Bioactive Cytokinins Are Selectively Secreted by *Sinorhizobium meliloti* Nodulating and Nonnodulating Strains

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Submitted 22 February 2013. Accepted 26 April 2013.

Bacteria present in the rhizosphere of plants often synthesize phytohormones, and these signals can consequently affect root system development. In legumes, plants adapt to nitrogen starvation by forming lateral roots as well as a new organ, the root nodule, following a symbiotic interaction with bacteria collectively referred to as rhizobia. As cytokinin (CK) phytohormones were shown to be necessary and sufficient to induce root nodule organogenesis, the relevance of CK production by symbiotic rhizobia was questioned. In this study, we analyzed quantitatively, by liquid chromatography-tandem mass spectrometry, the production of 25 forms of CK in nine rhizobia strains belonging to four different species. All bacterial strains were able to synthesize a mix of CK, and bioactive forms of CK, such as iP, were notably found to be secreted in bacterial culture supernatants. Use of a mutant affected in extracellular polysaccharide (EPS) production revealed a negative correlation of EPS production with the ability to secrete CK. In addition, analysis of a nonnodulating *Sinorhizobium meliloti* strain revealed a similar pattern of CK production and secretion when compared with a related nodulating strain. This indicates that bacterially produced CK are not sufficient to induce symbiotic nodulation.

Soil bacteria surrounding the plant root system, so-called rhizospheric bacteria, frequently produce plant hormones that can affect root growth and development. Plant growth-promoting rhizobacteria (PGPR) producing auxins, cytokinins (CK), ethylene inhibitors, and gibberellins influence root system growth (Saharan and Nehra 2011). This includes *Bacillus* spp., *Pseudomonas* spp., and *Azotobacter* spp. that produce the indole acetic acid (IAA) auxin modulating root growth. Another example is pathogenic bacteria manipulating plant hormonal balances to affect host growth and development. *Agrobacterium* spp. can transfer into the plant genome a DNA fragment encoding for auxin and CK hormonal biosynthetic genes, leading to the formation of “crown galls” or “hairy roots” on various host plants. In the case of *Rhodococcus fascians*, production of a specific mix of different types of CK hormones was linked to its pathogenicity by altering plant defense, stress, and developmental responses (Depuydt et al. 2009; Pertry et al.

2009; Stes et al. 2011). Concerning symbiotic bacteria, rhizobia are among the most active IAA producers and, therefore, PGPR inducers of root system development. In addition, rhizobia interact with specific legume host plants to generate new organs on the root system, the root nodules, in which differentiated symbiotic bacteria will fix atmospheric nitrogen for the benefit of the host plant. *Bradyrhizobium japonicum* mutants overproducing auxin are not only beneficial for root growth but they also promote symbiotic nodulation. Conversely, *Bradyrhizobium elkanii* mutants deficient in IAA production have decreased nodulation (Kaneshiro and Kwolek 1985; Fukuhara et al. 1994). A more recent study, however, revealed that mutants inhibiting IAA production in the broad-host-range *Rhizobium* sp. strain NGR234 did not show an altered nodulation efficiency (Theunis et al. 2004). Qualitative analyses additionally showed that *Rhizobium leguminosarum* and *B. japonicum* were able to produce and secrete bioactive CK, such as zeatin, at concentrations that could affect root development (Phillips and Torrey 1972; Sturtevant and Taller 1989; Boiero et al. 2007). However, no direct evidence, such as identification of CK-deficient bacterial mutants, links rhizobial CK production to plant nodulation ability. In contrast, production by rhizobia of lipochitooligosaccharide signaling molecules, the Nod(ulation) factors, was genetically demonstrated to be essential for nodulation and, additionally, responsible for the high host specificity of symbiotic interactions with rhizobia (Roche et al. 1991). Indeed, mutation in any bacterial enzyme essential to generate Nod factors, encoded by *nod* genes, leads to a strict nonnodulating (*nod*[−]) phenotype on host plants. The critical role of CK in nodulation was, however, more recently unambiguously revealed through the identification of plant MtCRE1/LHK1 (CK response 1/histidine kinase 1) CK receptors as necessary and sufficient for nodule organogenesis (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Plet et al. 2011; Tirichine et al. 2007). Together with the identification of specific photosynthetic *Bradyrhizobia* species that do not encode *NOD* genes in their genome (Giraud et al. 2007), these studies renewed interest for analyzing relevance of bacterially produced CK in triggering symbiotic nodulation (Frugier et al. 2008).

In this study, we have performed a detailed quantitative analysis of CK synthesis and secretion in four different rhizobia species, using a very sensitive liquid chromatography (LC)-positive electrospray ionization (ES⁺) tandem mass spectrometry (MS/MS) methodology. This revealed that all nine rhizobia strains tested are able to produce and secrete a mix of CK, including bioactive forms such as iP and *trans*-zeatin. A *nod*[−] *Sinorhizobium meliloti* strain was, however, still able to

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secrete a similar CK mix as that of a related nodulating strain, indicating that bacterially produced CK are not sufficient to allow nodulation.

RESULTS

Reference strains of *S. meliloti*, *S. fredii*, *S. medicae*, and *Mesorhizobium loti* (Table 1) were cultured in vitro until they reached a density at which there were sufficient cells for LC-MS/MS analysis of endogenous CK production and of CK release in the culture medium. In contrast to previous studies, bacteria were grown in a GTS minimal medium containing only mineral compounds (Kiss et al. 1979) and, therefore, not contaminated by CK, such as iP, their precursors or derivatives, as this is the case for media containing yeast extract (data not shown).

All the wild-type (WT) strains analyzed were able to synthesize CK, and among the 25 different compounds analyzed, 11 were successfully detected and classified as four CK types (free bases, ribosides, nucleotides, and methyl-thiol derivatives; Table 2). In contrast to previous studies, we determined relative CK content after centrifugation separately in bacterial cell pellets (Fig. 1A) and bacterial supernatants (Fig. 1B). All samples were normalized against the number of bacterial cells. Cell pellets of the four *S. meliloti* strains as well as the *S. medicae* strain presented similar CK production patterns. They differed slightly from *S. fredii* and *M. loti*, for which the free bases (CK-FB) exceeded 10% of total CK, two to three times greater than that of the other five strains (Fig. 1A). The predominating CK type in bacterial cells (pellet) of all tested rhizobia strains were methyl-thiols (CK-MET), which consistently made up more than 60% of all CK detected (Fig. 1A). The relative levels of CK-MET released to the medium were, however, only about 1.1-fold greater than those detected in the bacterial cells, while the CK-FB level in supernatants were at least 1.6-fold higher in the case of Sm10234R, around three times higher for *S. fredii* and *M. loti*, and almost 10-fold higher for Sm1021 (Fig. 1A and B). Interestingly, CK profiles of all bacterial supernatants were found to contain markedly more CK-FB than cell pellets, and in the case of *S. fredii* and *M. loti*, this type of CK represented over 40% of the total CK released (Fig. 1B). By contrast, nucleotides (CK-NT) and ribosides (CK-RB) were present almost exclusively in bacterial cells and were detected in the medium only in minute quantities.

To investigate the potential role of CK produced by rhizobia in relation to symbiotic nodulation in addition to the well-described function of Nod-factor signaling molecules, a detailed analysis of CK produced by *S. meliloti* nodulating strains (Rm41 and the AK631 *exo*⁻ derivative) versus a nonnodulating strain showing a large deletion affecting several *nod* genes (ZB138; Kondorosi et al. 1984) was performed. The LC-

(ES+)-MS/MS analyses revealed that all three *S. meliloti* strains were able to synthesize and secrete a mix of CK (Table 3; Fig. 2). The most abundant CK type again corresponded to MET forms, represented mainly by 2MeSZ and 2MeSiP. CK-FB were also identified in all cases within bacterial cells and as secreted in the medium, corresponding mainly to iP. Comparative analysis of these rhizobia mutant strains reveals that mutations in extracellular polysaccharide (EPS) extracellular polysaccharides and Nod factor production do not abolish rhizobial production of CK. In the cell pellet of the *nod*⁻ mutant ZB138, a CK mix similar to that of the WT and AK631 *exo*⁻ nodulating strains was recovered (no significant differences identified between strains for the four main CK groups; Duncan's multiple range test, *P* < 0.05 [Table 3; Fig. 2]). Interestingly, com-

Table 2. Cytokinins (CK) scanned for by liquid chromatography-positive electrospray ionization tandem mass spectrometry in rhizobia cells and supernatants^a

CK	Labeled CK standard
Nucleotides	
<i>Trans</i>-zeatin riboside-5'-monophosphate	
<i>Cis</i>-zeatin riboside-5'-monophosphate	² H ₃ [9RMP]DHZ
Dihydrozeatin riboside -5'-monophosphate	
N⁶-isopentyladenosine-5'monophosphate	² H ₆ [9RMP]iP
Ribosides	
<i>Trans</i>-zeatin riboside	² H ₃ [9R]Z
<i>Cis</i>-zeatin riboside	
Dihydrozeatin riboside	² H ₃ [9R]DHZ
N⁶-isopentyladenosine	² H ₆ [9R]iP
Free bases	
<i>Trans</i>-zeatin*	² H ₃ Z
<i>Cis</i> -zeatin	
Dihydrozeatin	² H ₃ DHZ
N⁶-isopentyladenine	² H ₆ iP
Glucosides	
<i>trans</i> -Zeatin- <i>O</i> -glucoside	² H ₃ ZOG
<i>cis</i> -Zeatin- <i>O</i> -glucoside	
Dihydrozeatin- <i>O</i> -glucoside	² H ₇ DHZOG
<i>trans</i> -Zeatin- <i>O</i> -glucoside riboside	² H ₃ ZROG
<i>cis</i> -Zeatin- <i>O</i> -glucoside riboside	
Dihydrozeatin- <i>O</i> -glucoside riboside	² H ₇ DHZROG
<i>trans</i> -Zeatin-9-glucoside	² H ₃ Z9G
<i>cis</i> -Zeatin-9-glucoside	
Dihydrozeatin-9-glucoside (DHZ9G)	² H ₃ DHZ9G
Methyl-thiols	
2-Methylthio-<i>trans</i>-zeatin	² H ₃ MeSZ
2-Methylthio-<i>trans</i>-zeatin riboside	² H ₃ MeSZR
2-Methylthio-N⁶-isopentyladenine	² H ₆ MeSiP
2-Methylthio-N⁶-isopentyladenosine	² H ₆ MeSiPA

^aDeuterated internal standards purchased from OlChemim Ltd. (Olomouc, Czech Republic) were used for the analysis. CK actually detected in rhizobia cells and supernatants are highlighted in bold. * indicates CK only detected in supernatants.

Table 1. Wild type and mutant strains of *Rhizobium* spp. used in this study

Strain	Description	Reference or source
<i>Sinorhizobium (Ensifer) meliloti</i>		
Rm41 (Exo+ Nod+)	Wild type (WT), nodulating <i>Medicago</i> spp.	Kondorosi et al. 1984
AK631 (Exo-Nod+)	Mutant derived from Rm41, nodulating <i>Medicago</i> spp.	Kondorosi et al. 1984
ZB138 (Exo-Nod-)	Nonnodulating mutant derived from AK631	Kondorosi et al. 1984
Sm2011	WT, nodulating <i>Medicago</i> spp.	Meade and Singer 1977
Sm1021	WT, nodulating <i>Medicago</i> spp.	Leigh et al. 1985
Sm102F34R	WT, nodulating <i>Medicago</i> spp.	Pocard et al. 1997
<i>Sinorhizobium (Ensifer) fredii</i>		
HH103	WT, nodulating <i>Medicago</i> spp.	Noreen et al. 2003
<i>Sinorhizobium (Ensifer) medicae</i>		
LMG19920 (=A321= Willems R-916)	WT, nodulating <i>Medicago</i> spp.	Rome et al. 1996
<i>Mesorhizobium loti</i>		
NZP2235	WT, nodulating <i>Lotus</i> spp.	Szczygłowski et al. 1998

pared with the WT, significantly lower levels of secreted CK were detected in both *exo*⁻ mutants (AK631 and ZB138) (Table 3; Fig. 2), independently of their ability to nodulate. This suggests that, whereas none of the mutants tested directly suppress bacterial CK synthesis, a lack of EPS secretion is correlated with a reduced CK release.

DISCUSSION

The aim of this study was to analyze the ability of different rhizobia species to synthesize and secrete CK, which are essen-

tial to induce the formation of symbiotic nitrogen-fixing nodules on legume host plants. We first showed that the four different species and nine strains analyzed were able to produce and release mixtures of CK in the medium. This is consistent with earlier publications that reported CK in supernatants of various rhizobial strains (Boiero et al. 2007; Phillips and Torrey 1972; Sturtevant and Taller 1989). However, these previous studies tracked only a few selected CK forms (only up to four different CK detected in a single assay, depending on analyses). More efficient techniques of hormone extraction and considerably greater sensitivity are now available (Farrow and

Fig. 1. Cytokinin (CK) distribution in seven *Rhizobia* strains. Bacterial cells of different WT strains nodulating *Medicago* spp. (*S. meliloti*, *S. fredii* and *S. medicae*) or *Lotus* spp. (*M. loti*) host plants were centrifuged. **A**, Bacterial cell pellets and **B**, supernatants were analyzed by liquid chromatography-tandem mass spectrometry separately for CK contents. The concentration of CK types (FB, RB, NT, MET) in different wild-type (WT) strains is expressed as a percentage share of the total CK pool that was produced (A) and secreted (B). A Duncan's multiple range test was performed to determine significant differences between strains for each CK type. Means are followed by letters only when significant differences were detected at the rejection level $P = 0.05$. NS indicates lack of significant differences between the strains.

Emery 2012), allowing us to determine the profile of 25 different CK compounds, out of which 11 were successfully detected in bacterial samples. In addition, the present study aimed to analyze bacterial supernatants and cell pellets separately, to more completely address the complexity of rhizobial CK metabolism.

CK-MET was the main CK type detected in all rhizobia cells tested as well as in most supernatants, and this was independent of the species (*S. meliloti*, *S. fredii*, *S. medicae*, or *M. loti*) or strains. This agrees with previous reports showing that high levels of CK-MET are characteristic of several rhizospheric microorganisms, including symbionts such as rhizobia (Phillips and Torrey 1972; Sturtevant and Taller 1989) and pathogens such as *Rhodococcus fasciens* when they interact with plants (Eason et al. 1996; Pertry et al. 2009). The high contribution of CK-MET relative to the total amount of CK detected in supernatants likely reflects their high production rate. The relative levels of CK-MET released to the medium were, however, proportionally much reduced compared with levels of CK-FB detected in supernatants. This indicates that CK-MET are not preferentially secreted from the bacterial cells, suggesting that, despite being the predominant type of rhizobial CK, they may not, by themselves, be the most important signaling molecules

acting in the rhizosphere. Similarly, nearly all CK-NT and CK-RB, considered to be less biologically active, were found as retained in bacterial cells, in which they could possibly be used as storage forms (Romanov 2011; Sakakibara 2006). Accordingly, *cisZ* derivatives were detected in CK-NT and CK-RB fractions, as observed in earlier reports, confirming that *cis* isomers of CK are abundant in microorganisms (Eason et al. 1996; Lichter et al. 1995; Pertry et al. 2009; Stevens and Berry 1988; Timmusk et al. 1999). The clearest differences between CK types found in bacteria versus those secreted in the medium were observed with CK-FB. Interestingly, a negative correlation between amounts of CK-FB and CK-MET released in the supernatants was identified, revealing that bacteria selectively secrete relatively high levels of CK-FB, which are considered the most biologically active forms of CK. No glucosyl-CK conjugates were detected, and this is consistent with previously reported evolutionary patterns of CK in prokaryotic organisms compared with angiosperms (Stirk and van Staden 2010). Overall, these results suggest that bacteria process CK-NT and CK-RB into CK-FB and that this biologically active form is preferentially secreted outside bacterial cells. At this point, it would be interesting to compare our results with similar data available in different bacteria tested in other studies. However,

Table 3. Cytokinin (CK) concentration in bacterial cells and surrounding media of *Sinorhizobium meliloti* strains nodulating *Medicago* spp. host plants (Rm41 and AK631) or nonnodulating (ZB138)^a

	Rm41Nod ⁺ Exo ⁺	Rm41Nod ⁺ Exo ⁻ AK631	Rm41Nod ⁻ Exo ⁻ ZB138
Cell pellets			
Nucleotides (NT)			
<i>trans</i> -Zeatin riboside-5'-monophosphate (<i>t</i> ZNT)	1.36E-11 ± 0.69E-11 NS	0.75E-11 ± 0.08E-11	0.64E-11 ± 0.12E-11
<i>cis</i> -Zeatin riboside-5'-monophosphate (<i>cis</i> ZNT)	7.16E-11 ± 1.81E-11 a	4.11E-11 ± 0.64E-11 b	3.68E-11 ± 0.00E-11 b
N ⁶ -isopentyladenosine-5'-monophosphate (iPNT)	7.20E-11 ± 3.28E-11 NS	9.24E-11 ± 0.60E-11	9.21E-11 ± 0.97E-11
Total NT	15.7E-11 ± 5.78E-11 NS	14.1E-11 ± 1.32E-11	13.5E-11 ± 1.09E-11
Ribosides (RB)			
<i>Cis</i> -zeatin riboside (<i>cis</i> ZR)	0.73E-11 ± 0.21E-11 a	0.15E-11 ± 0.02E-11 b	0.33E-11 ± 0.07E-11 b
N ⁶ -isopentyladenosine (iPR)	2.65E-11 ± 0.46E-11 NS	2.06E-11 ± 0.40E-11	1.62E-11 ± 0.27E-11
Total RB	3.38E-11 ± 0.67E-11 a	2.21E-11 ± 0.42E-11 ab	1.95E-11 ± 0.34E-11 b
Free Bases (FB)			
N ⁶ -isopentyladenine (iP)	29.5E-11 ± 10.7E-11 NS	15.9E-11 ± 3.24E-11	19.5E-11 ± 2.50E-11
Total FB	29.5E-11 ± 10.7E-11 NS	15.9E-11 ± 3.24E-11	19.5E-11 ± 2.50E-11
Methyl-thiols (MET)			
2-Methylthio- <i>trans</i> -zeatin (2MeSZ)	60.0E-11 ± 8.93E-11 NS	70.9E-11 ± 19.3E-11	40.1E-11 ± 6.52E-11
2-Methylthio- <i>trans</i> -zeatin riboside (2MeSZR)	0.90E-11 ± 0.25E-11 NS	1.65E-11 ± 0.25E-11	0.87E-11 ± 0.38E-11
2-Methylthio-N ⁶ -isopentyladenine (2MeSiP)	19.7E-11 ± 3.58E-11 b	51.8E-11 ± 7.55E-11 a	26.3E-11 ± 22.6E-11 b
2-Methylthio-N ⁶ -isopentyladenosine (2MeSiPA)	1.03E-11 ± 0.32E-11 b	1.40E-11 ± 0.30E-11 a	0.44E-11 ± 0.18E-11 b
Total MET	81.6E-11 ± 13.1E-11 ab	126E-11 ± 27.4E-11 a	67.7E-11 ± 29.7E-11 b
Supernatants			
NT			
<i>t</i> ZNT	2.33E-11 ± 1.08E-11 NS	0.58E-11 ± 0.36E-11	1.89E-11 ± 1.89E-11
<i>cis</i> ZNT	50.5E-11 ± 8.57E-11 a	22.3E-11 ± 10.1E-11 b	17.3E-11 ± 1.83E-11 b
iPNT	110E-11 ± 73.6E-11 a	1.21E-11 ± 0.81E-11 b	9.04E-11 ± 10.4E-11 b
Total NT	163E-11 ± 83.2E-11 a	24.1E-11 ± 11.3E-11 b	28.2E-11 ± 14.1E-11 b
RB			
<i>cis</i> ZR	6.61E-11 ± 2.96E-11 NS	2.65E-11 ± 0.78E-11	2.15E-11 ± 0.69E-11
iPR	29.2E-11 ± 9.89E-11 a	3.30E-11 ± 0.62E-11 b	3.33E-11 ± 0.34E-11 b
Total RB	35.8E-11 ± 12.8E-11 a	5.95E-11 ± 1.4E-11 b	5.48E-11 ± 1.03E-11 b
FB			
<i>trans</i> -Zeatin	55.1E-11 ± 18.4E-11 NS	43.7E-11 ± 5.79E-11	45.8E-11 ± 2.42E-11
iP	675E-11 ± 112E-11 a	410E-11 ± 59.3E-11 b	511E-11 ± 34.1E-11 ab
Total FB	730E-11 ± 130E-11 a	454E-11 ± 65.1E-11 b	557E-11 ± 36.5E-11 ab
MET			
2MeSZ	159E-10 ± 278E-11 a	983E-11 ± 57.7E-11 b	978E-11 ± 69.2E-11 b
2MeSZR	76.5E-11 ± 20.1E-11 a	22.0E-11 ± 3.97E-11 b	13.0E-11 ± 1.26E-11 b
MeSiP	220E-11 ± 64.5E-11 a	82.6E-11 ± 10.7E-11 b	160E-11 ± 10.6E-11 ab
MeSiPA	54.8E-11 ± 26.9E-11 a	8.07E-11 ± 5.43E-11 b	4.51E-11 ± 1.15E-11 b
Total MET	194E-10 ± 38.9E-10 a	109E-10 ± 7.78E-10 b	115E-10 ± 8.22E-10 b

^a After centrifugation, bacterial cell pellets and supernatants were analyzed by liquid chromatography tandem mass spectrometry separately for CK contents. Concentrations are given in picomoles per cell ± standard error. Results shown are the mean values of three biological replicates. A Duncan's multiple range test was performed to determine significant differences between strains for each CK type. Means are followed by letters only when significant differences were detected at the rejection level $P = 0.05$. NS indicates lack of significant differences between the strains.

past studies about bacterial CK profiling were fragmentary and did not discriminate CK production versus secretion and, thus, were not amenable to insightful comparisons.

In addition to Nod factor bacterial signals, CK were shown to be essential for nodule organogenesis (Frugier et al. 2008; Oldroyd 2007). In addition to plant-synthesized hormones, a contribution of bioactive CK produced by rhizobia could exist, so we compared CK produced by *S. meliloti* nodulating strains (Rm41 and the AK631 *exo*⁻ derivative) and a nonnodulating strain (ZB138) (Kondorosi et al. 1984; Putnoky et al. 1988). The *ExoB* mutation was initially introduced in Rm41, generating the AK631 strain, in order to allow an easier in vitro growth of bacteria, leading to a more compact structure of the colonies than for the Rm41 WT strain, which produces large amounts of EPS. This mutant strain, however, still shows a WT nodulation on *Medicago sativa* or *Medicago truncatula* host roots (Kondorosi et al. 1984; this study). All three *S. meliloti* strains were able to synthesize and secrete CK, with CK-MET types being, once again, the most abundant in bacteria, together with CK-FB in the supernatants. This revealed that the *nod*⁻ strain still produces and secretes bioactive CK despite its inability to initiate nodule organogenesis on *Medicago truncatula* or *Medicago sativa* host plant roots. A similar *nod*⁻ *S. meliloti* mutant engineered to synthesize large amounts of *trans*-zeatin was previously demonstrated to induce the formation of nodule-like structures devoid of bacteria on *Medicago sativa* roots (Cooper and Long 1994). These pseudo-nodules may, therefore, be formed because of the high level of CK production attained in this transgenic strain. Accordingly, exogenous application of CK, without any bacteria, is sufficient to trigger initial steps of nodule organogenesis in *Medicago sativa* (Bauer et al. 1996) or even to induce nodule-like structures in *Lotus japonicus* (Heckmann et al. 2011). The differential response to exogenous CK observed depending on species or even *Lotus* genotypes was, however, not correlated to host-plant nodulation efficiency.

Overall, this study demonstrates that all symbiotic rhizobia tested were able to produce and secrete a similar CK mix, including biologically active hormones. This ability is comparable to that observed for other rhizospheric bacteria including pathogens. A *nod*⁻ mutant unable to nodulate *Medicago truncatula* or *Medicago sativa* host plants retains its ability to produce similar amounts of CK, suggesting that bacterial CK production is not, in itself, sufficient to induce nodulation. The question remains whether bacterial CK could contribute to the symbiotic interaction in a WT context or during interactions with specific symbiotic bacteria that do not produce Nod factors, such as some *Bradyrhizobia* spp. (Podlešáková et al. 2013). In addition, as all analyses were performed in bacterial liquid culture, it is possible that, under rhizospheric soil conditions, some plant exudates such as flavonoids may affect either CK synthesis, secretion by rhizobia, or both, as shown for auxin synthesis, which depends on activation of Nod factor synthesis (Theunis et al. 2004). Finally, as described for auxin or CK production by rhizospheric pathogenic and PGPR bacteria, potential functions for rhizobia-produced CK on root system development other than those that pertain to symbiotic nodulation may exist, and these have not yet been explored.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Rhizobium strains from four species (*S. meliloti*, *S. fredii*, *S. medicae*, *M. loti*) were used in this study, as described in Table 1. Bacterial strains were maintained as 15% (vol/vol) glycerol stocks at -80°C. The strains were recovered by streaking directly onto GTS agar plates (Kiss et al. 1979) and were incu-

bated for 48 h. Liquid cultures were started by suspending the bacterial single colonies with a wire loop in 50-ml polypropylene culture tubes containing 12.5 ml of liquid GTS medium. Prior to CK extraction, bacteria were cultured for 72 h on a rotary shaker (250 rpm). All the cultures were grown at 30°C. To determine cell concentration in the liquid culture, the number of colony-forming units per milliliter on agar plates was determined, using a standard serial dilution technique. Three technical replicates were analyzed for all samples, each independently repeated three times.

Extraction and purification of bacterial CK.

The profiles of 25 CK metabolites were analyzed, including free bases, their riboside and nucleotide derivatives, as well as glucoside and methyl-thiol conjugates. Rhizobial CK were isolated and purified with a method modified from Quesnelle and Emery (2007), designed to prevent enzyme activities that could cause CK nucleotide degradation and CK isomerization.

Fig. 2. Cytokinin (CK) concentration in bacterial cells and surrounding media of *Sinorhizobium meliloti* strains nodulating *Medicago* host plants (Rm41 and AK631) or nonnodulating (*nod*⁻; ZB138). Three related *S. meliloti* strains were analyzed in parallel: two nodulating strains, Rm41 (*nod*⁺, *exo*⁺) and AK631 (*nod*⁺, *exo*⁻), as well as one nonnodulating strain, ZB138 (*nod*⁻, *exo*⁻). After centrifugation, **A**, bacterial cell pellets and **B**, supernatants were analyzed by liquid chromatography-tandem mass spectrometry separately for CK contents. The concentration of CK types (FB, RB, NT, MET) is expressed in picomoles per cell. Results shown are the mean values of three biological replicates. A Duncan's multiple range test was performed to determine significant differences between strains for each CK type. Means are followed by letters only when significant differences were detected at the rejection level *P* = 0.05. NS indicates lack of significant differences between the strains.

Independent extractions were performed for bacterial pellets and cell-free supernatants after 16 min of centrifugation at $4,000 \times g$. Medium not inoculated with rhizobium was also prepared and subjected to analysis to determine whether any interfering media components co-eluted with CK standards.

Fresh weight of cell pellets and volume of supernatants were recorded. Cell pellet and clarified supernatant samples were vortexed in 1 ml of cold (-20°C) modified Bielecki extraction solvent ($\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{HCOOH}$ [15:4:1, vol/vol/vol]) with zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada). Ten nanograms of each of the deuterated internal standard CK (Table 2) (OlChemim Ltd., Olomouc, Czech Republic) were added to the samples. Since deuterated standards of *cisZ* and *cisZR* are not commercially available, the levels of these two compounds were quantified based on the recovery of the deuterated standards of the corresponding *trans*-compounds. Samples were allowed to extract passively overnight at -20°C . Cell pellets were removed by centrifugation (10 min at $2,800 \times g$), the obtained extracts were collected, and samples were re-extracted with 1 ml of Bielecki buffer at -20°C . The remaining solids were separated by centrifugation and discarded, and the pooled extracts were evaporated to dryness at 35°C under vacuum (Model SPD111V; Thermo Scientific, Ottawa, Canada).

To purify and concentrate metabolites for LC-MS/MS, the extraction residues were reconstituted in 200 μl of 1 M HCOOH (pH 1.4). The hormone-containing fraction was purified and concentrated on a mixed mode, reverse-phase, cation-exchange SPE cartridge (Oasis MCX 6cc; Waters, Mississauga, Canada) as described by Kojima and associates (2009), with modifications (Farrow and Emery 2012). Cartridges were activated with high-performance liquid chromatography (HPLC) grade CH_3OH and were equilibrated using 1 M HCOOH (pH 1.4). After equilibration, each sample was loaded and washed with 1 M HCOOH (pH 1.4). CK fractions were eluted based on their chemical properties. CK-NT were eluted first with 0.35 M NH_4OH . CK-FB, CK-RB, CK-MET, and glucosides were retained based on charge and hydrophobic properties and, thus, were eluted last, using 0.35 M NH_4OH in 60% [vol/vol] CH_3OH . Each fraction was evaporated to dryness at 35°C under vacuum.

Because CK-NT cannot be analyzed directly with our methods, they were dephosphorylated by overnight incubation with about 3 units of alkaline phosphatase (CIP; New England BioLabs Ltd., Pickering, Canada) in 1 ml of 0.1 M ethanolamine-HCl (pH 10.4) at 37°C . The resulting CK-RB were dried at 35°C under vacuum. The samples were reconstituted in double-distilled water ($\text{dd-H}_2\text{O}$) and were further isolated on a reversed-phase C_{18} solid phase extraction column (Canadian Life Science, Peterborough, Canada), which was first activated with CH_3OH and equilibrated using $\text{dd-H}_2\text{O}$. Samples containing nucleosides were loaded onto the C_{18} cartridge and were allowed to pass through the column by gravity. The sorbent was washed with $\text{dd-H}_2\text{O}$. CK-RB were eluted using 100% CH_3OH and eluents were evaporated at 35°C under vacuum.

Prior to LC-MS/MS analysis, all samples were rinsed with starting conditions buffer ($\text{CH}_3\text{CN}:\text{CH}_3\text{COOH}:\text{ddH}_2\text{O}$ [5:0.08:94.92, vol/vol/vol]) and were centrifuged at $2,300 \times g$ for 10 min to remove debris (Thermo Scientific). The supernatants were stored at -20°C until further processing.

LC-(ESI+)-MS/MS conditions for bacterial CK analysis.

Purified CK were identified and quantified by LC-(ESI+)-MS/MS (Agilent 1100 series HPLC connected to a Sciex Applied Biosystem 5500 API mass spectrometer), according to the conditions outlined in Farrow and Emery (2012). An aliquot was injected on a Luna C_{18} reversed-phase column (3 μm ,

150 $\times$ 2.0 mm; Phenomenex, Torrance, Canada), and the CK were eluted with an increasing gradient of 0.08% CH_3COOH in CH_3CN (A) mixed with 0.08% CH_3COOH in ddH_2O (B), at a flow rate of 0.2 ml min^{-1} . The initial conditions were 5% A and 95% B, changing linearly in 17 min to 95% A and 5% B. Conditions remained constant for 5 min, and then, immediately returned back to initial conditions for 18 min. The effluent was introduced into the electrospray source using conditions specific for each CK, and quantification was obtained by multiple reaction monitoring of the protonated intact CK molecule $[\text{M}+\text{H}]^+$ and the specific product ion (Farrow and Emery 2012).

Statistical analysis.

The data were processed with the Analyst (v. 1.5) quantification software (AB Sciex, Framingham, MA, U.S.A.) to determine peak area according to isotope dilution analysis. Concentration of CK types (FB, RB, NT, MET) in the different WT strains is expressed as a percentage share of the total CK pool that was produced and secreted (Fig. 1A and B) or as picomoles per cell for the bacterial cells pellet and picomoles per contributing cell for the cell-free supernatant for the comparison between Rm41 WT and mutant strains (Table 3). A Duncan's multiple range test was performed to determine significant differences for each CK type between the tested strains ($P = 0.05$, $n = 3$).

ACKNOWLEDGMENTS

We would like to thank K. Szczygłowski from Agriculture & Agri Food Canada, London for providing the *M. loti* strain, P. Mergaert from ISV, Gif-sur-Yvette, France for providing *S. meliloti*, *fredii* and *medicae* strains, and M. Brault (ISV, Gif-sur-Yvette, France) for helpful discussions. Work in the F. Frugier laboratory was supported by the LEGUROOT French ANR project. Work in the R. J. Neil Emery laboratory was supported by a Natural Science and Engineering Research Council of Canada Discovery Grant. A. Kisiala and C. Laffont participated in the design, analysis and interpretation of the data, as well as in drafting the article. R. J. N. Emery and F. Frugier participated in the conception, design, and interpretation of the data, as well as revised the article. All authors have approved the final version of the article.

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