-galactosidase activity. The same growth conditions were used to prepare cultures of strain MG1655 and its ΔuxuR, ΔuxuR and ΔexuR derivatives for RNA extraction but no antibiotic was added to the medium. To investigate the possibility of the exuR transcription initiation at the orf2-dependent promoter, additional cultures were grown at 42 °C.

**ExuR promoter mapping.** Promoter-like signals in *E. coli* MG1655 (U00962.2) were found by pattern-recognition software PlatProm (Shavkovskov et al., 2009). Transcript starts were localized by single round transcription in vitro as previously described (Ozoline et al., 2001) and by measuring the lengths of primer extension products in vivo. RNA was extracted from 10 ml of cell culture (Favre-Bonte et al., 1999) and treated with DNase I (New England Biolabs, USA). For primer extension experiments, 5 μg of total RNA was incubated with 4 pmol of the primer exuR_prom_R2 and Super Script II reverse transcriptase (Invitrogen, USA) following the manufacturer’s protocol. The resulting samples were preheated and loaded onto a 6 % denaturing polyacrylamide gel calibrated with G-sequenceing products and [γ-32P]ATP-labeled 50 bp DNA ladder (New England Biolabs, USA). Gels were scanned using PMI Molecular Imager (Bio-Rad, USA) and autoradiographed.

**ExuR protein production.** Recombinant exuR was overexpressed from pGEMEhis vector that was constructed on the basis of pGEMAXbX (Igashiri & Ishihama, 1991) using primers exuR_beg and exuR_end_his (Table S2), with six histidine codons added on its 3′-end. As the DNA-binding domain of ExuR is located on the N-terminal end, addition of His-tag did not affect its functionality. Thus, the growth of the exuR deletion mutant complemented by the recombinant gene was exactly the same as the growth of the wild-type culture in all conditions used in this study.

To avoid possible toxicity of uncontrollable synthesis of the transcription factor at the early stages of cultivation (Shimada et al., 2013), the exuR gene was cloned under the control of its own Shine-Dalgarno sequence and operator region. This approach allowed suitable amount of soluble protein to be generated without any significant reduction in the culture growth. Conditions for protein production were optimized using several growth media and IPTG concentrations from 10 μM to 4 mM. High yields of ExuR protein were obtained during growth of BL21(DE3) cells in Terrific broth with 20 μM IPTG induction for 5 h. The ExuR protein was purified on Ni-NTA agarose (Qiagen, Germany) following the manufacturer’s protocol with minor modifications (Shimada et al., 2013). The same approach was used to purify UxuR protein.

**β-galactosidase assays.** To assay transcription initiation from the exuR, yjjM and yjjN promoters, corresponding DNA fragments were cloned into the lac expression vector pRW224 using EcoRI and HindIII sites (Table S2). β-Galactosidase activities in *E. coli* strain M182 carrying these recombinant plasmids were measured as described by Vine et al. (2011) and calculated as nanomoles of *ortho*-nitrophenyl-β-galactoside (ONPG) hydrolysed per min per mg of dry cell mass. Results reported are the means of at least three biological replicates each analysed in two assay repeats. The error bars are the standard deviations of all assays.

Electrophoretic mobility shift assays (EMSA) were used to test DNA binding efficiency of the ExuR protein. DNA fragments containing the same promoter regions of yjjM, exuR and exuR as were cloned in pRW224 for β-galactosidase assays were PCR-amplified (Table S2), extracted from the gel and used for subsequent experiments. The promoter region of the hns gene, amplified with primers hns_prom_F and hns_prom_R (−262/+66, Table S2), was used as a control template. One pmol of DNA was incubated at 37 °C in 1× Transcription buffer in the absence or presence of 100 mM of either d-glucose or d-galacturonate (Bates Utz et al., 2004; Ozoline et al., 1998), for 10 min. Then 1–4 pmol of purified ExuR and/or UxuR was added. After a further 30 min at 37 °C, complexes were loaded onto a 5 % polyacrylamide gel that had been pre-warmed to 37 °C. Protein complexes were separated from free DNA by electrophoresis. To estimate the efficiency of binding, free DNA

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fragments and proteins were loaded on separate lanes. Gels were stained by ethidium bromide to reveal bands containing DNA, while subsequent staining by AgNO₃ allowed both free protein and nucleoprotein complexes to be detected with high sensitivity (Tutukina et al., 2010). Western blotting was used for specific detection of proteins in the complexes. In brief, band-shifts were performed as above in duplicate, one of which was stained with ethidium bromide and another was transferred onto a PVDF membrane (Immobilon, Sigma-Aldrich, USA) using the Mini trans-blot protocol (Bio-rad, USA). The membrane was then blocked with 0.5 % skimmed milk (Oxoid, UK) and incubated with either anti-UxuR or anti-ExuR antibodies in Tris-buffered saline (TBS) supplemented with 0.1 % Tween-20 and 0.5 % skimmed milk for 2 h at 37 °C. After 3 × 10 min washes with TBS-T, secondary antibodies (A3687, anti-rabbit IgG, Sigma, USA) were added and incubation was allowed for 1 h at room temperature. Membranes were stained with Western-blue stabilized substrate for alkaline phosphatase (Promega, USA) and scanned. Polyclonal antibodies against UxuR and ExuR were produced in rabbits in the Institute of Cell Biophysics RAS using purified proteins.

**DNAse I footprinting.** For DNAse I footprinting, the same DNA fragments containing exuR and yjjMN promoter regions were amplified with [γ-³²P] ATP-labeled primers. Complexes were formed exactly as described earlier, and footprinting was performed as described in Ozo-line et al., (2001). Samples were preheated and loaded onto a 8 % denaturing polyacrylamide gel calibrated with G-sequencing products and [γ-³²P] ATP-labeled 50 bp DNA ladder (New England Biolabs, USA). Gels were scanned using a PMI Molecular Imager (Bio-Rad, USA) and autoradiographed.

**Quantitative PCR.** A DT-322 thermocycler (DNA-Technology, Russia) and SYBR Green I as a fluorescent dye (Invitrogen, USA) were used for quantitative PCR (qRT-PCR). Primers used for reverse transcription and amplification are listed in Table S2. No PCR products were detected in negative controls in the absence of reverse transcriptase. Data obtained from at least three biological samples and three statistical replicates were calculated by the ΔCt method. The error bars indicate the standard deviations of corresponding mean values.

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**Fig. 1.** Model for the regulation of hexuronate metabolism by ExuR and UxuR based upon data published before the start of the current work.

**Fig. 2.** Effects of uxxR and exuR deletions on growth in the presence of 0.2 % glucose (a) or glucuronate (b). Square symbols: the parent strain; circles: ΔuxuR mutant; triangles: ΔexuR mutant. Dashed lines correspond to the cultures complemented with recombinant genes.
RESULTS

Opposite effects of *uxuR* and *exuR* deletions on growth in the presence of glucose or glucuronate

Preliminary experiments were designed to compare the effect of the *uxuR* and *exuR* deletions on growth in the presence of glucose or glucuronate. Bacteria were grown in MS medium supplemented with 5% LB and either 0.2% glucose or 0.2% glucuronate as the main source of carbon and energy. All three strains grew equally well in the presence of glucose (Fig. 2a). The parent strain grew more slowly with glucuronate than with glucose as the carbon source, but deletion of *exuR* resulted in more rapid growth than the parent with glucuronate (Fig. 2b). In contrast, growth of the *exuR* mutant on glucuronate almost stopped as soon as the LB had been consumed. Both growth phenotypes were complemented by plasmids encoding either ExuR or UxuR (dashed lines in Fig. 2b). These results are in line with previous reports suggesting that UxuR is required for growth on glucuronate (Ritzenthaler & Mata-Gilsinger, 1982; Robert-Baudouy *et al.*, 1981) and reveal a functional difference between UxuR and ExuR.

Identification of the *exuR* transcription start sites

As a result of genome-wide expression analysis using the 5’-end specific RNA-seq approach, a potential $\sigma^{70}$-dependent promoter was suggested 144 bp upstream of the *exuR* coding sequence (Gama-Castro *et al.*, 2008; Huerta & Collado-Vides, 2003; Keseler *et al.*, 2013). A further four possible $\sigma^{70}$ promoters have been predicted in *silico* (Shavkunov *et al.*, 2009; Fig. 3a). Two methods were used to determine whether any of these predicted transcription start points might be physiologically relevant. First, PCR-amplified fragments of the *exuR* regulatory region were used in single round transcription assays with the $\sigma^{70}$-containing RNA polymerase. The results revealed a prominent transcription start site located 40 bases upstream of the *exuR* coding sequence that corresponds exactly to the predicted promoter P3 (Fig. 3b) and the major RNA detected by the 5’-end specific RNA-seq (Dornenburg *et al.*, 2010). Minor transcripts possibly originating from the predicted promoter P4 were also detected (Fig. 3b); however, they were not observed in any *in vivo* experiments. Activity of P3 was confirmed by a primer extension assay *in vivo* (Fig. 3c), which also revealed a shorter RNA, possibly attributable to P1. However, no transcript corresponding to the predicted $\sigma^{70}$-dependent promoter was found in these assays, even when bacteria had been grown at 42°C (Fig. 3c). Thus it was concluded that under the conditions used in these experiments, ExuR is primarily transcribed from the $\sigma^{70}$-dependent promoter P3, with possible minor contributions from P1 and/or P4.

Binding of ExuR to the *exuR* regulatory region

Genes for many transcription factors are autoregulated. It might therefore be expected that ExuR regulates its own synthesis. However, in view of homology between UxuR and ExuR, it is possible that UxuR also regulates *exuR* expression. Histidine-tagged ExuR protein was purified on Ni-NTA agarose virtually to homogeneity (Fig. 4a). Gel binding assays were then used to show that ExuR binds to the *exuR* regulatory region in the presence, but not in the absence, of glucuronate (Fig. 4b, lanes 4 and 3, respectively). If glucuronate was added, two-fold molar excess of ExuR reproducibly trapped all the DNA fragments containing *exuR* promoter. This result was consistent with the prediction that ExuR is a glucuronate-sensitive repressor of ExuR synthesis (Suvorova *et al.*, 2011). UxuR binding to the *exuR* regulatory region was also detected only in the presence of glucuronate, but the same molar excess of the protein left more unbound DNA as compared to ExuR (Fig. 4b, lanes 5–7). When both ExuR and UxuR were added to the reaction mixture with glucuronate, the fraction of free DNA increased compared to the ExuR sample (compare tracks 4 and 9, Fig. 4) indicating at least partial inhibition of ExuR binding by UxuR. This interference was not dependent on the order of addition of the purified proteins to the DNA (data not shown). Control experiments showed that, as expected, ExuR did not bind non-selectively to the *hns* promoter (Fig. 4c).

The gel binding assays were extended to identify which protein was bound to the DNA. Two identical gels were prepared and band-shifts assays were made exactly as mentioned earlier. After electrophoresis, one gel was stained with ethidium bromide to detect DNA, and proteins in the second one were transferred to a PVDF Immobilon membrane for Western blotting with both anti-ExuR and anti-UxuR antibodies. These experiments clearly show the presence of ExuR both in DNA–protein and protein–protein complexes (Fig. 5b), while in four independent assays made with anti-UxuR, the signal was detected only in protein–protein complexes, with very weak signal in DNA–protein complexes, or at the very top of the gel (data not shown). The latter observation will be discussed in one of the following paragraphs. DNA–ExuR complex was formed in the presence of glucuronate but not in its absence (Fig. 5). Less of the ExuR–DNA complex was detected in the presence of both UxuR and ExuR, but this was replaced by a DNA-free complex that was recognized by both anti-ExuR and anti-UxuR antisera. We propose that this complex is due to heterodimer formation between ExuR and UxuR, as originally proposed by Robert-Baudouy *et al.* (1981). If so, it would indicate that UxuR is required to release ExuR from the repression complex at the *exuR* promoter.

DNase I footprinting experiments were used to locate the site at which ExuR binds to the *exuR* regulatory region. Two binding sites were detected (Fig. 6a, b). One of them coincided with the predicted binding site that overlaps the transcription start point, suggesting the ExuR represses its own synthesis. A second binding site was located 160–190 bases upstream of the transcription start and better corresponded to the inverted ExuR consensus sequence (Fig. 6b).
Regulation of transcription at the exuR promoter

The exuR regulatory region was fused to lacZ in the low copy number translational fusion vector, pRW224. The resulting plasmid was used to compare the effects of mutations in exuR, uxuR and crp on exuR transcription during growth in the presence of glucose or glucuronate with those of the parent strain. In the parent strain β-galactosidase activity was about 60% higher during growth with glucuronate compared with growth with glucose as the main carbon source (Fig. 7a). No similar increase in β-galactosidase activity was detected in the exuR mutant. Higher activities were also found in the uxuR mutant during growth in the presence of glucose, consistent with weak repression of the exuR promoter by UxuR. Note, however, the unexpected absence of glucuronate induction in the uxuR mutant. This point will be discussed later in the paper. Promoter activity in the crp mutant was approximately 60% higher than in the parent strain during growth either in the presence of glucose or glucuronate.

Differences in levels of exuR transcripts assessed by qPCR were greater than the changes in β-galactosidase activity, but essentially confirmed the conclusions from the galactosidase data. Glucuronate induced exuR transcription approximately two-fold in the parent strain (Fig. 7b), and the requirement

Fig. 3. Identification of the exuR transcript start sites. The figure shows the predicted (a) and experimentally determined (a–c) transcription start points in the regulatory region of exuR. Grey horizontal arrows show positioning of genes. Bars represent the transcription start points predicted by PlatProm on both DNA strands. The vertical dashed arrow shows the location of the transcription start, predicted in (b). Black arrows point out the 5′-end of RNA detected by primer extension (panel c). Light grey arrows show transcription start points defined by the 5′-end-specific RNA-Seq (7). The length of these arrows reflects the number of recorded sequence reads. (b) Single round transcription assays using DNA-fragments amplified with primer pairs F1–R1 (lane 1), F1–R2 (lane 2) and F2–R2 (lane 3). M: Single-stranded DNA ladders. (c) Primer extension in vivo with primer R2. Dashed arrows indicate the expected location if exuR-RNA is transcribed from Ps32. G: G-sequence ladder from R2.
for a functional UxuR protein for glucuronate induction was confirmed (Fig. 7b). The most striking effect was the fourfold increase in transcript levels in the Δcrp derivative, irrespective of whether the bacteria were grown with glucose or glucuronate as the main carbon source (light grey bars in Fig. 7b). We propose that sugar-independent effects of CRP on transcription might be due to its cAMP-independent binding to a non-specific site leading to only a secondary effect (Kolb et al., 1993).

**Binding of ExuR to the uxuR regulatory region and the effects of ExuR on levels of uxuR transcription**

Transcription at the uxuR promoter is known to be auto-regulated by UxuR repression (Ritzenthaler & Mata-Gilsinger, 1982; Ritzenthaler et al., 1983). It was predicted that ExuR might also bind to the uxuR promoter and repress uxuR transcription. We therefore investigated whether ExuR binds to the uxuR regulatory region and, if so, whether this binding is also dependent upon glucuronate. As for the exuR promoter, ExuR binds to the uxuR promoter only in the presence of glucuronate, but not in its absence or in the presence of glucose (lanes 14–16 in Fig. 4d). In contrast, UxuR binds its own promoter in all conditions; however, addition of D-glucuronate to the sample significantly changed the binding pattern (compare lanes 17–19 in Fig. 4d). Although the amount of the binary complex was unchanged, the amount of unbound DNA decreased and was replaced by stuck complexes at the top of the gel. This might be due to glucuronate-induced UxuR oligomerization (Tutukina et al., 2016).

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Fig. 4. Purification of recombinant C-6xHis-tagged ExuR and its ligand-dependent interaction with the exuR regulatory region. (a) ExuR was purified by Ni-NTA affinity chromatography. (b) Electrophoretic mobility shift assays demonstrating glucuronate-dependent binding of ExuR, UxuR and a mixture of both proteins to the exuR regulatory region in a ligand-dependent manner. (d) The same experiment for the uxuR regulatory region. (c) Control experiment to show the absence of ExuR binding to the hns promoter. Additions to each sample are shown above the lanes. Molar protein : DNA ratios were 2 : 1 (as monomer) or 1 : 1 (as dimer). Gels were stained with (a) Coomassie R250 or (c) AgNO₃. Panels (b) and (d) show the results of band-shift experiments with [γ-³²P]ATP-labeled DNA fragments.
The effect of an \textit{exuR} deletion on the levels of \textit{uxuR} transcription was determined by qPCR (Fig. 7c). Contrary to expectation, deletion of \textit{exuR} resulted in total loss of glucuronate induction of \textit{uxuR} expression (Fig. 7c). High level expression of \textit{uxuR} in response to glucuronate is therefore dependent upon \textit{ExuR}. Conversely, glucuronate induction of \textit{exuR} expression was shown to be dependent upon \textit{UxuR}, so \textit{ExuR} and \textit{UxuR} are interdependent. Levels of \textit{uxuR} mRNA were identical in the parent strain and its \textit{crp} derivative during growth in the presence of glucuronate; so glucuronate induction is not CRP-dependent.

**Interaction of \textit{ExuR} with the regulatory region of genes known to be regulated by \textit{UxuR}**

It was previously predicted by bioinformatic analysis that \textit{UxuR} represses expression of the divergent \textit{yjjM} and \textit{yjjN} genes that have been implicated in the metabolism of...
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L-galactonate (Suvorova et al., 2011). YjjN (LgoD) is L-galactonate oxidoreductase, which converts L-galactonate to D-tagaturonate that is further converted to 2-keto-3-deoxygluconate by D-altronate oxidoreductase UxaB and the dehydratase UxaA (Fig. 1). Both of these enzymes are controlled by ExuR (Mata-Gilsinger & Ritzenenthaler, 1983; Nemoz et al., 1976; Portalier et al., 1980), and thus it was intriguing to check if the first enzyme in the pathway was also under the control of this transcription regulator. Only one binding site for both UxuR and ExuR was predicted in the yjjM/yjjN intergenic region, making it an ideal target for investigation of cross-talk between these two transcription regulators. We therefore compared the binding of ExuR and UxuR to the yjjM/yjjN intergenic region (Fig. 8a). Only low affinity binding of ExuR was detected in the presence or absence of glucose (lanes 2 and 3), but complete binding occurred in the presence of D-glucuronate (lane 4). UxuR also did not bind to this intergenic region in the presence of D-glucose (lane 11), but glucuronate stimulated weak interaction (lane 12). As for the exuR promoter, addition of UxuR to the binding assays resulted in less ExuR binding than in its absence (compare lanes 4 and 6), implying the same heterodimerization-mediated interference (Fig. 5). Interaction of both proteins with a common site in the yjjM/yjjN intergenic region was strongly supported by DNase I footprinting (Fig. 8b, c). It is located 161/146 bases upstream from the transcription start point of yjjN or 39/54 bases downstream of the yjjM start, respectively (Fig. 8).

**Effects of ExuR and UxuR on yjjM and yjjN transcription**

Having established by gel-retardation assays that ExuR can bind to a promoter region that is known to be regulated by UxuR (Suvorova et al., 2011), two methods were used to determine the effects of ExuR on yjjM and yjjN transcription. First the yjjM and yjjN promoters were fused to lacZ in the promoter-probe vector, pRW224. The resulting plasmids were transformed into E. coli M182 and its ΔuxuR, ΔexuR and Δcrp derivatives. During growth in the presence of glucose, only a basal level of transcription from the yjjM promoter was detected (Fig. 9a), and this level was unaffected by the deletion of either exuR or uxxuR, but was approximately two-fold lower in the crp deletion mutant. During growth in the presence of glucuronate, yjjM transcription in the parent strain was slightly higher than in the presence of glucose, but was significantly higher in the uxxuR mutant. In contrast, no glucuronate induction was detected in the exuR mutant, suggesting that glucuronate-mediated regulation of yjjM is mainly dependent upon a functional ExuR protein. Induction by glucuronate was also dependent upon a functional CRP protein.

The effects of ExuR and UxuR on yjjM transcription were confirmed by qRT-PCR experiments (Fig. 9b), but the differences in mRNA levels were more marked than those from the galactosidase assays. During growth in the presence of glucose, mRNA levels were 1.5–2-fold higher in the ΔexuR derivative than in the parent strain, but were not significantly affected by deletion of uxxuR. Glucuronate induced yjjM transcription four-fold in the parent strain, and almost nine-fold in the uxxuR deletion mutant, but strongly suppressed transcription of this gene in the ΔexuR and Δcrp strains.

The effects of exuR or uxxuR deletions in both the β-galactosidase experiments for the yjjN::lacZ fusion, and qPCR data for levels of yjjN mRNA (Fig. 9, panels c and d) were similar to those for yjjM transcription, except for some quantitative differences. For instance, in the parent strain glucuronate stimulated yjjN transcription initiation more than RNA synthesis from yjjM promoter (Fig. 9a, c), and the effect of crp deletion was less pronounced. The combined data confirmed that ExuR binds to the same site within the yjjM/yjjN intergenic region as UxuR, and is essential for glucuronate-induced transcription of these two genes.

![Fig. 7. Autoregulation of exuR expression and the effects of UxuR and cAMP-CRP. (a) β-Galactosidase activity under the control of the exuR promoter in pRW224 in E. coli strain M182 with or without the deletion of uxxuR, exuR or crp; (b) exuR- and uxxuR-mRNA levels in the wild type, ΔuxuR, ΔexuR and Δcrp K-12 MG1655 grown on different carbon sources. mRNA of hns was used as a reference.](http://mic.microbiologyresearch.org)
DISCUSSION

From the first report of properties of exuR mutants of *E. coli*, it was obvious that the ExuR regulon extends beyond galacturonate metabolism, and that ExuR partially represses the *uxuA-uxuB* genes that are required for glucuronate utilization (Portalier et al., 1980). The presence of a second regulator, UxuR, homologous to ExuR led to the conclusion that ExuR and UxuR are both repressors of at least some of the genes for hexuronate metabolism (Ritzenthaler & Mata-Gilsinger, 1982; Ritzenthaler et al., 1980; Robert-Baudouy et al., 1981). The observation that overexpression of either gene from a multi-copy plasmid is equally effective in repressing expression of the *uxuAB* operon provided direct evidence for such interchangeable roles (Ritzenthaler et al., 1983).

The demonstration of ExuR and UxuR cooperatively repressing *uxuAB* synthesis has led to the suggestion that ExuR and UxuR might interact to form a repression complex (Robert-Baudouy et al., 1981). However, such a simple explanation is inconsistent with the observation that unlike ExuR, UxuR is essential for rapid growth with glucuronate as the main source of carbon and energy (Fig. 2), implying that UxuR directly or indirectly plays a positive role in glucuronate metabolism. It is also inconsistent with the observation that glucuronate promotes rather than prevents binding of either ExuR or UxuR to the *exuR* promoter (Fig. 4b). Such ligand-
Fig. 9. Effects of ExuR, UxuR and cAMP-CRP on \( yjjM \) and \( yjjN \) transcription. \( \beta \)-galactosidase activity under the control of \( yjjM \) (a) and \( yjjN \) (c) promoters in pRW224 in \( E. coli \) strain M182 with or without the deletion of \( \Delta uxuR, \Delta exuR \) or \( \Delta crp \); (b) and (d) panels represent qRT-PCR data for \( yjjM \) and \( yjjN \), respectively, in the parent, \( E. coli \) K-12 strain MG1655, and its \( exuR, uxuR \) or \( crp \) mutants. No changes were detected in \( hns \)-mRNA levels that were used as controls. Promoter activity or mRNA levels are expressed relative to the parent strain during growth on glucose.

Fig. 10. Revised model to explain how ExuR and UxuR in the presence of glucuronate regulate expression of genes for glucuronate metabolism. ExuR-gln: ExuR with glucuronate bound; UxuR-gln: UxuR with glucuronate bound.
induced binding is more consistent with a transcription activation function than with relief of repression. Neither does it explain why ExuR binds less of its DNA targets in the presence of UxuR than in its absence (Figs 4, 5 and 8). This paradox was resolved by the detection of soluble ExuR–UxuR heterodimers by Western blotting (Fig. 5). We suggest that rather than cooperative binding of ExuR–UxuR heterodimers to their DNA target, as proposed by Robert-Baudouy et al. (1981), glucuronate promotes the formation of soluble heterodimers that are inactive as transcription repressors (Fig. 10). This proposal also explains nicely why ExuR is essential for induced expression of genes that have previously been shown to be repressed by UxuR (Figs 7 and 9).

We therefore propose a working model for glucuronate metabolism and its regulation that provides an explanation for the interplay between the two transcription factors, ExuR and UxuR (Fig. 10). According to this model, when glucuronate is present, UxuR is required to form a heterodimer with ExuR, thus relieving repression not only of exuR but also of uxaC, encoding the first enzyme for glucuronate metabolism that generates fructuronate, the inducer to which UxuR responds. In the absence of ExuR, transcription of uxaC will be derepressed, and more fructuronate will be produced. This in turn will relieve UxuR repression of the downstream genes, not only because fructuronate might derepress UxuR-binding, but also because less UxuR is produced in the absence of ExuR than in its presence. In the same manner, ExuR relieves the UxuR repression of yjjN (lgoD) which encodes the enzyme responsible for conversion of l-galactonate to D-tagaturonate, the first step of parallel way for 2-keto-3-deoxygluronate synthesis. If this model is correct, it can be predicted that ExuR is essential for expression of other genes that are strongly repressed by UxuR, and vice versa. Experiments to determine the global response to loss of either of these transcription factors are in progress as well as assays aimed to determine the effects of other hexuronic acids such as galacturonate and fructuronate on expression of genes regulated by UxuR and ExuR.

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