Transcriptomic Insight in the Control of Legume Root Secondary Infection by the *Sinorhizobium meliloti* Transcriptional Regulator Clr

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The cAMP-dependent transcriptional regulator Clr of *Sinorhizobium meliloti* regulates the overall number of infection events on *Medicago* roots by a so-far unknown mechanism requiring *smc02178*, a Clr-target gene of unknown function. In order to shed light on the mode of action of Clr on infection and potentially reveal additional biological functions for Clr, we inventoried genomic Clr target genes by transcriptome profiling. We have found that Clr positively controls the synthesis of cAMP-dependent succinoglycan as well as the expression of genes involved in the synthesis of a so-far unknown polysaccharide compound. In addition, Clr activated expression of 24 genes of unknown function in addition to *smc02178*. Genes negatively controlled by Clr were mainly involved in swimming motility and chemotaxis. Functional characterization of two novel Clr-activated genes of unknown function, *smb20495* and *smc02177*, showed that their expression was activated by the same plant signal as *smc02178* ex planta. In planta, however, symbiotic expression of *smc02177* proved independent of *clr*. Both *smc02177* and *smb20495* genes were strictly required for the control of secondary infection on *M. sativa*. None of the three *smc02177*, *smc02178* and *smb20495* genes were needed for plant signal perception. Altogether this work provides a refined view of the cAMP-dependent Clr regulon of *S. meliloti*. We specifically discuss the possible roles of *smc02177*, *smc02178*, *smb20495* genes and other Clr-controlled genes in the control of secondary infection of *Medicago* roots.

Keywords: rhizobium, legume, symbiosis, infection, adenylate cyclase, cAMP, transcriptome

INTRODUCTION

*Sinorhizobium meliloti* is a gram-negative bacterium that alternates between a free-living saprophytic life in the soil and the rhizosphere of plants, and an occasional symbiotic life within nodules of *Medicago* spp. Establishment of symbiosis requires the coordinated bacterial infection of the root epidermis and initiation of nodule organogenesis in the root cortex (Oldroyd, 2013). Neo-formed nodules are invaded intracellularly by bacteria that differentiate into nitrogen-fixing bacteroides. Infection of root hair cells takes place via specialized structures called epidermal Infection Threads (eIT) (Miri et al., 2016). Two bacterial effector molecules are required for eIT formation: Lipo-chitooligosaccharides (LCOs, also known as Nod factors), which are also needed for nodule organogenesis, and exopolysaccharides such as succinoglycan in *S. meliloti* (Cheng and Walker, 1998; Jones et al., 2007; Gibson et al., 2008).
Mechanisms negatively controlling the symbiotic interaction have also been identified. The best known is AON (autoregulation of nodulation) by which the plant systemically adjusts the number of nodules to its metabolic needs (Magori and Kawaguchi, 2009; Mortier et al., 2012). We recently obtained preliminary evidence that eIT formation was also negatively autoregulated in the *S. meliloti–Medicago* symbiosis (Tian et al., 2012). Specifically, we isolated *S. meliloti* mutants that displayed a hyper-infection phenotype on *Medicago* roots, resulting from a relaxed control of eIT formation. Whereas eIT formation by wild-type bacteria is transient, mutants kept infecting roots for an extended time-lapse, thus allowing increased secondary infection. Noteworthy, nodulation by hyper-infecting bacterial mutants was normal thus indicating that autoregulation of infection and nodulation are distinct mechanisms.

In *S. meliloti*, the control of secondary infection is mediated by a bacterial cAMP-cascade involving three receptor-like adenylate cyclases (ACs), CyaD1 (SMc02176), CyaD2 (SMc04307) and CyaK (SMb20776), a cAMP-dependent transcriptional regulator of the Crp family (Green et al., 2014), called Clr (SMc02175), CyaK (SMb20776), a cAMP-dependent transcriptional regulator of the Crp family (Green et al., 2014), called Clr (SMc02175), and *smc02178*, a gene of unknown biochemical function located nearby *cydD1* and *clr* on the chromosome. In nodules, a so-far unknown plant signal activates cAMP production by CyaD1, CyaD2 and CyaK, which in turns allows activation of *smc02178* transcription by Clr. This signal is also present in shoots but very low in roots (Tian et al., 2012). We and others have shown that purified Clr is as a 3′cAMP-dependent DNA-binding protein that binds a Clr-box in the promoter region of the *smc02178* gene (Mathieu-Demaziere et al., 2013; Krol et al., 2016). Inactivation of *clr*, of *smc02178* or of the three AC genes altogether, resulted in a hyper-infection phenotype on *M. sativa* (Tian et al., 2012). Noteworthy, individual mutants of any of the three ACs had no conspicuous hyper-infection phenotype thus indicating all three genes contribute full infection control, probably at different stages of the symbiotic interaction (Tian et al., 2012).

In order to shed light on the mechanism by which *S. meliloti* bacteria control secondary infection, we have identified here additional Clr targets by transcriptome profiling. We have found that Clr positively controls expression of 25 genes of unknown function as well the synthesis of succinoglycan and of a putative unknown polysaccharide. We compare our results with those recently obtained upon over-expression of CyaJ (Krol et al., 2016). Functional characterization of two genes of unknown function, *smc02177* and *smb20495*, demonstrated their implication in the control of secondary infection. We discuss the possible roles of *smc02177*, *smc02178*, *smb20495* and Clr-controlled surface polysaccharides in the control of secondary infection of *Medicago* roots.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

Bacterial strains used in this study are listed in Supplementary Table 1. Unless otherwise indicated, strains were grown at 28°C in VMG medium, i.e., Vincent minimal medium (Becker et al., 2004), supplemented with mannitol (1%w/vol) and glutamate (0.1%) as carbon and nitrogen sources, respectively. The concentrations of antibiotics used for *S. meliloti* were 200 µg/ml for streptomycin, 100 µg/ml for neomycin and 10 µg/ml for tetracycline in both liquid and solid media. Gentamicin was used at 10 µg/ml in liquid medium and 30 µg/ml on solid medium.

Primers used for DNA amplification are listed in Supplementary Table S1. *S. meliloti* Rm1021 was used as template for DNA amplification. The *smb20495* and *smc02177* single mutants were constructed by site-specific integration of the suicide pVO155 plasmid. *smb20495* and *smc02177* internal PCR fragments were amplified using 20495L-20495R, and L02177-R02177 primers (Supplementary Table S2), cloned into pCR2-1-TOPO and digested with BamHI and XbaI before cloning into pVO155. The resulting pVO155 derivatives were introduced into *E. coli* DH5α by transformation and then conjugated in *S. meliloti* using pRK600 as helper plasmid. All constructs were verified by PCR and Sanger sequencing in *E. coli* and by PCR in *S. meliloti*.

**Plasmids Construction**

Plasmids used in this study are listed in Supplementary Table S1. The *clr*-overexpressing construct pFA2175 was obtained after PCR amplification of the *clr* gene coding region using *S. meliloti* Rm1021 genomic DNA as template and the REco2175 and LBamH2175 primers (Supplementary Table S2). The PCR fragment was digested with BamHI and EcoRI and ligated into a BamHI-EcoRI digested pFAJ1708 plasmid. For pGD926-ExoY construction, the promoter region and the first 297nt of *exoY* coding region were PCR-amplified from pstb-LAFR5-ExoY plasmid DNA using the ExoY-fusHindIII and ExoY-fusBamHI primers. The PCR fragment was digested with BamHI and HindIII and ligated into a BamHI-HindIII digested pGD926 plasmid.

For Lsmc02178-lacZ construction (pGM150322), the *smc02178* coding region including signal peptide was amplified using the 2178H and 2178BamHIlacZ primers. The HindIII-fragment was ligated into a pGAJ1708 plasmid using the HindIII-BamHI restriction enzymes. The resulting plasmid was transformed into *E. coli* DH5α and *S. meliloti* Rm1021. The PCR fragments obtained from the *smb20495* gene using the primers smb20495F-smb20495R were ligated together, PCR-amplified, digested by HindIII and BamHI and cloned into a pGEM plasmid. The *phoA* gene from *E. coli* genomic DNA was amplified using the primers phoAEcoRV and phoABamHI and cloned into pGEM plasmid. The *phoA* and Lsmc02178 or *smb20495* fragments were ligated together, PCR-amplified, digested by HindIII and BamHI before cloning into HindIII/BamHI digested pGD926. Constructs were conjugated into a *phoA S. meliloti* derivative (Rm8002) to minimize phosphatase alkaline background activity. For the construction of pGD20495, a 934 bp PCR fragment encompassing the *smb20495* promoter region (full intergenic region) and 26 nucleotides of the *smb20495* gene was amplified using the SMB20495-HindIII and SMB20495-BamHI primers.
### TABLE 1 | Clr-induced and Clr-repressed genes.

| Gene ID | Gene product | M-value Clr++/clr− Aerobic | M-value Clr++/clr− Microaerobic |
|---------|--------------|----------------------------|-------------------------------|
| **Induced genes** | | | |
| smb20495* | Conserved hypothetical protein | 4.585 | 5.5721 |
| smc04190* | Conserved hypothetical protein | 4.0758 | 4.8696 |
| smb21224* | NodQ2 sulfate adenylyltransferase protein | 2.0767 | 3.8393 |
| smc01589 | Hypothetical protein | 2.1773 | 3.7637 |
| smc02278* | Hypothetical transmembrane protein | 4.0335 | 3.523 |
| smc02276 | NodP1 ATP sulfurylase | 1.453 | 3.5207 |
| smb21671* | Hypothetical protein | 2.2859 | 3.3252 |
| smc03985 | CyaF2 adenylate/guanylate cyclase protein | 2.4864 | 3.1647 |
| smb21223* | NodP2 sulfate adenylyltransferase protein | 1.3894 | 3.116 |
| smc02178* | Hypothetical protein | 2.1725 | 2.9945 |
| smc02175 | Clr, transcription regulator protein | 2.4063 | 2.95 |
| smb20907* | Hypothetical protein | 2.1938 | 2.688 |
| smc00198* | Hypothetical protein | 2.0852 | 2.5524 |
| smc04164* | Hypothetical protein | 1.4981 | 2.466 |
| smc03100* | Hypothetical protein | 1.556 | 2.463 |
| smc01210* | Hypothetical protein | 2.7891 | 2.4427 |
| smb20960* | ExoN UDP glucose pyrophosphorylase protein | 1.1766 | 2.3588 |
| smc02177* | Hypothetical protein | 1.5143 | 2.3533 |
| smb21240* | Putative surface saccharide export protein | 1.5351 | 2.2783 |
| smb21242* | Putative glycosyltransferase protein | 1.6205 | 2.2734 |
| smb20409 | Putative hydroxybutyrate dehydrogenase | 1.9187 | 2.2027 |
| smc01764 | Hypothetical protein | 1.383 | 2.1944 |
| smb21243 | Putative sulfotransferase protein | 1.0488 | 2.14 |
| smc03810 | Conserved hypothetical protein | 1.8922 | 2.1328 |
| smb21248 | Putative aminotransferase protein | 1.5844 | 2.1101 |
| smb21329 | Hypothetical exported peptide protein | 1.8956 | 2.066 |
| smb21069* | Hypothetical beta-glucosidase protein | 1.3609 | 2.0346 |
| smb21247* | Conserved hypothetical protein | 1.7569 | 2.0059 |
| smc01003* | Hypothetical protein | 1.0864 | 1.9794 |
| smb20954 | ExoH succinyltransferase protein | 1.093 | 1.8886 |
| smc00639 | Putative heat resistant agglutinin 1 protein | 1.2183 | 1.8819 |
| smb20946* | ExoY galactosyltransferase protein | 1.1735 | 1.8564 |
| smc01855 | Hypothetical transmembrane protein | 1.7977 | 1.7555 |
| smc04267 | LpsS LPS sulfotransferase | 1.0996 | 1.6751 |
| smb20945* | ExoF1 polysaccharide export protein | 1.1785 | 1.6738 |
| smc01136* | Hypothetical transmembrane protein | 1.0215 | 1.6632 |
| smb21690* | ExoW glucosyltransferase protein | 1.2603 | 1.4455 |
| smc01241 | Hypothetical protein | 1.3629 | 1.3209 |
| smb20948 | ExoU glucosyltransferase protein | 1.1547 | 1.3011 |
| smb20908 | Hypothetical protein | 1.1748 | 1.2806 |
| smc01580* | Hypothetical transmembrane protein | 1.0209 | 1.0248 |
| smc00925* | Hypothetical protein | 1.378 | 0.9256 |
| **Repressed genes** | | | |
| smc03027* | FlgB flagellar basal body rod protein | −2.2764 | −2.9643 |
| smb20745 | GlnK putative glutamine synthetase II protein | −2.4136 | −2.7335 |
| smc03009 | CheR chemotaxis protein methyltransferase | −1.4759 | −2.4545 |
| smc03049* | Flg, putative flagellar hook associated protein | −1.08 | −2.1779 |
| smc02251 | Putative aminotransferase protein | −1.3324 | −1.9797 |
| smc04114* | PIA1 putative pilin subunit protein | −1.3701 | −1.9591 |
| smc01122 | Conserved hypothetical protein | −1.2814 | −1.7944 |

(Continued)
digested with BamHI and HindIII and cloned in-frame with lacZ in the translational fusion plasmid pGD926. For pGD2177, a 303 bp PCR fragment encompassing the full intergenic region between smc02177 and smc02178 and 32 nucleotides of the smc02177 gene was amplified using the p2177HindIII and p2177BamHI primers, digested with BamHI and HindIII and cloned in-frame with the lacZ gene. For pGD2178, pGD20495 or pGD2177 plasmids were grown at 28°C in VMG medium, diluted to an OD600 of 0.12 in 20 ml of same medium and incubated overnight at 28°C. RNA preparations were as described in Bobik et al. (2006). Microarray raw data have been deposited in the ArrayExpress database under accession number E-MTAB-4780.

Transcriptome and Quantitative PCR Analyses

For transcriptome experiments, S. meliloti clr⁻ (GMI11567) and clr-overexpressing (GMI11896) strains (Table 1) were grown overnight at 28°C in VMG medium, diluted to an OD600 of 0.12 in 20 ml of same medium and incubated under either oxic or microoxic (2%O₂) conditions in the presence of caffeine (2.5 mM), to inhibit endogenous phosphodiesterase activity (Essayan, 2001). Microoxic conditions were as described before (David et al., 1988). When OD600 reached 0.3, 15 ml were filtered on Supor Membrane Disk Filters (Pall) and cells were frozen in liquid nitrogen and stored at −80°C. All experiments were performed in triplicate.

Reverse transcription were performed from 1 μg of RNA using the Transcriptor Reverse Transcriptase (Roche), and random hexamers as primers. cDNAs were used for running real-time PCR on a LightCycler 1.5 system (Roche) using the FastStart DNA Master PLUS SYBRGreen I kit (Roche) according to the manufacturer’s instructions. The rplM gene was used as reference gene for data normalization. Gene expression was assessed using the second derivative maximum analysis.

β-Galactosidase and Alkaline Phosphatase Assays

Sinorhizobium meliloti strains carrying the pGD926-ExoY, pGD2178, pGD20495 or pGD2177 plasmids were grown at
28°C in VMG medium. Overnight cultures were diluted to an OD600 of 0.1 in 10 ml Vincent medium and additionally grown for 2 h. Cultures were then supplemented with 10 mM exo\textit{cAMP} for pGD926-ExoY (Figure 1B) and grown for an additional 24 h. \textit{S. meliloti} strains carrying plasmids pGM150322, pGM150323, pGM150324, and pGD2178 were grown overnight in 1 ml-cultures in VMG medium supplemented with 100 µl of \textit{Medicago} shoot signal fresh extract prepared as described in Tian et al. (2012). Although a plant shoot extract is slightly less active than a nodule extract (Tian et al., 2012) it was preferred as it is easier to prepare \textit{ex tempore}.

The assays for beta-galactosidase activity were carried out using the protocol of Miller (1972), whereas alkaline phosphatase was assayed in a \textit{S. meliloti} \textit{phoA} background as described before (Brickman and Beckwith, 1975). All experiments were performed in triplicate.

Symbiotic detection of beta-galactosidase activity in \textit{Medicago} nodules was conducted as described before (Tian et al., 2012).

Calcofluor Dye Detection of Succinoglycan
\textit{Sinorhizobium meliloti} strains were grown at 28°C in LB-MC (LB+2.5 mM MgCl$_2$ and 2.5 mM CaCl$_2$). Overnight cultures were diluted to an OD600 of 0.15 and 10 µl were dropped off as a spot on LB-MC plates supplemented with 0.02% calcofluor white (Fluorescent Brightener 28, Sigma). After 2 and 13 days incubation, the fluorescence was monitored under UV light with a G:BOX BioImaging System (Syngene).

Motility Assays
10$^4$ cells (ca 5 µl) from overnight cultures in LB-MC medium were jabbed on top of soft (0.2%) agar plates. Motility (i.e., size of the colony) was measured after 5 days at 28°C.

RESULTS

Transcriptome Profiling of the Clr Regulon
We compared the transcriptomes of a \textit{S. meliloti} strain (GMI11896) expressing \textit{clr} from a constitutive \textit{nptII} promoter on a low copy-number plasmid and of a nearly isogenic \textit{clr} null mutant strain (GMI11567). Experiments were performed under both free-living aerobic and microoxic conditions, as low oxygen is a known symbiotic signal (Soupene et al., 1995). Both experiments, performed in biological duplicate, revealed a similar list of genes with the same extent of induction or repression thus indicating that oxygen did not affect Clr activity. We identified 72 genes displaying at least a twofold change in gene expression in both oxic and microoxic conditions, i.e., in 4 independent assays (Table 1). Most of these genes are probably indirect Clr targets (see below).

\textit{Sinorhizobium meliloti} has a tripartite genome consisting of one main chromosome (3.65 Mb) and two symbiotic replicons called pSymB (1.68 Mb) and pSymA (1.35 Mb) (Galibert et al., 2001). The 42 genes activated by Clr were all located on pSymB and chromosome with the exception of the pSymA-located \textit{nodP1} gene which was likely a false positive that was detected because of its very strong (99.7%) sequence similarity with \textit{nodP2} (see below).

Targets included six genes involved in succinoglycan synthesis (exoN, Y, H, F1, W, U), an exopolysaccharide that plays a key role in IT formation and stress adaptation.

![Figure 1](image-url)
Seven genes belong to a large ca. 30-kb cluster on pSymB between genes nOD2 and smb21248 that contains many genes involved in polysaccharide metabolism (see S. meliloti genome browser⁴), (Galibert et al., 2001).

Clr-targets in this cluster encompass two categories of genes: three genes involved in activated sulfate (PAPS) biosynthesis and sulfate transfer (NodP2, NodQ2, SmB21243) and four genes involved in sugar/polysaccharide metabolism including a glycosyl transferase (SmB21242), a beta-glucosidase (SmB21069), a sugar (GlcNAc-containing) deacetylase (SmB21247) and SmB21240, a protein with a tyrosine kinase domain resembling surface exopolysaccharide export protein of the ExoP family. Altogether, Clr target genes in this cluster may contribute synthesis of a sulfated surface polysaccharide (see Discussion).

Additional Clr targets included the lpsS-encoded sulfotransferase (Cronan and Keating, 2004), cyaF2 encoding an adenylate/guanylate cyclase of unknown function, as well as 25 genes of unknown function (including smc02178), most of which were located on the main chromosome.

Thirty genes were repressed by Clr under both oxic and microoxic conditions, most of which (25) were chromosomal (Table 1). Ten genes belonged to a large chemotaxis/motility cluster (from smc03004 to smc03072) involved in flagella synthesis, swimming motility and chemotaxis. Related genes were smc031104 (mcpX), smc01468 (cheW2) and smc04114 (pilA1). We also detected two genes involved in nitrogen metabolism (glnK, glnII) and 11 genes of unknown function. We identified a conspicuous Clr-box in the promoter region of 12 Clr target genes (Table 1 and Supplementary Table S3, see Discussion).

Clr and cAMP Drive Succinoglycan Synthesis

RT-qPCR analysis of four exo genes (exoH, exoM, exoN, and exoY) in S. meliloti wt and clr mutant cells supplemented with exogenous cAMP (abbreviated thereafter as exoCAMP) (Figure 1A) confirmed that the clr gene indeed mediated exoCAMP-activation of these genes. Inactivation of the direct Clr-target smc02178 had no significant effect on exoY and exoN expression but slightly decreased exoM and exoH gene expression (Figure 1A). Expression of a translational exoY-lacZ reporter fusion (Figure 1B) confirmed the clr-dependency and smc02178-independency of the exoCAMP-mediated exoY-lacZ expression. Noteworthy, exoY-lacZ ex planta expression was not induced by a plant shoot extract (Figure 1C) nor nodule extract thus indicating that cAMP-dependent exo gene expression and smc02178 expression are under different genetic control. Instead a plant extract had a negative effect on exoY expression that was, however, independent of Clr.

In order to directly assess the implication of Clr in succinoglycan biosynthesis, we monitored fluorescence of bacterial colonies on agar plates containing the calcofluor white dye which is specific for succinoglycan in strain Rm1021 (Jones, 2012). Under conditions where wt S. meliloti showed only background fluorescence, bright fluorescence was triggered (Figure 2) in a wt strain carrying the pGMI50127 plasmid that constitutively synthesizes endogenous cAMP (endcAMP) (Tian et al., 2012). Bright fluorescence was almost completely abolished in the clr mutant in accordance with previous results (Krol et al., 2016) whereas inactivation of the smc02178 gene had no detectable effect on overall succinoglycan synthesis (Figure 2).

The presence of a halo of similar size corresponding to diffusible low molecular weight succinoglycan around the wt and smc02178 bacterial colonies indicated that succinoglycan secretion was not impaired in the smc02178 mutant (Figure 2).

clr Negatively Controls Motility

exo and fla genes show opposite regulation in many biological instances, including symbiosis and stress responses (Mendrygal and Gonzalez, 2000; Ruberg et al., 2003; Hellweg et al., 2009). We confirmed by RT-qPCR that clr regulates flaB transcription negatively, in accordance with transcriptome experiments (Supplementary Figure S2). A swimming motility assay showed a reduced swim radius of the wild type strain producing endcAMP that was not due a reduced growth of the strain carrying plasmid.

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⁴https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi
Two Novel Clr-Target Genes Involved in the Control of Secondary Infection

Among the 25 genes of unknown biological function activated by Clr we characterized two of them; smb20495, which was the most highly-induced gene in the transcriptome experiment (Table 1) and smc02177, the gene located next to smc02178 on the chromosome.

We first tested activation of smb20495- and smc02177-lacZ translational gene fusions in free-living cultures supplemented by a plant shoot extract known to activate smc02178 expression (Tian et al., 2012). We found that free-living expression of both genes was activated by the plant signal in a clr- and cyaD1D2K-dependent manner, although to a lower level as compared to smc02178 (Figure 3A).

In planta, the pattern of smb20495-lacZ expression in nodules was similar to the one previously described for smc02178 (Figure 3B) and expression strictly depended on clr. However, although 30% of the nodules induced by the cyaD1D2K mutant were colorless, 70% stained pale blue. Probably one of the many ACs in the S. meliloti genome (25 besides CyAD1CyaD2CyaK; Galibert et al., 2001) allows low level of cyaD1D2K-independent expression of the smb20495 gene in planta.

The situation was even more contrasted for the smc02177-lacZ fusion. Although smc02177 displayed very little clr-independent expression ex planta (Figure 3A), its expression in mature
nODULES was essentially clr and cyaD1D2K-independent (Figure 3B). A likely explanation is that a Clr-independent promoter gets strongly activated in nodules.

Null mutants of *S. meliloti* in smb20495 and smc02177 genes displayed a full hyper-infection phenotype on *M. sativa* (Figure 4) comparable to the one previously observed for a smc02178 or clr mutant (Tian et al., 2012). However, no difference in nodule number was observed (Figure 4A). Nodules were pink and leaves were green thus indicating that the mutants were not affected for nitrogen fixation, as demonstrated before for a triple cyaD1D2K mutant (Tian et al., 2012). Both mutants synthesized endoCAMP-dependent HMW and diffusible LMW succinoglycan on calcofluor white dye plates, similar to the smc02178 mutant (Figure 2).

Altogether these results indicate that smb20495 and smc02177 expression are prone to plant signal regulation under clr and cyaD1D2K control. Nevertheless smc02177 showed a high-level of plant signal-independent symbiotic expression in nodules. Both genes were required for the control of secondary infection.

**Sequence Analysis of the SMc02177, SMc02178 and SMb20495 Proteins**

PSORTb 3.0 analysis (Yu et al., 2010) predicted a non-cytoplasmic location for all three SMc02177, SMc02178 and SMb20495 proteins without specifying more precisely their subcellular localization. SignalP 4.1 analysis (Petersen et al., 2011) indicated the presence of a cleavable signal peptide in SMc02178 and SMb20495. As for SMc02178, we experimentally demonstrated the localization of the main portion of the protein in the bacterial periplasm using a combination of lacZ and phoA reporter fusions (Supplementary Figure S3).

Contrary to Smc02178 for which sequence inspection gave no structural or functional clues, we identified a FecR domain (IPR006860, PF04773) in SMc02177. In the *E. coli* full-length FecR protein, the FecR domain interacts with the amino-terminal periplasmic part of the FecA outer membrane protein involved in iron citrate signaling. Sequence inspection of the large SMb20495 protein indicated the presence of several TPR domains (IPR019734) known to mediate protein–protein interactions.

**The smc02177, smc02178 and smb20495 Genes Are Not Required for Plant Signal Perception**

The predicted extra-cytoplasmic location of the three proteins and the presence of protein–protein interaction motifs in two of them suggested to us the possibility that these proteins were involved in the perception/transduction of the plant signal that activates the CyaD1, CyaD2 and CyaK ACs (Tian et al., 2012). We therefore monitored plant shoot signal-dependent expression of plasmidic smc02177- and smc02178-lacZ fusions in the three mutant backgrounds. For the smc02177-lacZ fusion no statistically significant difference in expression was observed in any mutant background as compared to wild-type. For the smc02178 mutation a weak but significant (*p* < 0.05) effect was observed on smc02178-lacZ expression (Figure 5).

**DISCUSSION**

The Clr Regulon

In this work, we compared the transcriptome of a strain moderately overexpressing *clr* (6- to 8-fold, Table 1) to that of a *clr* null mutant. As a result we identified 72 genes that showed at least a twofold up- or down-regulation in two biological replicates of the two biological conditions tested, i.e., aerobic and microaerobic (2% O<sub>2</sub>) conditions. Krol et al. (2016) recently determined the transcriptome of a strain overexpressing the CyaJ AC to a control strain carrying an empty vector plasmid with the purpose of identifying cAMP-dependent genes in *S. meliloti*. Of the 42 genes induced by Clr in both aerobic and microaerobic conditions in our experiments, 27 were also identified as being activated by CyaJ using similar thresholds (*M* > 1 and...
These 27 genes are thus bona fide cAMP-dependent Clr targets. Five additional genes in our experiments were found in the immediate vicinity of these bona fide targets. *cyf2* is a Clr target solely detected in our experiments but for which a Clr-binding site was experimentally identified (Krol et al., 2016). Cyf2 is thus probably a genuine Clr (direct) target too. Altogether, we believe that at least 32 genes (over 42) detected here are bona fide Clr- and cAMP-activated targets.

In contrast the list of Clr-repressed genes in our experiments and in previous (Krol et al., 2016) experiments (33 and 82 genes, respectively) showed a very limited overlap; 7 genes all involved in chemotaxis and swimming motility. Metabolic genes evidenced by manipulating intracellular cAMP concentration (Krol et al., 2016) were not detected here and thus may not be Clr targets.

Our data also provide direct evidence that the Clr regulon is wider than the *cydD1D2K* symbiotic signaling cascade since Clr targets such as *exo* and *fla* genes are not regulated by the plant signal nor its cognate ACs, CyaD1D2K. This is best explained by the fact that they are 28 ACs in the *S. meliloti* genome beside CyaD1D2K (Galibert et al., 2001). Altogether this suggests that Clr is a central transcriptional regulator that integrates different environmental signals via different ACs, both in the free-living and symbiotic life of *S. meliloti*.

We initially identified a functional palindromic (TGTNN_AACA) Clr-box in the *smc02178* promoter region (Tian et al., 2012). Based on DNA-binding assays, Krol et al. (2016) identified a relaxed consensus (HGTYHCNNNGRWACA) that we have found in the promoter regions of nine predicted Clr targets [*smb0906*, *smb20908*, *smb00864*, *smb00925*, *smb01136*, *smb01210*, *smb02177*, *smb03985* (*cyf2*), *smb04164* and *smb04190*]. Upon studying *smb20495* expression, we have observed that a promoter extending 500 bp upstream of the start ATG codon and carrying the Clr-like box (HGTYHCNNNGRWACG) was indeed inducible by Clr and cAMP (Figure 3A) whereas a shorter promoter version (285 bp upstream of ATG) lacking this putative Clr-box was not (Krol et al., 2016). On this ground we tentatively propose a novel HGTYHCNNNGRWACW Clr-box consensus that we have also found in the *smb21329* promoter (Table 1 and Supplementary Table S3). We acknowledge that a functional binding assay is needed to validate this prediction. No functional (e.g., ncRNA) or structural element (e.g., RIME) was identified so far in the 500 nt region between the Clr-box and the *smb20495* start codon.

In summary, 10 to 12 Clr-activated genes identified in this work could be direct Clr targets. Nine of them are chromosomal and three located on pSymB. Noteworthy pSymB-located *exo* genes and the gene cluster encoding the putative polysaccharide (*nodP2-smb21248*) may be indirect Clr targets. Similarly, none of the Clr-repressed genes involved in flagellin synthesis and chemotaxis showed a potential Clr-box matching the [HGTYHCNNNGRWACW] consensus. So, Clr-repressed

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2https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi
genes are likely indirect targets. Although more work is needed to precisely define the full Clr (direct) regulon our data suggest that the Clr-regulon mainly consists of chromosome-located genes positively regulated by Clr.

Clr-Mediated Control of Legume Root Secondary Infection

We have described here two novel Clr-target genes strictly required for the control of secondary plant infection. Inactivation of any of these two genes led to a hyperinfection phenotype similar to the one observed with a smc02178, clr or a triple cyaD1cyaD2cyaK mutant. Hence, both genes have a unique and essential role in the control of secondary infection. Similar to smc02178, smb20495 and smc02177 were expressed at a very low level ex planta in standard culture conditions and were activated by the plant signal ex planta in a cyaD1D2K- and clr-dependent manner. All three genes were expressed in nodules. Yet smc02177 expression in mature nodules was essentially independent of clr and cyaD1cyaD2cyaK (Figure 3B), likely reflecting a dual regulation of this gene.

The mechanism by which the three Clr-targets restrict secondary infection now needs to be elucidated. No enzymatic function was predicted for any of the corresponding proteins. Instead two of them displayed protein–protein interaction motifs (TPR) or domain (FecR).

We speculated that (some of) these proteins could be involved in plant signal perception. Our present results, however, do not lend support to this possibility (Figure 5). However, the observation that the CyaD1, CyaD2 and CyaK ACs act at successive stages of nodule development suggested that different signal molecules could be associated with them (Tian et al., 2012) and indeed we have obtained genetic evidence for other(s) signal in addition to the one evidenced so far in nodules and shoots. Hence the role of the smc02178, smb20495 and smc02177 genes in signal sensing would have to be reevaluated if more signals were discovered.

A second possibility is that the smc02178, smb20495 and smc02177 genes are involved in the secretion of an effector molecule or MAMP whose recognition by a plant receptor may dampen root susceptibility to infection. In this respect, the transcriptome profiling experiment pointed to two candidate MAMP molecules. The first candidate is cAMP-dependent succinoglycan. Considering the well-established (positive) role of succinoglycan in primary infection it is tempting to speculate that succinoglycan may play a negative role during secondary infection as well. Two lines of evidence, however, argue against implication of cAMP-dependent succinoglycan in the control of secondary infection: (i) the individual smc02177, smc02178 and smb20495 mutants—although displaying a full hyperinfection phenotype—were not affected in cAMP-dependent succinoglycan synthesis nor secretion (Figure 2) (ii) cAMP-dependent exoY gene did not depend on the plant shoot signal (Figure 1C) for expression ex planta, contrary to genes (smc02177, smc02178, and smb20495) involved in infection control. Altogether the data suggest that succinoglycan by itself is not the MAMP molecule but does not exclude succinoglycan is part of the MAMP biosynthetic or export pathway.

The second candidate molecule is a polysaccharide compound putatively encoded by a ca. Thirty kilobyte nodP2-smc21248 gene cluster in which we identified at least seven potential Clr target genes by transcriptome profiling. This cluster altogether encompasses 13 glycosyltransferases, 2 sugar epimerases (SMb21228 and SMb21232), 2 tyrosine kinases (SMb21240 and ExoP2) and 3 succinoglycan-related transport proteins (Wx1, ExoF2, and ExoF3) potentially involved in surface polysaccharide synthesis. Activation by Clr of nodP2Q2 involved in sulfate activation as well as three sulfortransferases (SMb21237, SMb21243, and SMb21249) in this large cluster as well as the lpsS-encoded sulfortransferase (Cronan and Keating, 2004) on the chromosome suggests a sulfated polysaccharide. Both (cAMP-independent) sulfated LPS and KPS have been described in S. meliloti (Cronan and Keating, 2004; Townsend et al., 2006). Although highly unlikely, the synthesis of a modified Nod factor molecule cannot be completely excluded at this stage.

The structural characterization of cAMP-dependent polysaccharide molecule and their possible implication in the control of secondary infection is now under investigation. This together with the precise subcellular localization of the SMc02177 and SMB20495 proteins as well as the identification of their interacting partners should shed further light to the control of secondary infection in the S. meliloti–Medicago symbiosis.

AUTHOR CONTRIBUTIONS

LZ, AG, CMD, FS and AMG performed experiments. AMG, JB and CMB designed the research. CMB, AMG and JB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01236/full#supplementary-material
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