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Dissimilatory nitrate reduction processes in *Bacillus azotoformans* LMG 9581^T

INTRODUCTION

Genome analyses of the soil organism *Bacillus azotoformans* LMG 9581^T, a phenotypically denitrifying strain, surprisingly revealed genes encoding all necessary enzymes for denitrification and dissimilatory nitrate reduction to ammonium (DNRA), previously thought mutually exclusive pathways. In addition, it is devoid of the assimilatory nitrate and nitrite reductase genes (no *nirBD*), suggesting that DNRA might serve nitrogen assimilation. However, it also lacks an ammonium-transporter gene (AmtB-type). So we suppose that ammonium in the media can't be utilized and DNRA, if functional, it's only used for energy conservation and not for assimilation. To confirm this, growth experiments were performed both anaerobically and aerobically in various growth media with excess of carbon compared to nitrogen and with/without ammonium.

MATERIALS & METHODS

Topological model of enzymes involved in dissimilatory nitrate reduction (DNR) pathways

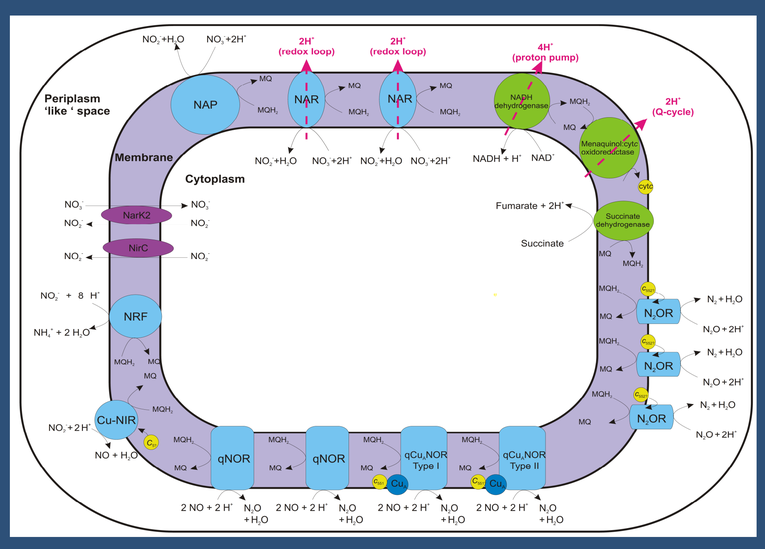

Figure 1. Menaquinol (MQH_2) donates electrons to a menaquinol:cytochrome c oxidoreductase (related to bc_1/b_6f), NAR, NIR, NRF, NOR and N_2OR. Reduction of all the membrane-bound *c*-type cytochromes is coupled with menaquinol oxidation mediated by the menaquinol:cytochrome *c* oxidoreductase. Membrane-bound *c*-type cytochromes subsequently donate electron to their corresponding enzymes.
The location of the substrate-binding sites are hypothesized to be similar to other bacteria. Enzymes involved in respiratory nitrate reduction are depicted in blue. Nitrate/nitrite transport system are in purple. Menaquinol:cytochrome *c* oxidoreductase (related to bc_1/b_6f), NADH dehydrogenase and succinate dehydrogenase are in green. Charge displacements contributing to the proton motive force are in pink. Cytochrome *c* lipoproteins are in yellow.
Deduced from genome data or taken from Suharti & De Vries (2005).

Hypothesis 1: DNRA is not for nitrogen assimilation

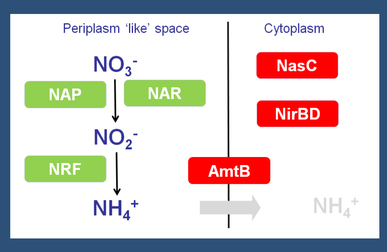

Figure 2. Enzyme (complexes) in green are present in the genome of *B. azotoformans*, in red are absent. Without genes coding enzymes for assimilation (NasC, NirBD), and the genes of ammonium transporter (AmtB-type), it's suggested that *B. azotoformans* is not able to use NH4+ from periplasm as nitrogen source. Therefore, the DNRA, if functional, it's only used for energy conservation, not for assimilation.

Hypothesis 2: energy conservation anaerobically is based on denitrification and DNRA

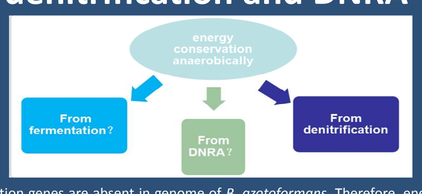

Figure 3. Fermentation genes are absent in genome of *B. azotoformans*. Therefore, energy supporting growth of *B. azotoformans* may only come from dissimilatory nitrate reduction process , including denitrification and DNRA.

Growth experiments

- Anaerobic
- mineral media ± NH_4^+ ± yeast extract
- Measurement of OD_{600}, NO_3^-, NO_2^- , NH_4^+ , N_2O

- Aerobic and anaerobic
- Tryptone soy broth(TSB), nutrient broth(NB) ; mineral media ± NO_3^-
- Measurement of OD_{600}, NO_3^-, NO_2^- , NH_4^+ , N_2O

RESULTS

Aerobic growth experiment

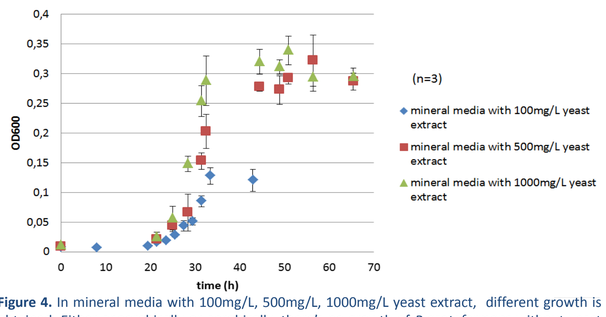

Figure 4. In mineral media with 100mg/L, 500mg/L, 1000mg/L yeast extract, different growth is obtained. Either anaerobically or aerobically, there's no growth of *B.azotoformans* without yeast extract in mineral media , neither replacing with Vitamin Mix, vitaminB1,B2,B12 (data not shown).

Anaerobic growth experiment

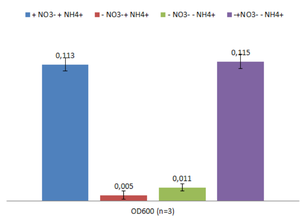

Figure 5. In mineral media with nitrate and ammonium, highest OD600 is obtained after 4 days' growth; while in mineral media with nitrate without ammonium, similar highest OD600 is obtained after a slower growth of 14 days. No obvious growth is obtained in media without nitrate.

Yeast extract composition (%, w/w):

- B-complex vitamin;
- Amino nitrogen 5.1;
- Sodium chloride 0.3

1. Yeast extract is proved pivotal and unreplaceable for growth of *B.azotoformans*.

- Same growth is obtained in media without ammonium.
- No ammonium consumption is observed during aerobic and anaerobic growth.
- No obvious growth is obtained in media without nitrate.
- Low concentration Yeast extract(100mg/L) is included in media which is necessary.

2. Ammonium can not be consumed as nitrogen source for assimilation supporting growth of *B.azotoformans*.

N equilibrium of anaerobic growth

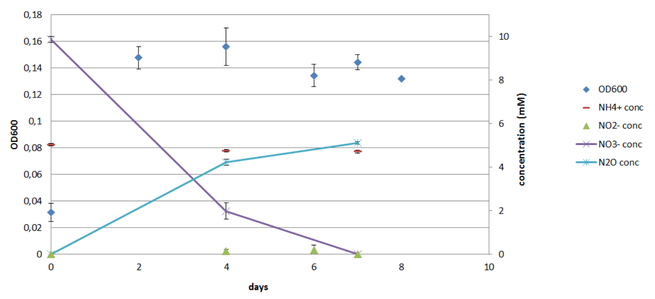

Figure 6. *B.azotoformans* grows anaerobically with 4.6mM NH4+, 10mM NO3-, 100mg/ml Yeast extract, 60mM sodium acetate in the mineral media(n = 3). After 4 days, it reaches stationary phase while denitrification continues until initial 10mM NO3- is completely consumed after 7 days. No obvious consumption of NH4+ is observed. No NO2- is measured at sampling time. NO3- is consumed during growth and all converts to 5mM N2O after 7 days.

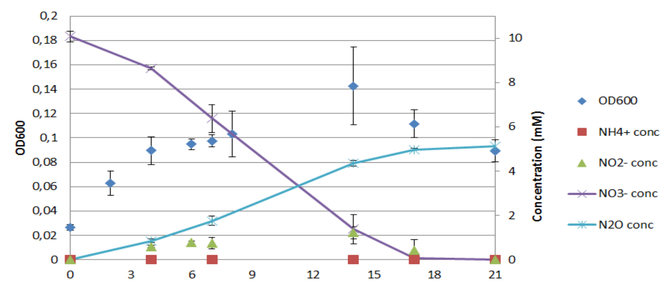

Figure 7. *B.azotoformans* grows anaerobically with 10mM NO3-, 100mg/ml Yeast extract, 60mM sodium acetate in the mineral media without NH4+). OD600 value goes up and starts to fall down after 14 days, while denitrification continues until initial 10mM NO3- is completely consumed after 21 days. No obvious consumption of NH4+ is observed. low amount of NO2- is measured at sampling time. NO3- is consumed during growth and all converts to 5mM N2O after 21 days.

- Growth of . *B.azotoformans* with and without ammonium reached same cell densities but it showes an extended lag phase if ammonium is absent in the media.
- During anaerobic growth of . *B.azotoformans* , initial nitrate all converts to nitrous oxide
- No ammonium is produced, DNRA doesn't take place in experimental conditions.

3. DNRA doesn't take place in *B.azotoformans* under experimental conditions performed.

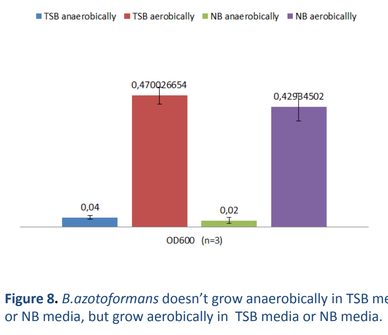

Figure 8. *B.azotoformans* doesn't grow anaerobically in TSB media or NB media, but grow aerobically in TSB media or NB media.

5. *B.azotoformans* can not ferment in TSB or NB media.

CONCLUSIONS & FUTURE PERSPECTIVES

Concluding remarks

- Yeast extract is proved pivotal for growth of *B. azotoformans*, probably provide as organic nitrogen source.
- No ammonium is consumed for assimilation. This confirms the absence of an ammonium transporter in *B. azotoformans.*
- DNRA, if expressed in *B. azotoformans*, is only for energy conservation, not for assimilation.
- Dissimilatory nitrate reduction is the only process that contributes to energy conservation of *B. azotoformans* in anaerobic condition.

Future perspectives

- Other conditions favouring DNRA will be tested to verify whether DNRA exists in *B. azotoformans* or not.

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