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Analysis of the secretome of the soybean symbiont *Bradyrhizobium japonicum*

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ABSTRACT

Proteins from the supernatant of *Bradyrhizobium japonicum* were separated by two-dimensional gel electrophoresis and stained with Coomassie. This revealed more than 100 protein spots. Sixty-eight proteins were identified by mass spectrometry. Thirty-five are predicted to contain an N-terminal signal peptide characteristic for proteins transported by the general secretory pathway. Most of these appear to be substrate-binding proteins of the ABC transporter family. Ten proteins were categorized as unclassified conserved or hypothetical. None of the proteins has similarity to proteins transported by a type I secretion system or to autotransporters. Three of the proteins might be located in the outer membrane. The addition of genistein led to changes in the spot pattern of three flagellar proteins and resulted in the identification of the nodulation outer protein Pgl. Moreover, the application of shot-gun mass spectrometry resulted in the first-time identification of NopB, NopH and NopT, which were present only after genistein induction. Replacing genistein with daidzein or coumestrol reduced the amount of the type III-secreted protein GunA2.

1. Introduction

Many bacteria live in close relationship with host organisms resulting in symbiotic or pathogenic interactions. Secreted proteins are important determinants for the outcome of such an interaction. Proteins that are secreted by pathogenic organisms are, e.g., adhesins, degradative enzymes or toxins (Finlay and Falkow, 1989; Gohar et al., 2005). The extracellular proteomes of several Gram-negative and Gram-positive pathogenic bacteria have been analysed (Nouwens et al., 2002; Gohar et al., 2005; Watt et al., 2005; Hansmeier et al., 2006). In contrast, extracellular proteomes of rhizobia have not been described. A few reports about type I-secreted proteins are available. Bladergroen et al. (2003) identified a secreted protein of *R. leguminosarum* strain RBL5523, which is similar to ribose-binding proteins. *R. leguminosarum* bv. *viciae* 3841 secretes at least 12 proteins via the type I secretion system PrsDE (Finnie et al., 1998; Krehenbrink and Downie, 2008). In *S. meliloti*, TolC is required for the secretion of three proteins and a mutant is defective in symbiosis (Cosme et al., 2008).

An alternative export route for proteins is the type IV secretion system, which has been identified, e.g., in *Mesorhizobium loti* strain R7A (Hubber et al., 2007). Best studied in rhizobia, however, is the transport of proteins via the type III secretion system (T3SS). The T3SS has been identified in *Rhizobium* sp. NGR234 (Freiberg et al., 1997), *Sinorhizobium fredii* USDA257 (Krishnan et al., 2003), *S. fredii* HH103 (de Lyra et al., 2006), *M. loti* MAFF303099 (Kaneko et al., 2000) and *Bradyrhizobium japonicum* USDA110 (Göttfert et al., 2001; Krause et al., 2002). Type III-secreted proteins of rhizobia are in general designated Nop (*n*odulation *o*uter *p*rotein; Marie et al., 2001). *Rhizobium* sp. NGR234 encodes at least seven secreted proteins: NopA, NopB, NopC, NopL, NopP, NopT and NopX (Ausmees et al., 2004; Dai et al., 2008; Deakin et al., 2005; Marie et al., 2004; Saad et al., 2005; Viprey et al., 1998). *S. fredii* USDA257 is very similar to NGR234 and secretes a similar set of proteins (Krishnan, 2002; Krishnan et al., 2003; Lorio et al., 2004). Recently, the secretion of NopM and NopD by *S. fredii* HH103 was reported (Rodrigues et al., 2007) and in *B. japonicum*, several type III-secreted proteins were identified, including GunA2 and NopP (Süß et al., 2006). Depending on the host, the secreted proteins may be beneficial or detrimental for symbiosis or appear not to affect the plant. Most of the genes that encode the type III-secretion system or type III-secreted proteins contain a *tts* box motif in their promoter regions (Krause et al., 2002; López-Baena et al., 2008; Wassem et al., 2008; Zehner et al., 2008). These genes are regulated by *ttsI*, which itself is downstream of a *nod* box promoter.

Bioinformatic analyses, as summarized in the *CBS Genome Atlas Database* (Hallin and Ussery, 2004), indicate that rhizobia are very well equipped with respect to protein secretion systems, and the potential number of secreted proteins is high. For *B. japonicum*, 961 (12%) of the proteins are predicted to contain a signal sequence,

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Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Reference/source
Escherichia coli		
DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA*1 *endA*1 *araD*139 Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL nupG*, Sm^r	Invitrogen, Karlsruhe, Germany
Bradyrhizobium japonicum		
110*spc*4	Referred to as wild type, Sp^r	Regensburger and Hennecke (1983)
BJDΔ283	Derivative of the wild type Δ(*fliCI, fliCII*), Sp^r, Km^r	Süß et al. (2006); Kanbe et al. (2007)
BJD567	Derivative of the wild type, cointegration of plasmid pBJD567, *gunA2-cmyc*, Sp^R, Tc^R, Km^R	This study
Plasmid		
pUC-4-KIXX	Source of the Km^r cassette, Ap^r, Km^r	Pharmacia, Uppsala, Sweden
pSUPPOL2SCA	Derivative of pSUP202 used for cointegration, Tc^r	Krause et al. (2002)
pBJD567	pSUPPOL2SCA derivative containing a *gunA2- cmyc* fusion[a], Tc^r Km^r	This study

Abbreviations: Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracyclin.
[a] Only the 3′-end of *gunA2* is present.

95 proteins (1%) might use the TAT pathway for export and 391 proteins (5%) might reach the extracellular environment by a non-classical pathway as defined by Bendtsen et al. (2005).

Abovementioned studies have shown that extracellular proteins can significantly influence the interaction of rhizobia and legumes. To obtain a better understanding of potential symbiosis-relevant proteins in the supernatant of *B. japonicum*, we decided to analyse the secretome in more detail.

2. Materials and methods

2.1. Microbiological and molecular techniques

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* was cultured in Luria-Bertani (LB) medium (Sambrook and Russel, 2001) at 37 °C. Arabinose-gluconate (AG) medium (Sadowsky et al., 1987) was used for growth of *B. japonicum*. If appropriate, induction with genistein, daidzein or coumestrol (10 μM) was done as described (Süß et al., 2006).

Molecular cloning techniques were applied according to established protocols (Sambrook and Russel, 2001). The *cmyc* tag was inserted into the mobilizable plasmid pSUPPOL2SCA by oligonucleotide insertion. The 3′-end of *gunA2* (bp 304-805) was amplified by PCR using chromosomal DNA of *B. japonicum* as template. Primers were designed to allow cloning of the fragment upstream of the *cmyc* tag. The *gunA2-cmyc* fusion of the resulting plasmid pBJD567 was verified by nucleotide sequencing. For selection purposes, a kanamycin resistance cassette was inserted into the vector. pBJD567 was mobilized into *B. japonicum* by conjugation as described (Krause et al., 2002). Cointegration was verified by PCR.

2.2. Isolation of extracellular proteins

B. japonicum strains were grown in AG medium at 28 °C to late logarithmic phase. Proteins for Western analysis were isolated from a 200 ml culture. Cells were pelleted by centrifugation (4000 × *g*, 4 °C, 60 min). For better removal of exopolysaccharides, the supernatant was centrifuged again (8000 × *g*, 4 °C, 30 min). Proteins were precipitated by addition of TCA (10%, w/v) and incubation over night at 4 °C. Subsequently, proteins were pelleted by centrifugation (10,000 × *g*, 4 °C, 30 min). The precipitate was washed twice with 5 ml cold 80% acetone and resuspended in 100 μl rehydration buffer (8 M urea, 2% w/v CHAPS, 0.01% w/v bromophenol blue). Proteins for 2D-PAGE were isolated from a 1.4 l culture as described (Süß et al., 2006).

2.3. Isolation of soluble cell proteins

The cell pellet was washed twice with cell wash buffer (10 mM Tris pH 8.0, 5 mM magnesium acetate). Cells were disrupted using 0.1 mm zirconia/silica beads (ROTH, Karlsruhe, Germany) and vigorous vortexing (five times for 1 min) in the presence of proteinase inhibitor complete (Roche, Mannheim, Germany). Soluble proteins were separated from cell fragments by centrifugation (12,000 × *g*, 4 °C, 15 min).

2.4. Western blot analysis and two-dimensional gel electrophoresis

Aliquots of proteins were separated on a 12% SDS polyacrylamide gel, transferred to Hybond™-P membrane (GE Healthcare, Chalfont St. Giles, Great Britain), probed with anti-cmyc mouse monoclonal antibody (Roche) and ECL™ Anti-mouse linked with horseradish peroxidase (GE Healthcare). The chemiluminescence reaction was carried out with ECL plus Western Blotting Detection Reagents (GE Healthcare).

2D-PAGE was essentially done as described (Süß et al., 2006). For isoelectric focussing, about 350 μg protein were loaded onto an IPG Strip (pH 3–11 NL or pH 4–7, 13 cm, GE Healthcare). The second dimension was done with 16% polyacrylamide gels. Proteins were stained with Coomassie Brilliant Blue R250.

2.5. Mass spectrometry and computer analysis

MALDI-TOF-MS analysis of proteins separated by 2D-PAGE was done as described (Watt et al., 2005). ESI-MS/MS and LC–MS/MS were carried out at the MS facility at the MPI-CBG (Dresden, Germany). For LC–MS/MS analysis, protein bands separated by SDS-PAGE (5–20% gradient) were visualized by staining with Coomassie Brilliant Blue. Full lanes were cut into ca. 30–40 slices while, to enhance the detection dynamic range, visible bands were always sliced separately. Excised gel plugs were cut into ca. 1 mm cubes and in-gel digested with sequencing grade modified porcine trypsin (cat. number V5111, Promega, Mannheim, Germany) as described (Shevchenko et al., 2006). Peptide material was extracted from the gel pieces with 5% formic acid and acetonitrile. Recovered peptides were dried down in a vacuum centrifuge. Dried peptide extracts were re-dissolved in 20 μl of 0.05% (v/v) trifluoroacetic acid (TFA). 4 μl were injected using a FAMOS autosampler into a nanoLC–MS/MS Ultimate system (Dionex, Amsterdam, The Netherlands) interfaced on-line to a linear ion trap LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA) and analysed under conditions described (Waridel et al., 2007).

MS/MS spectra were exported as *dta* (text format) files using BioWorks 3.1 software (Thermo Fisher Scientific) under the following settings: peptide mass range: 500–3500 Da; minimal total ion intensity threshold: 1000; minimal number of fragment ions: 15; precursor mass tolerance: 1.4 amu; group scan: 1; minimum group count: 1. *dta* files were merged into a single MASCOT generic format (*mgf*) file and searched against a MSDB database restricted to bacterial proteins using MASCOT software version 2.2 installed on a local 2 CPU server. Tolerance for precursor and fragment masses was 2.0 and 0.5 Da, respectively; instrument profile: ESI-Trap; variable modification: oxidation (methionine); allowed number of miscleavages: 1; peptide ions score cut-off: 15. Hits were considered