FEMS Microbiology Letters 19 (1983) 281–284
Published by Elsevier

Nicotinic acid requirement and degradation by *Sesbania rhizobium* strain ORS571

Claudine Elmerich, Bernard Dreyfus * and Jean-Paul Aubert

Unité de Physiologie Cellulaire, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France

Received and accepted 4 May 1983

1. SUMMARY

Sesbania rhizobium strain ORS571, which grows in the free-living state at the expense of N_2 or ammonia, requires nicotinic acid as a growth factor and also uses this vitamin as a nitrogen source in culture medium devoid of ammonia. Consequently, under nitrogen-fixing conditions, *Sesbania rhizobium* requires about 10-times more nicotinic acid than when grown in the presence of ammonia. The physiological implications of this property are discussed.

2. INTRODUCTION

The *Rhizobium* strain ORS571 is associated with the tropical legume *Sesbania rostrata* and it proliferates in the free living state with either ammonia or N_2 as the sole nitrogen source for growth [1]. Under both conditions, the strain requires 3 vitamins: biotin, nicotinic acid, and panthothenic acid [1]. However, the amount of vitamins (0.02 mg biotin/l, 4 mg nicotinic acid/l and 4 mg calcium panthothenate/l), sufficient for exhausting the carbon source in the presence of ammonia, limits growth under conditions of nitrogen fixation.

This observation prompted our reexamination of the vitamin requirements for *S. rhizobium*. We report here that nicotinic acid can be used as a nitrogen source by *S. rhizobium* and that the vitamin is metabolized at a much higher rate under conditions of nitrogen fixation than under conditions of ammonia assimilation.

3. MATERIALS AND METHODS

3.1. Bacterial strains

The wild-type *Rhizobium* strain ORS571 [2] and the Nif⁻ mutant 5740 [1] were used.

3.2. Media, growth conditions and assays

The minimal (LSO) and complete (YLS) media were previously described [1]. All cultural studies except those with [^{14}C]nicotinic acid, were performed in a 1.5 l Biolafitte fermentor containing 1 l of LSO medium at 30°C. Air was used when ammonia was the nitrogen source, and under conditions of nitrogen fixation the gas phase was a mixture N_2–O_2 (97 : 3, v/v). In both cases, the gas flow rate was 1.0 l/min and the pH was regulated at 7.2 by 1 N HCl addition. Cultures in LSO medium were inoculated at A_{570} 0.05–0.1 with bacteria grown overnight in YLS medium. Experiments in the presence of [^{14}C]nicotinic acid were

* Permanent address: Laboratoire de Biologie des Sols, Centre ORSTOM, P.O. Box 1386, Dakar, Sénégal.

0378-1097/83/$03.00

performed in 1-l serum bottles containing 10 ml of LSO medium. [Carboxyl-^{14}C]nicotinic acid (56 mCi/mmol, Amersham) was used at the concentration of 0.15 μCi/ml. Unlabelled nicotinic acid was from Sigma Chemical Co. Absorption spectra were recorded in a DB-GT Beckman Spectrophotometer. Radioactive samples were counted in a Prias (Packard) Liquid Scintillation Counter. High pressure liquid chromatography (HPLC) analysis of nicotinic acid was performed in a Perkin-Elmer HS5 C18 column (12.5 cm × 4.6 mm) eluted by an acetonitrile-ethylammonium 20 mM pH 7.0 gradient (from 0.1 to 50% acetonitrile in 20 min).

4. RESULTS

4.1. Identification of nicotinic acid as a limiting growth factor

As shown in Table 1, a 10-fold increase in the concentration of the 3 vitamins or of nicotinic acid alone resulted in a large increase in growth. However, growth was not increased by the addition of excess biotin, panthothenic acid or by several other growth factors tested. The purity of nicotinic acid was checked as a control by HPLC to exclude a possible contamination of the vitamin by another active compound. No impurity was detected in significant amounts.

Table 1

Influence of vitamins on growth of *Sesbania rhizobium* strain ORS571 cultured under conditions of nitrogen fixation

Vitamin addition	Growth (Maximal A_{570})
1 × BNP [a]	0.3
10 × BNP	3.3
1 × BNP + other vitamins [b]	0.4
1 × NP + 10 × B	0.3
1 × BP + 10 × N	3.5
1 × BN + 10 × P	0.5

[a] B : 0.02 mg biotin/l, N : 4 mg nicotinic acid/l, P : 4 mg calcium panthothenate/l.

[b] 2 mg riboflavin/l, 0.2 mg folic acid/l, 20 mg inositol/l, 0.5 mg ergosterol/l, 2 mg vitamin B12/l. The doubling time of growing cultures was about 6 h.

Table 2

Influence of nicotinic acid concentration on growth of *Sesbania rhizobium* cultures under conditions of N_2 fixation or ammonia assimilation

Strain	Nicotinic acid concentration (mg/l)	Growth (Maximal A_{570})	
		with N_2	with ammonia
ORS571	4	0.4	2.8
	10	2.0	3.7
	20	3.3	3.7
	40	3.5	3.8
5740	4	0.3	2.5
	40	0.5	3.6

4.2. Nicotinic acid requirement of the wild-type strain ORS571 and of the Nif⁻ mutant 5740

Results reported in Table 2 show that, to reach a maximal A near 3.5, the wild-type strain ORS571 required between 4 and 10 mg nicotinic acid/l in the presence of ammonia, and between 20 and 40 mg/l in the presence of N_2. The lower requirement in the presence of ammonia was independent of the oxygen tension and the same results were obtained whether the gas phase was air or a mixture N_2–O_2 (97 : 3, v/v) (data not shown). Control experiments with the Nif⁻ mutant 5740 showed that nicotinic acid, even at the concentration of 40 mg/l did not account alone for the full growth of strain ORS571.

4.3. Metabolism of nicotinic acid

Nicotinic acid has a maximum absorption at 263 nm (pH 7.0) with an $E^{1\%}_{1cm}$ of 260. Absorption spectra of culture media supplemented with 40 mg nicotinic acid/l were recorded before inoculation and after growth in the presence of ammonia or N_2. Results reported in Fig. 1 show that in ammonia-grown cultures the peak at 263 nm was still present at the end of growth and corresponded to a concentration of about 30 mg nicotinic acid/l. The same result was obtained with the Nif⁻ mutant 5740 (data not shown). Under conditions of nitrogen fixation, this absorption peak completely disappeared after growth of either the Nif⁺ strain ORS571 or the Nif⁻ mutant 5740.

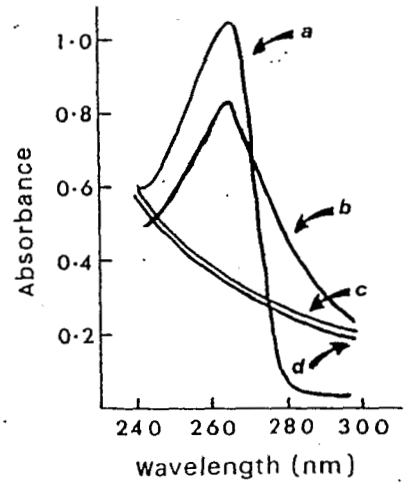

Fig. 1. Absorption spectra of culture media. (a) LSO medium (40 mg nicotinic acid/l) before inoculation; (b) supernatant at the end of growth of strain ORS571 in LSO (NH_4^+); (c) supernatant of strain ORS571 in LSO (N_2); (d) supernatant of mutant 5740 in LSO (N_2).

To differentiate between the possibility of a higher intracellular accumulation or of a degradation of nicotinic acid when the bacteria were grown under conditions of nitrogen fixation, experiments were performed with strain ORS571 in the presence of [^{14}C]nicotinic acid. Results reported in Table 3 show that there were no differences in the radioactive content of the bacteria, regardless of the nitrogen source. There was a very small incorporation of the [^{14}C]carboxyl group of nicotinic acid into cellular material in both experimental conditions. However, the net loss of radioactivity was about 10%/ml *A* 1 with ammonia-grown cells and 40%/ml *A* 1 with N_2-grown cells. This observation suggested that nicotinic acid was more effectively metabolized by N_2-growing cells than by ammonia-growing cells.

These results were confirmed by studying the ability of nicotinic acid to be used as a growth substrate by strain ORS571. Nicotinic acid (at concentration of 10 or 20 mM) was a good nitrogen source for aerobic growth in the LSO medium, a poor carbon source in the presence of ammonia, and supported growth at slower rates when used alone as both nitrogen and carbon sources.

Table 3

Distribution of radioactivity in cultures of *Sesbania rhizobium* strain ORS571 grown in the presence of [^{14}C]nicotinic acid

Analyses were performed 23 h after inoculation with ammonia-grown cells (A_{570}:3.8) and 28 h with N_2-grown cells (A_{570}:1.65). The LSO medium contained 40 μg unlabelled nicotinic acid/ml and 270000 cpm of [^{14}C]nicotinic acid/ml.

Distribution	Radioactivity (cpm/ml)	
	Ammonia-grown cells	N_2-grown cells
Supernatant	170000	90000
Untreated cells	3300	1800
5% cold TCA-treated cells	2200	1500
% label recovered	64.2	34.0

5. DISCUSSION

In bacteria, a variety of nitrogen-containing compounds can be used as a source of nitrogen and eventually as source of carbon and energy. As a general rule, the enzymes involved in the degradative pathways are repressed when the bacteria are grown in the presence of high concentrations of ammonia. The genetic control, which includes both the ammonia-providing and ammonia-assimilating enzymes is now well-documented [3]. With regard to nicotinic acid catabolism, the pathway of degradation was studied in *Pseudomonas fluorescens* [4–7], *Clostridium barkeri* [8–11] and in a *Bacillus* species [12,13]. In all cases, the first step is a hydroxylation of the molecule in the 6-position. Proposed pathways that lead to maleic acid, formic acid, CO_2 and NH_3 are described in [12]. To our knowledge, no such study with a *Rhizobium* species has been reported.

Results reported here are consistent with the hypothesis that *Sesbania rhizobium* strain ORS571 is able to degrade nicotinic acid and to utilize the end products as a nitrogen source and, to a lesser extent, as a carbon source. The enzymes involved in the degradative pathway are likely repressed or inhibited when the bacteria are grown in the presence of ammonia. These properties seem to have no direct relationship with nitrogen fixation since the Nif$^-$ mutant 5740, that is devoid of a func-

tional Mo-Fe protein [1], behaves as the wild-type strain. In addition, the results obtained with the Nif$^-$ mutant show that nicotinic acid at a concentration of 40 mg/l (0.3 mM) accounts only for about 1/10 of the total growth observed when nitrogen fixation is not impaired. This suggests that, in the wild-type strain, N_2 is preferentially used over nicotinic acid as a nitrogen source.

In general, the ability to utilize various nitrogen sources may be considered as beneficial for bacteria. The particular case of *S. rhizobium*, for which nicotinic acid is a growth factor, raises an interesting problem of physiology, since under conditions of nitrogen fixation the organism destroys the growth factor it requires. The results reported here clearly demonstrate that nicotinic may be a limiting growth factor when the bacteria are fixing nitrogen in the free-living state. Thus one wonders if this is also occurs when the organism fixes nitrogen within the root or the stem nodules of its plant host and if this phenomenon plays a role in the stability of the symbiosis. Further studies of a mutant impaired in the first enzyme of the degradative pathway may provide some answer to this question.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Patrice Allard who performed the HPLC assays. This work was supported by a Grant (N° GB1-5-028-F (SD)) from the Commission of the European Communities and by research funds from University Paris 7.

REFERENCES

[1] Elmerich, C., Dreyfus, B.L., Reysset, G. and Aubert, J.-P. (1982) The EMBO J. 4, 499–503.
[2] Dreyfus, B.L. and Dommergues, Y.R. (1981) FEMS Microbiol. Lett. 10, 313–317.
[3] Magasanik, B. (1982) Annu. Rev. Genet. 16, 135–168.
[4] Behrman, E.J. and Stanier, R.Y. (1957) J. Biol. Chem. 228, 923–945.
[5] Hughes, D.E. (1955) Biochem. J. 60, 303–310.
[6] Hunt, A.L., Hughes, D.E. and Löwenstein, J.M. (1958) Biochem. J. 69, 170–173.
[7] Hunt, A.L., Rodgers, A. and Hughes, D.E. (1959) Biochim. Biophys. Acta 34, 354–372.
[8] Harary, I. (1957) J. Biol. Chem. 227, 815–822.
[9] Harary, I. (1957) J. Biol. Chem. 227, 823–831.
[10] Pastan, I., Tsai, L. and Stadtman, E.R. (1964) J. Biol. Chem. 239, 902–906.
[11] Holcenberg, J.S. and Stadtman, E.R. (1969) J. Biol. Chem. 244, 1194–1203.
[12] Ensign, J.C. and Rittenberg, S.C. (1964) J. Biol. Chem. 239, 2285–2291.
[13] Hirschberg, R. and Ensign, J.C. (1972) J. Bact. 112, 392–397.