Quantification of Flavin Production by Bacteria

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[Abstract] This protocol provides a simple and fast method of quantification for intracellular flavin content, and for flavin secretion by bacteria. Intracellular flavins are extracted from bacterial pellets, and secreted flavins are examined in the cell growth medium. Flavins are separated and measured using HPLC with fluorescence detection, and quantified based on a comparison to standards.

Materials and Reagents

1. *Sinorhizobium meliloti* (*S. meliloti* 1021, Galibert *et al.*, 2001)
   Note: The protocol can be also applied to other bacteria.
2. Bio-Rad Protein Assay Kit I (Sigma-Aldrich, catalog number: 500-0001)
3. Flavin standards:
   a. Riboflavin (Sigma-Aldrich, catalog number: 95170)
   b. FMN (Sigma-Aldrich, catalog number: F2253)
   c. FAD (Sigma-Aldrich, catalog number: F6625)
   d. Lumichrome (Acros, catalog number: 146930010)
   Note: FMN and FAD were further purified as described previously (Sandov et al., 2008).
4. Ammonium formate
5. Formic acid
6. Methanol
7. YMB medium (Somerville and Kahn, 1983) (see Recipes) (1 plate per sample)
8. $MMNH_4$ medium (Somerville and Kahn, 1983) (see Recipes) (6 ml per sample)
9. Extraction buffer (see Recipes)
10. HPLC mobile phase (see Recipes)
    Note: Except as otherwise noted, all other chemicals were obtained from Sigma-Aldrich.

Equipment

1. Aluminum foil
2. Eppendorf 1.7 ml tubes (Thermo Fisher Scientific, catalog number: 14-222-168)

3. 14 ml Falcon tubes (BD Biosciences, catalog number: 352059)
4. 0.22 µm syringe filter for HPLC sample preparation (Microsolv Technology, catalog number: 58022-N04-C)
5. Scale (Ohaus Corporation, model: E10640)
6. Mini-centrifuge
7. Shaker
8. HPLC: Waters Alliance 2695 HPLC system linked to a 2475 fluorescence detector
9. SunFire C18 reverse-phase column (4.6 x 150 mm, 3.5 µm)

Procedure

A. Cell growth

1. Grow *S. meliloti* on YMB plate for 48 h at 30 °C. The optimal temperature for *S. meliloti* growth is between 28 °C and 30 °C.
2. Inoculate *S. meliloti* from the stock YMB plate to an OD_{600} of ~0.1 in 3 ml $MMNH_4$ medium.
3. Grow cells for 48 h at 30 °C, 250 rpm in 14 ml Falcon or glass tubes completely wrapped with foil to prevent light-induced flavin degradation. In case of testing flavin levels in light-grown culture or flavin degradation by light, the foil could be omitted.
4. Dilute the cells 20-fold into 3 ml fresh $MMNH_4$ medium and grow for 1 or 3 days at 30 °C, 250 rpm in 14 ml Falcon or glass tubes covered with aluminum foil. The length of cell growth could vary from several hours to 10+ days depending on the specific question of the research.
5. Remove 1 ml of the growing culture for protein quantification. These 1 ml samples can be frozen at -20 °C for later protein quantification (the rest 2 ml of cell cultures will be used for flavin detection, steps B, C and D).
6. For protein assay, break the cells by sonication (Fisher Sonic Dismembrator 300 with the intermediate size attachment and a power setting of 60%, three times for 3 min at 4 °C).
7. Remove cells debris by centrifugation (≥ 3,000 *x g*; room temperature; 20 min).
8. Measure protein concentration in the cultures by using Bio-Rad Protein Assay Kit.
   Note: Carry out the rest of the procedure for flavin extraction and analysis under as limited lighting as possible since flavins are extremely light sensitive. Protect extracted and filtered flavins from light by storing in a light-tight container. For long-term storage, samples can be stored below 0 °C in a light-tight container.

B. Separation of cells from conditioned media

1. Harvest cells from 1 ml culture by centrifugation (≥ 10,000 *x g*; room temperature; 20 min) in pre-weighed Eppendorf tubes.
2. Transfer the supernatant to clean Eppendorf tubes. It will be used in step C and can be stored at -20 °C in foil wrapped Eppendorf tubes for later analysis.
3. Wash the pellets with 1 ml MMNH4 buffer and centrifuge (≥ 10,000 *x g*; room temperature; 20 min).
4. Pipet out all liquid and re-weigh the tube to measure the pellet mass. The pellet mass is expected to be at the range between 5 mg and 10 mg depending on the time of growth.
5. The pellet will be used in step D can be stored at -20 °C in foil wrapped Eppendorf tubes for later analysis.

C. To measure secreted flavins

1. Filter the supernatant left after harvesting the bacteria (step B) using Microsolv 0.22 µm syringe filters.
2. For riboflavin, FMN, and FAD detection, separate the samples by reverse-phase chromatography using a Waters Alliance 2695 HPLC system with a Waters SunFire C18 column (4.6 x 150 mm, 3.5 µm) maintained at 35 °C linked to a Waters 2475 fluorescence detector. Detect riboflavin, FMN, and FAD by using an excitation wavelength of 470 nm and an emission wavelength of 530 nm. Run mobile phase at a flow rate of 1 ml/min for 12 min per sample. Maintain the samples at 10 °C.
3. For lumichrome detection, use excitation and emission wavelengths of 260 nm and 470 nm respectively. Use the mobile phase gradient program indicated in Table 1. The column and flow rate are the same as indicated above. Run mobile phase for 15 min. Column temperature 35 °C.
4. Determine the flavin concentration by comparison to standards (0.05, 0.1, 0.5, 1.0 and 5.0 µM FAD, FMN and riboflavin).
5. Normalize the flavin concentration against the protein concentration (as calculated above in step A8).

Table 1. Solvent gradient program parameters for lumichrome detection

Time (min)	Water (%)	Methanol (%)
0	77	23
8	40	60
11	40	60
12	0	100
13	0	100
14	77	23
15	77	23

*Flow rate 1 ml/min

D. To measure intracellular flavin concentration
   1. Resuspend the cell pellets from step B in extraction buffer (1: 10 w/v), heat at 80 °C for 10 min, then centrifuge at 20,000 *x g* for 20 min at 4 °C.
   2. Filter the supernatants using a 0.22 µm syringe filter.
   3. Measure riboflavin, FMN, FAD, and lumichrome as described for the secreted flavins.

Representative data

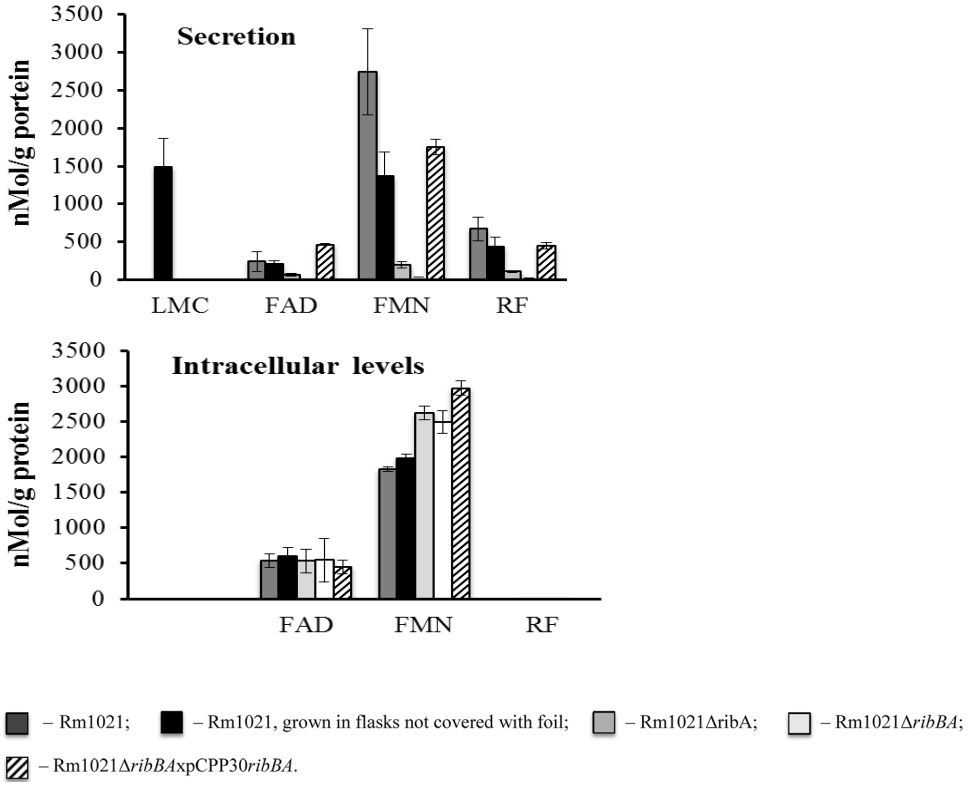

Figure 1. Flavin secretion and accumulation by the *S. meliloti* strains. *S. meliloti* strains

were grown in $MMNH_4$ media. 1 ml of cell cultures was taken after 3 days of growth. The cells were spun down and the mass of the pellets was measured. Lumichrome (LMC), FMN, FAD and riboflavin (RF) concentration in the supernatants and the pellets were measured using HPLC. The flavin concentration was normalized against the protein concentration. Rm1021 - wild type strain; Rm1021Δ*ribA* and Rm1021Δ*ribBA* - Rm1021 mutants with decreased ability to secret flavins; Rm1021ΔribBAxpCPP30*ribBA* - Rm1021 mutant with restored ability to secret flavins. Data are averaged from at least three independent experiments.

Recipes

1. YMB media for *Rhizobium*

	1 L	Concentration
Yeast Extract	1 g	
Mannitol	10 g	54.9 mM
Agar	15 g	

Autoclave, cool to 55 °C, then add

YMB Salt I	10 ml
YMB Salt II	10 ml

YMB Salt I	1 L	Concentration
K_2HPO_4	50 g	287.06 mM
NaCl	10 g	171.15 mM
d-H_2O	960 ml	

YMB Salt II	1 L	Concentration
$MgSO_4 \cdot 7H_2O$	20 g	81.11 mM
d-H_2O	1 L	

2. MMNH4 (minimal mannitol ammonia media for *Rhizobium*)

	per 1 L	Concentration
Mannitol	10.0 g	54.9 mM
NH_4Cl	0.5 g	9.34 mM
Agar (for plates preparation)	15.0 g	
d-H_2O	970 ml	

Autoclave, cool to 55 °C, then add:

Biotin (0.2 mg/ml in 50% EtOH)	1.0 ml
Thiamine (2 mg/ml), filter sterilized	1.0 ml
Min Man Salts I	10.0 ml
Min Man Salts II	10.0 ml

Min Man Salts I

	per 1 L	Concentration
K_2HPO_4	100 g	574.12 mM
KH_2PO_4	100 g	734.8 mM
Na_2SO_4	25 g	174.8 mM
d-H_2O	1 L	

Min Man Salt II

	per 1 L	Concentration
$FeCl_3 \cdot 6H_2O$	1.0 g	3.7 mM
Concentrated HCl	adjust pH to ~7.0 (~1 drop)	
$CaCl_2 \cdot 2H_2O$	10.0 g	68 mM
$MgCl_2 \cdot 6H_2O$	25.0 g	123 mM
d-H_2O	1 L	
Autoclave		

3. Extraction buffer

   100 mM ammonium formate

   100 mM formic acid

   25% methanol

4. HPLC mobile phase

   100 mM ammonium formate

   100 mM formic acid

   25% methanol

   For 1 liter of extraction buffer/ mobile phase, add the following components in the order below. Mix well after adding all the components.

   550 ml water

   100 ml ammonium formate, 1 M, filtered through a 0.22 µm filter

   100 ml formic acid, ≥98% pure, 1 M

   250 ml methanol, HPLC grade

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References

1. Galibert, F., Finan, T. M., Long, S. R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F. J., Weidner, S., Wells, D. H., Wong, K., Yeh, K. C. and Batut, J. (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* 293(5530): 668-672.
2. Sandoval, F. J., Zhang, Y. and Roje, S. (2008). Flavin nucleotide metabolism in plants: monofunctional enzymes synthesize fad in plastids. *J Biol Chem* 283(45): 30890-30900.
3. Somerville, J. E. and Kahn, M. L. (1983). Cloning of the glutamine synthetase I gene from *Rhizobium meliloti*. *J Bacteriol* 156(1): 168-176.
4. Yurgel, S. N., Rice, J., Domreis, E., Lynch, J., Sa, N., Qamar, Z., Rajamani, S., Gao, M., Roje, S. and Bauer, W. D. (2014). *Sinorhizobium meliloti* flavin secretion and bacteria-host interaction: role of the bifunctional RibBA protein. *Mol Plant Microbe Interact* 27(5): 437-445.

