

Differentiation of *Rhizobium japonicum*, III. Inhibition of Nitrogenase Derepression by Chloramphenicol and Rifampicin Concentrations, Not Inhibiting Growth

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Development of nitrogenase ($40\text{--}140\text{ nmol C}_2\text{H}_4\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$) in *Rhizobium japonicum* 61-A-101 after transfer to special culture conditions (medium 20 P, 2% O₂, 10% CO₂, 88% N₂ in the gas phase) is inhibited by chloramphenicol ($6\times 10^{-4}\text{--}10^{-3}\text{ M}$) and by rifampicin (10^{-5} M). These concentrations do not inhibit the slow growth of the cells under these conditions with a doubling time of the cell protein and living cell number of 3–5 d. Nitrogenase activity of previously derepressed cells is not inhibited by chloramphenicol. Growth of the cells under air in yeast extract-mannitol-glycerol medium (8 h doubling time) is affected significantly more by chloramphenicol ($2.5\times 10^{-4}\text{ M}$) than growth under nitrogenase derepressed culture conditions.

Introduction

For the cowpea *Rhizobium* strain 32 H 1 [1, 2] and the *Rhizobium japonicum* strain 61-A-101 [3] various culture conditions are now available that allow a nitrogenase activity well above $100\text{ nmol C}_2\text{H}_4\cdot\text{h}^{-1}\cdot\text{mg protein}^{-1}$ in pure culture. Systematic studies of combinations of various amino acids, organic acids, sugars and inorganic mineral concentrations showed remarkable differences between the cowpea strain and *Rhizobium japonicum* 61-A-101 in their effect on growth and nitrogenase activity [4–6]. A number of 37 out of 77 strains of *Rhizobium japonicum* and cowpea *Rhizobium* tested on agar surfaces for nitrogenase activity were even completely negative [7], indicating that the right culture conditions have not been found for these strains to fix nitrogen.

The development of an enzyme activity always raises the question, of de novo synthesis or activation of a previously synthesized protein. Synthesis of a new protein can be itself regulated at six or seven different levels [8]. In bacterial cells transcriptional control by synthesis of the cognate m-RNA is the dominating type of regulation. Intracellular protein degradation in growing or starving bacterial cells in regulating the amount of special proteins per cell has additionally gained attention [9]. By use of chloramphenicol and rifampicin as inhibitors of peptidyl-transferase [10] and DNA dependent RNA poly-

merase [11] we studied in the following experiments with *Rhizobium japonicum* whether nitrogenase activity develops also after inhibition of de novo protein synthesis. We tried further to find those inhibitor concentrations, that still allow an undisturbed growth of the cells without losing the potential effect on derepression of nitrogenase.

Materials and Methods

Cultivation of *Rhizobium japonicum* 61-A-101 in suspension culture as nitrogenase repressed and derepressed cells was as described [3]. To evaluate the effect of chloramphenicol and rifampicin (Serva, Heidelberg) on the development of nitrogenase activity, 100 ml cell suspension, grown in medium 20 E [5] was centrifuged, resuspended in 50 ml water and mixed with 500 ml medium 20 N and 50 ml inhibitor solution or 50 ml water to give 600 ml medium 20 P [3]. This suspension was sparged in a 1 l flask continuously with a gas mixture of 2% O₂, 10% CO₂ and 88% N₂ at a rate of $12\text{ ml}\cdot\text{min}^{-1}$ and additionally stirred with a magnetic stirrer (330 rpm). These culture conditions are called "Nitrogenase Derepressing Culture Conditions" (NDCC).

The effect of chloramphenicol on nitrogenase activity of previously derepressed cells was studied by use of suspensions 5 days after transfer to NDCC. To 5 ml samples (5 parallels) inhibitor dissolved in 0.5 ml medium 20 P (or 0.5 ml pure medium) was added.

Assay of nitrogenase activity, protein determinations and microbiological control tests were as described [3].

Results

After transfer from a medium with yeast extract and nitrate as nitrogen sources (medium 20 E) cultivated under aerobic conditions to a special medium with several carbon sources and leucine as single source of combined nitrogen (medium 20 P) sparged with a gas mixture of only 2% oxygen with 10% CO₂ and 88% N₂ cells of *Rhizobium japonicum* 61-A-101 develop after several days a significant nitrogenase activity (Fig. 1). Addition of 6×10^{-4} M chloramphenicol prevents this development (Fig. 1). The same concentration of chloramphenicol does not inhibit the increase in cell protein and turbidity (Fig. 2). Both parameters increase in fact in the cultures with chloramphenicol significantly more, with the largest difference 6 d after transfer to medium 20 P. Also half the concentration of chloramphenicol (3×10^{-4} M) inhibits the derepression of nitrogenase activity in this culture system. Nitrogenase activity of *Rhizobium japonicum* cells, previously derepressed, is not affected by chloramphenicol (Fig. 3). The activity proceeds linearly in the closed test bottles (with 2% O₂ in argon in the gas phase) for two hours with a specific activity of 40–48 nmol C₂H₄ · h⁻¹ · mg protein⁻¹.

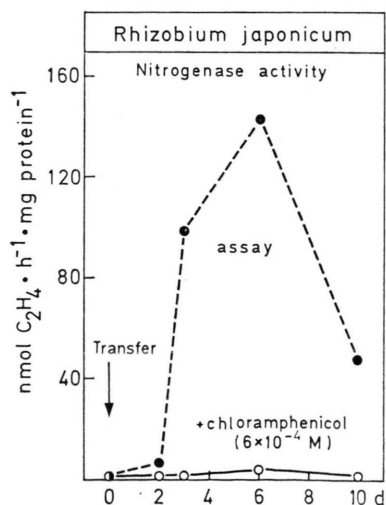


Fig. 1. Effect of chloramphenicol (6×10^{-4} M) on the development of nitrogenase activity in *Rhizobium japonicum* 61-A-101 after transfer from medium 20 E under air to medium 20 P under 2% O₂, 10% CO₂ and 88% N₂ in the gas phase in 600 ml batch culture (28 °C).

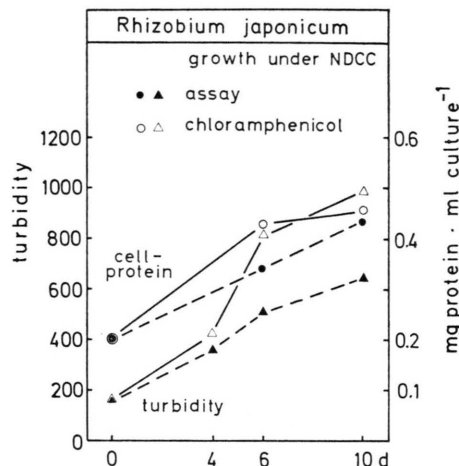


Fig. 2. Effect of chloramphenicol (6×10^{-4} M) on growth (cell protein, turbidity) of *Rhizobium japonicum* 61-A-101, conditions as Fig. 1: NDCC: nitrogenase derepressing culture conditions.

Inhibition of DNA dependent RNA polymerase by rifampicin (10^{-4} – 10^{-5} M) prevents also the development of nitrogenase activity after transfer to nitrogenase derepressing culture conditions (NDCC) (Fig. 4). With the lower concentration of 10^{-5} M a small but significant nitrogenase activity is found only 7–10 d after transfer. Growth measured as optical density is not inhibited by 10^{-5} M rifampicin, but even slightly enhanced (Fig. 5) as in the experiments with chloramphenicol (Fig. 2). Increase in total cell protein is, however, slightly affected.

The concentrations of chloramphenicol used in the experiments described are comparatively high. In a batch culture under air, 2.5×10^{-4} M chlorampheni-

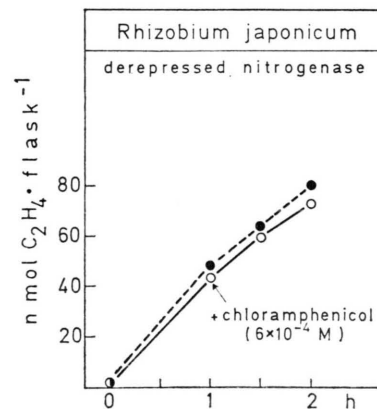


Fig. 3. Effect of chloramphenicol (6×10^{-4} M) on nitrogenase activity of previously derepressed cells of *Rhizobium japonicum* 61-A-101.

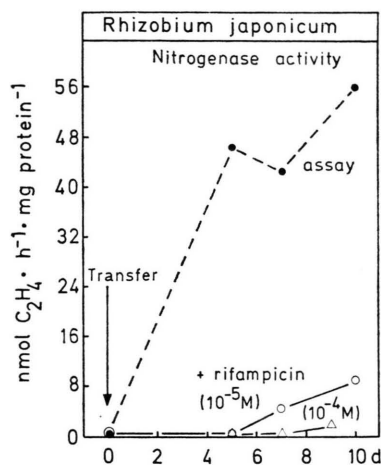


Fig. 4. Effect of rifampicin (10^{-4} and 10^{-5} M) on the development of nitrogenase activity in *Rhizobium japonicum* 61-A-101, conditions as Fig. 1.

col affects the growth significantly only during the first 3 days (Fig. 6). Then the growth rate in the presence and the absence of the inhibitor is almost the same with 8–10 h doubling time. A sixty times lower concentration of chloramphenicol (4×10^{-6} M) stops the growth of *Klebsiella pneumoniae* K 11 almost completely in the same medium at the same temperature (28°C). Within the *Rhizobium japonicum* cultures we observed another differential effect of chloramphenicol. Under aerobic conditions, 2.5×10^{-4} M significantly effect the growth of the cultures (Fig. 6), however under NDCC even 1×10^{-3} M concentrations do not effect the increase in living cell number during a 10 d period (Fig. 7).

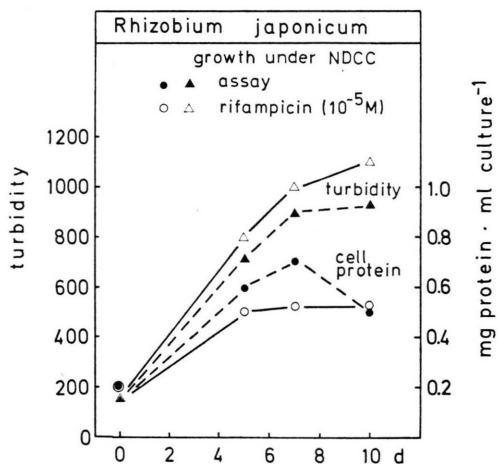


Fig. 5. Growth (cell protein, turbidity) of *Rhizobium japonicum* 61-A-101 in the experiment for Fig. 4.

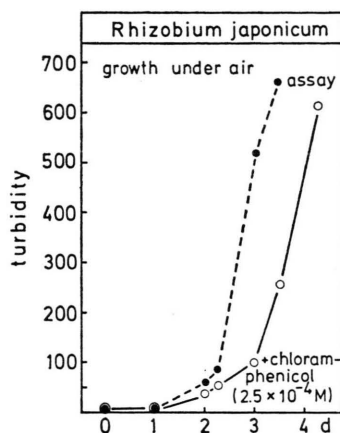


Fig. 6. Growth of *Rhizobium japonicum* 61-A-101 in medium 20 E under air as affected by chloramphenicol (2.5×10^{-4} M).

Discussion

The effect of chloramphenicol and rifampicin on the development of nitrogenase after transfer of *Rhizobium japonicum* cells to special culture conditions indicate a de novo protein synthesis. This finding is consistent with previous reports, that *e.g.* anaerobically grown rhizobia contained neither the molybdenum iron protein nor the iron protein component of nitrogenase [12]. Which of the various factors or factor combinations in the media as oxygen concentration [3], amino acids [5], pentose sugars or organic acids [6] could be considered as essential for depression of nitrogenase is wide open. There is good evidence, that the same biosynthetic pathway *e.g.* for the amino acids synthesized from

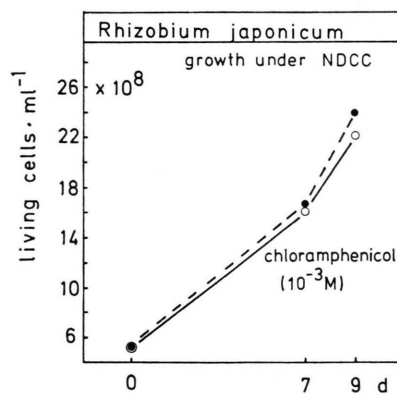


Fig. 7. Growth (living cell number) of *Rhizobium japonicum* 61-A-101 under NDCC as affected by chloramphenicol (1×10^{-3} M).

aspartate are regulated in different bacteria by different mechanisms [13] and the model for the lac repressor [14] is too simple to fit for more complicated biosynthetic pathways and enzymes [15]. Regulation of nitrogenase in *Klebsiella* [16] and *Rhizobium* [3] is affected in a different way e.g. by ammonia and in the relation to glutamine synthetase [17–20]. In the discussion of the pool concept in regulation of enzyme synthesis [15] several examples are given, that exogenously supplied and endogenously synthesized amino acids behave differently in suppression [21, 22]. At present, we have some information about the optimum combinations of exogenous supplied metabolites for nitrogenase in *Rhizobium japonicum* [6], however, we have almost no data about the endogeneous pools in nitrogenase derepressed cells compared to repressed cells. We can assume, that these pools are also effected significantly by any change in the oxygen supply to the cells and by the growth rate of the bacteria.

The inhibitor concentrations used in the experiments of this study are, compared to those used for other bacteria [23] rather high. Nevertheless, they do not inhibit growth and protein synthesis of the cells under nitrogenase derepressing culture conditions. Induced enzyme synthesis is more sensitive to chloramphenicol inhibition compared to constitutive enzymes also in other cells and with

several enzymes [23, 24]. The doubling time of *Rhizobium japonicum* 61-A-101 under nitrogenase derepressed culture conditions (NDCC) with oxygen limitation and leucine as sole source of fixed nitrogen is 3–5 days, compared to about 8 h under aerobic conditions in yeast extract mannitol medium. This difference in growth rate is correlated with the effect, that under NDCC 1×10^{-3} M chloramphenicol is not inhibiting growth, whereas cells from the other culture conditions are significantly affected by 2.5×10^{-4} M concentrations. A selection of chloramphenicol resistant cells after transfer to NDCC is unlikely, since it has been shown, that this is a result of several cooperative gene mutations [25]. Somewhat more effective chloramphenicol degrading enzymes [26] in *Rhizobium japonicum* under NDCC compared to fast growing cells under air is a hypothesis, which has to be proven in other experiments. A reduced K_0 value for uptake of the inhibitor, as demonstrated for the uptake of 2-oxoglutarate and L-glutamic acid in bacteroids [27] of *Rhizobium japonicum* compared to free living cells is also a possibility, that has to be studied.

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