Effect of L-methionine-DL-sulfoximine on acetylene reduction and vesicle formation in derepressed cultures of *Frankia* strain D11

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Accepted February 23, 1983

GAUTHIER, D. L. 1983. Effect of L-methionine-DL-sulfoximine on acetylene reduction and vesicle formation in derepressed cultures of *Frankia* strain D11. Can. J. Microbiol. **29**: 1003–1006.

L-Methionine-DL-sulfoximine, a glutamate analog known to inhibit glutamine synthetase (GS) and glutamate synthase (GOGAT), allowed nitrogenase biosynthesis by *Frankia* strain D11 in presence of NH_4^+. GS activity, detected in cell-free extracts of D11, was higher in N_2-fixing than in nonfixing conditions. These facts suggested that nitrogenase biosynthesis in *Frankia* was probably regulated by GS, GOGAT, or a product of their reaction, but regulation of GS by an adenylylation–unadenylylation system was probably not involved. Similarities between *Frankia* and *Anabaena cylindrica* were stressed.

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Un analogue du glutamate, la L-methionine-DL-sulfoximine, inhibiteur des activités glutamine synthétase (GS) et glutamate synthase (GOGAT), permet la dérépression de la biosynthèse de la nitrogénase en présence de NH_4^+ chez la souche de *Frankia* D11. En ce qui concerne l'activité GS, cette dernière est plus importante dans les extraits bruts de cultures de D11 réduisant l'acétylène que dans ceux provenant de cultures réprimées. D'autre part, il n'a pas été possible de mettre en évidence un système d'adénylation de la GS chez la souche D11. Ces résultats suggèrent que le NH_4^+ *per se* n'est pas l'effecteur de la régulation de la biosynthèse de la nitrogénase mais que cette dernière est probablement sous le contrôle des enzymes de l'assimilation de l'azote (GS et GOGAT). Enfin, quelques points de similarité entre *Frankia* et *Anabaena cylindrica* sont relevés.

In an earlier paper (Gauthier *et al.* 1981), we reported that a strain isolated from *Casuarina equisetifolia* (D11) reduces acetylene *in vitro* and NH_4^+ inhibits nitrogenase biosynthesis, a fact also related by Tjepkema *et al.* (1980, 1981). Using strain D11 we want to know if NH_4^+ is the actual effector for nitrogenase repression or if nitrogenase repression is governed by the enzymes of nitrogen assimilation, namely, glutamine synthetase (GS) and glutamate synthase (GOGAT) as in other nitrogen-fixing microorganisms such as *Klebsiella pneumoniae* (Tubb 1974), *Azospirillum* (Okon *et al.* 1976; Gauthier and Elmerich 1977), and *Anabaena cylindrica* (Dharmawardene *et al.* 1973). To determine if NH_4^+ assimilation is required for the inhibition of nitrogenase biosynthesis, we added methionine sulfoximine (MSX), a glutamate analog known to inhibit both GS and GOGAT (Gordon and Brill 1974; Stewart and Rowell 1975). We also explored the effect of MSX on vesicle production.

Material and methods

Strain

Strain D11 of *Frankia* isolated from nodules of *Casuarina* was that described by Gauthier *et al.* (1981) and Diem *et al.* (1982).

Growth conditions

D11 was aerobically grown in liquid QMOD medium (Lalonde and Calvert 1979) at 30°C for 10 days without shaking. In this medium vesicles were produced. When QMOD medium was supplemented with NH_4Cl (20 m*M*), no vesicles were formed.

Derepression of nitrogenase biosynthesis

Cultures of D11 from QMOD or QMOD + NH_4Cl were centrifuged and resuspended in an equal volume of the following nitrogen-free medium (NFM) (grams per litre): KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.01; $FeSO_4$, 0.01; Na succinate, 1.2; $NaMoO_4 \cdot 2H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.025; $ZnSO_4 \cdot 7H_2O$, 0.007; $CuSO_4 \cdot 5H_2O$, 0.00125; $CoSO_4 \cdot 7H_2O$, 0.0014; H_3BO_3, 0.0003; pH 6.8. Portions (25 mL) of this suspension were placed in 145-mL vials with serum stoppers in air containing 10% C_2H_2 and maintained at 30°C without shaking.

Acetylene reduction activity (ARA)

ARA was measured daily by the method of Postgate (1972) for 8 days and was expressed as nanomoles of C_2H_4 produced per hour and per milligram of dry weight. Supplementation with NH_4Cl and (or) MSX was performed at the rate of 20 and 10 m*M*, respectively.

Glutamine synthetase activity (GS)

GS was determined in two cultures of D11: (*i*) a culture of D11 grown in QMOD medium for 10 days (repressed culture), and (*ii*) a culture of D11 grown in QMOD for 10 days and then derepressed in NFM for 8 days (derepressed culture). Cell-free extracts were obtained by passage through a French press at 18 000 psi (1 psi = 6.894 757 kPa) in Imidazol–HCl buffer (10 m*M*, pH 7.1) containing 10 m*M* $MnCl_2$ and 1 m*M* mercaptoethanol.

GS activity was estimated by the glutamyltransferase assay (Shapiro and Stadtman 1970). Relative adenylylation of GS in crude extracts was estimated by the snake venom phosphodiesterase (SVD) method (Bishop *et al.* 1975) and also from the transferase assay conducted in the presence or absence of 60 m*M* $MgCl_2$ (Bishop *et al.* 1975).

Protein content

Protein content was estimated by the method of Lowry *et al.* (1951).

Enumeration of vesicles

D11 cultures were centrifuged, resuspended in a known volume of phosphate buffer, homogenized, and counted in a Petroff–Hausser chamber under phase illumination.

Results

When one compares the effect of different media on ARA and vesicle production by D11 (Table 1), one finds that QMOD medium allowed the production of vesicles without ARA expressed. NFM allowed both ARA and the production of vesicles. Supplementation with a nitrogen source, such as NH_4Cl or glutamine, inhibited both ARA and vesicle production.

A culture of D11 grown in QMOD medium, thus containing vesicles, could not derepress its nitrogenase biosynthesis in the presence of NH_4Cl (20 m*M*), but this inhibition could be partly overcome by addition of MSX (Table 2).

An experiment similar to that reported in Table 2 was made using a culture of D11, which being grown in QMOD medium supplemented with NH_4Cl (20 m*M*) was thus devoid of preexisting vesicles. NH_4^+ inhibited both ARA and vesicles production, but adding MSX restored ARA and vesicle production even in the presence of NH_4^+ (Table 3).

When one compares the GS activity in cell-free extracts from D11 cultures grown on QMOD medium or from the same culture derepressed in NFM medium, one observes that this activity was higher in the derepressed culture than in the repressed one (Table 4).

In contrast with what was observed with other nitrogen-fixing organisms, such as *Azospirillum* (Okon *et al.* 1976), addition of SVD did not markedly alter the relative adenylylation (+Mg/−Mg) of GS (Table 4).

TABLE 1. Effect of different media on acetylene reduction activity (ARA) and vesicle production in *Frankia* strain D11

Medium[a]	ARA	Vesicle production
QMOD	−	+
QMOD + NH_4Cl (20 m*M*)	−	−
QMOD + glutamine (250 μg/mL)	−	−
NFM	+	+
NFM + NH_4Cl (20 m*M*)	−	−

NOTE: NFM, nitrogen-free medium.
[a]In all assays *Frankia* was grown aerobically.

TABLE 2. Derepression (under air) of nitrogenase biosynthesis in a culture of D11 with preexisting vesicles (obtained in QMOD medium)

Medium	ARA[a]
NFM + NH_4Cl (20 m*M*)	0
NFM + MSX (10 m*M*)	25
NFM + NH_4Cl(20 m*M*) + MSX(10 m*M*)	10

NOTE: NFM, nitrogen-free medium; MSX, L-methionine-DL-sulfoximine.
[a]Expressed as nanomoles C_2H_4 per hour per milligram dry weight.

Discussion

Two facts suggest that NH_4^+ was not the actual effector and that nitrogenase biosynthesis was possibly regulated by GS, GOGAT, or a product of their reaction. First MSX, a glutamate analog known to inhibit GS and GOGAT, allowed nitrogenase biosynthesis in the presence of NH_4^+. Second, GS activity was actually detected in cell-free extracts of D11, this activity being higher in nitrogen-fixing conditions (derepression in NFM medium) than in nonfixing conditions (QMOD medium) (Table 4). However, the hypothesis that nitrogenase biosynthesis is regulated by GS should be further tested using GS mutants of strain D11 as in the case of *Klebsiella pneumoniae* (Streicher *et al.* 1974) or *Azospirillum* (Gauthier and Elmerich 1977).

The fact that adding SVD to cell-free extracts of D11 did not affect the relative adenylylation (+Mg/−Mg) suggests that GS in strain D11 is not regulated through the adenylylation–unadenylylation system found in *E. Coli* (Wuff *et al.* 1967).

Vesicle formation was always required for the expression of nitrogenase activity, a conclusion already stressed by Tjepkema *et al.* (1981); however, from our data we

TABLE 3. Derepression (under air) of nitrogenase activity and vesicle production in a culture of D11 devoid of preexisting vesicles (obtained in QMOD medium supplemented with 20 m*M* NH_4Cl

Medium	ARA[a]	No. of vesicles[b]
NFM + NH_4Cl (20 m*M*)	0	0
NFM + MSX (10 m*M*)	18	13
NFM + NH_4Cl(20 m*M*) + MSX(10 m*M*)	7	5

NOTE: NFM, nitrogen-free medium; MSX, L-methionine-DL-sulfoximine.
[a]Expressed as nanomoles C_2H_4 per hour per milligram dry weight.
[b]Expressed as $\times 10^6$ per milligram dry weight.

cannot infer that vesicle formation is regulated in the same way as nitrogenase biosynthesis.

The general behaviour of strain D11 was reminiscent of that of *Anabaena cylindrica*, since the biosynthesis of nitrogenase by the latter organism is restored by MSX in presence of NH_4^+ (Stewart and Rowell 1975) and its GS has no adenylylation–unadenylylation system (Dharmawardene *et al.* 1973; Rowell *et al.* 1979). Another similarity between *Frankia* and *Anabaena cylindrica* is that both organisms are filamentous and able to develop specialized structures which are the site of nitrogen fixation: vesicles in *Frankia* and heterocysts in *Anabaena cylindrica*.

TABLE 4. Specific glutamine synthetase activity in strain D11

Medium	Transferase assay[a]				Relative adenylylation[b]	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$ + SVD	$+Mg^{2+}$ + SVD	$+Mg^{2+}/-Mg^{2+}$	$(+Mg^{2+}/-Mg^{2+})$ + SVD
QMOD (repressed culture)	7.00	0.70	2.80	0.24	0.09	0.08
NFM (derepressed culture)	12.00	0.60	8.20	0.55	0.05	0.07

NOTE: $+Mg^{2+}$, transferase assay with Mn^{2+} (3 m*M*) and Mg^{2+} (60 m*M*); $-Mg^{2+}$, transferase assay with Mn^{2+} (3 m*M*) only; +SVD, cell-free extract incubated 3 h in snake venom phosphodiesterase (10 μg/mL) at 37°C; NFM, nitrogen-free medium.

[a]GS activity measured by transferase assay is expressed as micromoles γ-glutamyl-hydroxamate per 10 minutes per milligram of protein. The assay mixture was as follows in a final volume of 1.0 mL: glutamine, 30 m*M*; hydroxylamine–HCl, 30 m*M*; sodium ADP, 0.4 m*M*; sodium arsenate, 20 m*M*; $MnCl_2$, 3 m*M* in Imidazol–HCl buffer, 80 m*M*; pH 7.1.

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Reprinted from

Canadian Journal of Microbiology

Réimpression du

Journal canadien de microbiologie

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Volume 29 • Number 8 • 1983

Pages 1003–1006

National Research Council Canada

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