OXYGEN INFLUENCE ON THE b-OXIDATIVE METABOLISM OF METHYL RICINOLEATE FOR LACTONES PRODUCTION BY THE YEAST *YARROWIA LIPOLYTICA*

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Abstract. The yeast *Yarrowia lipolytica* is able to carry out the peroxisomal β-oxidative catabolism of methyl ricinoleate, a process leading notably to the production of γ-decalactone, a high-value aroma compound. In fact, this metabolic pathway gives rise to different C_{10} lactones and their accumulations report the efficiency of the peroxisomal β-oxidation enzymes. γ-Decalactone or 3-hydroxydecan-4-olide accumulate when acyl-CoA oxidase or 3-hydroxyacyl-CoA dehydrogenase, respectively, control the flux within the pathway. We investigated the accumulation of C_{10} lactones in various aeration conditions. An important accumulation of 3-hydroxydecan-4-olide was observed when increasing the aeration up to a certain point, above it its concentration was lowered, which indicates a stimulation of the activity of 3-hydroxyacyl-CoA dehydrogenase. Cultures under high-pressure were performed in order to increase the oxygen in the medium by an alternative mean. *Y. lipolytica* growth in glucose medium was accelerated at 0.5 MPa of total air pressure. A decrease in γ-decalactone production was observed with the increase in pressure. Curiously, applying a pressure of 0.5 MPa during growth and then during biotransformation resulted in high decenolides concentrations.

Keywords: β-Oxidation Flux Regulation, Oxygen, *Yarrowia lipolytica*.

1. Introduction

γ-Decalactone is an aroma compound of industrial interest that can be produced biotechnologically by some microorganisms. The yeast *Yarrowia lipolytica* is able to achieve this by biotransformation of methyl ricinoleate, using its peroxisomal β-oxidation machinery. The amount of γ-decalactone in the medium is the difference between the production by the yeast cells and its degradation, which was shown to imply also the β-oxidative pathway. Indeed, several compounds proceeding from the first step of degradation of the direct precursor of the lactone, i.e. 4-hydroxydecanoate, (3-hydroxy-decalactone, 2- and 3-decenolides), were identified in biotransformation media (Waché *et al*., 2003).

The activities of acyl-CoA oxidase and 3-hydroxyacyl-CoA dehydrogenase, two enzymes of the yeast peroxisomal β-oxidation, are influenced by oxygen, which is necessary in the regeneration of the required cofactors (Fig. 1). *Y. lipolytica* possesses a five-member acyl-CoA oxidase family and it was shown previously that it is possible to change genetically the profiles of lactones production (Waché *et al*., 2001). However, at least in Europe, aroma compounds from genetically modified organisms are currently avoided by the consumers. For this reason and also in order to better understand the β-oxidation fluxes in that yeast, we investigated the influence of the oxygen on the production of γ-decalactone and on its further degradation during the

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biotransformation process: a better understanding of the regulation of the C_{10} compounds degradation through β-oxidation in this yeast species, should bring new insights to improve the lactone production yields and moreover this aspect appears of great general interest, due to the importance of the β-oxidation pathway in the production of many compounds.

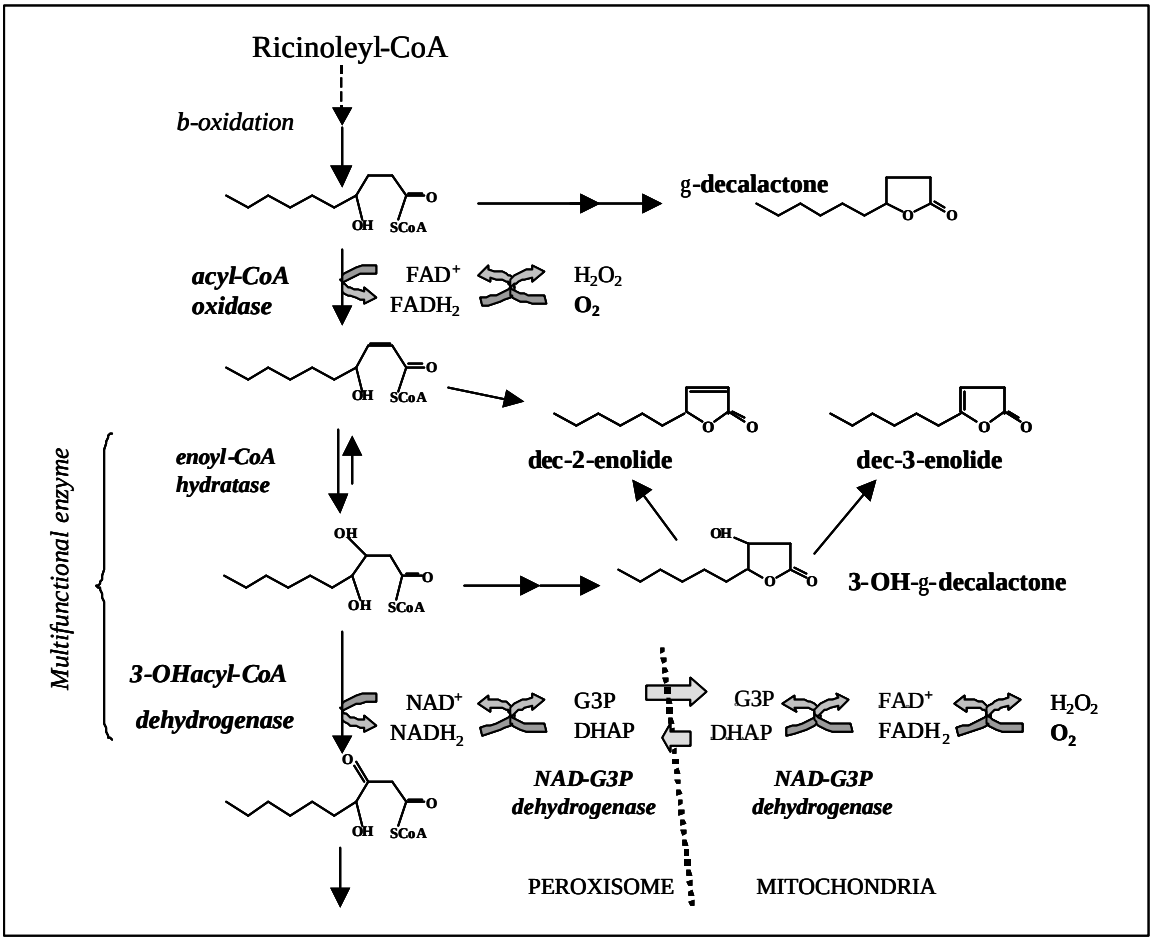

Fig. 1. β-Oxidation cycle at the level of 4-hydroxy-decanoate (the direct precursor of γ-decalactone) during the degradation of ricinoleyl-CoA. The enzymatic activities whose cofactor requirements need oxygen are indicated in bold. The names of the different lactones whose concentrations were determined in the biotransformation medium are also indicated in bold.

Oxygen transfer rate (OTR) from the gas to the liquid medium can be enhanced by increasing the oxygen solubility, notably by raising the total air pressure (Belo *et al*., 2003). Here, the OTR improvement was carried out by using hyperbaric air in a high-pressure reactor. The effect of pressure on the growth of *Y. lipolytica* in glucose medium was evaluated and the production of γ-decalactone and of three other C_{10} lactones coming from its further metabolism was followed in biotransformation medium.

2. Materials and Methods

2.1. Microorganism and Media

The strain used in this study is *Y. lipolytica* W29 (ATCC 20460). The composition of the glucose growth medium and the experimental conditions were described elsewhere (Waché *et al.*, 2000 and 2001).

The biotransformation medium was composed of 5 $g.L^{-1}$ methyl ricinoleate, 6.7 $g.L^{-1}$ yeast nitrogen base, 5 $g.L^{-1}$ NH_4Cl and 0.2 $g.L^{-1}$ Tween 80.

2.2. Culture under Atmospheric Conditions

A 2 L reactor (Biolab, B. Braun, Germany) was used, with 1.7 L of biotransformation medium.

2.3. Culture under Hyperbaric Air

A 600 mL stainless steel high-pressure bioreactor (model 4563, Parr, USA) was used. Cells were harvested (10000 g, 5 min) from the glucose growth medium, washed twice and resuspended in the reactor containing 400 mL of biotransformation medium. Agitation rate was of 400 rpm and aeration was 0.36 $L.min.^{-1}$ (0.9 vvm).

2.4. Lactones Quantification

Analyses were carried out on 2 mL samples collected from the culture medium. They were acidified to pH < 2 with HCl and γ-undecalactone was added as internal standard before extraction with diethyl ether. The organic phase was analyzed by GC-MS composed by a Varian 3400 GC gas chromatograph (CPWAX-52-CB column with l = 50 m, d = 0.25 mm and film thickness = 0.2 μm; Helium was the carrier gas) and a ion-trap Varian Saturn II mass spectrometer (detector used in electronic impact mode, ionization energy of 70 eV and mass acquisition spectrum between 29 and 360 m/z, with 610 ms of time intervals).

3. Results

3.1. Effect of oxygen on the production of C_{10} lactones

Modifying the agitation and air flow rates within the reactor influences the oxygen mass transfer rate to the medium, as shown by the values of the oxygen transfer volumetric coeficient (K_La) calculated (Table 1) by a theoretical model previously defined for our biotransformation medium (Gomes *et al*., 2005).

Table 1: K_La values predicted according to the empirical model at normal pressure (Gomes *et al.*, 2005).

Bioreactor used	Agitation (rpm)	Air flow (vvm)	K_La (h^{-1})
2 L (Biolab, B. Braun, Germany)	300	0.3	26
	600	0.9	123
	600	1.8	162

The production of γ-decalactone and of the other C_{10} lactones coming from 4-hydroxydecanoate were determined for these different conditions. The concentration of γ-decalactone in the medium reached a maximum after 10 hours; the maximum for 3-hydroxy-decalactone and dec-2 and 3-enolides concentrations was after more than 20 hours. Following these maximums, the concentration of the compounds decreased and dissapeared totally around 50 hours, corresponding to a degradation by the yeast cells.

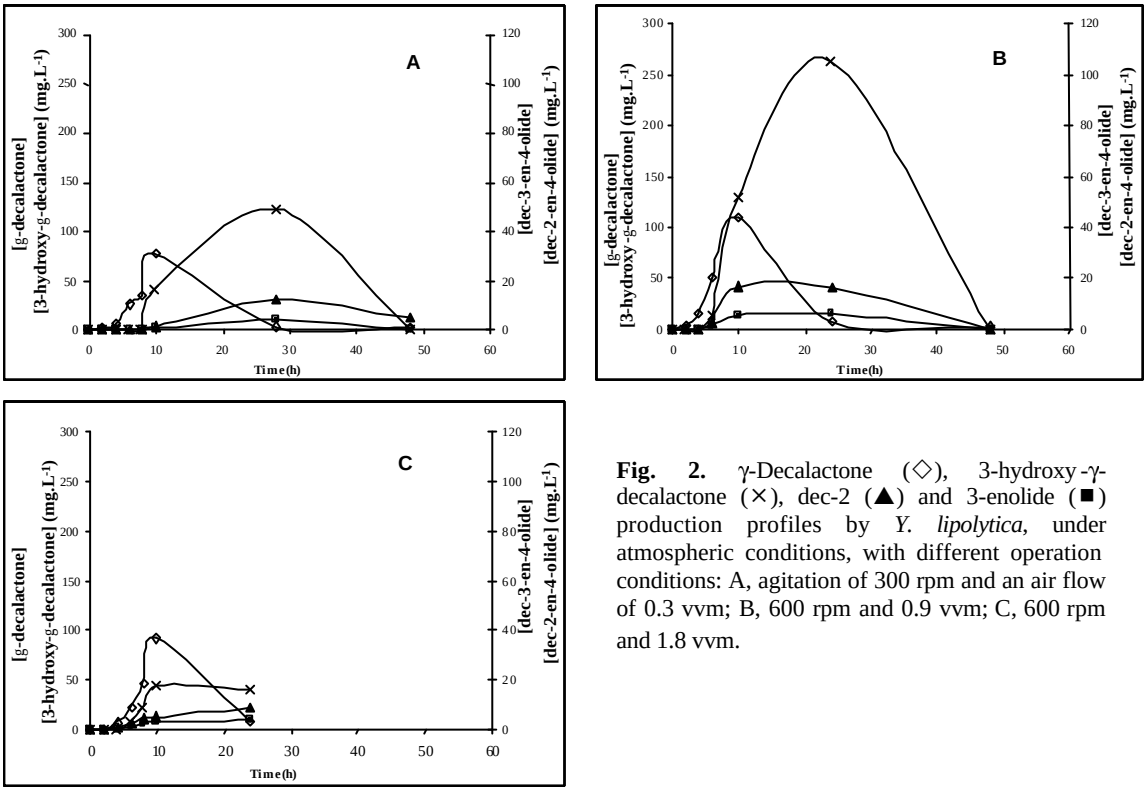

Fig. 2. γ-Decalactone (◇), 3-hydroxy-γ-decalactone (×), dec-2 (▲) and 3-enolide (■) production profiles by *Y. lipolytica*, under atmospheric conditions, with different operation conditions: A, agitation of 300 rpm and an air flow of 0.3 vvm; B, 600 rpm and 0.9 vvm; C, 600 rpm and 1.8 vvm.

In all cases the production of γ-decalactone was in the same concentration range (between 80-110 $mg.L^{-1}$). Increasing the K_La from 26 to 123 h^{-1}, prevented the depletion of O_2 from the medium during the highest aroma production phase and resulted in an important increase in the concentration of 3-hydroxy-decalactone (from 122 to 263 $mg.L^{-1}$), and in a lesser extent of 2- and 3-decenolides (Fig. 2A and B). A further increase of K_La by an air flow rate of 1.8 vvm, maintained the dissolved oxygen at its maximum level and caused a drastic reduction of the concentrations of the three compounds mentionned above (Fig. 2C).

3.2. Effect of pressure on the growth of *Y. lipolytica*

The application of 0.5 MPa stimulated the cell growth compared to the atmospheric conditions (Fig. 3). Such a pressure level, increases the oxygen mass transfer to the medium and to the cells leading to a positive effect on the metabolism of this strictly aerobic yeast. When the cells were grown under a pressure of 1 MPa, the growth was inhibited, mainly after 10 h of exposition, and the level of biomass attained was decreased.

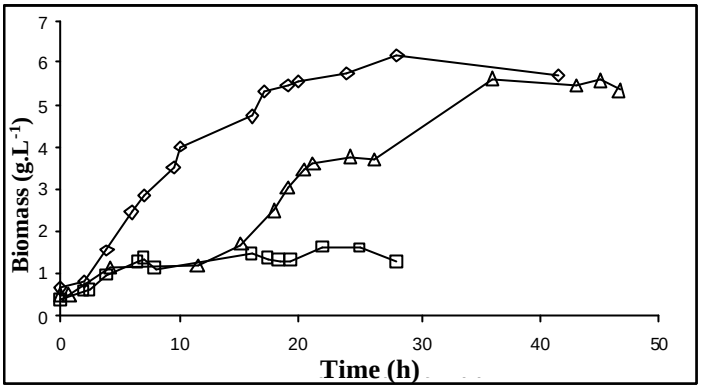

Fig. 3. Growth of *Y. lipolytica* with an agitation of 400 rpm and an air flow of 0.9 vvm, under atmospheric conditions (△), under pressures of 0.5 MPa (◇) and 1 MPa (□).

3.3. Effect of pressure on the metabolic pathway of lactones production

First the production of lactones was followed in this reactor under atmospheric pressure in order to compare with what was obtained in the former reactor (above described). With these experimental conditions, the production of the lactones was much slower, the maximal concentration being reached after 25-30 hours; the production of γ-decalactone was higher (around 300 mg.L^{-1}) and that of 3-hydroxy-γ-decalactone was in the same concentration range (Fig. 4A). When an increased pressure was applied, the production kinetics were improved, the production of 3-hydroxy-γ-decalactone was stimulated and the concentrations of decenolides were high (Fig. 4B and C).

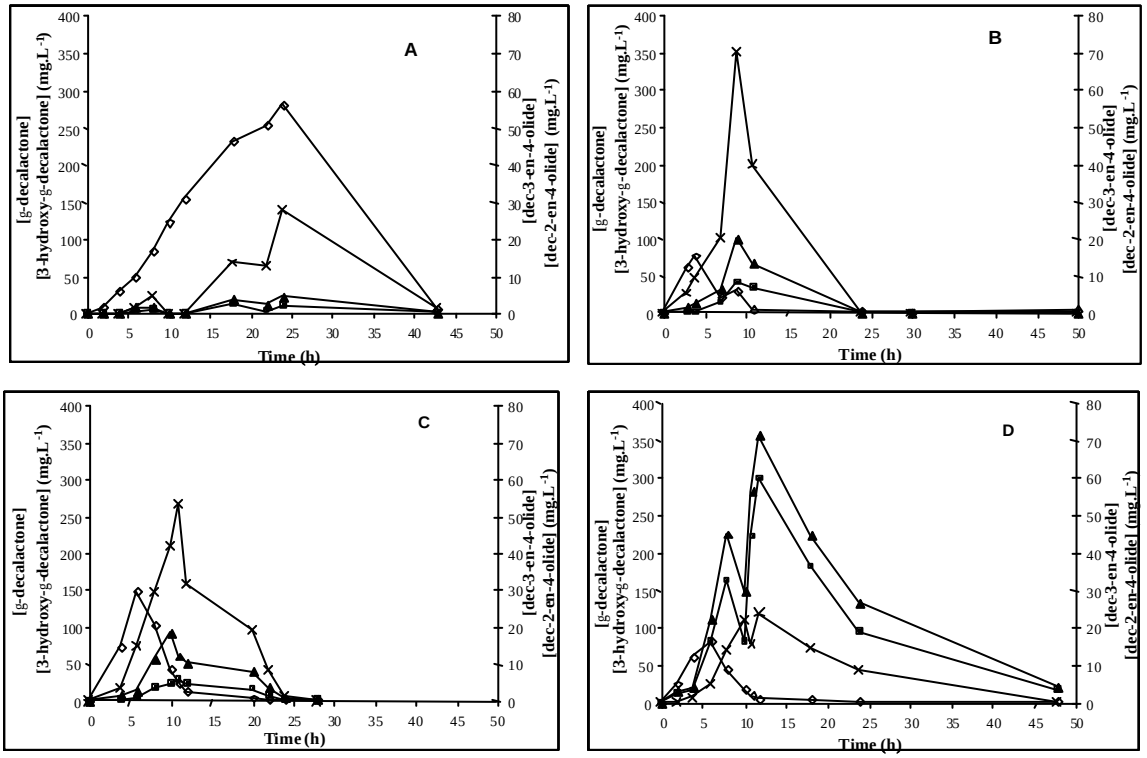

Fig. 4. γ-Decalactone (◇), 3-hydroxy-γ-decalactone (×), dec-2 (▲) and 3-enolide (■) production profiles by *Y. lipolytica*, in the high-pressure reactor with an agitation of 400 rpm and an air flow of 0.9 vvm, at different pressures: A, 0.1 MPa; B, 0.5 MPa; C, 1 MPa and D, 0.5 MPa during growth and 0.5 MPa during biotransformation.

The effect of the pressure on the growth of the cells in the glucose medium before their transfer to the biotransformation medium under different pressure conditions was also tested and it appeared that this point had no influence on the C_{10} lactones production profiles (not shown), however curiously, an important difference was observed when both the cells growth and then the biotransformation were performed under a pressure of 0.5 MPa: the concentration of 3-hydroxy-γ-decalactone was equal to 120 $mg.L^{-1}$ and that of both decenolides reached concentrations of 300-350 $mg.L^{-1}$ (Fig. 4D).

4. Discussion

For the production of γ-decalactone by *Y. lipolytica*, the compound has to exit β-oxidation pathway before being transformed to acetyl-CoA. The lactone concentration in the medium is in fact the difference between the production and its degradation by the yeast. As previously shown, peroxisomal β-oxidation is also implicated in the degradation of γ-decalactone (Waché *et al*., 2001).

During the β-oxidative metabolism of ricinoleyl-CoA by yeast, the intermediates of the pathway can freely exit from the peroxisomes into the medium (Blin-Perrin *et al*., 2000). Thus, the concentrations of 3-hydroxy-γ-decalactone, dec-2 and 3-enolides in the medium give an indication of the control of the pathway at the C_{10} level.

As shown in figure 1, the main cycle exits come from the oxidation steps catalyzed by the oxidase and by the dehydrogenase, both requiring cofactors. The acyl-CoA oxidase utilizes molecular oxygen and requires FAD^+. The dehydrogenase step is generally not considered as exerting a high control on the pathway. This activity could depend on the regeneration of NAD^+ (Osmundsen *et al*., 1994). The re-oxidation of the $NADH,H^+$ used in the peroxisomes remains unclear but it could be linked to the mitochondrial respiration pathway through shuttle mechanisms (Fig. 1). Thus, these two enzymatic steps of the pathway may be influenced by the oxygen concentration in the medium.

Here the oxygen availability for the cells was modified experimentally (Table 1). Increasing oxygenation first led to an increase in the formation of 3-hydroxy-γ-decalactone and of both decenolides (Fig. 2B), which indicates that the activity of acyl-CoA oxidase was stimulated. Then, for a higher O_2 level, the concentrations of these three compounds was drastically reduced (Fig. 2C), indicating this time a stimulation of 3-hydroxy-acyl-CoA dehydrogenase.

So, It seems that acyl-CoA oxidase controls the pathway up to a certain O_2 level (possibly corresponding to a K_La around 120 h^{-1}), and for higher values 3-hydroxy-acyl-CoA dehydrogenase regulates the pathway (Fig. 1).In all the cases, the concentration of dec-2-enolide was higher than that of dec-3-enolide, which can be explained by the two possible pathways leeding to the first compound (Fig. 1).

At higher pressure, the driving force for mass transfer of oxygen from the gas phase to the liquid phase, and thus to the cells, is increased. A pressure of 0.5 MPa stimulated the growth of *Y. lipolytica*, however 1 MPa induced a stress for the cells, decreasing their growth.

The application of pressure improved the production kinetics of γ-decalactone, but increased the concentration of its oxidation compounds: this indicates that the activity of acyl-CoA oxidase is much more stimulated than that of 3-hydroxy-acyl-CoA dehydrogenase, being in that case the rate-limiting step of the pathway.

The high concentrations of both decenolides observed when a pressure of 0.5 MPa was applied during cell growth and then during biotransformation (Fig. 4D), constitute an unclear point, however it could be linked to an induction of the dehydroxylase activity that transforms 3-hydroxy-γ-decalactone into both decenolides.

4. Conclusions

Since *Yarrowia lipolytica* possesses a family of five acyl-CoA oxidases, each exhibiting some specificities in relation to the chain length of the substrate, a good solution would be to block or inactivate the oxidase activity which is the most effective on C_{10} compounds (Aox 3), thus a degradation of γ-decalactone would be avoided. This was attempted, but no real improvement in the lactone production rates was observed (Waché *et al*., 2001). The better understanding of the metabolic pathway is an alternative solution: here, with that aim, the effect of oxygen was evaluated.

Our results confirm that β-oxidation is sensitive to the oxidative state of the medium. From a single substrate, it can give rise to different amounts of compounds with ten carbons. The dehydrogenase seems to increase its control of the pathway when the O_2 increases above certain levels. The non-improvement of γ-decalactone production indicates that O_2 is not a limiting factor in that step of the process, however it is a very important factor in the further metabolism of the C_{10} compounds, avoiding the further formation of γ-decalactone from 4-hydroxydecanoate. Increasing the air flow in the reactor or the pressure to improve the O_2 mass transfer, globally stimulates the oxidation of 4-hydroxydecanoate by acyl-CoA oxidase. A good solution to obtain higher γ-decalactone yields would be to decrease the O_2 in the medium, which would also reduce the cell activity, but in that case the process would be much longer (Fig. 4A). Thus, an equilibrium has to be established in order to optimize the production without impairing this strictly aerobic yeast.

3-Hydroxy-γ-decalactone has no peculiar aroma properties, however dec-3-en-4-olide was shown to exhibit a strong fruity note, close to that of γ-decalactone but much more powerfull: the optimization of the production of this compound is another opened possibility, however with the condition to find a simple solution to separate it from its isomer dec-2-en-4-olide, which exhibits a mushroom taste (Gatfield *et al*., 1993). Here, an important production of both decenolides was achieved under a pressure of 0.5 MPa (Fig. 4D), moreover with the use of conditions (high-pressure reactor) that have potential advantages in biotechnological processes, such as lowering the energy costs and the shear forces, compared to the mechanical agitation necessary to reach the same OTR (Belo *et al*., 2003).

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