

MICROBIAL DIVERSITY SUMMER CORSE 1995

Research project title:

“The effect of respiration on the phototactic behaviour of the purple nonsulfur bacterium *Rhodospirillum centenum* “

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INTRODUCTION

In a recent review on phototaxis and other sensory phenomena in purple photosynthetic bacteria, Howard Gest (1) reports and interesting consideration by Presti and Delbruk (2) about the use of light by living organisms: “ as a source of *energy* and as a means of obtaining information about their environment”. Photosynthetic organisms would therefore be expected to use light for sensory functions as well as for energy-requiring processes, and many are know to exhibit the phenomenon of phototaxis, defined as the oriented movement of a locomotive organisms with respect to the *direction* of light. The movement can either toward a light source (positive phototaxis) or away from the source (negative phototaxis).

It has long been proposed that phototaxis provides the photosynthetic organisms with the capability of orienting itself at light intensities optimal for growth. However, the fact that phototxis has been observed also in non-photosynthetic organisms, e.g. *Isosphaera pallida* (3), clearly indicates that this phenomenon is *per se* independent of “photosynthesis”. It is evident that the *energy* required for light-induced movements can be supplied in various ways, namely: by photosynthetic electron transport, by respiration and, possibly, by fermentation.

Phototaxis has been studied and unambigously documented in algae and cyanobacteria (2); surprisingly, in over a century of research on this topic, the phototactic behaviour of a nonsulfur purple bacterium, *Rhodospirillum centenum*, has clearly been demonstrated only in 1994 (4). Indeed, “swarm agar-colonies” of *Rsp.centenum* are capable of macroscopically visible phototactic behaviour. This is a remarkable and unique feature of this bacterial species and it is paralleled by a production of numerous lateral flagella (5).

In this study we analyze the effect of respiration on the phototactic behaviour of *Rsp.centenum*. To this purpose we have been using both biochemical and genetic approaches, namely: a) detection of the respiratory capability of “single agar-

colonies" by means of a oxygen microelectrode (see Revsbech 1986) under variable respiration-inhibited conditions either in the light or in the dark; b) correlation of the respiratory activities by isolated membranes, with the "photomovement" of the agar-colonies c) isolation of mutants altered in respiratory electron transport obtained by Tn5 transposon mutagenesis with an IncP plasmid vector as described by Yildiz et al. (1991).

MATERIALS AND METHODS

1. Bacterial strains, media and routine growth conditions.

The bacterial strains used in this study were *Escherichia coli* SM10/ Lpir/ pTn5-spR and *Rhodospirillum centenum* SW (ATCC 51521) and YB707 (bacteriochlorophyll deficient mutant). *E. coli* cells were grown at 37°C in Luria broth (7). For liquid dark-aerobic growth, cells of *Rsp. centenum* were cultivated at 42°C in either 30ml or 1 liter flasks using PYVS (0.3% peptone, 0.3% yeast extract, 0.4% soytone, 20ng/ml vitamin B12 and 1.5 µg/ml biotin) medium as described previously (5).

2. Membrane isolation procedure, respiratory assays.

Cells of *Rsp. centenum* SW and YB707 were grown in CENMED medium as previously described (5) and harvested at late log phase (0.8-0.9 A at 660nm) and cells resuspended in MOPS (pH7.4), MgCl₂ 10mM buffer at approx 0.1g (wet wt)/ml. Membrane fragments were prepared by following the French-pressure cell method (see 8).

Respiratory activities were determined by optical spectroscopy as described previously (8).

3. Mutagenesis.

Transposon mutagenesis was accomplished by using a transposon delivery system of *E. coli* involving mobilizable IncP plasmids delivering resistance to spectinomycin; as reported, *Rsp. centenum* is naturally resistant to high levels of kanamycin. Coniugal transfer of the IncP plasmid was performed by mixing appropriate aliquots of donor and recipient (8:1 and 10:1 ratios) and then concentrating them by filtration through a 0.45 µm-size-pore filter (Gelman GN-6). The filter was placed onto PYVB agar medium, incubated at 37 C for 24 h and then washed with 3ml of PYVB medium. 0.2 ml of the resuspended cells were then plated onto selective plates (PYVS + Km 50µg/ml + Sp7.5µg/ml + 1.5% agar). The plates were incubated at 37°C for 60 h to obtain isolated colonies. The colonies were subsequently streaked out on CENMED agar plates (+Km 50µg/ml + Sp 7.5µg/ml) to further reduce the *E. coli*-background. Respiratory deficient mutants were selected through the use of the NADI-oxidase test (alfa-naphthol+DMPD) which specifically identify colonies defient in cyt c oxidase activity

4. Oxygen microelectrode technique.

Oxygen consumption by single swarm colonies was measured using a Clark-microelectrode (diameter of the tip approx 12 μ m) by essentially following the procedure described by Revsbech (9). This electrode is insensitive to stirring and allows to determine oxygen variations within a 1/2time of 0.4sec.

RESULTS AND DISCUSSION

The photosynthetic purple nonsulfur bacterium *Rsp.centenum* can grow either in illuminated anaerobic environment by using photosynthetic electron transport to generate an electrochemical proton gradient or heterotrophically in dark aerobic conditions by using respiratory electron transport. In contrast to other purple non sulfur species, e.g. *Rb.capsulatus* and *Rsp.rubrum*, in which the synthesis of the photochemical apparatus is controlled by the oxygen partial pressure (see Zannoni 1995) *Rsp.centenum* synthesizes a functional photosystem under both aerobic and anaerobic conditions (Yildiz et al. 1991). This peculiarity allows to observe light-driven colony mobility (phototaxis) under both aerobic and anaerobic conditions. For simplicity, we conducted all of the mobility experiments shown here, under aerobic conditions at 37-40°C (light intensity of 3 μ E m⁻² sec⁻¹).

It has previously been shown that in a low light intensity (approx 5 μ E) the so called "phototactic" response of *Rb.sphaeroides* cells is negatively affected by oxygen (Armitage et al. 1985). Although the phototactic behaviour of this bacterial species can more properly be described as a "scotophobic" effect since the cells are moving toward a gradient of light intensity, it is apparent that under non-saturating light intensities (\ll 50 μ E) oxygen affects the cell movement. This conclusion is in line with an early study (Rugolo and Zannoni 1983) on intact cells of semiaerobically dark-grown *Rb.capsulatus*, demonstrating that the light-generated membrane potential is inhibited by respiratory electron transport. The rationale for this result is that photosynthetic and respiratory apparatuses are intermingled in such a way they share a series of redox components, i.e. soluble cyt c and ubiquinone molecules.

The results obtained in both *Rb.capsulatus* and *Rb.sphaeroides* predict that if the phototactic response of *Rsp.centenum* strictly depends upon the electrochemical proton gradient generated by light, inhibition of respiration would enhance the locomotive capacity of this bacterial species.

1. The redox chain of aerobically dark-grown *Rsp.centenum*.

1.1 Spectral analysis.

Figure 1 shows the reduced-minus-oxidized difference spectra of both isolated membranes and the 140.000xg supernatant from dark-grown cells of *Rsp.centenum*. It is apparent that the "soluble" fraction contains a consistent amount of cytochrome(s) c (peak at 552 nm and a shoulder at 550.5 nm) while the membrane fraction shows the presence of both c- (peaks at 552 and 556nm) and b-

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type haems (peaks at 558 and 560nm). Using extinction coefficients of 19mM^{-1} and 22mM^{-1} for cyt *c* and *b*, respectively, we calculated that also isolated membranes contains high amounts of *c*- and *b*-cytochromes (2.9 and 1.8 nmol mg prot⁻¹, respectively); notably, the amount of *c*-type haem released in the 140.000xg supernatant during the cell-fractionation by mechanical treatment was equal to the one present in intact membranes (see Materials and Methods). The latter finding strongly suggests that intact cells of *Rsp.centenum* are endowed with a periplasmically-located cytochrome(s) *c* which is likely to be involved in connecting the redox-chain to the photosynthetic reaction center and/or to a high-potential membrane-bound oxidase. This conclusion is supported by evidence that addition of Na-ascorbate ($E_m = +65\text{mV}$), which poises the redox-potential of an open-cuvette sample at approx $+150\text{mV}$, reduces 1/3 of the membrane-bound cyt *b* and 90% of the soluble *c*-type cytochrome(s) (see trace c).

1.2 Respiratory activities

Table 1 shows a series of respiratory activities measured in isolated membranes from aerobically dark-grown *Rsp.centenum*.

Table 1.

Electron donors	Inhibitors	Electron acceptors	Activities (*)	% of the control
NADH	/	oxygen	1.60	100
NADH	rotenone	oxygen	0.12	8
NADH	antimycin A	oxygen	0.47	29
NADH	myxothiazol	oxygen	0.32	20
NADH	/	cyt c ^(a)	3.03	100
NADH	rotenone	cyt c ^(a)	0.59	19
NADH	antimycin A	cyt c ^(a)	1.57	52
NADH	myxothiazol	cyt c ^(a)	0.60	20
Cit C ^(b)	/	oxygen	25.80	100

(*) Activities are expressed as μ moles of either electron donor or acceptor h⁻¹ mg of protein⁻¹

(a) Measured in the presence of 5mM cyanide to avoid cyt *c* oxidation after reduction by NADH.

(b) Horse-heart cytochrome *c* was 80% reduced by addition of Na-ascorbate.

Additions and non-standard abbreviations: NADH, 0.1mM; cyt *c* (horse-heart cytochrome *c* SIGMA) 0.1mM; rotenone and myxothiazol, 1 μ M each; antimycin A, 3 μ M.

The data of Table 1 clearly show that membranes from dark-grown *Rsp.centenum* present a NADH oxidase activity which is fully sensitive to rotenone (1 μ M) and partially sensitive to antimycin A and myxothiazol, two specific inhibitors of complex III. This suggests that the redox chain is endowed with a ND-1 type dehydrogenase delivering reducing equivalents to an orthodox bc1-redox complex (Gennis et al. 1993). On the other hand, the presence of a significant residual respiratory activity insensitive to both antibiotics is indicative for the presence of a branched chain in which one of the two branches is formed by a membrane bound quinol oxidase (see Zannoni 1995).

In Fig.2, the patterns of the NADH and cyt c oxidation activities as a function of increasing concentrations of cyanide, are shown. It is evident that oxidation of exogenously added reduced-cyt c is completely inhibited by 50 μ M cyanide, a concentration which considerably reduces (80%) also the NADH consumption, the remaining 20% of activity being inhibited only by 5-10mM cyanide. Notably, the respiratory inhibitor NaN₃ is far less efficient in inhibiting the total respiratory activity of *Rsp.centenum* (75% inhibition at 1-2mM) being however more specific in blocking the cyt c oxidase activity although used at mM concentrations.

2. Phototactic response of *Rsp.centenum*.

The experiments to test the swarming properties of *Rsp.centenum* (reported in Table 2) were performed as following: cells (1ml harvested at 0.9 A) were concentrated and resuspended in 40 μ l of PYVS medium. For inhibition experiments, the inhibitors were incubated approx 5 min at room temperature before centrifugation. The concentrated cells (40 μ l) were placed onto side of PYVS squared-plates and let the spot dry for a few minutes at room temperature. The plates were subsequently incubated in the dark at 42°C for 4 h before subjecting them to light source (3 μ E m⁻¹ sec⁻¹) at approx 37-40°C for 3 h. As previously described, the dark incubation period is required to induce formation of lateral flagella (Ragatz et al. 1995). The approx swarming rates of wild type cells were 10-15 mm/h on 0.8% agar-PYVS plates. Occasionally we observed swarming rates of approx 20 mm/h.

The results summarized in Table 2, rows 2 and 5, indicate that the swarming capability of *Rsp.centenum* is strongly repressed by those inhibitors affecting the photosynthetic electron transport such as antimycin A and myxothiazol. Conversely, Na-azide which inhibits the cyt c oxidase (see Table 1) significantly stimulates (120%) the swarming capacity of *Rsp.centenum*. This indicates that in the light the oxygen consumption by the respiratory electron transport reduces the swarming rate of this bacterial species so to suggest that respiration plays a minor role in phototaxis. This is confirmed by the data obtained with YB707 cells, a mutant which is proficient in aerobic growth but it has also no phototactic response (Tab. 2); thus, the light-dependent electron flow is required for locomotion of

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Rsp.centenum. In this respect, the effect of rotenone (20% inhibition) is interesting because rotenone is not expected to affect photosynthetic electron transport unless to consider that it might also inhibit the light-dependent formation of NADH (by reversal electron flow) which is presumably required for the cell movement. It is noteworthy that addition of asc/DCIP slightly reduces the swarming rate of the cells; this is consistent with the fact that reduced DCIP is likely to stimulate respiration (see below, paragraph 3).

Table 2.

Swarming rates of *Rsp.centenum* w.t. and YB707 strains on 0.8% agar-PYVB plates. The rates are expressed as mm/h

Additions (concentrations)	STRAINS		
	W.T.	% of the control	YB707
1. ----	14.0	100	0.0
2. antimycin A (5µM)	7.4	53	/
3. Na N ₃ (10mM)	16.8	120	/
4. Rotenone (10µM)	11.2	80	/
5. Myxothiazol (5µM)	0.0	0	/
6. CCCP (10µM)	0.0	0	/
7. Myxo+ Asc/DCIP	0.1	0	/
8. Asc/DCIP	10.1	72	/

The data are the average of two independent sets of experiments

3. Effect of light on respiration of swarming colonies on agar-plates as detected by a oxygen microelectrode.

Changes in oxygen concentration occurring within a swarm colony on agar plates during light-dark cycles can be analyzed using a oxygen microelectrode (see Revsbech and Ward 1983). A series of oxygen profiles obtained by quickly advancing the electrode (tip size of 12µm, 80% response time 0.5sec) from the colony surface and downward, stopping for a 3s period at 25µm intervals, was performed. The average depth of the colonies was approx 100µm and the electrode was fixed at 50-70µm depth corresponding to a oxygen concentration of 80-130µM. As previously described (see Rev. by Revsbech and Jorgensen 1986) the slope of the oxygen profile results from the difference between the rate of the oxygen diffusion and the rate of oxygen consumption by the cells on condition that the depth of the agar-colonies does not vary. The oxygen profile of myxothiazol treated cells was considerably different (decrease of the slope-profile of 38%) from the control while a

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slight, but significant increase (120%) of the slope-profile was seen in the presence of the protonophore CCCP and of the redox couple ascorbate/DCIP (not shown). Figure 3 shows the oxygen pattern by the swarming colonies when subjected to short periods (30 sec) of IR light ($0.3 \mu\text{E m}^{-2} \text{sec}^{-1}$). As expected, light has a strong inhibitory effect on respiration by the swarming colony resulting in a rapid increase of the oxygen concentration (from 95 to $148 \mu\text{M}$) around the microelectrode tip (approx $10 \mu\text{m}$ radius). Conversely, in myxothiazol treated colonies (no phototactic response, see Tab.2), the light-period seems to stimulate the respiratory rate so that the oxygen concentration drops from 81 to $56 \mu\text{M}$; this result was confirmed by the pattern shown in trace (d) in which the oxygen concentration of myxothiazol treated cells is strongly reduced by light in the presence of ascorbate/DCIP (from 110 to $64 \mu\text{M}$). Conversely, when the cells were treated with the uncoupler CCCP (no phototaxis, see Tabl.2) the respiratory activity of the colony was still inhibited by light leading to an increase in oxygen concentration (trace c, from 75 to $114 \mu\text{M}$). This latter finding is clearly in contrast with the proposal that inhibition of respiration by light is due to a thermodynamic control upon the rate of oxygen consumption (Cotton et al. 1983) but instead it supports the idea that in some circumstances reducing equivalents may switch between photosynthetic and respiratory components (Zannoni et al. 1978; Rugolo and Zannoni 1983).

4 . Transposon mutagenesis

The transposon mutagenesis protocol described under Materials and Methods led to isolate a series of colonies carrying both kanamycin and spectinomycin resistance. However, the low frequency (5-6 pigmented colonies per plate) and the strong background on PYE plates suggested to select for those colonies able to grow on CENMED agar (Km50+Sp7.5) plates in the dark. A screening for NADI-negative (pigmented colonies) was performed and two mutants that presented pink and brown colonies, respectively, were streaked out on CENMED-agar plates and let them incubate at 37°C for 6 days. After this period we obtained two different types of colonies, both NADI-minus, of approx 1mm (brown) and 0.3mm (pink) diameter with a very poor background. No further characterization of these mutants has been performed (the Microbial Diversity summer course was over!!).

CONCLUSIONS

An important conclusion of this work is that the positive phototactic behaviour of *Rsp.centenum* is negatively affected by respiration. This result, obtained through the use of the oxygen microelectrode technique (Table 2 and Fig.3) strongly suggests that photosynthetic electron flow is essential for cells movement as also demonstrated by the lack of phototaxis of the bacteriochlorophyll deficient mutant YB707 which is however proficient in dark-growth. We have also been able to demonstrate that the facultative phototroph *Rps.centenum* synthesizes a branched respiratory chain when grown aerobically in the dark. A variety of different branched respiratory chains has been found in both facultative phototrophs, such as for examples *Rb.capsulatus* and *Rsp.rubrum*, and aerobes (see

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Zannoni 1995). The high-amount of membrane-bound c-type cytochromes (Fig.1) found in *Rsp.centenum* suggests the presence of a c-type tetrahaem/reaction center; this type of reaction center resembles that of *Rps.viridis* in which the role of the periplasmically located loosely bound cyt c is however still a matter for debate due to its low concentration. Thus, *Rsp.centenum* could be the first facultative phototroph in which the interaction between soluble-c and the c-tetrahaem/RC could hopefully be demonstrated easily due to the presence of high-concentrations of soluble c. Another aspect to be further investigated is the effect of the oxygen tension on the synthesis of cytochromes since we know that, in contrast to other facultative phototrophs, the pigment synthesis by *Rsp.centenum* is naturally insensitive to oxygen.

Acknowledgements

I would like to thank my wife (Michela) and my wonderful son (Luca) for giving me the opportunity to spend 47 exciting days at the MBL (Woods Hole) working at the bench after so long time!

I want also to thank all persons (faculty staff and TAs) involved in the Microbial Diversity summer course for their advice, competence and ... patience.

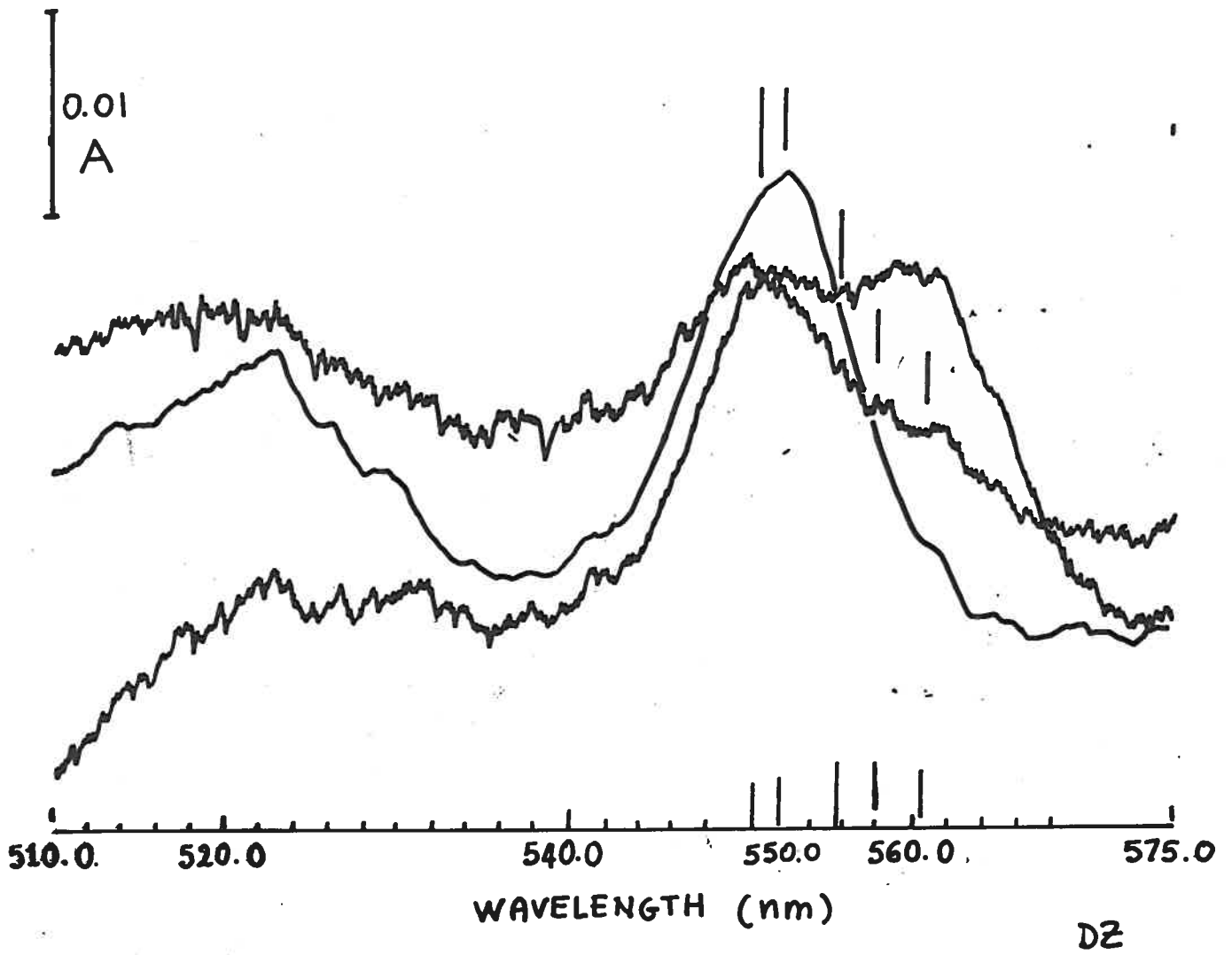
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July 24 1995....."the game is over, no more time to play!"

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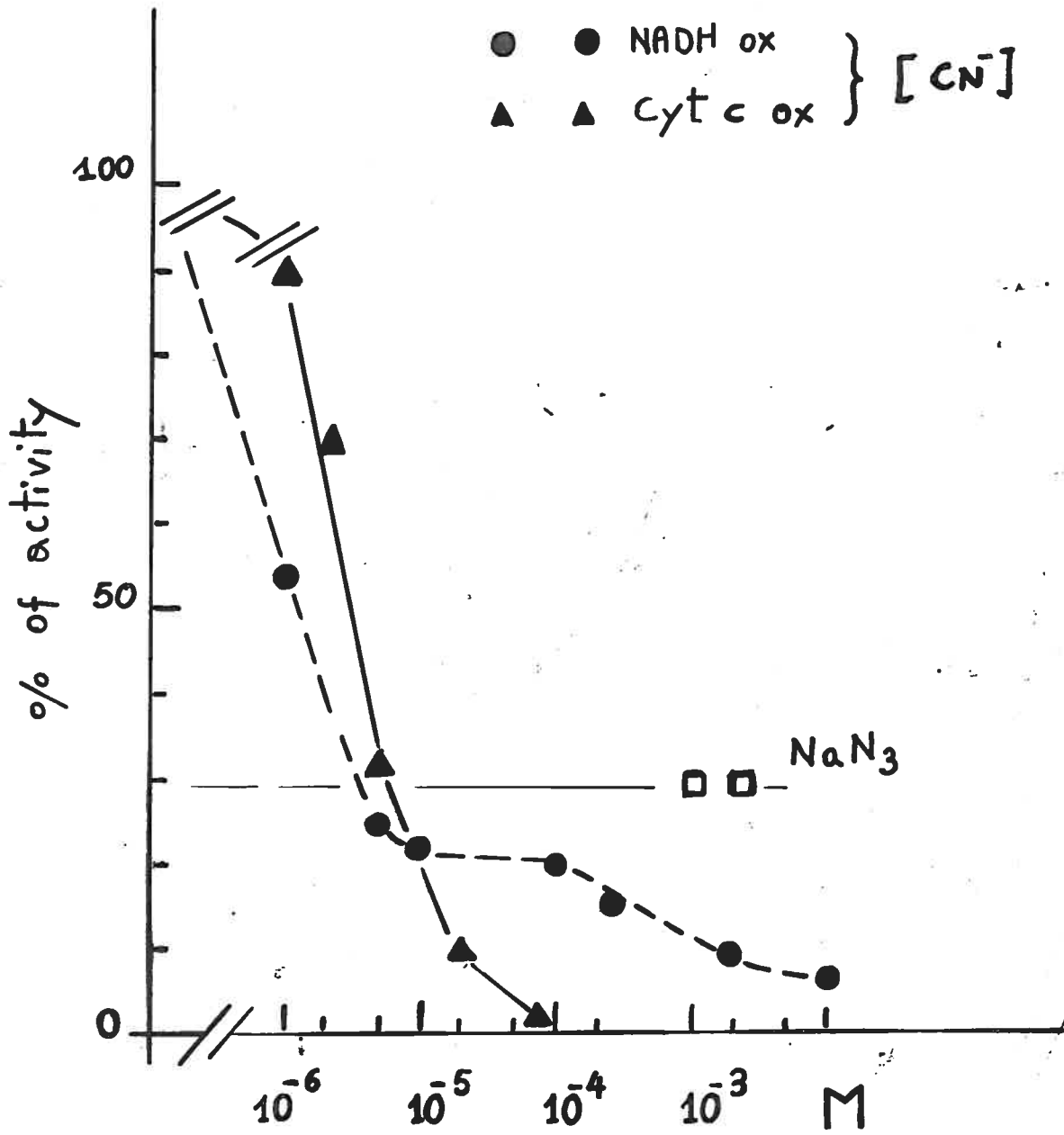
Fig 1



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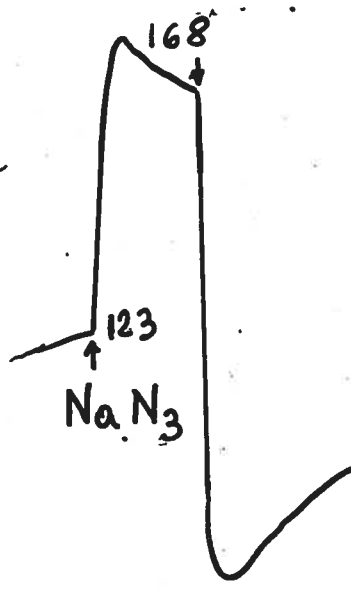
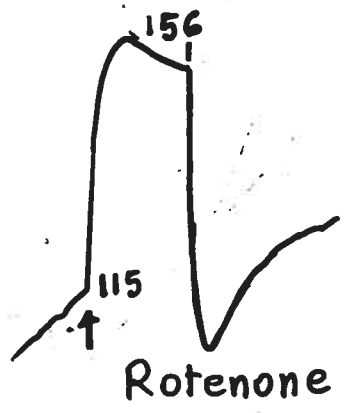
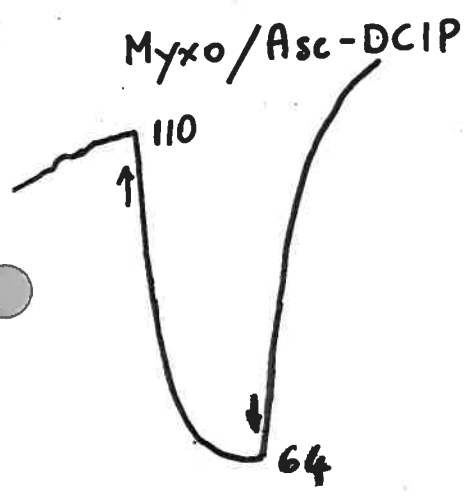
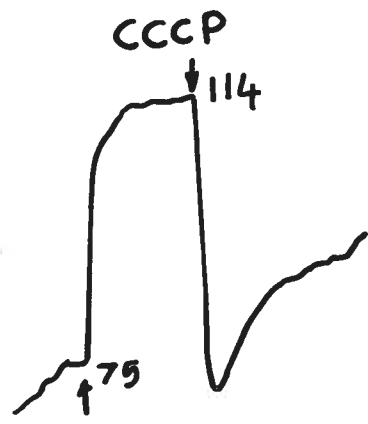
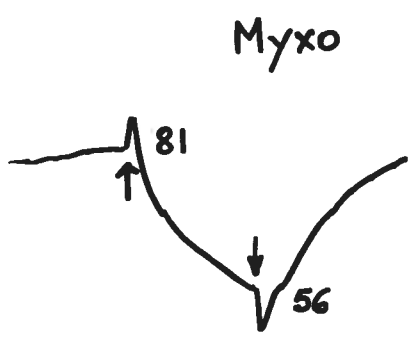
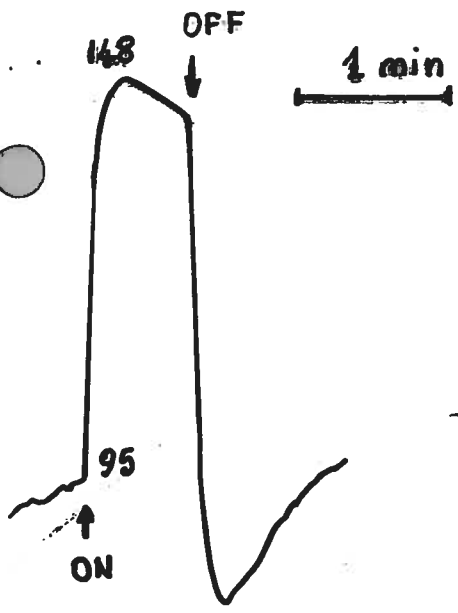
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Fig. 2



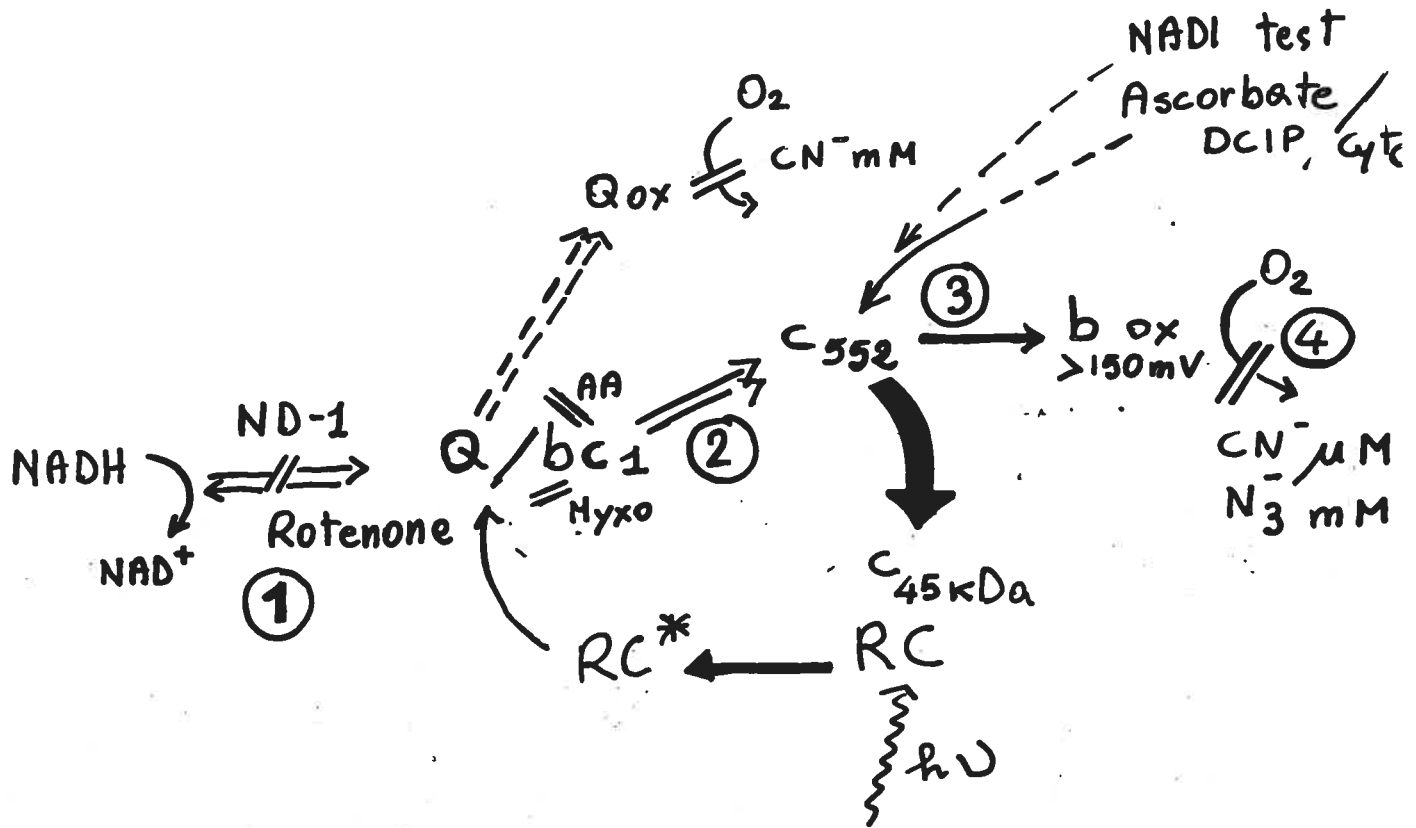
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tig3



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Fig. 4



Asp. centenum redox chain
D. Zannoni 7/27 '95 WH

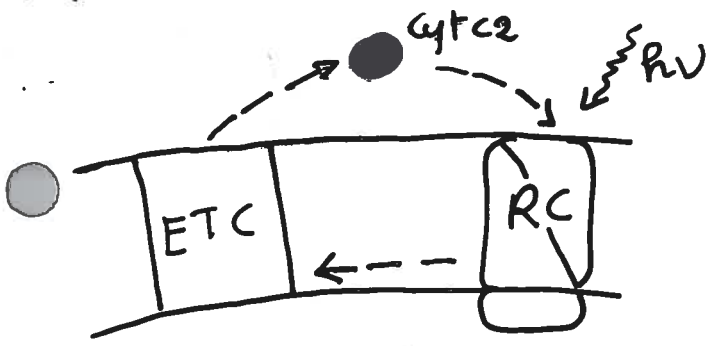
Redox steps affecting positive phototaxis

- ① Slight inhibition
- ② Complete inhibition
- ③ Slight inhibition
- ④ Stimulation

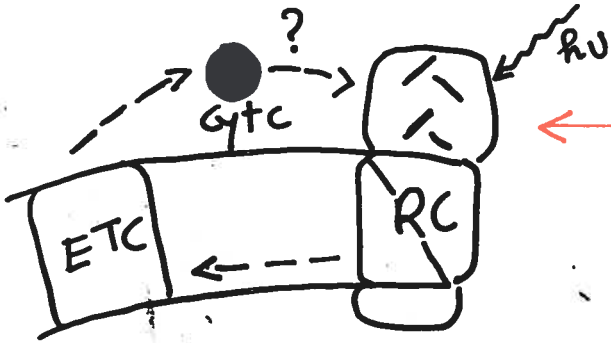
CONCLUSION

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Respiration through the cytochrome oxidase pathway inhibits *Aspergillus centenum* phototactic movement.

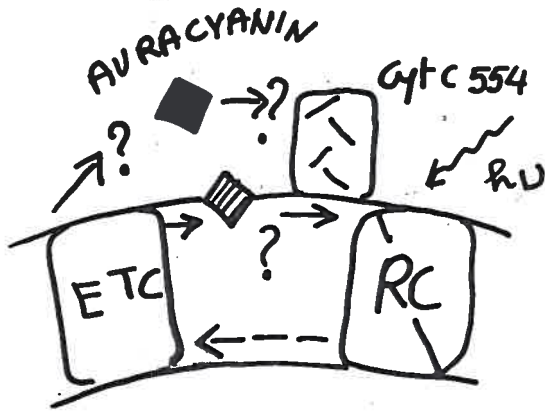


Rb. sphaeroides
Rsp. rubrum

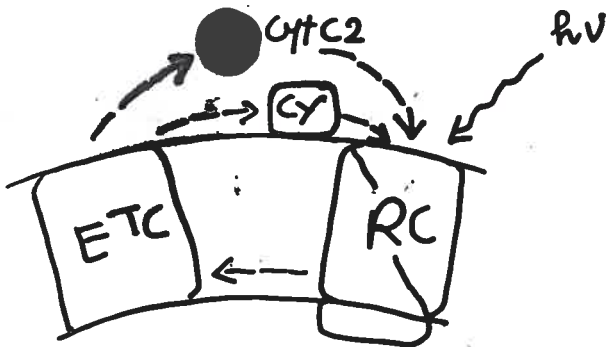


Rsp. centenum

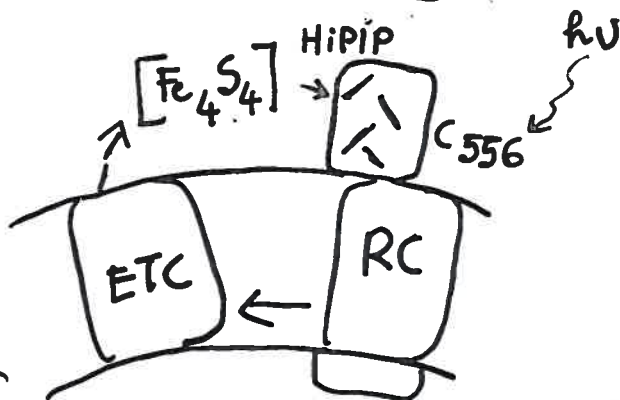
Rps. viridis



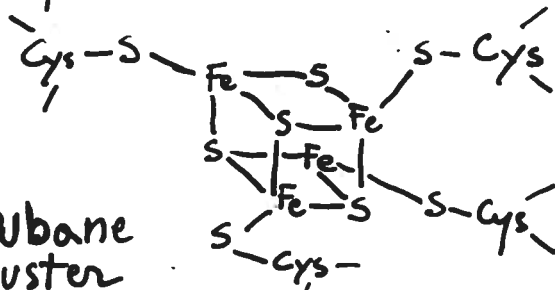
Chloroflexus aurantiacus



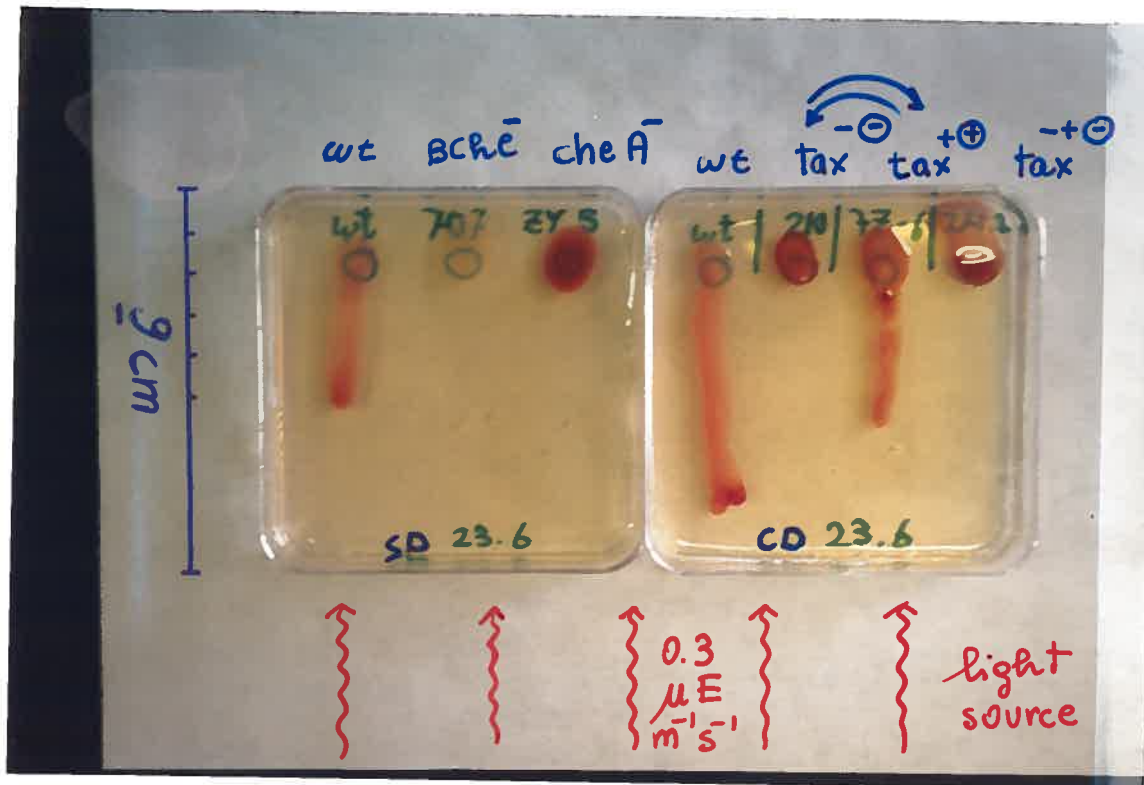
Rb. capsulatus



Rhodospirillum rubrum



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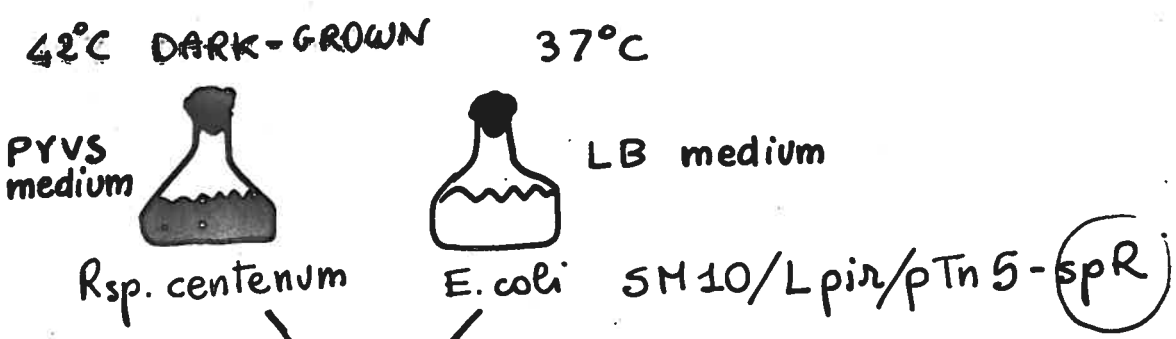


Legend

- wt, *Rsp. centenum* wild type
- 707, BChl deficient mutant
- ZY5, cheA deficient mutant
- YB 2-10, deficient in negative taxis
- YB 77-6, deficient in positive taxis
- YB 271-2, deficient in both positive and negative taxis

Note : labels of 77-6 and 271-2 mutants were mixed up.

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* 8:1 or 5:1
 mating-filter

PYVS plate
 24 hr - 37°C

resuspend and rinse with
 PYVS medium

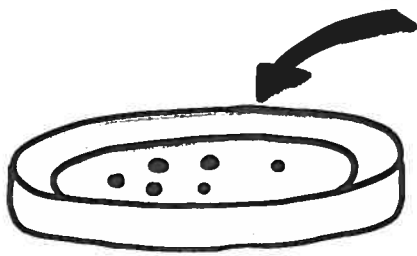
0.2 ml n times !!!

spreading on PYVS (Kanamycin 50 µg/ml
 Spectinomycin 7.5 µg/ml)

5-6 pigmented colonies each plate
 plus a strong background of E. coli and possibly

* control plates
 8:1 and 5:1
 Rsp. centenum/
 E. coli
 mix

07



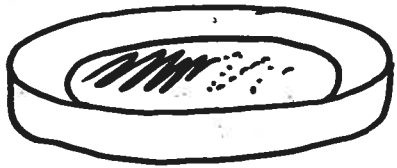
NADI-reaction

(α -naphthol + DMPD)

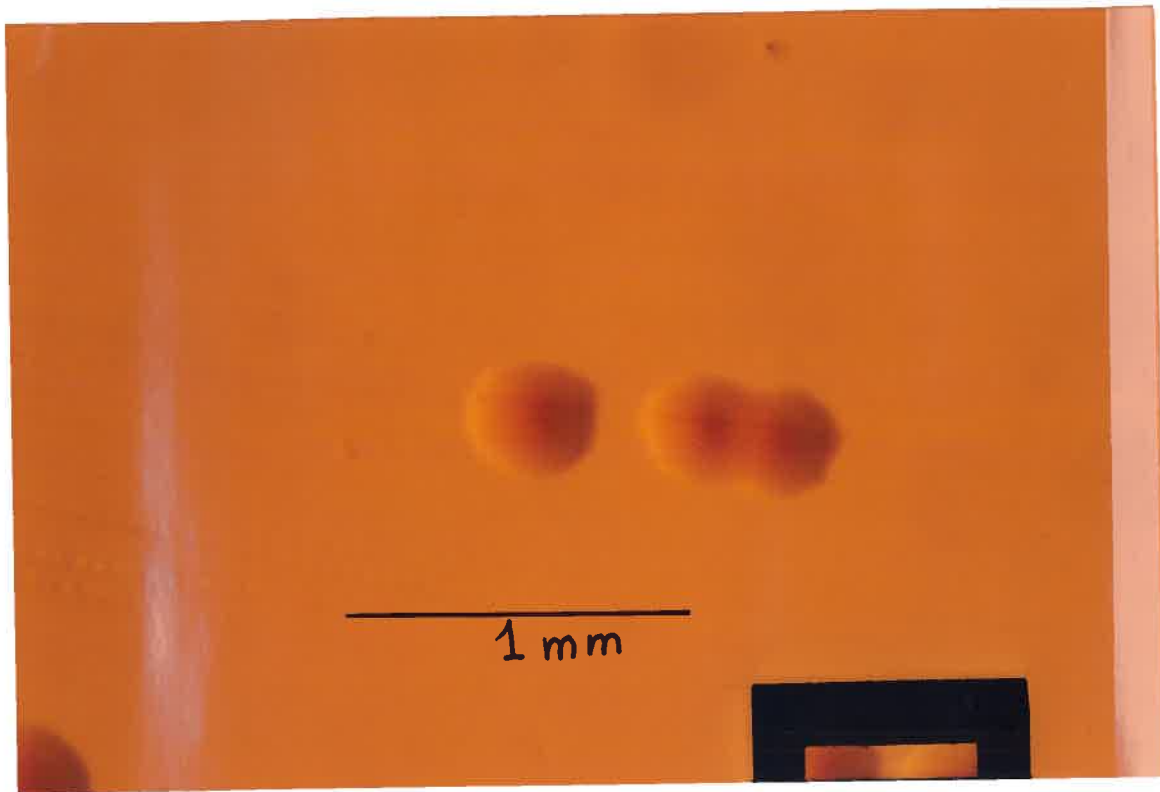
avoid blue colonies and pick up
(if any) pigmented brown-red-pink
colonies (only 2!!!) showing NADI⁻



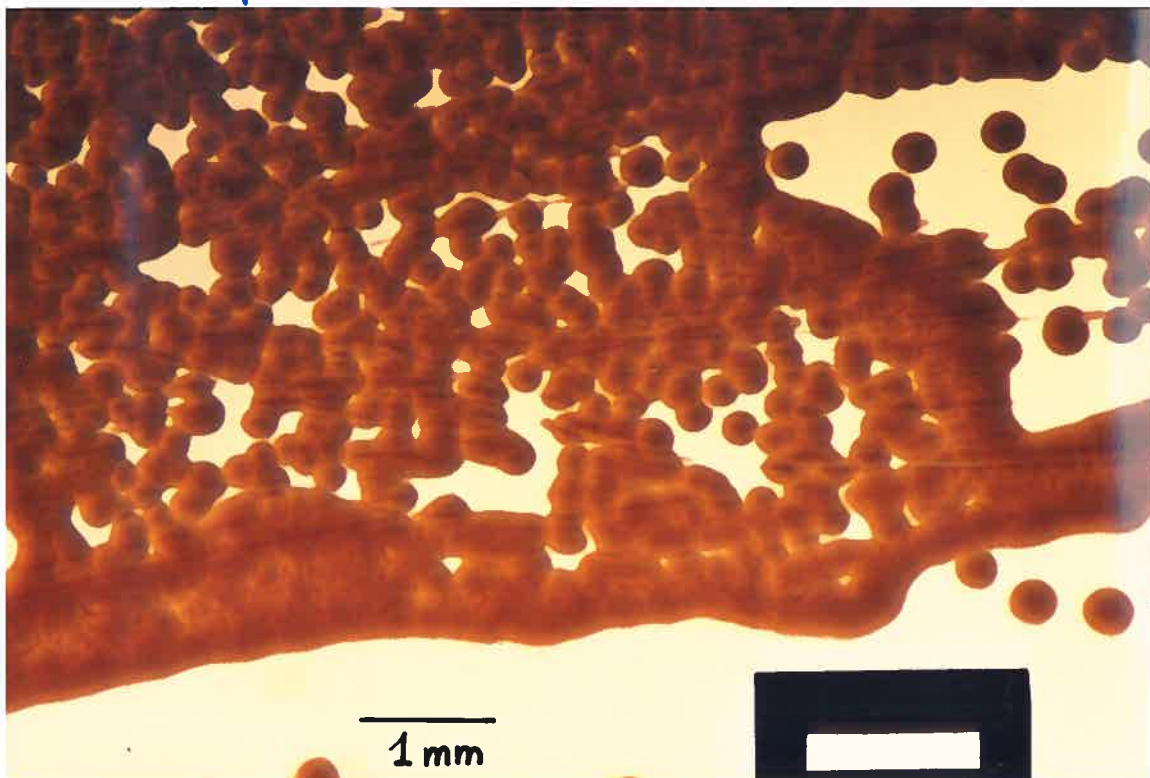
streak them out
on CENMED plates (Km 50, Sp 7.5)



That's it !!



Tn 5 mutagenized colonies of
Rsp. centenum (KmR + SpR)



Note: the actual color of the colonies was red-pink

DZ NAD⁻ mutant (24 h after NAD⁻ addition)