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Analysis of the adaptation to alkanes of the marine bacterium *Marinobacter hydrocarbonoclasticus* sp 17 by two dimensional gel electrophoresis

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Abstract – To better understand the molecular mechanisms involved in the biodegradation of hydrocarbon compounds from the "*Erika*" oil-spill, we have studied the ability of *Marinobacter hydrocarbonoclasticus* strain sp 17 to cope with hexadecane as sole carbon and energy source. Growth kinetics of cultures shifted from acetate to hexadecane revealed the presence of a 20 hours adaptation phase. Changes in global protein expression in response to hexadecane was analyzed by two-dimensional gel electrophoresis. Of the 370 proteins detected 42 had their expression level altered in presence of hexadecane indicating that alkane adaptation may involve many cellular functions.

Key words: Alkanes / Biodegradation / Proteomic / 2D Electrophoresis

1 Introduction

Marine microbial communities in coastal zones, are responsible for biogeochemical cycles important to maintain the integrity of the whole marine ecosystem (Cohen 2002; Castenholz et al. 1994). Since marine microbial communities are exposed to oil pollution, particulary during oil spills such as the one from the wreck of the "*Erika*", it is of interest to determine how these bacteria respond to this changed environment. Alkanes are very common pollutant as they constitute 20−50% of crude oil (Rosenberg and Ron 1996). Many bacteria able to grow on alkanes have been isolated and in some cases like *Pseudomonas putida* GP01 degradation pathway has been elucidated metabolites identified and enzymes purified (Van Beilen et al. 1994; Smits et al. 2002; Van Beilen et al. 2003). Despite the large amount of data accumulated on the metabolism of alkanes by bacteria, many aspects of alkane degradation such as uptake and regulation of degradation pathway are still poorly understood at the molecular level.

The genus *Marinobacter* is well represented among hydrocarbon degrading bacteria isolated from marine sediment of polluted coastal zones. One strain, *Marinobacter hydrocarbonoclasticus* sp 17 isolated for its ability to degrade long chain alkanes has been chosen to investigate the molecular mechanisms of alkanes degradation (Gauthier et al. 1992). Comparison of lipid composition of *M. hydrocarbonoclasticus* sp 17 grown on acetate and icosane suggested the following pathway for alkane degradation: hydroxylation to the C_{20} primary alcohol, transformation into the C_{20} β-hydroxy acid and subsequent degradation into lower homologues (Lattuati et al. 2002).

In this study, we have determined protein expression profiles, by two-dimensional gel electrophoresis of cells grown hexadecane. Many proteins have their expression altered in presence of hexadecane revealing the occurrence of a molecular response to alkane in *M. hydrocarbonoclasticus* sp 17.

2 Materials and methods

2.1 Growth conditions

M. hydrocarbonoclasticus sp 17 was grown in 120 mL of EMS (Gauthier et al. 1992) medium supplemented with acetate at 20 mM or hexadecane at 0.2% (v/v) in 1 L Erlenmeyer flask at 30 °C in an orbital shaker set at 180 rpm. Growth was monitored by measuring the optical density of the culture at 600 nm.

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2.2 Protein extraction and 2D electrophoresis

Extracts were performed on 40 mL of culture. During the entire procedure cell suspension or protein extracts were kept at 4 °C. Cells in exponential growth were harvested by centrifugation, pellets were washed twice with 30 mL of sonication buffer (10 mM tris pH 7.5, 5 mM $MgCl_2$) and stored at −80 °C.

Pellets were resuspended in 1 mL of sonication buffer, thawed on ice and sonicated twice for 30 s with a Brandson 250 sonicator. 10 μL of DNaseI (Sigma) at 1 mg mL^{-1} and 10 μL of RNaseA (Sigma) at 250 μg mL^{-1} in 50 mM $MgCl_2$ were added to the extract and incubated 10 minutes on ice. Proteins were precipitated with 10% (v/v) TCA (trichloroacetic acid). Protein pellets were washed twice with 10% TCA, twice with cold acetone and then air-dried. Proteins were solubilized in 1 mL of solubilization buffer (8 M urea, 4% (w/v) CHAPS (Amersham Biosciences) 60 mM DTT (dithiothreitol Amersham Biosciences, 2% (v/v) Pharmalyte pH 3–10 (Amersham Biosciences) and 0.0002% (w/v) bromophenol blue (Sigma). Protein concentration in extracts was determined by the Bradford method using the Biorad kit.

2.3 Two dimensional electrophoresis

The first dimension IEF, was carried out on IPGphor (Amersham Biosciences) according to the instructions of the manufacturer. Immobiline dry strip pH 3–10 (Amersham Biosciences) were rehydrated with 450 mL of protein extract at 1 mg/mL for 12 hours at 20 °C and run for 1 hour at 500 V, 1 hour at 1000 V and 8 hours at 8000 V.

Prior to running in the second dimension, the strips were equilibrated 15 min in 50 mM tris pH 8.8, 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol, 0.0002% (w/v) bromophenol blue and 65 mM DTT.

For the second dimension strips were applied on 12% SDS polyacrylamide gel using Ettan Dalt Six (Amersham Biosciences) apparatus. Gels were prepared and run according to manufacturer instructions.

2.4 Protein detection and gel analysis

Gels were stained with Sypro Orange (Molecular Probes) dye according to the procedure described by Malone et al. (2000).

Gel images were acquired with fluorescent scanner Typhoon 9200 (Amersham Biosciences) and analyzed using the software Image Master 2D (Amersham Biosciences). Analysis was done on triplicate gels. Protein spots not appearing on the three gels were removed from the data set.

3 Results and discussion

In our study of hexadecane degradation by *M. hydrocarbonoclasticus* sp 17, we first focused on the events occurring during the transition between acetate and hexadecane utilization. Bacteria were grown up to exponential phase in a medium containing acetate as sole carbon source. Cells were harvested washed and placed in medium containing hexadecane as sole carbon source. After the shift from acetate to hexadecane growth stopped and resumed after a 20 hours lag period (Fig. 1a). In the control experiment where acetate-grown cell were transfered to acetate medium, no lag was observed. These results shows that cultures growing on acetate need a 20 hours adaptation phase before being able to use hexadecane. Adaptation of bacteria to a new carbon source usually takes a few minutes or tens of minutes. The much longer lag phase observed in the case of adaptation of *M. hydrocarbonoclasticus* to hexadecane is probably due to the insoluble nature of the substrate. It has been reported that hydrocarbon uptake can occurs via two mechanisms: direct interaction between the cell and the water-hydrocarbon interface or by emulsification of the hydrocarbon (Rosenberg et al. 1982; Ron and Rosenberg 2001). In both cases this would required the setting up of specific functions for alkane uptake which could account for the long lag observed. We next wanted to see if cultures growing on hexadecane would

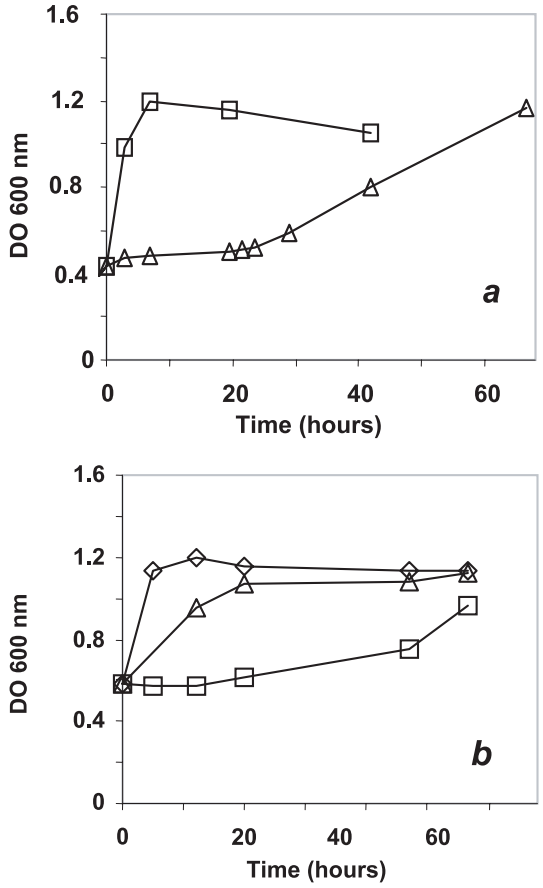

Fig. 1. Growth kinetics of *M. hydrocarbonoclasticus* sp 17 after carbon source exchange. Cells were grown up to exponential phase ($A_{600\ nm}$0.4–0.6) before changing the carbon source. A: transfer from acetate to acetate □, transfer from acetate to hexadecane △. B: transfer from hexadecane to acetate ◊, transfer from hexadecane to hexadecane □, culture on hexadecane without carbon source exchange △.

need an adaptation phase to use acetate. Figure 1b shows that cell in exponential growth on hexadecane maintained their growth, without lag, after transfer to acetate medium. The cells were able to use acetate without adaptation.

In contrast, when cells growing on hexadecane were shifted to fresh medium containing hexadecane a 20 hours lag phase was observed before the growth resumed. This was surprising since one would expected that cells already adapted to growth on hexadecane would not have needed further adaptation to grow on fresh medium containing hexadecane. The occurrence of this lag phase suggests that utilization of hexadecane required the establishment of interaction between the cell and hexadecane. Such interaction would be destroyed during washing and medium transfer and growth only resumed only after restoration of the hexadecane-cell interaction in the fresh medium.

These results show that utilization of hexadecane as carbon source requires an adaptation phase corresponding most likely to the establishment of a new physiological state involving cellular functions specific for hydrocarbon degradation such as metabolic pathways and functions involved in hydrocarbon uptake.

Modifications of physiological state result from an alteration in gene expression of the cell. Proteomic analysis has been proven to be the most powerful method to study the alteration of protein expression in a cell under different environmental conditions (Grave and Haystead 2002). We therefore used this approach to evaluate variation of physiological state of *M. hydrocarbonoclasticus* sp 17 during hexadecane adaptation. Proteins were extracted from *M. hydrocarbonoclasticus* sp 17 cells grown on acetate or hexadecane and then separated by two-dimensional gel electrophoresis. The gels obtained represent the protein expression profile of the cell grown in acetate or hexadecane. About 370 protein spots were reproducibly detected (Fig. 2). Gels comparison revealed that 11% (42) of all the detected proteins underwent a significant change (more than 2 fold) in their expression level in response to hexadecane (Table 1). In hexadecane grown bacteria, 26 proteins were found up regulated, among them 6 were novel proteins not detected in the acetate condition (spots 1416, 1614, 1404, 1488, 1478 and 1560). Protein 1416 is of particular interest since it is only expressed in presence of hexadecane and is one of the most abundant proteins (relative amount 2.3%). It is therefore a good candidate for a protein directly involved in hexadecane utilization. Three highly abundant proteins, 476, 101 and 181 representing each more than 1% of the total amount of protein found in acetate were induce by a factor two in presence of hexadecane. These proteins are likely required for growth on both acetate and hexadecane and could be general metabolism, stress or housekeeping proteins.

Alkanes metabolic pathway has been extensively studied in *Pseudomonas pudita* and genetic organization of the genes involved, the *alk* genes, has been elucidated in many bacteria. To date ten *alk* genes are known, their products include the alkane hydroxylase, two types of rubredoxin, the rubredoxin reductase, the alcohol deshydrogenase, the aldhehyde dehydrogenase, an acyl-CoA synthase, a regulatory protein, an outer membrane protein and a putative methyl-accepting transducer protein (van Beilen et al. 2003; van Beilen et al. 2001).

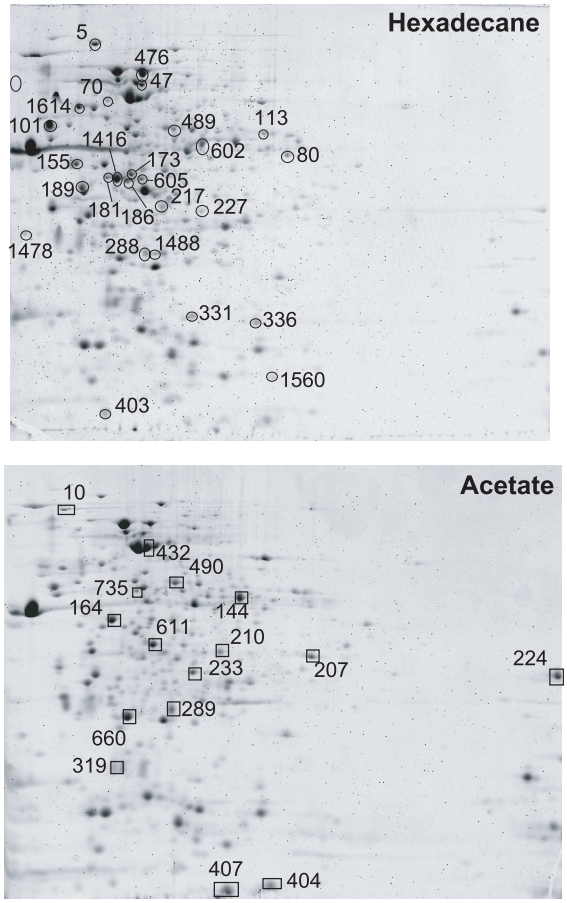

Fig. 2. Two-dimensional gel electrophoresis of cells grown on acetate and hexadecane. Circle: protein up-regulated in hexadecane-grown cells. Square: protein down-regulated in hexadecane-grown cells.

Our results show that alkanes utilization involve many others proteins than the known Alk proteins. This supports the concept that other cellular functions than the metabolic pathways participate in this process. Such functions could be chemotactism transport across membranes, stress response, biosurfactants production and cell-cell communications. Identification of proteins playing a role in alkane response would lead to a better understanding of the molecular and biochemical basis of microbial behavior and physiological responses to alkanes and the impact of these responses on bioremediation. Moreover, such a complex process is most likely to be affected by environmental variations. Studies on the impact of environmental conditions on hexadecane adaptation could help to predict what would be the conditions favorable or unfavorable to hydrocarbon degradation in marine coastal zone.

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Table 1. Protein induced or repressed in *M. hydrocarbonoclasticus* by hexadecane, (a) Relative amounts are expressed in % of total protein, (b) The induction ratios are relative to acetate condition and means of the values from at least 3 gels. ND: not detected, NA: not applicable.

	Spot number	Relative Amount[a]	Induction Ratio[b]
Proteins induced in hexadecane	1416	2.3	novel
	1614	0.5	novel
	1404	0.2	novel
	1488	0.1	novel
	1478	0.06	novel
	1560	0.07	novel
	47	0.7	21.6
	155	0.4	19.0
	186	0.4	8.0
	217	0.1	7.7
	489	0.2	6.4
	288	0.3	5.0
	113	0.1	4.9
	189	0.7	4.6
	173	0.5	4.1
	227	0.08	3.6
	70	0.08	3.6
	605	0.2	3.5
	336	0.2.	3.5
	5	0.8	3.1
	403	0.2	3.0
	331	0.1	2.8
	476	2.9	2.4
	101	2.3	2.2
	181	2.7	2.2
	602	0.7	2.1
Protein repressed in hexadecane	319	0.1	0.83
	144	0.8	0.48
	289	0.3	0.48
	660	0.9	0.48
	432	1.3	0.45
	233	0.2	0.37
	164	0.7	0.34
	490	0.3	0.33
	404	0.2	0.31
	210	0.06	0.18
	207	0.07	0.13
	407	0.1	0.11
	224	0.1	0.09
	735	ND	NA
	10	ND	NA
	611	ND	NA

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