EFFECT OF O_2, ERGOSTEROL AND STEROL PRECURSORS ON THE MATING ABILITY OF Kluyveromyces lactis

Jaime Mas, Esteban Celis, Enrique Piña and Aurora Brunner

(Depto. de Biología Experimental, Instituto de Biología and Depto. de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, México 20, D. F., México)

Received October 10,1974

SUMMARY

It was found that the difference in the partial pressure of oxygen (pO_2) resulting from the distinct altitude over the sea level, between Baltimore, Md., USA and Mexico City, about 2,240 meters, is sufficient to cause variance in conjugation frequency in two haploid strains of Kluyveromyces lactis.

Further studies confirm that sexual conjugation in K. lactis is highly sensitive to changes in the pO_2. Ergosterol which depends on O_2 for its biosynthesis, stimulates the mating process, as does the ergosterol precursor, squalene. Ergosterol and O_2 effects were found to be additive, suggesting that O_2 may influence conjugation in more than one way. Culture, storage and transfer frequency modify both, mating efficiency and the response to O_2 and lipid supplement.

The present study has an interesting international basis, two haploid strains of Kluyveromyces lactis that conjugated normally in Baltimore, Md., USA (sea level) mated poorly if at all in Mexico City, Mexico (altitude over the sea level, 2,240 meters). A search for the cause of this difference has shown that its basis is ecological and has led to the development of a new experimental system for studying biological activities of sterols.

MATERIALS AND METHODS

Strains.- K. lactis strains W600B (α, ade, leu) and WM37 (a, his) were used throughout this work. These were a generous gift from Dr. James R. Mattoon.

Media.- YPAD: 1% Bacto yeast extract, 2% Bacto-peptone, 2% dextrose, 1.7% Bacto-agar and adenine sulfate (80mg/liter). ME agar: 5% Difco malt extract broth plus 2.6% Bacto-agar. This medium was supplemented with different concentrations of ergosterol and squalene as described in the text. SD.: 0.67% Difco yeast nitrogen base w/o amino acids, 2% dextrose, 1.7% Bacto-agar.

A stock solution of ergosterol (40 mg/ml in absolute ethanol plus 1% Tween 80) and squalene (0.2 or 0.4 ml/100 ml of medium) were added to the appropriate media after autoclaving.

Diploid formation and isolation.- Diploids were obtained in the following manner: 2 loopfuls of each strain were suspended and mixed in 0.5 ml of 50 mM potassium phosphate buffer, pH 7. The heavy suspension was mixed with the help of a vortex mixer and one drop of this suspension put on ME agar. The plates were incubated at 30°C to allow conjugation under different conditions as indicated in Results, and diploids were selected by prototrophic growth on selective media.

Conjugation in the presence of O_2.- ME plates containing the drop of mating mixture were put in a vacuum desiccator connected to a manometer which was itself connected to a vacuum pump and to an oxygen tank. After maximum evacuation was attained pure oxygen was admitted until the desired pressure was reached; pure nitrogen was then admitted until the desiccator reached atmospheric pressure. The desiccator was kept at 30°C and dilutions of the mating mass were made at the desired intervals of time.

Dilutions of the mating mass were made so as to have approximately 100 to 300 diploids per plate (a preliminary estimation was made by microscopic observation). The cell suspension was spread on SD, plates were then incubated at 30°C for 48 hours, and the resulting diploid clones were counted. Sometimes small colonies appeared on the plates, but these were not considered as diploid clones but as wild type segregants, since K. lactis diploids sporulate fairly rapidly and resulting asci break easily.

Total number of cells was estimated spectrophotometrically after the appropriate dilutions.

Number of zygotes is always expressed per million cells.

The same procedures were used with supplemented media.

Experiments with "fresh" and "old" cultures.- Two series of experiments were performed in some instances: In the first, cultures were subcultured daily on solid YPAD and sampled at intervals to obtain cultures which had undergone

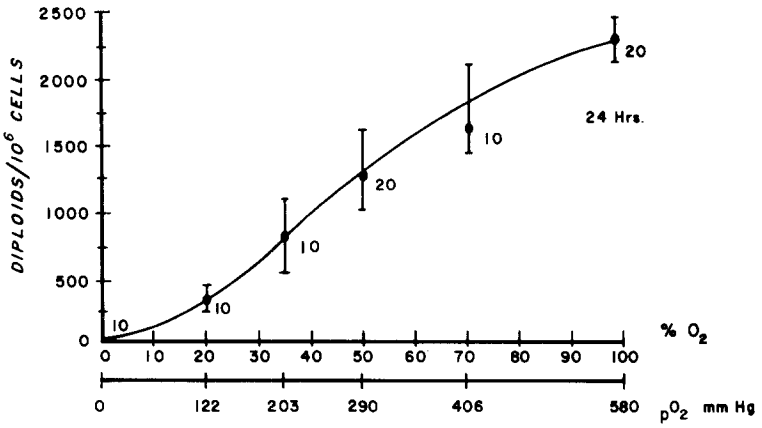

Figure 1. Effect of different O_2 concentrations on the number of diploids obtained. Two strains of different mating type were crossed (as indicated in methods) on ME agar and the cross kept under different O_2 concentrations at 30°C. Diploids were obtained by prototrophic selection on SD medium. Bars represent the standard error of the mean. Number of determinations is given in parenthesis.

different numbers of serial transfers. In the second, cultures were not transferred, but were maintained for one month at 5°C on the same medium. We will refer to the first group as "fresh" and to the second as "old" cultures.

Fresh 24 hours slants from either kind of culture were always prepared before crosses were made.

RESULTS

The number of diploids formed in a mating mass increases with increase in the concentration of oxygen (pO_2) present during conjugation (Fig. 1). During the course of these experiments it was observed that the O_2 effect tended to disappear with time until few if any diploids were formed, even in the presence of O_2. Subsequently haploid strains were kept in the cold at 5°C for a month, and when the experiment was repeated with these "old" strains, the stimulatory effect of oxygen reappeared. The results obtained in 5 successive experiments with 7 or 8 independent crosses in each one, are given in Table I. Although there is some dispersion in the results, there was an average 5-fold increase in mating frequency in 100% oxygen compared to 21% oxygen, the normal atmospheric concentration.

Since it is well known that molecular O_2 is required in certain reactions

TABLE I.

Effect of oxygen on the number of diploids obtained with "old" cultures.

		DIPLOIDS IN 10^6 CELLS / 24 HOURS									
EXPERIMENT		1		2		3		4		5	
O_2 CONCENTRATION		100%	21%	100%	21%	100%	21%	100%	21%	100%	21%
Cross No.	1	352	83	336	43	342	115	321	112	295	48
	2	348	79	398	61	410	89	280	123	283	53
	3	392	113	374	69	383	110	300	128	324	36
	4	386	98	386	64	374	122	297	81	299	0
	5	424	84	412	112	338	96	336	96	338	93
	6	413	91	356	52	412	81	283	61	263	34
	7	-	-	343	29	350	112	340	0	322	58
	8	-	-	-	-	-	-	329	0	343	66

Strains were maintained in the cold (5°C) for one month. Fresh cultures were grown 24 hrs then mass mated in the presence of 21 and 100% O_2.

Five successive experiments were made with 7 to 8, independent crosses in each one.

in the biosynthesis of unsaturated fatty acids and sterols (1, 2) we tested the direct effects of Tween 80 (as a source of oleate) and ergosterol on the efficiency of mating (Table II). The effect of different ergosterol concentrations on the number of diploids produced starting from "old" and "fresh" cultures respectively. Whereas ergosterol has only a slight effect on "old" cultures, with "fresh" ones 100 times as many diploids are obtained at low ergosterol concentrations. With both kind of cultures an inhibition can be observed at higher concentrations so that with 2 or 2.5 mg/ml there are no detectable zygotes produced among $1x10^6$ cells plated. Tween 80 alone did not stimulate zygote formation (Table III).

Since both oxygen and ergosterol stimulate mating in K. lactis, it was reasoned that an ergosterol precursor together with oxygen should give rise to

TABLE II

Effect of different ergosterol concentrations on the number of diploids obtained.

Ergosterol µg/ml	"Old"	"Fresh"
0.0	520	5
0.1	628	552
0.2	640	584
0.4	848	648
0.6	400	387
0.8	374	254
1.0	57	11
1.5	27	0
2.0	2	0
2.5	0	0

Mass-matings with 24 hour old precultures were made on ME agar media containing different ergosterol concentrations and 1% Tween 80. After 24 hrs incubation at 30°C, dilutions of the mating mixtures were prepared and aliquots plated on SD media. After 48 hrs incubation at 30°C the number of diploid clones were counted. An experiment with Tween 80 at 1% gave essentially the same value as the control.

TABLE III.

Effect of ergosterol, squalene and different O_2 concentrations on the number of diploids obtained.

	DIPLOIDS IN 10^6 CELLS					
EXPERIMENT	1	2	3	4	5	
					100%O_2	21%O_2
"Old" + Ergosterol	423	418	368	439	792	329
"Old" + Squalene	-	-	1856	1642	2868	950
"Old" + Tween 80	376	338	343	383	584	273
Control	354	342	350	391	514	198
"Fresh"+ Ergosterol	342	329	320	331	418	223
"Fresh"+ Squalene	-	-	648	525	1316	556
"Fresh"+ Tween 80	0	0	0	4	32	19
Control	0	0	6	12	38	24

The experimental conditions were the same as those described for Tables I and II. Squalene was also included in ME media at the concentration of 0.4 ml/100 ml of medium.

ergosterol and might possibly cause greater stimulation than oxygen alone. Table III shows that squalene is more effective than either ergosterol or oxygen. The effect is more dramatic with the "fresh" cultures than with the "old" ones, although with the latter there is still a 4 to 5 -fold increase, an effect much greater than that obtained with ergosterol. In the fifth experiment we can see that 100% oxygen caused stimulation in addition to that obtained with ergosterol or squalene.

If we compare the diploid frequencies obtained in control cultures in Table III we see that higher values were obtained in experiments 4 and 5. This might indicate an adaptation to ergosterol synthesis in these "fresh" cultures. Results presented in Fig. 2 illustrate this same effect; sometime after 40 days of a daily subculturing on fresh medium, the number of diploids obtained begin to rise again. This figure also illustrates the progressive decline obtained in producing "new" strains from "old" strains noted earlier.

DISCUSSION

Jakob (3) showed that mating frequency in S. cerevisiae could be increased 2.5 times by rapid agitation which consequently improved aeration. Also Hunt and Carpenter (4) found that treatment of Hansenula wingeii with organic solvents or

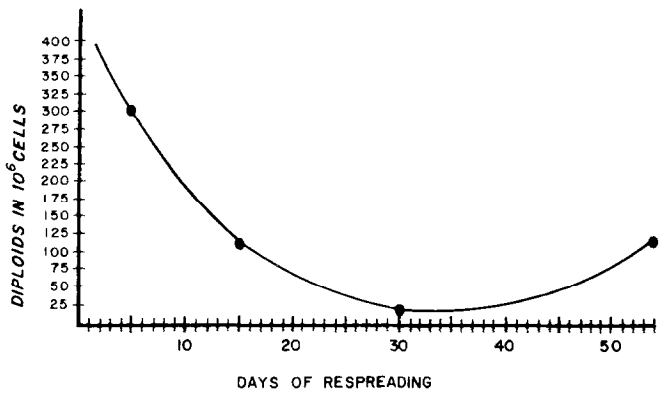

Figure 2. Effect of a daily spreading on YPAD media on the number of diploids obtained. Cells were transferred daily on fresh YPAD media for the number of days indicated. At different intervals of time a mass mating was performed, and the number of diploids determined by spreading aliquots on SD media.

nystatin inhibited mating, and they suggested a possible role for sterols in this process.

It is likely that oxygen improves mating efficiency in more than one way, since, in the presence of optimal ergosterol concentration, an increase in pO_2 further enhanced sexual conjugation. This could hardly indicate stimulation of ergosterol production, since higher concentrations are inhibitory.

Of special interest is the observation that the sterol precursor, squalene, is far more effective than ergosterol itself. This result suggests the possibility that some sterol other than ergosterol is the active agent in zygote formation.

Several of the metabolic steps in the conversion of squalene into ergosterol require oxygen (1), and one of the various sterol intermediates in this complex pathway (5) may be the active agent or "hormone" responsible for efficient mating. Sex hormones in yeast were postulated by Levi (6). Later the existence of "steroid hormones" in Saccharomyces cerevisiae was suggested by Yanagishima's group (7, 8). On the other hand, a diffusible peptide has been recently isolated which also seems to be implicated in conjugation in S. cerevisiae (9). These findings point to the fact that several factors or "hormones" are implicated in this process.

The inhibitory effect of higher ergosterol concentrations might be related to feed-back control of sterol biosynthesis at the level of HMG-CoA reductase, which is known to be the case in liver (10). If the active compound is a sterol intermediate in ergosterol synthesis, excess ergosterol might limit the synthesis of this sterol by feed-back control.

The basis for the ageing effects depicted in Fig. 2 are currently under investigation. An obvious possibility could be that the culture least active in conjugation has the lowest content of ergosterol. However, when the total sterol content of different cultures was determined by the method of Breivik and Owades (11), no significant differences appeared. Chromatographic analysis of the various sterols of K. lactis may reveal the existence of a mating-control sterol.

The importance of sterols in membrane structure and function is well known.

Another possible basis for the ergosterol effects could be that for efficient fusion of membranes during mating there is an optimal ergosterol concentration.

ACKNOWLEDGEMENTS

We are greatly indebted to Dr. James R. Mattoon for his encouragement and help in the preparation of the manuscript.

REFERENCES

1. David, H., and Kirsop, B. H. (1973) J. Gen. Microbiol. 77; 529-531
2. Bloomfield, D. K. and Bloch, K. (1960) J. Biol. Chem. 235; 337-531
3. Jakob, H. (1962) C. r. hebd. Seanc. Acad. Sci. (Paris) 254; 3909-3911
4. Hunt, D. E. and Carpenter P. L. (1963) J. Bacteriol. 86; 845-847
5. Fryberg, M., Oehlschlager, A. C. and Unrau, A. M. (1974) Arch. Biochem. and Biophys. 160; 83-89
6. Levi, J. D. (1956) Nature 177; 753-754
7. Yanagishima, N. (1969) Planta 87; 110-118
8. Takao, N., Shimoda, C. and Yanagishima, N. (1970) Develop. Growth and Differ. 12; 199-205
9. Duntze, W., Mc Kay, V. and Hanney, T. R. (1970) Science 168; 1472-1473
10. Harper, H. A. (1969) Review of Physiological Chemistry, pp. 315, Lange Medical Publications, Los Altos, California.
11. Breivik, O. N. and Owades, J. L. (1957) Agric. and Food Chem. 5; 360-363