Characterization of LgnR, an IclR family transcriptional regulator involved in the regulation of L-gluconate catabolic genes in Paracoccus sp. 43P

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Five genes encoding enzymes required for L-gluconate catabolism, together with genes encoding components of putative ABC transporters, are located in a cluster in the genome of Paracoccus sp. 43P. A gene encoding a transcriptional regulator in the IclR family, lgnR, is located in front of the cluster in the opposite direction. Reverse transcription PCR analysis indicated that the cluster was transcribed as an operon, termed the lgn operon. Two promoters, P\textsubscript{lgnA} and P\textsubscript{lgnR}, are divergently located in the intergenic region, and transcription from these promoters was induced by addition of L-gluconate or D-idonate, a catabolite of L-gluconate. Deletion of lgnR resulted in constitutive expression of lgnA, lgnH and lgnR, indicating that lgnR encodes a repressor protein for the expression of the lgn operon and lgnR itself. Electrophoretic mobility shift assay and DNase I footprinting analyses revealed that recombinant LgnR binds to both P\textsubscript{lgnA} and P\textsubscript{lgnR}, indicating that LgnR represses transcription from these promoters by competing with RNA polymerase for binding to these sequences. D-idonate was identified as a candidate effector molecule for dissociation of LgnR from these promoters. Phylogenetic analysis revealed that LgnR formed a cluster with putative proteins from other genome sequences, which is distinct from those proteins of known regulatory functions, in the IclR family of transcriptional regulators. Additionally, the phylogeny suggests an evolutionary linkage between the L-gluconate catabolic pathway and D-galactonate catabolic pathways distributed in Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Actinobacteria.

INTRODUCTION

L-Gluconate is an enantiomer of D-gluconate belonging to the C6 aldonic acid family of saccharides. While D-gluconate is commonly found as a catabolic intermediate of the Entner–Doudoroff pathway, L-gluconate has only recently been found in nature (Shimizu et al., 2012). An L-gluconate catabolic pathway and a gene cluster encoding enzymes required for this catabolism, termed the lgn gene cluster, were identified in Paracoccus sp. strain 43P through an analysis of its L-glucose catabolic pathway (Shimizu et al., 2012). This catabolic pathway begins with the C-5 epimerization of L-gluconate to form D-idonate, and is catalysed by L-gluconate dehydrogenase (L-GnDH; LgnH) and 5-keto-L-gluconate reductase (LgnI). Dehydration of D-idonate produces 2-keto-3-deoxy-D-galactonate (KDGal) and is catalysed by D-idonate dehydratase (LgnE). Finally, the conversion of KDGal to D-glyceraldehyde-3-phosphate and pyruvate is catalysed by KDGal kinase (LgnF) and 2-keto-3-deoxy-6-phospho-D-galactonate (KDPGal) aldolase (LgnG).

Although genes and biochemical functions involved in this L-gluconate catabolic pathway have been characterized, the transcriptional organization and regulation of these genes are still poorly understood. Based upon nucleotide sequence data, lgnR, which is located upstream of the cluster in the opposite direction, has amino acid sequence similarity to IclR family transcriptional regulators, such as YiaJ (Ibáñez et al., 2000) and KdgR (Pouyssegur & Stoebner, 1974). This suggests that lgnR is responsible for transcriptional regulation of the lgn gene cluster.

IclR was first characterized as a repressor for the Escherichia coli aceBAK operon responsible for acetate utilization (Maloy & Nunn, 1982; Sumnarborg et al., 1990). Further studies revealed that IclR autologously represses transcription of iclR itself (Gui et al., 1996), and two different modes of repression have been proposed for repression of aceBAK transcription by IclR (Yamamoto & Ishihama, 2003). Recent expansion of genome databases revealed that iclR orthologues are distributed in a wide range of prokaryotes, including Archaea (Krell et al., 2006), and the proteins are characterized by a DNA-binding helix–turn–helix motif in
the N-terminal region and an effector binding site in the C-terminal region (Molina-Henares et al., 2006; Zhou et al., 2012). Members of this family are involved in diverse physiological functions, such as quorum sensing (Kim et al., 2007), multidrug resistance (Rojas et al., 2003), secondary metabolism (Yamazaki et al., 2003), degradation of aromatic compounds (Gerischer et al., 1998) and pathogenicity in plants (Lu et al., 2011). Unlike the LysR family of transcriptional regulators that possess a conserved operator sequence motif (TN_11A; Maddocks & Oyston, 2008), operator sequences of genes regulated by the IclR family proteins are more variable in their base composition and secondary structures, which may include palindromes, pseudopalindromes and direct repeats. Moreover, the regulatory mechanisms through which IclR proteins regulate transcription are also diverse, as they act as either repressors or activators, and some family members possess dual functions including autoregulation (Chao & Zhou, 2013). It is therefore challenging to predict the operator sequences, as well as regulatory mechanisms, important for the function of uncharacterized proteins in the IclR family of transcriptional regulators. Therefore, we undertook a biochemical characterization of a newly discovered member of this family involved in a novel physiological process in order to explore the physiological roles and functional diversity present within the IclR family.

In this report, we investigated the transcriptional organization of the lgn gene cluster as well as its regulation by LgnR, which belongs to the IclR family of transcriptional regulators. Moreover, we discuss the evolutionary origin of L-glucanate catabolic genes.

METHODS

Bacterial strains, plasmids, media and chemicals. Bacterial strains and plasmids, and primers, used in this study are listed in Tables 1 and 2, respectively. E. coli strains DH10B, BL21(DE3) and S17-1pir (Simon et al., 1983) were used for subcloning; production of His-tagged LgnR and conjugation, respectively. E. coli strains were grown in Luria–Bertani (LB) medium. Pseudomonas sp. strain 43P and its mutant strain were grown in LB medium or a synthetic minimal medium [20 mM NH₄Cl, 20 mM potassium phosphate (pH 7.0), 5 mM MgSO₄, 10 mM KCl, 0.2 % Hutner’s trace element solution (Hutner et al., 1950)] supplemented with the carbon sources described below. L-Glucanate was synthesized from L-glucose (Tokyo Chemical Industry) using hypoxioido-methanol oxidation as described elsewhere (Moore & Link, 1940). D-Idonate was synthesized by base hydrolysis of D-idon-1,4-lactone (Carbosynth). L-5-Keto-glucanate, KDGal and KDPGal were prepared as previously described (Shimizu et al., 2012).

Construction of the ΔlgnR strain. To obtain a mobile suicide plasmid for disruption of lgnR, PCR was performed using genomic DNA isolated from strain 43P and the LgnR_SF/LgnR_SR primer set. The amplified 2665 bp fragment was cloned into the Sphl–BanII site of pUC-mob (Shimizu et al., 2012) to obtain pUClgnRM. The resultant plasmid was digested with HindIII, for which there is a restriction site located within the lgnR ORF, and a kanamycin-resistance gene cassette, amplified by PCR using the kanF/kanR primer pair and pBBR1MCS2 (Kovach et al., 1995) as a template, was inserted into the restriction site, generating the pUClgnRMK conjugative suicide plasmid. Introduction of pUClgnRMK into strain 43P and selection of transconjugants were conducted according to our previous report (Shimizu et al., 2012), and gene disruption was confirmed by PCR with Lgn_SF and Lgn_SR primers.

L-GnDH activity assay. Strain 43P and its ΔlgnR mutant were cultured in minimal medium supplemented with 15 mM L-glucanate, 15 mM D-ido-nate or 20 mM succinate until the O.D.₆₀₀ of the culture reached 0.5–0.7. Cells were harvested by centrifugation and disrupted by sonication with a Sonifier 250 (Branson) in 100 mM Tris/HCl, 10 % (v/v) glycerol, 1 mM DTT pH 8.0. After the cell debris was removed by centrifugation at 20,400 g for 15 min, the supernatant was used as a cell-free extract. L-GnDH activity was assayed using a DU 800 spectrophotometer (Beckman-Coulter) by measuring the reduction of NAD⁺ at 25 °C in 200 μl reaction mixture composed of 1 mM potassium L-glucanate, 1 mM NAD⁺, 2.5 % (v/v) cell-free extract, 100 mM Tris/HCl pH 9.0. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol NAD⁺ min⁻¹ under the defined assay conditions.

RNA techniques. Strain 43P and its ΔlgnR mutant were cultured as described above until the O.D.₆₀₀ of the cultures reached 0.5–0.8. Total RNA was prepared using a hot SDS/phenol method (Jahn et al., 2008), and cDNA was prepared from total RNA using a Quantitect reverse transcription kit (Qiagen) according to manufacturer’s instructions. Reverse transcription PCR (RT-PCR) was performed with 20 ng total RNA as a template with or without reverse transcription using the primer sets listed in Table 2. Rapid amplification of cDNA ends (RACE) analysis was performed using a 5’ RACE system (Invitrogen) and the primers in Table 2. Quantitative RT-PCR (Q-RT-PCR) was carried out with SYBR Green reaction mix (Bio-Rad) using the primer sets in Table 2 and a reverse transcription product from 5 ng total RNA as a template. Amplification of PCR products was monitored using a MiniOpticon real-time PCR system (Bio-Rad). For each sample, expression of lgn genes was standardized to 16S rRNA as an internal control. Q-RT-PCR experiments were conducted as three technical replicates from each of two independent cultures for each gene analysed. The relative expression level of each gene for each culture condition was compared using the 2⁻ΔΔCT method (Livak & Schmittgen, 2001).

Expression and purification of His₆-LgnR. To construct an lgnR expression plasmid, the ORF was PCR amplified with primers LgnR_NdeF and LgnR_EcoR from 43P genomic DNA, and the resultant fragment was cloned into the NdeI–EcoRI site of pET28a(+). The resultant plasmid was designated pET-lgnR. E. coli BL21(DE3) harbouring pET-lgnR was cultured in LB medium containing kanamycin at 37 °C until the O.D.₆₀₀ of the cultures reached 0.5. IPTG was added to the cultures at a final concentration of 0.2 mM, and bacteria were grown for a further 3 h at 28 °C. Cells were harvested by centrifugation and disrupted by sonication in 50 mM Tris/HCl (pH 8.0), 10 % (v/v) glycerol, 0.5 M NaCl, and 40 mM imidazole. After the cell debris was removed by centrifugation at 24,000 g for 30 min, the supernatant was passed through a 0.45 μm filter. His₆-LgnR was purified using a HiTrap FF column (1 ml) (GE Healthcare) according to the manufacturer’s instructions. Purity of the protein was confirmed by SDS-PAGE as described elsewhere (Laemmli, 1970). The purified proteins were dialysed against 50 mM HEPES-KOH (pH 7.2), containing 50 % (v/v) glycerol, 0.1 % (v/v) Triton X-100 and 1 mM DTT, then stored at −20 °C until further use.

Size exclusion chromatography. Protein aliquots (25 μl) containing 14 mg purified His₆-LgnR ml⁻¹ were loaded onto a Superose 12 10/300 GL (GE Healthcare) column installed on an AKTA system (GE Healthcare) pre-equilibrated with buffer containing 50 mM HEPES-KOH, 10 % glycerol, 250 mM NaCl, 1 mM DTT (pH 7.2). Gel filtration was performed at a flow rate of 0.5 ml min⁻¹ at room temperature.
Characterization of LgnR

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source</th>
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<td>BL21(DE3)</td>
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<td>Novagen</td>
</tr>
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<td>S17-1pir</td>
<td>RP4-2-Tc::Mu-Km::Tn7 λpir; host for conjugal transfer</td>
<td>Simon <em>et al.</em> (1983)</td>
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<td><em>Paracoccus</em> sp.</td>
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<td></td>
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<td>43P</td>
<td>Isolated from soil lgnR: Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Shimizu <em>et al.</em> (2012)</td>
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<td>ΔlgnR</td>
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<td><strong>Plasmids</strong></td>
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<td>pUC-mob</td>
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<td>Novagen</td>
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<td>pET-LgnR</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; PT7lac-lgnR</td>
<td>This study</td>
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**Phylogenetic analysis of LgnR.** Using the complete amino acid sequence of LgnR as the query sequence, a *BLAST* search was performed in the online interface module of the *KEGG* genome database (http://www.genome.jp/kegg/). Amino acid sequences showing scores higher than 170 bits in the *BLAST* search (89 sequences), in addition to YiaJ and KdgR from *E. coli* as representative proteins in the Icr family of transcriptional regulators, were multiply aligned using CLUSTAL_X (Chenna *et al.*, 2003). A phylogenetic tree was generated with the MEGA4 package (Tamura *et al.*, 2007) using the neighbour-joining method and 1000 replicates. Comparisons of gene organization surrounding *lgnR* orthologues were done by manual validation using the genome map tool in the *KEGG* database.

**RESULTS AND DISCUSSION**

**Transcriptional mapping of the lgn gene cluster**

A schematic of the gene organization of the *lgn* cluster, as well as the positions of the primer sets used for the RT-PCR analysis, are shown in Fig. 1(a). Our RT-PCR analysis showed that the nine genes expressed from the same strand (*lgnA–I*) were co-transcribed (Fig. 1b), indicating that these ORFs constitute an operon. However, as suggested by its reverse orientation, the *lgnR* gene, which encodes a putative IcR family transcriptional regulator, was transcribed separately, because no RT-PCR product was observed with the primer set designed to amplify the 2650 bp fragment containing the intergenic region between *lgnA* and *lgnR*. Based on these data, we focused on the intergenic region between *lgnA* and *lgnR*, which is likely to contain promoter regions for both the *lgn* gene as well as the *lgn operon*.

*5′*-RACE analysis revealed that the transcriptional start sites of *lgnR* and the *lgn* operon were located at 22 and 157 bp upstream of the *lgnR* and *lgnA* ORFs, respectively (Fig. 1c). We identified sequences similar to the consensus sequence of the *E. coli* Eco<sup>76</sup> promoter (Harley & Reynolds, 1987) further upstream of these transcriptional start sites.
<table>
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<th>Primer</th>
<th>Sequence (5’→3’)†</th>
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<td>LgnR_NdeF</td>
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<td>lgnA_CD_nonlabel</td>
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†Introduced restriction sites are underlined.
–35 and −10 regions for the lgnR promoter, 5′-TTCTACA-3′ and 5′-CATTTAT-3′, and those for the promoter of the lgn operon, 5′-TGAGA-3′ and 5′-CATTTAT-3′. This result is consistent with the early observation that the genus Paracoccus possesses typical σ70-dependent transcription (Baker et al., 1998). Taken together, we concluded that lgnR and the lgn operon are transcribed under the direction of separate promoters, P_lgnR and P_lgnA, located in this intergenic region. Genes encoding LgnABCD, putative ABC transporter components, were co-transcribed with lgn catabolic genes, suggesting that they might be involved in incorporation of L-gluconate.

**Effect of lgnR disruption on the expression of lgn genes**

To determine whether LgnR functions as a transcriptional regulator of the lgn operon, we constructed a ΔlgnR strain and examined the effect of deleting this gene on biochemical production of L-GnDH as well as expression of genes in the lgn operon. Insertion of the Km′ cassette to the lgnR ORF was confirmed by PCR using primer set LgnR_SF/LgnR_SR, as the fragment amplified from the genomic DNA of the ΔlgnR strain was shown to be 1000 bp larger than that from the wild-type strain (data not shown).
When the wild-type strain was cultured in minimal medium supplemented with L-gluconate, D-idonate or succinate as a control, we detected L-GnDH activity catalysed by LgnH at 0.272, 0.322 and 0.002 unit (mg protein)$^{-1}$, respectively (Fig. 2a). Q-RT-PCR analysis showed that the expression of $lgnA$ and $lgnH$ in the $lgn$ operon was induced approximately 60-fold by L-gluconate and 70-fold by D-idonate when compared with expression when cells were grown in succinate (Fig. 2b). This result was in good correlation with the LgnH activity measured (136- and 161-fold with L-gluconate and D-idonate, respectively, compared with that of succinate); although, the induction level was about twice in the enzyme activity measurement. In contrast, the $\Delta lgnR$ strain exhibited similar L-GnDH activity irrespective of the carbon source used for culturing the cells, the activity of which was almost equal or slightly higher than that of the wild-type cultured with L-gluconate or D-idonate (Fig. 2a). Additionally, the transcriptional levels of $lgnA$ and $lgnH$ in the $\Delta lgnR$ strain cultured with succinate were significantly higher than those in the wild-type cultured under the same conditions (170- and 340-fold for $lgnA$ and $lgnH$, respectively), and reached the levels of those in the $\Delta lgnR$ and the wild-type strains cultured with L-gluconate or D-idonate (Fig. 2b). These results suggest that the expression of the $lgn$ operon is induced by either L-gluconate or D-idonate, and LgnR functions as a repressor for its expression.

As the $\Delta lgnR$ strain was constructed by inserting a Km$R$ cassette into the $lgnR$ ORF, we measured $lgnR$ expression from $P_{lgnR}$ in the $\Delta lgnR$ strain by using a primer set ($lgnR\_RT\_F$ and $lgnR\_RT\_R$) located in a region upstream of the Km$R$ cassette. Interestingly, expression of $lgnR$ was regulated by LgnR itself. Expression of the gene was induced by L-gluconate or D-idonate in the wild-type strain, and deletion of $lgnR$ resulted in higher levels of

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**Fig. 2.** Effects of $lgnR$ disruption on production of L-GnDH, and expression of $lgnR$ and the $lgn$ operon. (a) L-GnDH activity in cell-free extracts prepared from wild-type (white bars) and $\Delta lgnR$ (grey bars) strains cultured with succinate, L-gluconate or D-idonate. Cell-free extracts were prepared from cells in late-exponential growth phase ($OD_{600}$ 0.5–0.8). Mean values ± SD of three independent experiments are shown. Note that the activity in the wild-type cultured with succinate was 0.002 unit (mg protein)$^{-1}$. (b) Relative expression of $lgn$ genes in wild-type (white bars) and $\Delta lgnR$ strain (grey bars). Values shown are fold-increases in relative mRNA levels in comparison with the wild-type strain cultured with 20 mM succinate.
Characterization of LgnR

Fig. 3. Binding of His<sub>6</sub>-LgnR to the lgnR–lgnA intergenic region. (a) Gel filtration analysis of the purified His<sub>6</sub>-LgnR. Open circles indicate elution points of molecular marker proteins (aldolase, 158 kDa; conalbumin, 75 kDa; carbonic anhydrase, 29 kDa; RNase A, 13.7 kDa; aprotinin, 6.5 kDa), and the filled circle indicates that of His<sub>6</sub>-LgnR. (b) Schematic representation of the lgnA–lgnR intergenic region and positions of the biotin-labelled DNA fragments used in EMSA. The locations of labelled DNA fragments are shown as lines with numbers. The length of each fragment is shown on the right. Grey bars indicate the positions of −10 and −35 boxes of P<sub>lgnA</sub> and P<sub>lgnR</sub>. (c) EMSA with 1 nM biotin-labelled DNA fragments in the presence (+) or the absence (−) of 500 nM His<sub>6</sub>-LgnR. The fragments used are indicated with numbers corresponding to those shown in (b). (d) EMSA with 1 nM biotin-labelled DNA fragments in the presence of different amounts of His<sub>6</sub>-LgnR. Experiments were conducted in triplicate, and representative results are shown. F, Unbound DNA; C1, complex 1; C2, complex 2. (e) Hill plots of the data obtained in (d). Circles, triangles and squares indicate the results of three independent experiments. Note that for fragment 1 >99.8% of the fragment was shifted at 400 nM LgnR, and therefore the data for 600 nM LgnR were omitted from the plot.
expression (Fig. 2b). These observations suggest that LgnR functions as a repressor for the lgn operon and lgnR itself, and that L-glucuronate, D-idonate, or related compounds may act as an inducer for the expression of the lgn operon and lgnR.

**LgnR binds to both the P<sub>lgnR</sub> and P<sub>lgnA</sub> regions**

To further investigate the biochemical function of LgnR, we produced and purified a recombinant His<sub>6</sub>-LgnR, using the pET-system in *E. coli*. The purified protein was detected at a molecular mass of 27 kDa by SDS-PAGE (data not shown) and 48.6 kDa by gel filtration (Fig. 3a), suggesting that His<sub>6</sub>-LgnR may exist as a homodimer in vivo.

To determine whether LgnR binds to the P<sub>lgnR</sub> and P<sub>lgnA</sub> regions, EMSA was performed using His<sub>6</sub>-LgnR and several biotin-labelled DNA fragments containing the lgnA–lgnR intergenic region (Fig. 3b). Two band-shifts were observed using fragments containing both P<sub>lgnA</sub> and P<sub>lgnR</sub> in the presence of His<sub>6</sub>-LgnR (Fig. 3c, fragment 1 and 2), while no band-shift was observed using a fragment containing neither P<sub>lgnA</sub> nor P<sub>lgnR</sub> regions (Fig. 3c, fragment 3). Moreover, a single shifted band was observed using fragments lacking either of the –10 regions in P<sub>lgnA</sub> and P<sub>lgnR</sub> (Fig. 3c, fragment 4 and 5). These results indicate that there are two LgnR binding sites within the lgnA–lgnR intergenic region, and the hyper-shifted bands observed for fragments 1 and 2 may represent formation of higher-order complexes as a result of LgnR binding to two sites in the same DNA fragment. In addition, the –10 regions identified within both P<sub>lgnA</sub> and P<sub>lgnR</sub> seem to play a critical role in binding of LgnR.

The binding affinities of His<sub>6</sub>-LgnR to each fragment were compared by performing EMSA in the presence of various concentration of His<sub>6</sub>-LgnR (Fig. 3d). After quantification of the bound and unbound DNA, we plotted the data on Hill plot (Fig. 3e). The Hill coefficients for fragments 1, 4 and 5 were 5.2 ± 0.8, 4.1 ± 0.1 and 2.8 ± 0.2, respectively, indicating that binding of LgnR to these fragments is cooperative. LgnR exists as a dimer in solution, and its binding to fragments 4 or 5 produces a single shifted band. If we assume that fragments 4 and 5 contain only one binding site in each fragment, this result may indicate that two or more LgnR dimers cooperatively bind to a single site. In the IclR family of transcriptional regulators, BlcR is known to bind its binding site as tetramer; although, it exists as dimer in solution (Pan *et al.*, 2011). It is thus possible that LgnR also forms a homotetramer upon binding to its binding site.

Apparent dissociation constants of His<sub>6</sub>-LgnR to fragments 1, 4 and 5 were calculated from the plots as monomer to be 118 ± 11, 136 ± 13 and 248 ± 21 nM, respectively. His<sub>6</sub>-LgnR showed 1.8-fold higher binding affinity for fragment 4, containing the P<sub>lgnA</sub> region, than for fragment 5, containing the P<sub>lgnR</sub> region. This suggests that transcriptional repression mediated by LgnR on the lgn operon is stronger than the repression of lgnR. Also, there was only slight difference between binding affinity of His<sub>6</sub>-LgnR to fragment 4 and fragment 1, which contains both P<sub>lgnA</sub> and P<sub>lgnR</sub> regions, suggesting that binding of LgnR to P<sub>lgnA</sub> and P<sub>lgnR</sub> are independent of each other.

**Effector molecule of LgnR**

To identify potential effector molecule(s) that may be required for dissociation of LgnR from its binding sites, L-glucuronate and catabolic intermediates of this pathway were added as additional components in further EMSA assays. Addition of 100 μM D-idonate effectively dissociated LgnR from the DNA fragment containing both promoter regions (Fig. 4a). Other compounds, including L-glucuronate, had no effect on this dissociation. Our EMSA results with D-idonate agreed with those of the *in vivo* expression analyses (Fig. 2). However, we previously described that addition of L-glucuronate or D-idonate to culture media induced the expression of both lgnR and the lgn operon. As L-glucuronate is converted to D-idonate by LgnR–LgnI reactions in this pathway, it is possible that supplementary L-glucuronate acts as an inducer only after its conversion to D-idonate.

**Fig. 4.** Effect of D-idonate addition on the binding of His<sub>6</sub>-LgnR to P<sub>lgnA</sub> and P<sub>lgnR</sub>. (a) Effect of catabolic intermediates in the L-glucuronate pathway on binding of His<sub>6</sub>-LgnR to the lgnA–lgnR intergenic region. EMSA was performed with 400 nM His<sub>6</sub>-LgnR and 1 nM biotin-labelled fragment 1 in the presence of 100 μM of each catabolic intermediate. F, Unbound DNA; C1, complex 1; C2, complex 2; 5-KLGlu, 5-keto-L-glucuronate. (b) EMSA with 400 nM His<sub>6</sub>-LgnR and 1 nM fragment 4 and 5 in the presence of various concentrations of D-idonate. D-idonate was added at (from left to right) 0, 50, 100, 200 and 400 μM, respectively.
To further demonstrate that D-idonate acts as an effector on the dissociation of LgnR from both of its binding sites, we performed EMSA using fragments containing \( P_{lgnA} \) or \( P_{lgnR} \) (4 or 5) in the presence of various concentrations of D-idonate. As expected, dissociation of LgnR from both fragments was observed in a manner dependent on the concentration of D-idonate (Fig. 4b), suggesting that D-idonate is a genuine inducer molecule mediating expression from both \( P_{lgnA} \) and \( P_{lgnR} \).

**DNase I footprinting analysis**

To identify LgnR binding sequences within the two promoters, DNase I footprinting analysis was performed on a 345 bp fragment extending from the +120 region of \( P_{lgnA} \) to the +136 region of \( P_{lgnR} \). This DNA sequence was labelled with IRD-800 dye at each end individually. As shown in Fig. 5, protection was observed at the two promoter regions within both the \( lgnA \)- and \( lgnR \)-coding strands in the presence of 400 nM LgnR. In the \( P_{lgnR} \) region, sequence extending from +5 to −35, containing the transcriptional start site as well as the −10 and −35 boxes, was protected by the presence of LgnR. In the \( P_{lgnA} \) region, sequence extending from −10 to −36, containing the −10 and −35 boxes, was protected. These data indicate that LgnR represses transcription from these two promoters by binding to the promoter regions and thereby preventing the binding of RNA polymerase to the promoters with steric hindrance, as has been described for other bacterial repressor proteins (Browning & Busby, 2004). Both of these protected regions contain an inverted repeat (IR) sequence (5'-'CATAAATATG-3') at their −10 boxes, suggesting that these IR sequences are important for the binding of LgnR to \( P_{lgnA} \) and \( P_{lgnR} \). This was supported by the EMSA results that deletion of either of the −10 regions produced loss of the hyper-shifted bands.

In the presence of D-idonate, protection by LgnR at the −35 regions of the two promoters was only slightly

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**Fig. 5.** DNase I footprinting analysis using His6-LgnR and the \( lgnA \)-\( lgnR \) intergenic region. DNase I footprinting was conducted with 100 ng DNA fragment labelled with IRD-800 at the 5' end and 400 nM His6-LgnR in the presence or the absence of the indicated concentration of D-idonate. Positions of \( P_{lgnR} \) and \( P_{lgnA} \) are shown on the left. 'ACGT' indicates the sequencing ladders generated by the chain-terminating sequencing reactions. 'N' denotes DNase I reaction without LgnR addition. Black bars indicate regions protected by LgnR within the \( lgnA \)- and \( lgnR \)-coding strand, and the sequences of protected regions are shown on the right. Inverted repeat sequences found in both protected regions are indicated by arrows on the sequences. Grey bars indicate the regions where LgnR protection was slightly reduced by D-idonate addition.
reduced (Fig. 5, grey bars), while the $-10$ regions continued to be protected to the same degree. The surprisingly modest influence of D-idonate on the dissociation of LgnR from the operator sites may be due to the high concentration of LgnR used in our assays. A second possibility is that D-idonate requires a synergistic effector compound for complete dissociation of LgnR from its target DNA. A comparable mechanism has been described.
for the regulation of MhpR activator, where phenylpro-
propionate itself is not an effector for MhpR binding to DNA,
but rather phenylpropionate enhances the activity of its true
effectors, 3-hydroxy-phenylpropionate and 3-(2,3-
dihydroxyphenyl)propionate (Manso et al., 2009). To
examine this second possibility, we performed the DNase I
protection assay in the presence of D-idonate and
additional compounds, including all intermediates found
in the L-gluconate catabolic pathway. No synergistic effects
were observed with the compounds tested (data not
shown). Nevertheless, based on the results from our in
vivo expression and EMSA analyses, we propose that D-
idonate is a true effector for LgnR. However, we cannot
exclude the possibility that an unknown compound(s) may
also be involved in the induction of P_{lgnA} and P_{lgnR}, either
acting directly or synergistically.

**Phylogenetic analysis of LgnR and evolutionary
origin of the lgn operon**

In the KEGG genome database, LgnR orthologues are dis-
tributed in the classes Alphaproteobacteria, Betaproteobacteria
and Gammaproteobacteria, and in the phylum Actinobacteria.
Phylogenetic analysis based on the amino acid sequences of
LgnR orthologues, along with known members of the IclR family
of transcriptional regulators, revealed that LgnR orthologues
form a cluster with putative proteins from other genome
sequences, which is deeply branched from a cluster of
proteins of known function, YiaJ and KdgR, which are
responsible for the catabolism of acidic sugars like L-
gluconate. KdgR controls both the utilization of 2-keto-3-
deoxy-D-gluconate in *E. coli* (Nasser et al., 1992), as well as
pectin degradation by plant pathogens (Reverchon et al.,
1991), and YiaJ regulates the expression of L-ascorbate
catabolic genes in *E. coli* (Campos et al., 2008). The amino
acid sequence of LgnR shares only 28 and 32 % identity with
the sequences of *E. coli* KdgR and YiaJ, respectively.

In the cluster containing LgnR, two subgroups (groups I
and II) are apparent and LgnR is present in group I (Fig.
6a). Genes encoding group II proteins are often found in
the respective bacterial genomes in clusters with *rhamD*
orthologues encoding putative L-rhamnate dehydratases
(Rakus et al., 2008). All of the genes encoding group I
proteins, with the exception of two *Pseudomonas* genes
(PFA506_3986, PFLU4681), are located in gene clusters with
*lgnF* and/or *lgnG* orthologue(s) encoding putative
KDGal kinases and KDGPal aldolases, respectively (Fig.
6b). This may reflect the co-evolutionary history of *lgnF/
*lgnG* orthologues with group I *lgnR* orthologues. *dgoD*
orthologues encoding putative D-galactonate dehydratases
(Babbitt et al., 1995) are present in some of these gene
clusters, suggesting that these gene clusters are responsible
for D-galactonate utilization. Notably, proteins placed in
the downstream portion of the L-gluconate catabolic pathway
in *Paracoccus* sp. 43P and those of the D-galactonate catabolic
pathway reported in *E. coli* (Deacon & Cooper, 1977) and
non-pathogenic *Mycobacteria* (Szumilo, 1981) share identical
catabolic processes from KDGal, which are mediated by
LgnF/LgnG orthologues. Based on our previous phylogenetic
analyses, *lgnE, lgnH* and *lgnI* genes in the *lgn* operon form
respective clusters with sequences from other bacterial
affiliations, and therefore are assumed to be derived from
other bacterial origins (Shimizu et al., 2012). The catabolic
and genetic linkages between the L-gluconate catabolic
pathway in *Paracoccus* sp. 43P and the D-galactonate catabolic
pathways in other bacteria may indicate bottom-up evolution
of these catabolic pathways. The KDGal catabolic pathway,
which is regulated by an *lgnR* orthologue, was present in the
past as an ancestor, and this pathway may have been
subsequently adapted to D-galactonate utilization by acquiring
*dgoD* orthologues, or to L-gluconate utilization by acquiring
*lgnE, lgnH* and *lgnI* genes.

**Conclusions**

In this report, we determined the transcriptional organiza-
tion of genes involved in L-gluconate catabolism in
*Paracoccus* sp. 43P. We found that the *lgn* gene cluster is
co-transcribed as an operon. A transcriptional regulator in
the IclR family, encoded by *lgnR*, is located in the upstream
region of *lgnA* and negatively regulates transcription of the
*lgn* operon and *lgnR* itself. EMSA and DNase I footprinting
analysis revealed that LgnR binds to both P_{lgnA} and P_{lgnR},
indicating that LgnR inhibits transcription from these pro-
motors by competing with RNA polymerase. D-Idonate, an
intermediate of the L-gluconate catabolic pathway, is a
candidate inducer molecule for expression from these
promoters. Phylogenetic analysis revealed that LgnR belongs
to a cluster of putative proteins from genome sequences in
the IclR family of transcriptional regulators. Further gene
cluster analysis implied an evolutionary linkage between the
L-gluconate catabolic pathway and D-galactonate catabolic
pathways distributed in *Alphaproteobacteria, Betaproteo-
bacteria, Gammaproteobacteria* and *Actinobacteria*.

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