Expression of the O antigen gene cluster is regulated by RfaH through the JUMPstart sequence

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Abstract

O antigen genes are clustered, with a JUMPstart sequence located upstream. JUMPstart is a 39-bp sequence, present upstream of many polysaccharide gene clusters and also upstream of haemolysin and F factor gene clusters. RfaH is known to regulate the expression of *E. coli* group II capsule, haemolysin, F factor and the outer core of lipopolysaccharide all of which have the JUMPstart sequence, and has been shown to function as a transcriptional antiterminator in some cases. Using *lacZ* fusions to genes in the O antigen gene cluster of *Salmonella enterica* serovar Typhimurium, we found that RfaH also regulates the expression of O antigen. We showed that RfaH enhances expression of the 18-kb O antigen gene cluster, with promoter-distal genes affected more dramatically. We also showed that the JUMPstart sequence was required for RfaH function.

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1. Introduction

Lipopolysaccharides (LPS) are integral components of the outer membrane of Gram-negative bacteria. They consist of three structural regions: the lipid A moiety, oligosaccharide core and O antigen. O antigen is a polysaccharide comprised of repeat oligosaccharide units (O units), which comprise a few sugar residues. Characteristically, all genes specific to O unit synthesis are clustered in a region close to *his* and *gnd* in *Escherichia coli*, *Salmonella enterica* and related species (for reviews see [1,2]). Several O antigen gene clusters have been cloned and sequenced, and we found that a short conserved DNA sequence of 39 bp, termed JUMPstart, is located in the non-coding region upstream of all those O antigen gene clusters [3]. We also identified the JUMPstart sequence in the regulatory region in gene clusters of *waaQ-K* (*rfaQ-K* (LPS outer core)) and *kps* (*E. coli* group II capsules). *rfaH* encodes a positive regulatory protein for the LPS outer core, *E. coli* group II capsules, *E. coli* haemolysin (*hly* operon), and the F-factor (*tra* operon) [4–7].

Neito et al. [8] showed that the region upstream of the *hly* and *tra* operons had considerable similarity to the JUMPstart sequence and that an eight base part of it (which they named as the *ops* polar suppressor sequence) of the *hly* operon of plasmid pHly152 was required for the positive regulatory effect of RfaH. They claimed that the larger JUMPstart sequence is not conserved in the *hly* or *tra* op-
The JUMPstart consensus sequence, obtained from an alignment of 10 different polysaccharide-associated gene clusters [3], is shown above. Residues conserved in two or more of the four sequences are in upper case. The boxed segments were deleted by ourselves (LT2), Leeds and Welch [9] (hly\textsubscript{J96}) or Neito et al. [8] (hly\textsubscript{pHly152}) in experiments described or referred to in the text.

Fig. 1. Alignment of JUMPstart sequences from the LT2 O antigen cluster (GenBank entry X56793), tra operon (GenBank entry U01159), plasmid-borne hly\textsubscript{pHly152} operon (GenBank entry X07565) and chromosomal hly\textsubscript{J96} operon (GenBank entry M10133). The JUMPstart consensus sequence, obtained from an alignment of 10 different polysaccharide-associated gene clusters [3], is shown above. Residues conserved in two or more of the four sequences are in upper case. The boxed segments were deleted by ourselves (LT2), Leeds and Welch [9] (hly\textsubscript{J96}) or Neito et al. [8] (hly\textsubscript{pHly152}) in experiments described or referred to in the text.

RfaH has been shown to act as a transcriptional antiterminator in the kps (K5), hly and tra operons [6,7,10,11]. However although the JUMPstart sequence was first recognised in O antigen clusters, its role in O antigen expression has not been investigated. To do this, we first cloned the promoter of the LT2 O antigen gene cluster upstream of a reporter gene, and showed that the expression of the reporter gene is affected by the presence of RfaH. By use of lac\textsubscript{Z} fusions in genes in the O antigen gene cluster, we then showed that RfaH increases the expression of O antigen genes. We also showed that the JUMPstart sequence is the site of RfaH action on the O antigen gene cluster.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 1. P5096, a gal\textsubscript{E} derivative of E. coli K-12

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Lab stock no.</td>
</tr>
<tr>
<td>E. coli K-12 strains</td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td>P2398</td>
</tr>
<tr>
<td>P5096</td>
<td>same as JM101, gal\textsubscript{E}28, nad\textsubscript{A}50::Tn10</td>
</tr>
<tr>
<td>P5097</td>
<td>\text^{\text^}_3, Rel1, spo\textsubscript{T}1, th\textsubscript{I}, nad\textsubscript{A}50::Tn10</td>
</tr>
<tr>
<td>x2819</td>
<td>P3851</td>
</tr>
<tr>
<td>NK6033</td>
<td>P4877</td>
</tr>
<tr>
<td>PL2</td>
<td>P2945</td>
</tr>
<tr>
<td>Salmonella LT2 strain</td>
<td></td>
</tr>
<tr>
<td>SA2887</td>
<td>P9479</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Only given if different from strain name.
JM101, was constructed as follows: first P5097 (galE28, nadA50::Tn10(Tet')) was made by introducing nadA50::Tn10 from strain NK6033 into strain PL2 (galE28) using the protocol described by Wang and Reeves [12]. The galE28 allele was then moved from P5097 into JM101 using nadA50::Tn10 as marker to give P5906. The galE mutation in both P5096 and P5097 was confirmed by bacteriolysis of the cells in the presence of galactose. Promoter cloning vector pKK232-8 was from Pharmacia. Plasmid pPR1634 carries strain LT2 O antigen DNA from positions 2923 to 5338 cloned into the SalI and HindIII sites of pKK232-8. Plasmid pPR1786 was made by doing mutagenesis on pPR1634 to replace the JUMPstart sequence with an EcoRI site using oligonucleotide 858 (5'-tgcaacaagtcgatatgtacgaattctgtaaccgacttgagcaattaattt-3') and the Alter-a-site kit from Promega (see Fig. 1 for detail). Plasmid pPR1747 was made by PCR amplifying the E. coli K-12 rfaH gene and its upstream regulatory region using oligonucleotides 722 (5'-atagaattcatgcgacagccagcgtataatcag-3') and 723 (5'-tatgaattcatgcgcatggcctgcc-3'), and cloning the PCR product into the EcoRI site of plasmid pACYC184 [13]. The function of the rfaH gene in plasmid pPR1747 was confirmed by its ability to confer P22 sensitivity on the rfaH strain SA2887 [14]. Molecular positions are as in [15].

2.2. Transposon mutagenesis

Transposon Tn1737 on plasmid pRU868 [16] was used for insertional mutagenesis of cosmid pPR1009 (carrying the entire LT2 O antigen gene cluster [15]) following the protocol described by Ubben and Schmitt [16].

2.3. Enzyme assays

The activity of β-galactosidase was determined using the method described by Miller [17]. The activity of chloramphenicol acetyl transferase (CAT) was assayed using the Promega CAT Enzyme Assay System and [3H]chloramphenicol, following the manufacturer’s protocol. The CAT activity was calculated using a standard curve obtained from reactions using serial dilutions of purified CAT enzyme from Promega. Crude protein extract used for CAT assay was prepared as described before [18], and protein concentration was measured using the Kinetic BCA protein assay kit from Pierce. Each assay was carried out three times using different protein preps to confirm the reproducibility of the result: results were consistent and the result from one assay for each experiment has been used in the paper.

3. Results and discussion

3.1. Cloning of the main promoter(s) of the LT2 O antigen gene cluster

More than 20 O antigen gene clusters have been sequenced. The O antigen gene cluster of strain LT2 is the one studied most thoroughly with functions of all the genes identified [1,2]. To identify the functional promoter, plasmid pPR1009, which carries the entire LT2 O antigen gene cluster [15], was digested with HindIII and SalI and fragments cloned into pKK232-8, in which the insert is upstream of a promoter-less CAT gene. Eighty transformants, which were able to grow on nutrient agar plates containing chloramphenicol (25 μg ml⁻¹), were screened by restriction analysis of the plasmid. The clones carrying O antigen DNA in the correct orientation all have the same insert covering the region from positions 2923 to 5338. One such plasmid carrying this insert was named pPR1634.

DNA from positions 2923 to 5338 includes the potential promoter identified by sequence analysis, which is located just upstream of the JUMPstart sequence and the first O antigen gene with −35 and −10 regions at positions 3894–3899 and 3912–3917 respectively [3,15]. DNA from positions 3880 to 3986 was PCR amplified using oligonucleotide primers with BamHI and HindIII sites incorporated in the forward and reverse primers respectively (see Fig. 2), and cloned into the BamHI/HindIII sites of plasmid pKK232-8 to make plasmid pPR1730, which was shown to have promoter activity (data not shown). Zhang et al. [19] identified four functional promoters in the O antigen region of Yersinia enterocolitica O3: two (P1 and P2) within close distance to each other are located just upstream of the JUMPstart sequence, and the other two (also within close distance to each other) in the middle of the gene cluster. The
promoter of strain LT2 O antigen has −35 and −10 regions almost identical to those of P1 of Y. enterocolitica O3.

3.2. RfaH regulates the LT2 O antigen promoter

To determine if RfaH regulates expression of LT2 O antigen, pPR1634 was transformed into strains SA2887 (rfaH−, see Table 1) and SA2887 carrying pPR1747. SA2887 carrying pPR1634 has a CAT activity of 6.7 units protein, SA2887 carrying both pPR1747 and pPR1634 has 15 units protein, while SA2887 has no activity. This result demonstrates that RfaH acts as a positive regulator on the expression of genes downstream of the main promoter, although there is considerable activity in the absence of RfaH.

3.3. Effect of RfaH depends on location of O antigen genes in the gene cluster

To determine the manner in which RfaH affects the expression of the O antigen gene cluster itself, we carried out transposon mutagenesis to make transcriptional fusions in different O antigen genes. Transposon Tn1737 [16] was used: it contains a promoterless lacZ gene with its own Shine-Dalgarno (SD) sequence and translation stops in all reading frames upstream of the SD sequence. The transcription level of a given O antigen gene can be estimated by the β-galactosidase activity of the strain with the lacZ gene fused to it.

The position and orientation of the transposon insertion was determined by restriction mapping. Insertions with the lacZ gene in the correct orientation were obtained for 10 of the 16 O antigen genes (Fig. 3). These 10 genes are distributed throughout the gene cluster and taken to be representative. The plasmids carrying these insertions were transformed into SA2887 carrying plasmids pACYC184 or pPR1747.

The effects of RfaH on the activity of β-galactosidase from these fusions is shown in Fig. 3. The expression of β-galactosidase from all of the fusions was significantly less in the rfaH− strain (SA2887 carrying pACYC184) compared to that in the same strain carrying a functional rfaH plasmid (SA2887 carrying pPR1747), although the fusions in the first half of the gene cluster gave a smaller reduction. This indicates that RfaH is necessary for full expression of all the O antigen genes.

RfaH has been shown to function as a transcriptional antiterminator in the kps, waqQ-K, hly and tra operons, where it is necessary for the elongation of the mRNA (for a recent review, see [20]). Our results

Table 2
Effect of RfaH and JUMPstart sequence on O antigen gene cluster promoter activity

<table>
<thead>
<tr>
<th>RfaH strain</th>
<th>Plasmid carrying rfaH gene</th>
<th>Other plasmid</th>
<th>CAT activity (units µg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA2887</td>
<td></td>
<td>pPR1786</td>
<td>4.05 × 10⁻⁴</td>
</tr>
<tr>
<td>SA2887</td>
<td>pPR1747</td>
<td>pPR1786</td>
<td>4.2 × 10⁻³</td>
</tr>
<tr>
<td>SA2887</td>
<td></td>
<td>pPR1634</td>
<td>6.7 × 10⁻³</td>
</tr>
<tr>
<td>SA2887</td>
<td>pPR1747</td>
<td>pPR1634</td>
<td>15 × 10⁻³</td>
</tr>
<tr>
<td>SA2887</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. Regulatory region of the LT2 O antigen gene cluster. DNA sequence from positions 3880 to 4101 is shown with the −35 and −10 region of the promoter indicated. The JUMPstart sequence is boxed, and the first codon of the O antigen gene, rmlB, is underlined. The JUMPstart sequence is replaced by an EcoRI site in plasmid pPR1786. The PCR priming sites for amplification and clone of the promoter region to make plasmid pPR1730 are indicated by arrows.
support the possibility that RfaH is also a transcriptional antiterminator in the O antigen gene cluster.

Fusions in the second half of the gene cluster have lower β-galactosidase activity than those in the first half in the absence of RfaH. Plasmid pPR1634 carrying the promoter and a small fragment of downstream O antigen DNA (see Fig. 3) showed high level activity of the CAT gene in the absence of RfaH (see above). These results agree with the finding in the hly operon that RfaH has greater effect on promoter-distal genes [10]. It may indicate the presence of a terminator sequence in the region between ddhC and wzx (see Fig. 3). Gene abe is located between ddhC and wzx. Analysis of the sequence data of the intergenic regions between ddhC and abe, and abe and wzx revealed no stable hairpin loop.

3.4. RfaH action requires the JUMPstart site

To study the effect of the JUMPstart sequence in the action of RfaH on O antigen, plasmid pPR1786 was constructed and transformed into the rfaH strain SA2887 with and without pPR1747. As shown in Table 2, RfaH does not affect the level of CAT activity produced by plasmid pPR1786. Thus, we conclude that the JUMPstart sequence is involved in the antitermination action.

Bailey et al. [6] and Leeds and Welch [9] recently reported that RfaH and the JUMPstart sequence function together to enhance transcription through the four gene hly operon, and noted similarity at amino acid level between RfaH and NusG suggesting that RfaH could act like NusG. Transcriptional regulation during the early transcription of lytic growth of bacteriophage λ, involving the λ N protein in a controlled antitermination mechanism, has been well studied. Protein N and host factors NusA, B and G interact at the nut site, located between the promoter and the first terminator, and form a transcription complex with RNA polymerase and host factor S10. This complex enables the RNA polymerase to maintain a termination-resistant form. There is evidence that this complex is still associated with RNA polymerase when it reaches the downstream terminator (for a recent review, see Richardson and Greenblatt [21]).

LPS contains lipid A, core and O antigen. Core is synthesised on lipid A by products of waa genes. O antigen is synthesised separately on a lipid carrier, and the mature O antigen is then transferred to core lipid A to form LPS before being translocated to the outer membrane. It has long been known that the transcription of genes for the outer core synthesis is positively controlled by RfaH [5]. We have now shown that the expression of O antigen genes is also positively controlled by RfaH.

LPS is essential to the cell and replaces phospholipid in the outer leaflet of the outer membrane. A specific amount of it is needed for each cell. In contrast capsules, haemolysin, and F factor are not es-

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**Fig. 3.** Transposon insertions in the LT2 O antigen genes and the activity of the transposon β-galactosidase gene in rfaH<sup>−</sup> and rfaH<sup>+</sup> backgrounds. Each of the LT2 O antigen genes is represented by a box with the name in it (please note the use of a new nomenclature for bacterial surface polysaccharide genes. See [23] for details). The JUMPstart sequence and the main promoter are represented by a circle and an arrow respectively at their approximate positions. Transposon insertion in a gene is indicated by two bars, with the height of the bar indicating the β-galactosidase activity of that plasmid in rfaH<sup>−</sup> (cross-hatched bars) or rfaH<sup>+</sup> (hatched bars) background. The names of the insertion plasmids are listed. The insert of plasmid pPR1634 is indicated by a line.
sential and in some cases (such as the E. coli group II capsules) the level of expression can be varied substantially. It is not clear why the cell controls the expression of these genes in part at least using the same mechanism. Bauer and Welch [22] recently reported that a LPS mutant, which can only synthesise truncated LPS molecules, affects exccellular expression and activity of haemolysin and to a lesser extent the transcription of hly. A physical interaction between LPS and haemolysin was suggested, in which LPS participates in forming or maintaining an active conformation of haemolysin. This may offer some explanation for the hly operon, O antigen gene cluster, and waaQ-K being controlled by RfaH. It should also be noted that some of the operons are very large (e.g. the tra operon is 31 kb (JUMPstart is located 5.5 kb from the 5’ end) and the S. enterica LT O antigen gene cluster is 18 kb) and the role of RfaH may simply be to prevent premature transcription termination.

Acknowledgments

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