Multiple sensors provide spatiotemporal oxygen regulation of gene expression in a Rhizobium-legume symbiosis

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Abstract

Regulation by oxygen (O₂) in rhizobia is essential for their symbioses with plants and involves multiple O₂ sensing proteins. Three sensors exist in the pea microsymbiont Rhizobium leguminosarum Rlv3841: hFixL, FnrN and NifA. At low O₂ concentrations (1%) hFixL signals via FxkR to induce expression of the FixK transcription factor, which activates transcription of downstream genes. These include fixNOQP, encoding the high-affinity cbb₃-type terminal oxidase used in symbiosis. In free-living Rlv3841, the hFixL-FxkR-FixK pathway was active at 1% O₂, and confocal microscopy showed hFixL-FxkR-FixK activity in the earliest stages of Rlv3841 differentiation in nodules (zones I and II). Work on Rlv3841 inside and outside nodules showed that the hFixL-FxkR-FixK pathway also induces transcription of fnrN at 1% O₂ and in the earliest stages of Rlv3841 differentiation in nodules. We confirmed past findings suggesting a role for FnrN in fixNOQP expression. However, unlike hFixL-FxkR-FixK, Rlv3841 FnrN was only active in the near-anaerobic zones III and IV of pea nodules. Quantification of fixNOQP expression in nodules showed this was driven primarily by FnrN, with minimal direct hFixL-FxkR-FixK induction. Thus, FnrN is key for full symbiotic expression of fixNOQP. Without FnrN, nitrogen fixation was reduced by 85% in Rlv3841, while eliminating hFixL only reduced fixation by 25%. The hFixL-FxkR-FixK pathway effectively primes the O₂ response by increasing fnrN expression in early differentiation (zones I-II). In zone III of mature nodules, near-anaerobic conditions activate FnrN, which induces fixNOQP transcription to the level required for wild-type nitrogen fixation activity. Modelling and transcriptional analysis indicates that the different O₂ sensitivities of hFixL and FnrN lead to a nuanced spatiotemporal pattern of gene regulation in different nodule zones in response to changing O₂ concentration. Multi-sensor O₂ regulation is prevalent in rhizobia, suggesting the fine-tuned control this enables is common and maximizes the effectiveness of the symbioses.
Author summary
Rhizobia are soil bacteria that form a symbiosis with legume plants. In exchange for shelter from the plant, rhizobia provide nitrogen fertilizer, produced by nitrogen fixation. Fixation is catalysed by the nitrogenase enzyme, which is inactivated by oxygen. To prevent this, plants house rhizobia in root nodules, which create a low oxygen environment. However, rhizobia need oxygen, and must adapt to survive the low oxygen concentration in the nodule. Key to this is regulating their genes based on oxygen concentration. We studied one Rhizobium species which uses three different protein sensors of oxygen, each turning on at a different oxygen concentration. As the bacteria get deeper inside the plant nodule and the oxygen concentration drops, each sensor switches on in turn. Our results also show that the first sensor to turn on, hFixL, primes the second sensor, FnrN. This prepares the rhizobia for the core region of the nodule where oxygen concentration is lowest and most nitrogen fixation takes place. If both sensors are removed, the bacteria cannot fix nitrogen. Many rhizobia have several oxygen sensing proteins, so using multiple sensors is likely a common strategy enabling rhizobia to adapt to low oxygen precisely and in stages during symbiosis.

Introduction
Rhizobia are alpha-proteobacteria that engage in symbiosis with legume plants [1]. The bacteria convert inert atmospheric N\(_2\) into biologically accessible ammonia and provide it to their plant host in a process called nitrogen fixation [2,3]. All biological fixation is catalysed by the nitrogenase enzyme complex that evolved before the Great Oxygenation Event and requires near-anoxic conditions to function [4–6]. However, rhizobia are obligate aerobes and must respire to meet the high energy demands of nitrogen fixation [7,8]. These competing requirements create an ‘oxygen paradox’ in symbiotic nitrogen fixation [9,10]. To overcome this paradox, intricate cooperation between rhizobia and their plant partners has evolved (reviewed in [11,12]). Legume plants host rhizobia in dedicated root nodules which form where bacteria have entered the plant root, usually via infection threads (reviewed in [13,14]). Nodules create a near-anoxic internal environment suitable for nitrogenase activity [15–17]. To produce this environment, oxygen (O\(_2\)) is captured and shuttled to bacteroids by plant leghaemoglobins [18–21]. The concentration of remaining free O\(_2\) in the core nitrogen fixation zone of nodules is as low as 20–50 nM [22,23]. Rhizobia undergo a radical lifestyle change after nodule entry to survive and fix nitrogen in these conditions (reviewed in [24,25]). In indeterminate nodules, such as those produced by Pisum sativum (pea), rhizobia are initially free-living upon entry [26,27]. They then undergo irreversible lifestyle changes as they move from the nodule tip to its core [28,29]. Beginning in zone II and accelerating in the II–III interzone, rhizobia terminally differentiate into quasi-organelle bacteroids specialized for nitrogen fixation [30,31]. Zone III of indeterminate nodules contains differentiated bacteroids which are actively fixing nitrogen [32]. Rhizobial regulatory mechanisms sensitive to O\(_2\) tension are essential for successful differentiation into bacteroids and the establishment of a productive symbiosis [33–35].

Multiple O\(_2\) sensors have evolved in rhizobia, three of which are widespread and often coexist within the same organism [11,36]. The first is the membrane-bound FixL protein, which forms a two-component system (TCS) with the FixJ receiver protein (reviewed in [37,38]). Under microaerobic conditions, FixL phosphorylates FixJ, which in turn induces expression of the fixK transcription factor [39–41]. FixK induces expression of downstream genes by
binding as a dimer to an ‘anaerobox’ motif (TTGAT-N₄-ATCAA) upstream of their promoters [42,43].

The second common O₂ sensor is a variant of FixL called hybrid FixL (hFixL) [44,45]. This forms an alternative TCS with FxkR acting as the receiver protein. FxkR is not a FixJ homolog but similarly induces expression of fixK, by binding to an upstream ‘K-box’ motif (GTGACA-N₄-GTTACA) [46]. The third O₂ sensor is the FnrN transcription factor. Like FixK, FnrN binds the anaerobox motif as a dimer and both are close homologs of the E. coli anaerobiosis regulator FNR [47–49]. Unlike FixK but like FNR, FnrN contains an N-terminal cysteine-rich cluster that makes the protein a direct sensor of O₂ [50–53]. The FixL and hFixL sensors are known to become active at relatively mildly microaerobic conditions, including in free-living rhizobia [54–56]. FnrN is likely to be far less O₂ tolerant. The O₂ sensitivity of FnrN has not been determined, but the E. coli FNR homolog is active only under anaerobic conditions [57–59]. All symbiotic rhizobia studied to date employ at least one of these three sensors [11]. It is common for these sensors to coexist, notably in Rhizobium leguminosarum biovar viciae VF39, multiple strains of Ensifer meliloti (previously Sinorhizobium meliloti) and Rhizobium etli CFN42 [44,45,60–62].

Further emphasizing the importance of O₂ regulation in symbiotic nitrogen fixation, rhizobia also employ the O₂ sensing NifA transcription factor to regulate their final differentiation into nitrogen fixing bacteroids (for reviews see [38,63]). NifA oxygen sensitivity is thought to derive from a metal-binding cysteine-rich motif in an inter-domain linker of the protein [64–66]. The protein has a large regulon, notably including nitrogenase components such as nifH [67–70]. Expression of nifA is typically auto-regulated in rhizobia, often via read-through from an upstream gene or operon that is NifA regulated, in many cases fixABCX [69,71–73]. In Rlv3841, a fixABCX operon is found directly upstream of nifA, suggesting such a read-through NifA auto-activation mechanism. Usually, neither expression of nifA nor the activity of the protein is directly regulated by the three O₂ sensors described above [11]. One notable exception is E. meliloti, where nifA is regulated by the FixLJ system [74–76]. There is no evidence that FixK or FnrN directly regulates nifA expression in Rlv3841.

There appears to be a spectrum among rhizobia, with some species segregating oxygen sensors into separate pathways, whilst in other species these sensors have partially or completely merged into a combined hierarchical pathway [77,78]. Where oxygen sensors are in separated pathways, redundancy often exists. In these situations the loss of one oxygen sensor does not abolish nitrogen fixation activity [79,80]. By contrast, where sensors have been merged into a single regulatory pathway, some components are individually essential [11]. Thus, loss of one oxygen sensor can severely impair nitrogen fixation even if other sensors remain.

In R. leguminosarum bv. VF39, knocking out FnrN or hFixL reduced nitrogen fixation to 30% or 50% of WT respectively, suggesting a non-hierarchical, redundant arrangement [60]. The hFixL-FxkR-FixK pathway of R. etli CFN42 is dispensable as a double fixK mutant had no effect on nitrogen fixation, whilst a double fnrN mutant reduced fixation to 20% of WT levels. By contrast, R. etli CFN42 appears to employ a complex hierarchical pathway, with multiple homologs of FixK and FnrN regulating each other’s expression [61,62]. Species encoding homologs of only hFixL or FnrN have also been found. Rhizobium leguminosarum biovar viciae UPM791 contains two FnrN homologs but neither FixL nor hFixL [81]. It is unknown whether the two FnrN proteins respond to different O₂ concentrations or act in a redundant fashion. E. meliloti 1021 contains no FnrN homolog but a well-studied FixLJ system and appears to have homologs of hFixL and FxkR [82–84].

To examine the relationship between hFixL and FnrN, we studied the model organism Rhizobium leguminosarum biovar viciae 3841 (Rlv3841) which employs both sensors (Fig 1) [85,86]. Rlv3841 has a single chromosome whose gene names start with RL, and six
megaplasmids pRL7-12 whose gene names start with e.g. pRL9. The main symbiotic plasmid is pRL10, but many symbiotic genes are also found on pRL9 including a copy of the fixNOQP and fixGHIS operon. Rlv3841 encodes two copies of hfixL, which we named hfixL_9 (pRL90020 on pRL9) and hfixL_c (RL1879 on the chromosome). It has three putative fixK genes, which we designated fixK_9a (pRL90019), fixK_9b (pRL90025) and fixK_c (RL1880). The fixK_9a and fixK_9b sequences have 53% amino acid identity, whilst fixK_c shares 38% and 47% identity with these proteins, respectively. Both fixK_9a- hfixL_9 and fixK_c- hfixL_c appear to form operons (Fig 1B and 1D). Rlv3841 has one copy of fnrN (RL2818), regulated by two anaeroboxes. A similar dual-anaerobox arrangement exists in Rlv UPM791, where FnrN positively and negatively auto-regulates its own expression [87]. Binding of FnrN to the distal anaerobox induces fnrN transcription and binding to the proximal anaerobox represses it. Auto-activation of FnrN has also been reported in Rhizobium etli CNPAF512 [88]. FixK regulation of fnrN expression is likely as it also binds anaeroboxes, but this had not been investigated.

Fig 1. The integrated hFixL-FxkR-FixK and FnrN oxygen regulation systems of Rlv3841 form a single pathway and are genetically clustered. Oxygen is shown in red diamonds. Proteins are shown as ovals, operator sites as squares and genes as pointed rectangles. Transcription start sites are shown as right-angled arrows. Line endings indicate activation (arrows), inhibition (blunt end) and translation (circle). (A) The single pathway formed by the two sensors acts in two stages. Stage I starts under microaerobic conditions and can function outside the nodule. In this stage, hFixL is active but FnrN is not. hFixL activates FxkR, which binds to the K-box operator (orange “K” squares) to induce expression of fixK. FixK binds to anaerobox operators (blue “A” squares) to induce expression, including upstream of fixNOQP (dashed line) and fnrN. Once oxygen in the bacteria reaches near-anoxic levels, FnrN becomes active and stage II begins. Like FixK, FnrN binds anaeroboxes. It auto-regulates fnrN both positively and negatively and induces fixNOQP expression. (B) Rlv3841 has multiple copies of several oxygen regulation genes and many are arranged in clusters. On megaplasmid pRL9, fixK_9a forms an operon with hfixL_c regulated by a K-box. This operon is adjacent to fixNOQP_9, regulated by an anaerobox. (C) fixK_9b and fskR_9 are adjacent, with an anaerobox and a K-box in their intergenic region. (D) The Rlv3841 chromosome also has a cluster, containing fskR_c, fixK_c and hfixL_c. Unlike the similar clusters on pRL9, the intergenic region of this cluster contains no anaerobox or K-box operators. (E) The fnrN gene is not part of a cluster and is positively and negatively regulated by a distal and proximal anaerobox, respectively. Details of transcription start site, anaerobox and K-box locations can be found in Table 1.

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A study in *R. leguminosarum* VF39 found that microaerobic expression of *fnrN* also requires RpoN [89]. This finding has not been replicated elsewhere, and its significance remains unclear. Work in *R. etli* CNPAF512 showed that *fnrN* is not controlled by RpoN in that organism [88]. Rlv3841 encodes one putative rpoN gene (RL0422), but we found no RpoN binding sites upstream of the Rlv3841 *fnrN* transcription start site. RpoN therefore does not appear to be required for *fnrN* expression in Rlv3841.

A parallel arrangement of hFixL-FxkR-FixK and FnrN in Rlv3841 would produce redundancy, whereas an arrangement in series would create hierarchy between the two regulators. Our goal is therefore to understand how the two sensors interact in Rlv3841 and to provide insight into why they coexist.

**Results**

**Expression of *fnrN* is auto-regulated and controlled by the hFixL-FxkR-FixK pathway**

The hFixL-FxkR-FixK pathway is known to be active at relatively high O$_2$ concentrations, including in free-living rhizobia under microaerobic conditions [45,90]. The role of FnrN is less well understood and we began by investigating this sensor. The *fnrN* gene contains two anaeroboxes upstream of its promoter, a proximal site at -2 relative to the transcription start site (TSS) and a distal site at -34 (Fig 1E and Table 1). Binding at the proximal site inhibits transcription through steric hindrance, whilst the distal site activates it [87]. As expected from the presence of the distal site, *fnrN* was induced under microaerobic conditions in free-living Rlv3841 (Fig 2). Both FixK and FnrN bind to anaerobox operators and induce gene expression under microaerobic conditions [91,92]. Therefore, microaerobic induction of *fnrN* could be due to FnrN auto-activation and/or induction by the hFixL-FxkR-FixK pathway. To determine their respective importance, expression of *fnrN* was studied in Rlv3841 mutants defective in either hFixL or FnrN.

In a double *hfixL* mutant (LMB496; *hfixL*::ΩSpec *hfixL* c:pK19 single recombination), free-living microaerobic expression of *fnrN* was reduced to 25% of its wild-type (WT) level (Fig 3).

| Gene name     | TSS coordinate | Anaerobox 1 location | Anaerobox 2 location | K-box location |
|---------------|----------------|----------------------|----------------------|----------------|
| *fnrN* (RL2818) | 2978390        | -34                  | -2                   | Not present    |
| *fixK*$_{sa}$ (pRL90019) | 19878          | Not present          | Not present          | -62            |
| *fixK*$_{sb}$ (pRL90025) | 27090          | Not present          | Not present          | -62            |
| *fixK* (RL1880) | 1977111        | Not present          | Not present          | Not present    |
| *fxkR*$_{b}$ (pRL90026) | 27146          | Not present          | +38                  | +6             |
| *fxkR*$_{c}$ (RL1881) | 1977167        | Not present          | Not present          | Not present    |
| *fixNOQP*$_{a}$ (pRL90018-16) | 197210        | -33                  | Not present          | Not present    |
| *fixNOQP*$_{10}$ (pRL100205-207) | 206214       | -34                  | Not present          | Not present    |
| *fxGHIS*$_{a}$ (pRL90015-12A) | 15908         | -32                  | Not present          | Not present    |
| *fxGHIS*$_{10}$ (pRL100208) | 210004        | -32                  | Not present          | Not present    |

Locations of anaeroboxes and K-boxes are given relative to the TSS of each gene respectively. Anaeroboxes more than 90 bp upstream of transcription start sites are not included. The location of activating anaeroboxes is well conserved across the genes shown above (between -32 and -34 relative to the TSS). Likewise, both megaplasmid-encoded *fxk* copies have their respective K-boxes in the same position relative to the TSS (-62). The anaerobox downstream (+38) of the *fxkR$_b$* transcription start site likely represses it when bound. A single K-box is shared between *fxkR$_b$* and *fixK$_{sa}$* (see Fig 1). It is correctly located (-62) to induce *fixK$_{sa}$* when bound, and its location suggests it simultaneously represses *fxkR$_b$* (+6). The operators downstream of *fxkR$_b$* may create a negative feedback loop in the Rlv3841 hFixL-FxkR-FixK pathway. The downstream anaerobox also suggests FnrN repression of hFixL-FxkR-FixK via repression of *fxkR$_b$* transcription, but this requires further study.

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The single mutant of hfixL9 (LMB495; hfixL9::ΩSpec) individually reproduced most of this reduction whilst the single mutant of hfixLc (LMB403; hfixLc::pK19 single recombination) did not reduce fnrN expression. This suggests hFixL9 is the critical hFixL protein under free-living microaerobic conditions, with hFixLc playing little to no role.

hFixL acts through the FxkR intermediary in rhizobia and Rlv3841 contains two FxkR homologs [44]. fxkRc forms an O2 regulation cluster with fixK9c, which we labelled cluster 2 (Fig 1C) and fxkRc forms a cluster with fixKc-hfixLc, which we labelled cluster 3 (Fig 1D). Cluster 2 contains an anaerobox and a K-box. The anaerobox is relatively far from the fixK9c TSS.
(-94), but downstream of the fskR<sub>9</sub> TSS, suggesting its main role is to repress fskR<sub>9</sub> transcription (Table 1). Its effect on fixK<sub>9b</sub> transcription may be minimal, if any. The K-box is also downstream of the fskR<sub>9</sub> TSS, suggesting it also represses transcription. Simultaneously, this K-box also likely acts to induce fixK<sub>9b</sub>, as its location upstream of the fixK<sub>9b</sub> TSS (-62) is identical to the relative position of the upstream fixK<sub>9a</sub> K-box in cluster 1 (Fig 1B and Table 1). Both K-boxes therefore appear functional for fixK induction, and the cluster 2 K-box appears to have a dual function, repressing fskR<sub>9</sub> and inducing fixK<sub>9b</sub>. The second cluster, containing fskR<sub>c</sub>, has no anaeroboxes or K-boxes (Fig 1D). This could imply minimal expression from the genes in this cluster, or constitutive expression that is not O<sub>2</sub> regulated. Neither fixK<sub>c</sub> (1.6-fold upregulated, p = 0.140) nor fskR<sub>c</sub> (1.3-fold upregulated, p = 0.145) was significantly upregulated in 21 day old bacteroids compared to free-living Rlv3841 [93]. Based on these findings,
we speculated that FxkR<sub>9</sub> is the main FxkR protein and fskR<sub>9</sub> was deleted to produce strain OPS1808 (ΔfxkR<sub>9</sub>). This mutant reproduced the reduced induction of fnrN under free-living microaerobic conditions observed in the double hfixL mutant (Fig 3). This finding supports the role of FxkR<sub>9</sub> as the mediator of hFixL O<sub>2</sub> regulation in Rlv3841, in agreement with studies in other rhizobia [44,45]. Studying the role of the hFixL-FxkR-FixK pathway in fnrN expression in planta, we observed that the double hfixL mutant reduced fnrN expression to 28% of WT levels (Fig 4A). This indicates the pathway also plays an important role in inducing fnrN during symbiosis.

We next studied the role of FnrN auto-regulation. A mutant of fnrN (LMB648, fnrN::ΩTet) had no effect on expression of fnrN at 1% O<sub>2</sub> (Fig 3), indicating FnrN auto-activation does not occur under free-living microaerobic conditions. By contrast, in planta the fnrN mutant reduced fnrN expression to 22% of WT levels (Fig 4A), similar to the reduction observed in the hfixL double mutant. FnrN auto-activation is thus an important regulatory effect during symbiosis but not under microaerobic conditions. During symbiosis, expression of fnrN is driven both by auto-activation and the hFixL-FxkR-FixK pathway, and both are required to attain full WT-level expression of the gene.

**FnrN is critical for symbiotic gene expression but hFixL also plays an important role**

To understand the respective importance of FnrN and hFixL as regulators of anaerobox controlled genes during symbiosis, their role in fixNOQP expression was studied. The fixNOQP operon encodes a high-affinity cbb<sub>3</sub>-type terminal oxidase required for respiration during symbiosis [94–96]. It is typically regulated by an anaerobox [97]. Some rhizobia...
encode multiple redundant terminal oxidases controlled by different regulators, but no alternates appear to be encoded by Rlv3841 [98,99]. The strain therefore likely relies entirely on fixNOQP for respiration during symbiosis. Three putative homologs of fixNOQP exist in Rlv3841, which we labelled fixNOQP\(_9\) (encoded on pRL9), fixNOQP\(_{10}\) (encoded on pRL10) and fixNOQP\(_{10}\) (encoded on the chromosome) [85]. Rlv VF39 encodes two fixNOQP operons, and either was able to sustain nitrogen fixation activity [56]. In Rlv3841, the plasmid-encoded operons are near-identical (>90% protein identity) but diverge from the fixNOQP operon, with which they share approximately 50% identity. Only the plasmid-encoded fixNOQP\(_9\) and fixNOQP\(_{10}\) operons contain an upstream anaerobox, at -33 and -34 relative to their TSS respectively (Table 1). Past microarray work in our group found no significant upregulation of fixN\(_9\) expression (1.7-fold upregulated, p-value 0.101) in 21 day old bacteroids compared to free-living Rlv3841 [93]. This contrasts sharply with fixN\(_9\) (38.1-fold up, p = 0.010) and fixN\(_{10}\) (119.6-fold up, p = 0.003), both highly upregulated. Taken together, these findings suggest the two plasmid-encoded fixNOQP operons of Rlv3841 are functional and anaerobox-regulated, whilst fixNOQP\(_9\) is not. Rlv3841 also has two homologs of the fixGHIS operon (>90% protein identity), encoding the assembly machine for the fixNOQP terminal oxidase [51,100]. Like fixNOQP, fixGHIS operons are typically anaerobox regulated [47,62,101]. Both fixGHIS operons in Rlv3841 have a single upstream anaerobox, in a near-identical position to those of the fixNOQP operons [85] (Table 1). In line with findings in other rhizobia, the fixGHIS and fixNOQP operons are therefore likely regulated by oxygen in a similar way [89,102,103].

Both fixNOQP operons were induced in cultured cells under microaerobic conditions, confirming their regulation by O\(_2\) (Fig 2). In culture, the double hfixL\(_9\) mutant severely reduced this microaerobic induction, resulting in minimal expression of fixNOQP\(_{10}\) and 17% of WT fixNOQP\(_9\) expression (Fig 2). The single hfixL\(_9\) mutant significantly reduced expression of both operons whilst the hfixL\(_{10}\) mutant only reduced expression of fixNOQP\(_9\), to 71% of WT. These results indicate hFixL\(_9\) is the dominant protein and hFixL\(_{10}\) plays only a minor role in fixNOQP expression, matching their respective importance for microaerobic fnrN expression (Fig 2). In the Rlv3841 fskR\(_9\) mutant, expression of both fixNOQP operons was reduced to less than 25% of WT, indicating that the protein is required for hFixL regulation of fskK and hence fixNOQP, as found in other rhizobia [44]. The hFixL-FskK-FixK pathway is thus a key regulator of fixNOQP expression under free-living microaerobic conditions. The remaining expression of fixNOQP\(_9\) and fixNOQP\(_{10}\) in the fskR\(_9\) mutant may be due to redundancy via the fskR\(_{10}\) homolog, or the result of background FixK or FnrN activity. By contrast, in these conditions the fnrN mutant minimally affected fixNOQP expression, with only fixNOQP\(_9\) showing a small albeit statistically significant reduction (73% of WT). In line with our study of fnrN expression, the hFixL-FskK-FixK pathway is crucial for fixNOQP expression under free-living microaerobic conditions whilst FnrN plays a minimal role. It is likely that the FnrN protein remains mostly inactive at the O\(_2\) concentration (1% O\(_2\)) used in our free-living experiments.

We also checked the activity of NifA, a central activator of nitrogen fixation genes, in free-living microaerobic conditions [64,65,104]. NifA is O\(_2\) sensitive and in most rhizobia is active only in the near-anoxic core of nodules [32,55,105,106]. Work in E. meliloti has however suggested the protein may already be active in the early stages of nodule development [107]. Recent work has suggested some rhizobial nifA variants can be active outside of the nodule [108,109]. We checked the NifA dependant induction of nifH, a component of the nitrogenase complex [70,73,110]. As expected, nifH expression did not increase under microaerobic conditions (Fig 2), indicating Rlv3841 NifA is not expressed or is inactive under these conditions.

Next, the role of FnrN and hFixL on expression of fixNOQP in planta during symbiosis was studied. We found that nodules formed by the fnrN mutant expressed both fixNOQP operons at only 5% of WT (Fig 4B and 4C). The FnrN sensor is thus critical for fixNOQP expression.
inside the nodule. In nodules infected by the double hfixL mutant, expression of fixNOQP<sub>9</sub> and fixNOQP<sub>10</sub> was reduced to 68% and 58% of WT, respectively. Expression of fixK<sub>9a</sub> was abolished (S1 Fig), suggesting minimal FixK production in the absence of hFixL-FxkR TCS activity. Taken together, our results indicate that FnrN is critical for fixNOQP expression during symbiosis but the hfixL-FxkR-FixK pathway also plays a significant role. 

To assess the impact of FnrN and the hfixL-FxkR-FixK pathway on symbiotic nitrogen fixation, acetylene reduction assays were performed on pea plants inoculated with O<sub>2</sub> regulation mutants. In line with its poor expression of fixNOQP, the fnrN mutant was critically impaired in nitrogen fixation, reducing acetylene at only 15% of the WT level (Fig 5A). Plants inoculated with this mutant produced only small and unelongated pale or brown nodules indicative of poor development and low leghaemoglobin production (Fig 5D). Thus, FnrN is critical for effective nitrogen fixation by Rlv3841. Complementation restored 88% of WT acetylene reduction activity and produced nodules indistinguishable from WT (S2 Fig).

Plants inoculated with either individual hfixL mutants or the double mutant were also impaired in nitrogen fixation but retained approximately 75% of WT acetylene reduction activity (Fig 5A). No morphological changes were observed in these nodules (Fig 5C). Thus, the hfixL-FxkR-FixK pathway is also an important contributor to symbiotic fixation activity and is required to attain a WT level of fixation. Complementation of the double hfixL mutant was attempted but the gene was found to be toxic in E. coli (see Materials and Methods for details). The fskR<sub>9</sub> mutant impaired acetylene reduction rates but the decrease was insufficient to be significant (p = 0.0584). The FxkR<sub>c</sub> homolog is likely at least partially active and sufficiently produced to rescue hfixL in the absence of FxkR<sub>9</sub>. In the triple fnrN hfixL<sub>9</sub> hfixL<sub>c</sub> mutant (LMB673; fnrN::Ω tet hfixL<sub>9</sub>::Ω spec hfixL<sub>c</sub>·pK19) only negligible levels of fixation were recorded. This reinforces the importance of the contribution from both the hfixL-FxkR-FixK pathway and FnrN, suggesting no additional regulators exist which induce these anaerobox controlled genes in Rlv3841 during symbiosis.

hfixL and FnrN are active in spatially distinct nodule zones during symbiosis

Legume nodules create a large internal O<sub>2</sub> gradient, with semi-aerobic conditions at their tip and near-anoxic conditions as low as 20 nM O<sub>2</sub> in the central nitrogen fixing zone [26,111]. This gradient is typically split into four zones (Fig 6A) containing different O<sub>2</sub> concentrations and rhizobia in different stages of differentiation (for reviews, see [26,31,112]). To understand how hfixL-FxkR-FixK and FnrN operate in this context, we used confocal microscopy to map the spatial expression of fnrN and fixNOQP in nodules.

Expression of fnrN in nodules infected with WT Rlv3841 (Fig 6B) was visible throughout all nodule zones. This included expression in infection threads in zone I, indicating that low O<sub>2</sub> induction of fnrN begins when Rlv3841 first enters the nodule and before the bacteria have differentiated into bacteroids. By contrast, fnrN expression in zone I was greatly reduced in nodules infected with the double hfixL mutant (Fig 6B). This suggests the O<sub>2</sub> concentration in the relatively aerobic environment of zone I is sufficiently low to activate the hfixL-FxkR-FixK pathway. In the absence of this pathway, some fnrN expression was retained in zone II and interzone II-III, but this was weaker than WT. Minimal fnrN expression was observed in zone III in the hfixL double mutant.

In the fnrN mutant, expression of fnrN appeared to be localized primarily in infection threads, around the entire periphery of the nodule (Fig 6B). Nodules infected by this mutant were severely impaired in their development, failed to elongate and contained little to no leghaemoglobin (Fig 5D). Free O<sub>2</sub> concentration is unlikely to drop as much in these nodules
as it does in fully developed nodules. It is therefore noteworthy that the hFixL-FxkR-FixK pathway is nevertheless active, suggesting even poorly developed nodules produce a sufficiently low $O_2$ concentration to activate the pathway.

Expression patterns of $fixNOQP_9$ (Fig 7A) and $fixNOQP_{10}$ (Fig 7B) were similar. In WT Rlv3841, expression of both started abruptly in the II-III interzone of nodules, in agreement with past studies [55,56,113,114]. This abrupt start was absent in nodules infected with the double $hfixL$ mutant, indicating it requires the hFixL-FxkR-FixK pathway (Fig 7C and 7D). Without hFixL-FxkR-FixK, expression of $fixNOQP_9$ and $fixNOQP_{10}$ started gradually after the II-III interzone, presumably driven by FnrN. Expression was also weaker than in the WT. In
the fnrN mutant, we observed minimal expression of fixNOQP. This may be due in part to the poor development of these nodules, but the expression of fnrN in the fnrN mutant (Fig 6B) indicates that the hFixL-FxkR-FixK pathway is still relatively active in these underdeveloped nodules. The lack of fixNOQP expression in the fnrN mutant therefore indicates that hFixL-FxkR-FixK cannot directly induce much fixNOQP expression in zone III of mature nodules. Although FnrN is the main driver of fixNOQP expression, the hFixL-FxkR-FixK pathway is required for full fnrN expression and also plays an important role, albeit indirectly.

**Integration of FnrN and hFixL improves the O₂ response**

To study the dynamics of an integrated cascade containing both hFixL and FnrN, we constructed an ordinary differential equation model of their combined pathway based on past...
literature (S1 Text). A map of the regulatory connections incorporated in the model is given in S3 Fig. One each of hFixL, FxrR, FixK, FnrN and FixNOQP is considered in the model. hFixL was assumed to become active near a headspace concentration of 1% O₂ and FnrN near 0.01%
$O_2$, corresponding to dissolved $O_2$ concentrations of ~12 μM and ~120 nM respectively at equilibrium. Past studies have suggested that hFixL binds $O_2$ cooperatively, and that FnrN and FixK bind DNA as dimers [87,115–118]. Consequently all three of these binding processes were modelled using Hill functions with a Hill coefficient of 2 [119,120]. FixK and FnrN were assumed to have an identical induction effect on transcription when bound to the anaerobox motif. However, based on the critical role that FnrN but not FixK plays in regulation, FnrN was assumed to have a greater binding affinity for anaerobox motifs than FixK.

Our model reproduced the biphasic response of the integrated hFixL-FnrN cascade observed in WT Rifv3841 as $O_2$ dropped from atmospheric (~21%) to near-anoxic concentrations (0.001%) (Fig 8A and 8B). Expression of fnrN and fixNOQP first began in microaerobic
conditions (10–1% O₂) under the action of the hFixL-FxkR-FixK pathway. Subsequent activation of FnrN around 0.01% O₂ led to a further increase in fixNOQP expression, mirroring our findings in planta. Expression of fnrN near 0.01% O₂ initially increased due to auto-activation. Subsequently, as the O₂ concentration continued to drop, fnrN expression decreased due to auto-repression. Thus, the model correctly predicts a generally homogenous level of fnrN expression throughout the nodule, and increased fixNOQP expression in the core of the nodule relative to the tip.

In the absence of FnrN (Fig 8C and 8D), the model shows initial expression of fnrN and fixNOQP under microaerobic conditions due to the hFixL-FxkR-FixK pathway. There is however no further induction as O₂ continues to drop. This agrees with our finding that some fnrN expression takes place in the Rlv3841 fnrN mutant, albeit at a reduced level relative to WT. The model also predicts a lower level of fixNOQP expression in the fnrN mutant, consistent with our confocal microscopy results for fixNOQP expression (Fig 7A).

In the absence of hfixL (Fig 8E and 8F), an important new behaviour of the pathway is predicted by our model. As expected, no induction of fnrN or fixNOQP takes place under microaerobic conditions, in line with our experimental findings. However, once O₂ drops below 0.01%, our model suggests that the pathway may be bistable, with possible steady states at either high fnrN expression or near-zero expression. As the O₂ concentration continues to decrease, the disturbance needed to move from minimal expression to the high expression steady state becomes smaller. Thus, as the bacteria experience increasingly anaerobic conditions moving to areas of the nodule more proximal to the root, the model predicts that an increasing proportion of cells will transition from near-zero to high fnrN expression due to stochastic variations in expression of the gene. This agrees with the gradual increase in fixNOQP expression observed in the hfixL double mutant in Rlv3841 (Fig 7A and 7B).

**Discussion**

O₂ regulation is essential for rhizobia to establish a successful symbiosis with their legume partners. The model *Rhizobium* Rlv3841 employs three O₂ sensors in symbiosis: hFixL, FnrN and NifA. In the present study, we examined this multi-sensor arrangement through a combination of *in vitro*, *in planta* and *in silico* approaches. The hFixL-FxkR-FixK pathway is active in the earliest stages of symbiosis, followed by FnrN as the bacteria move to areas of the nodule more proximal to the root. Both regulate genes required for symbiotic survival, such as fixNOQP. NifA is active at a later stage, in zone III of nodules, and regulates activation of core nitrogen fixation machinery. The hFixL-FxkR-FixK pathway is the most O₂ tolerant of the three sensors, active in free-living bacteria under microaerobic conditions and *in planta* beginning in zone I of nodules. The FnrN protein is inactive under free-living microaerobic conditions and only becomes active from the II-III interzone onwards. FnrN is critical for expression of fixNOQP and nitrogen fixation activity. Indirectly, the hFixL-FxkR-FixK pathway also plays an important role by inducing fnrN expression under microaerobic conditions, priming it for auto-activation in the central nitrogen fixing zone. Our modelling results suggest the hFixL-FxkR-FixK pathway also prevents bistability in the low O₂ response, thereby ensuring all cells commit to fixNOQP expression in the central nitrogen fixing zone. Thus hFixL-FxkR-FixK and FnrN act as a single regulation pathway which integrates both O₂ sensors.

Rhizobia experience a drop in O₂ concentration of at least three orders of magnitude as they transition from a free-living lifestyle in soil to terminally differentiated bacteroids in nodules. Like Rlv3841, it is common for other rhizobia to employ multiple O₂ sensors during this transition. These multiple sensors may be used to create redundancy, a feature often found in
key regulatory pathways to improve their robustness. Elements of this redundancy are present in Rlv3841, including the multiple hFixL homologs and the overlap between their role and that of FnrN. However, our results also demonstrate that each sensor plays an important distinct role. Thus, integrating sensors into a single cascade in Rlv3841 also improves the responsiveness of regulation and allows the bacteria to respond appropriately across the entire range of O₂ concentrations experienced during symbiosis. A similar dual-sensor arrangement has also previously been described in *Rhodopseudomonas palustris*, which combines a FixLJ-FixK pathway with the FnrN homolog AadR [121–123]. *R. palustris* is not symbiotic but is noted for its ability to grow under both aerobic and anaerobic conditions [124]. The combined pathway in *R. palustris* was shown to provide fine-tuned regulation for adapting to the large range of O₂ concentrations it experiences.

The prevalence of multi-sensor O₂ regulation arrangements in rhizobia may also have arisen in response to competitive fitness pressures. Legume plants can sanction rhizobia based on their nitrogen fixation activity [125–127]. We speculate the bacteria may also be selected based on the speed with which they are able to adapt to life inside nodules and begin productively fixing nitrogen. This would create pressure for strains to rapidly demonstrate their effectiveness to their legume host. Past work has suggested that one of the benefits of FnrN compared to FixLJ-FixK is that it is more responsive to O₂ concentration, providing more flexible regulation [86,87,128]. By enabling more fine-tuned control, integrated multi-sensor O₂ regulatory pathways may speed up the symbiotic transition, providing a competitive advantage.

**Materials and methods**

**Bacterial strains and growth conditions**

*E. coli* strains were grown in liquid or solid LB medium[129] at 37°C supplemented with appropriate antibiotics (µg mL⁻¹): ampicillin 100, kanamycin 20, spectinomycin 50 and gentamicin 10. Rlv3841 strains were grown at 28°C in Tryptone-Yeast (TY) extract[130] or Universal Minimal Salts (UMS)[131] with glucose and ammonium chloride at 10 mM each. Antibiotics for Rlv3841 were used at the following concentrations (µg mL⁻¹): gentamicin 20, kanamycin 50, spectinomycin 100, streptomycin 500, tetracycline 2, neomycin 80 and nitrofurantoin 20. A list of the strains used is given in S2 Text.

**Cloning, colony PCRs and conjugations**

All routine DNA analyses were done using standard protocols [129]. PCR reactions for cloning were carried out according to the manufacturer’s instructions with Q5 High-Fidelity DNA Polymerase (New England Biolabs). Colony PCRs used OneTaq DNA Polymerase (NEB). Restriction enzymes (NEB) were used according to the manufacturer’s instructions. Sanger sequencing was carried out by Eurofins Genomics. Assemblies using BD In-Fusion cloning (Takara Bio) were performed according to the manufacturer’s instructions. Triparental conjugations, and transductions with bacteriophage RL38, were performed as previously described [132,133]. Tn7 integrations were performed according to the method described by Choi and colleagues [134,135]. A list of the plasmids and primers used is given in S2 Text.

**Mutant generation and complementation**

**Rlv3841 hfixL<sup>c</sup> (RL1879) mutant, LMB403.** A 1 Kb internal fragment of *hfixL<sup>c</sup>* was PCR amplified from Rlv3841 with primers pr0988/0989, adding XbaI sites at the 5’ and 3’ ends. This fragment was cloned into pK19mob digested with XbaI, using BD In-Fusion cloning, to
produce plasmid pLMB441. Triparental filter conjugation of pLMB441 into WT Rlv3841 was then performed using kanamycin selection. Colonies were screened by colony PCR using primers pr0482 and pK19A, which bind upstream of hfixL and inside the integrated pK19 backbone respectively. This gave mutant strain LMB403.

**Rlv3841 hfixL₉ (pRL90020) mutant, LMB495.** A 1 Kb region containing hfixL₉ was PCR amplified from Rlv3841 with primers pr1270/1271. This region was digested with XbaI/Xhol and cloned into pQ200SK using BD In-Fusion, digested with the same enzymes, to give plasmid pLMB585. A spectinomycin resistance cassette was digested out of the pH45QSpc plasmid with SmaI and cloned into pLMB585 at a unique StuI site blunted using the Klenow fragment to give plasmid pLMB590. Triparental filter conjugation of pLMB590 into WT Rlv3841 was then performed using spectinomycin selection. Colonies were screened by colony PCR using primers pr1272/1273. This gave mutant strain LMB495.

**Rlv3841 double hfixL₉ hfixL₉ mutant, LMB496.** An Rlv3841 mutant in both hfixL genes was generated by triparental filter conjugation of pLMB441 into strain LMB495, producing double mutant strain LMB496.

**Rlv3841 fnrN (RL2818) mutant, LMB648.** A 2.5 Kb region containing fnrN was PCR amplified from Rlv3841 with primers pr1381/1382. This fragment was digested with XbaI/Xhol and cloned using BD In-Fusion into pJQ200SK linearized with digestion by the same enzymes to make plasmid pLMB732. A tetracycline resistance cassette was then digested out of the pH45Q Tet plasmid with EcoRI and cloned into pLMB585 at a unique MfeI site to give plasmid pLMB733. Triparental filter conjugation of pLMB733 into WT Rlv3841 was then performed using tetracycline selection. Colonies were screened by colony PCR using primers pr1432/1433. This gave mutant strain LMB648.

**Rlv3841 triple hfixL₉ hfixL₉ fnrN mutant, LMB673.** A triple Rlv3841 mutant, in both hfixL genes and the fnrN gene, was generated by transducing fnrN::O Tet from LMB648 into LMB496 to produce strain LMB673.

**Rlv3841 fxxK₉ (pRL90026) mutant, OPS1808.** Two 1 Kb regions, one upstream and one downstream of fxxK₉, were PCR amplified from Rlv3841 with primer pairs oxp2874/2875 and oxp2876/2877 respectively. These were cloned with BD In-Fusion into pK19mobSacB digested with PstI and EcoRI to produce plasmid pOPS1199. Triparental filter conjugation of pOPS1199 into WT Rlv3841 was then performed using kanamycin selection. Colonies were screened by colony PCR using primers oxp3155 and pK19A. Colonies with correct integration were subsequently subjected to sucrose selection to remove plasmid pK19mobSacB as previously described [136]. Colonies were then screened for loss of kanamycin resistance and using colony PCR with primers oxp3155/3156 to isolate mutant strain OPS1808.

**Complemented Rlv3841 fnrN mutant (OPS2260).** The fnrN gene with its native promoter was amplified from Rlv3841 with primers oxp4115/4116 and cloned into BsaI-digested pOGG280 using BD In-Fusion. This plasmid was then genomically integrated with kanamycin selection and colonies screened with primers oxp2327/2328 and confirmed with sequencing. This produced strain OPS2260.

**Attempts at complementing the Rlv3841 hfixL₉ mutant.** Our hfixL₉ (LMB495) mutant showed the largest phenotypic effect out of the two single hfixL mutants, and we therefore attempted to complement this strain. We first attempted complementation via Tn7 integration using the pOGG280 backbone in which hfixL₉ was under Plac control. This construct assembled in E. coli but could not be successfully conjugated into Rlv3841. We theorized the protein was being produced to a toxic level in Rlv3841 due to poor LacI repression. We next attempted to rectify this problem by driving hfixL₉ from the native fixK₉ promoter and RBS instead (fixK₉ and hfixL₉ likely form an operon). However, this construct could not be transformed
into *E. coli*, suggesting *PfixK* was causing toxic levels of *hfixL* production. Finally, we sought to strike a balance between these two approaches by using the *Plac* promoter but the native *hfixL* RBS, in a pOGG250 backbone. This construct assembled in *E. coli* and could be conjugated into Rlv3841 but failed to complement the mutant. It is likely that this promoter-RBS combination avoided toxicity by reducing *hfixL* production but produced insufficient protein to achieve complementation.

**Microaerobic induction measurements in cultured cells**

Rlv3841 strains were first grown on TY slopes with appropriate antibiotics for three days. Cells were resuspended and washed three times by centrifugation at 5,000 RCF for 10 minutes. Washed cells were used to inoculate 10 mL liquid UMS cultures to OD<sub>600</sub> 0.01 and grown overnight without antibiotics. Cultures were then diluted to OD 0.1 in 400 μL UMS per well in a 24-well microtiter plate (4titude). A gas-permeable membrane (4titude) was applied to microtiter plates. Plates were then incubated in a FLUOstar Omega plate reader equipped with an Atmospheric Control Unit (both produced by BMG) to adjust O<sub>2</sub> concentration to 1% and CO<sub>2</sub> concentration to 0.1%. Readings were taken every 30 minutes and plates shaken at 700 rpm in double orbital mode between readings. Induction was measured at 18 hrs post-inoculation, when all cultures had reached stationary phase.

**Plate-based measurements**

sYFP2 measurements were made on a BMG FLUOstar Omega plate reader using the bottom optic with a gain setting of 2,000 and orbital averaging enabled (53 readings, 6 mm radius). Measurements were filter-based with excitation at 520 nm and emission recorded at 540 nm. Luminescence measurements were made on a Promega GloMax plate reader using the manufacturer’s protocol. Luminescence was used for S1 Fig as this reporter construct was already available.

**Plant growth and acetylene reduction**

*Pisum sativum* cv. Avola seeds were surface sterilized using 95% ethanol and 2% sodium hypochlorite before sowing. Plants were inoculated with 1 × 10<sup>7</sup> cells of the appropriate rhizobial strain and grown in 1 L beakers filled with sterile medium-grade vermiculite and nitrogen-free nutrient solution as previously described in a growth room (16h light/8h dark) [137]. Harvesting was 21 days later and acetylene reduction rate was determined as previously described [138]. All nodules for each plant were counted and their combined mass weighed; acetylene reduction rates were normalised by total nodule weight.

**Bacteroid isolation**

Bacteroids were isolated from root nodules after 21 days of plant growth following a differential spin protocol adapted from Tsukada et al. 2009 [139]. Approximately 100 mg of nodules were picked per plant. Nodules were immersed in 1 mL of sterile isolation buffer (1 M K<sub>2</sub>HPO<sub>4</sub>, 1 M KH<sub>2</sub>PO<sub>4</sub>, 300 mM sucrose, 2 mM MgCl<sub>2</sub>) and macerated. The mixture was spun down at 200 RCF for 5 minutes to remove plant debris. The supernatant was transferred to a fresh tube and spun down at 3,500 RCF for 5 minutes. The supernatant from this second spin was discarded and the pelleted fraction, containing the isolated bacteroids, was resuspended in isolation buffer and used for microtiter plate measurements.
Transcription start site mapping

The TSS data set referenced in this paper can be found in full on the NCBI SRA database, Bio-Project number PRJNA667846. A publication discussing the data in full is forthcoming. Protocol details can be found in S3 Text.

Statistical analysis

All analyses were performed using GraphPad Prism 8 (GraphPad Software). Significant differences were determined by Student’s t-test or one-way ANOVA followed by Dunnett’s multiple comparisons post-hoc test correction. A p-value less than 0.05 was considered statistically significant.

Confocal microscopy

Reporters were constructed by transcriptional fusion of promoters to an ORF of the sYFP2 fluorescent protein. Reporters were subsequently genomically integrated into Rlv3841 strains using the mini-Tn7 system [134]. Plants were inoculated with marked strains and grown as described above. After 21 days, nodules were picked and immersed in water then cut in half longitudinally. Images were taken with an LSM 880 confocal laser-scanning microscope equipped with the Axio Imager.Z2 (Zeiss), using the manufacturer’s ZEN Black software. A Plan-Apochromat 10×/0.45 M27 objective (Zeiss) was used. Excitation was at 514 nm with an Argon laser and emission measurements filtered to a range of 519–572 nm. Acquisitions were tile scans with 2×3 tiles per image. 31 Z-stack slices were taken for each tile, separated by a height of 10 μm. Images shown in this publication are maximum-intensity orthogonal projections produced with the ZEN Blue software (Zeiss).

Figure data

Data for main text Figs 2–5 and S1 and S2 Figs are given in S4 Text. Data for main text Fig 8 are given in S1 Spreadsheet.

Supporting information

S1 Fig. hfixL is required for in planta fixK9a expression in Rlv3841. A reporter (pOPS0136) was built with the luxCDABE reporter operon fused to the fixK9a promoter. The promoter was active in isolated WT Rlv3841 bacteroids (OPS0376), but no luminescence above no-reporter background was recorded in double hfixL mutant bacteroids (OPS0528). Data are averages (±SEM) from at least four plants. *P < 0.05 by one-way ANOVA with Dunnett’s post-hoc test for multiple comparisons.

S2 Fig. Complementation of the Rlv3841 fnrN mutant. (A) Acetylene reduction rates; the activity of the fnrN mutant (LMB648) was 20% of WT Rlv3841. The complemented strain (OPS2260) fixed at 88% of WT. Nodules colonized by Rlv3841 (B) WT, (C) the complemented fnrN mutant and (D) the fnrN mutant. Acetylene reduction rates are normalised to total weight of nodules per plant. Data are averages (±SEM) from seven plants, ns (not significant) P ≥ 0.05; ****P < 0.0001 by one-way ANOVA with Dunnett’s post-hoc test for multiple comparisons. Complementation also restored nodule morphology.

S3 Fig. Simplified map of the Rlv3841 dual sensor oxygen cascade used for modelling. Only one copy each is included of hfixL, fixK, fskR and fixNOQP. Both FnrN and FixK can
positively and negatively regulate expression of furN. FxKR negative auto-regulation is not included in the model. Regulation is indicated with lines ending in arrows (positive regulation) and ending in blunt ends (negative regulation). Translation is shown as lines ending in circles.

**S1 Text. Modelling oxygen regulation in Rlv3841.**
(PDF)

**S2 Text. Strains, plasmids and primers used in the study.**
(PDF)

**S3 Text. Further materials and methods details for transcription start site mapping.**
(PDF)

**S4 Text. Data tables for main text Figs 2–5 and S1 and S2 Figs.**
(PDF)

**S1 Spreadsheet. Data table for main text Fig 8.**
(XLSX)

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