BIOCHEMICAL COMPOSITION AND MODES OF SUBSTRATE UPTAKE TO ALKANE-GROWN MARINE BACTERIUM *Pseudomonas nautica* STRAIN 617

(Komposisi Biokimia dan Bentuk Transport Substrat *Alkane-Grown* pada Bakteri Laut *Pseudomonas nautica* Strain 617)

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ABSTRACT

The variation of the relative amounts of proteins, carbohydrates and lipids quantified from cells and supernatants of a marine bacterium *Pseudomonas nautica* strain 617, was a response to changes in growth substrates (acetate or eicosane). Modes of substrate uptake: adherence, emulsification and solubilization depend of its biochemical composition. The enchancement of extracellular compounds produced during the growth on eicosane. The solubilizing, emulsifying activities as well as their adherence were observed in relation to the biochemical changes. The biodegradation percentage of eicosane is 59% in 3.5 days-old culture.

Key word: biochemical composition, substrate uptake, biodegradation, marine bacterium, n-alkane

ABSTRAK

Komposisi protein, karbohidrat dan lemak dari kultur (sel dan supernatan) bakteri laut *Pseudomonas nautica* strain 617 dipengaruhi substrat pertumbuhan (*acetate* atau *eicosane*). Bentuk transport substrat ke permukaan sel: *adherence*, emulsifikasi dan solubilisasi dipengaruhi oleh komposisi biokimiawi yang diproduksi bakteri. Peningkatan konsentrasi senyawa ekstraseluler nampak selama pertumbuhan bakteri pada substrat *eicosane*. Aktifitas solubilisasi, emulsifikasi dan *adherence* berubah pada saat terjadi perubahan komposisi biokimiawi ekstraseluler. Diperoleh prosentase biodegradasi *eicosane* sebesar 59% setelah kultur berumur 3.5 hari.

Kata kunci: komposisi biokimiawi, transport substrat, biodegradasi, bakteri laut, n-alkana.

INTRODUCTION

Long chain n-alkanes are highly insoluble in water, and consequently various mechanisms have been proposed to explain the ability of microorganisms to metabolize such compounds. Of the aliphatic hydrocarbons, it is the n-alkanes that are claimed to be rapidly degraded components in both laboratory culture and the natural environment (Oudot *et. al.*, 1986).

Alkane-grown bacteria cultures exhibit physico-chemical properties which enable growth by increasing the contact between cells and hydrocarbons can be realized through: 1). adherence which depends on cell hydrophobicity and biochemical composition of the cell surface (Goswami and Singhs, 1991; Rosenberg and Rosenberg, 1985; Neu 1996). Lemke *et al* (1995). 2) the excretion of extracellular compounds with tensio-active properties, the so-called biosurfactants, also contribute for increasing the surface of contact between the cells and the hydrocarbon substrate; 3) they can either solubilize and/or emulsify the substrate to facilitate its transport into the cell (Haferburg *et. al*., 1986; Goswami & Singh, 1991; Zhang and Miller 1994; Hommel, 1994; *Marin et. al*., 1996).

The chemical composition of biosurfactants is highly variable (Haferburg *et al* 1986; Rosenberg, 1986; Georgiou *et al.,* 1992). A biosurfactant may have one of the following structures; mycolic acid, glycolipid, polysaccharide-lipid complex, lipoprotein or lipopeptide, phospholipid.

Most biosurfactant-producing microorganisms seem to produce biosurfactants in response to the presence of hydrocarbons in the grown medium. The yeast *Candida lipolytica*

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only produces biosurfactant when grown on hydrocarbon, and it is assumed that the presence of hydrocarbon may induce biosurfactant production (Parreilleux, 1979). *Acinetobacter* is morphologically different when grown with and without hydrocarbon in the growth medium (Zajic and Mahomedy, 1984). The general effects of hydrocarbon on biosurfactant production suggest that lipids, proteins and carbohydrates may play a role in hydrocarbon uptake.

A number of works have attempted to understand the processes such as adherence and/or biosurfactants release involved in hydrocarbon biodegradation. Most of these studies were performed in a range temperature from 25°C to 37°C. Furthermore, data related to marine bacteria are still scarce (Floodgate, 1978; 1984; Passeri, *et. al*., 1992; Bertrand *et. al.,* 1993; Fernandez-Linares *et. al*. 1996).

With the aim to evidence the biochemical adaptation of a marine bacterium growing on hydrocarbons, we conducted a serie of experiments on a strain *Pseudomonas nautica* 617 isolated from petroleum refinery outlets. The biochemical composition of cells and supernatants were investigated during the growth of *Pseudomonas nautica* 617 on n-alkanes at 32°C, and was related to the three modes of substrat uptake to the cell surface: adherence, emulsification and solubilization.

MATERIALS AND METHODS

Microorganism, Culture Condition and Degradation Percentage

The bacterium strain *Pseudomonas nautica* 617 was isolated from coastal area of Mediterranean sea at the mouth of a petroleum refinery outlet which is chronically polluted by hydrocarbons. It was deposited at the collection of the Institute Pasteur with reference No 617/1.85 (Bonin *et. al*., 1987). The marine bacterium was grown in synthetic sea-water medium with the following composition (in *g/1* distilled water): Tris (hydroxy-methly-aminomethane), 6.00, NaCI, 23.00; KCI, 0.75; CaC12, 2H2O, 1.50; NH4Cl, 3.74; MgSO4, 7H2O, 3.91, MgC12, 5.08, FeSO4, 0.002 and K2HPO4 0.036. The pH was adjusted to 7.8 with (1 N) HCI. The carbon source: Na-acetate (2 g 1-1) or eicosane (1 g 1-1). The cultures were incubated at 32°C on a reciprocal shaker (96 rev. min-1). Growth was monitored by following absorbance at 450 *nm* with a Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan, UV 240). Three cultures were conducted for each experiment characterized by the substrate and the growth temperature. Cells were harvested at the end of the exponential growth phase. For measurement of the percentage biodegradation, one set of cultures (50 ml) was basified with NaOH and then was extracted with chloroform for 12 hours in a liquid-liquid soxhlet type extractor (Linares *et al*., 1996)

Determination of the Different Modes of Substrat Uptake

Adherence was measured according to Rosenberg (1991), the emulsifying activity according to Roy *et. al*., (1979). Solubilizing activity was measured according to Reddy *et. al.,* (1983).

Biochemical Composition

Lyophilisats from cells and supernatant were resuspended in distilled water and separate into aliquots devoted to protein, carbohydrate and lipid analyses. Proteins were estimated by the method of Lowry *et. al*. (1951) using bovine serum albumin as standard. Total carbohydrates were quantified according to Dubois *et. al. (1956)*. Lipids were extracted from lyophilized cells and supernatant according to Bligh and Dyer (1959). Separation and identification of class of compounds were performed using thin-layer chromatography coupled with flame ionization detetion (TLC/FID) on latroscan apparatus TH 10-MK IV (serial number 492106, latron Laboratories, Tokyo) according to the multi step development scheme described in Goutx *et. al.* (1987) and Goultx *et. al.,* (1990a). Identification of the phospholipid compounds by the TLC/FID method was validated by bidimentional TLC in plates following the procedure developed by Gerin & Goutx (1993).

RESULTS

Growth

The generation time increased when growth on insoluble substrate (Table 1). Eicosane degradation percentage is 59%. The values was obtaine at the end of exponential phase, that is after 3,5 days. Protein content increased

after growth on eicosane compared to substrat acetate.

Table 1. Growth Characteristics of *P. nautica* 617 Cultured on Acetate (2 *g/l*) or Eicosane (*1 g/l*).

Substrate	Generation time (hour)	Protein	Degradation (%)	Growth Yield mg protein/mg degraded eicosane
Acetate	2	123.44 (13.91)	-	-
Eiconne	14	147.51 (13.91)	59 (6)	0.25

Values shown are the means of three replicates =/- standard deviation (in parentheses)

Biochemical Composition of Cell Pellets

Endocelluler carbohydrates did not exhibited any clear pattern of change with substrat. Concentration is 234.32 and 241.00 *ug. mg.l*$^{-1}$ protein for eicosane-grown cell and acetate grown cell respectively. Lipid content was affected by changes in substrat, it was higher in eicosane-grown cell (195.81 *ug mg*$^{-1}$ protein) than in acetate-grown (40.55 *ug mg*$^{-1}$ protein)

Polar lipid classes (AMPL and PL) varied in eicosane-grown cells than in acetate-grown cells (Table 2). WE, AMPL, and PL concentration enhanced in cells during growth on eicosane substrate. The main phospholipids of the bacteria were DPG + PG and PE according to Gerin and Goutx (1993) (Table 3) whatever the substrate. PE predominated in all the cultures. Both PE and DPG + PG concentration (231.24 *ug mg-1* protein). Lysophosphatidylethanolamine (LPE), the identification of which was confirmed by TLC analyses, characterized eicosane-grown cells.

Table 2. Lipid Class Composition of Cell Pellets (*ug/l*) *P. nautica* 617 Grown on Acetate or Eicosane.

Carbon Source	PL	AMPL	ALC	FFA	WE
Acetate	95.80 (11.90)	0.08 (0.00)	0.00	9.61 (5.14)	0.00
Eicosane	146.94 (26.34)	4.88 (0.47)	trace	4.54 (2.77)	6.37 (0.68)

Abbreviations: PL (phospholipids); AMPL (acetone mobile polars lipids); ALC (alcohols); FFA (free fatty acids); WE (wax esters). Values shown are the means of three replicates +/- standard deviation (in parentheses)

Table 3. Phospholipid Composition of Cell Pellets (*ug/mg* protein) of *P. nautica* 617 Grown on Acetate or Eicosane.

Carbon Sources	DPG + G	PE	LPE
Acetate	29.24 (0.26)	66.56 (11.64)	0.00
Eicosane	54.33 (8.88)	81.82 (16.10)	10.79 (1.36)

Abbreviations: DPG (disphosphatidylglycerides); PG (phosphatedylglycerides); PE (phosphatidylethanolamine); LPE (lyso-phosphatidylethanolamine); Values shown are the means of three replicates +/- standard deviation (in parentheses)

Biochemical Composition of Supernatant

Extracellular proteins did not exhibite any clear pattern of change with substrat. Supernatant from eicosane-grown cultures were enriched in both carbohydrates and lipids compared to acetate-grown.

Extracellular lipids were mainly composed of PL, AMPL, WE and FFA (Table 4). The proportions of AMPL in total lipids were higher in supernatant than in cells. All lipid classes concentrations increased after growth on eicosane.

Table 4. Lipid Classes Composition of Supernatant (*ug/l*) of *P. nautica* 617 Grown on Acetate or Eicosane.

Carbon sources	PL	AMPL	ALC	FFA	WE
Acetate	34.78 (19.01)	10.41 (2.51)	0.00	0.00	0.00
Eicosane	119.85 (30.50)	45.46 (19.89)	trace	8.29 (0.08)	44.23 (6.13)

Abbreviations: PL (phospholipid); AMPL (aceton mobile polars lipids); ALC (alcohols); FFA (free fatty acids); WE (wax esters). Values shown are the means of three replicates +/- standard deviation (in parentheses).

The phospholipids of the supernatant were phosphtidylglyceride, phospatidylethanolamine and lysophatidylethanolamine. They increased after growth on n-alkane (Table 5). Phosphotidylethanolamines exhibited a drastic increase (x 3) after growth on eicosane.

Modes of substrate uptake to the cells

Adherence activity of *Pseidomonas nautica* 617 show a significant increase (up to x 4.3) in eicosane-grown cells compared to acetate-grown cells. Up to 30 - 37% of the cells adhered to the hexadecane when cells were grown on eicosane.

Table 5. Phospholipid Composition of Supernatant (*ug/l*) of *P. nautica* 617 Grown on Acetate or Eicosane.

Carbon Sources	DPG + PG	PE	LPE
Acetate	17.29 (8.06)	17.49 (10.95)	0.00
Eicosane	64.04 (14.72)	55.81 (12.78)	trace

Abbreviations: DPG (diphosphatidylglycerides); PG (phosphatedylglycerides); PE (phosphatidylethanolamine); LPE (lyso-phosphatidylethanolamin. Values shown are the means of three replicates +/- standard deviation (in parentheses)

Emulsifying activity (EA) of supernatant was higher in eicosane-grown cultures nn (0.40 – 0.60) than in acetate-grown culture (0.20 – 0.24). A sizeable solubilizing activity was observed in eicosane-grown cultures (8.20 - 10.80 $mg.l^{-1}$) compared to acetate-grown cultures where it was neglectable.

DISCUSSION

Changes in the biochemical composition of cells and supernatants and the modes of substrates uptake were characteristics of *Pseudomonas nautica* 617 growth on eicosane. *Pseudomonas nautica* 617 cellular lipids, phospholipids in particular, increased up to 2.7 fold during the growth of the strain on the n-alkane, whereas carbohydrates concentrations remained stable as a consequence, these changes in the composition of lipids can influence some fundamental cellular function including membrane transport and the activy of membrane-bound enzymes. Two hyphotesis could explain these observations: 1) an enhancement of bacteria cells size during growth on eicosane or 2) a change in intracellular composition of cells. Microscopic observation of the cultures did not reveal any increase in cell size (Husain *et. al*., 1997) which could explain the phospholipid increase by a size/content relationship. From a qualitative point of view, cellular phosphatidylglyceride, where as lysophosphatidylethanolamine was present at trace level. Several authors already reported increased of phospholipid contents in hydrocarbon grown bacteria. Kennedy and Finnerty (1975) related this observation to the presence of intra-cytoplasmic membranes, necessary to the complex-enzymatic activity responsible for the oxidation of n-alkanes. Duppel, *et. al*. (1973) show that phosphatidylethanolamines were associated to this complex-enzymatic system. Makula *et. al*., (1975), Scott & Finnerty (1976) carefully described the distribution of the phospholipids in the membrane structure of *Acinetobacter* sp. H01-N, which is able to oxidise hydrocarbons. For these authors, the phosphatidylglycerides were the specific components of the intra cytoplasmic membrane of cells grown on hydrocarbon substrate. Such evidences support our hypothesis according to which intra cytoplasmic membrane formation would lead to phospholipid synthesis in *Pseudomonas nautica* 617 cells growing on eicosane.

Another difference in the biochemical composition of eicosane grown cells and acetate grown cells concern wax esters and acetone mobile polar lipids, both enriched in the former cells. These two lipid classes compounds as possible agents of cell hydrophobicity. The hydrophobic properties of wax esters are known in the cuticles of plants (Kolattukudy and Walton 1973) and crustaceans (Sargent, *et. al.,* 1981), although their function as energy storage in cells is usually described. Accumulation of wax esters in n-alkane grown cultures is reported by Gallagher (1971), Makula, *et. al*. (1975) and Goutx, *et. al.* (1990b). Its synthesis proceed from the metabolism of the alkane and a primary alcohol issued from alkane oxidation. Thus, the metabolism of cells growing on alkane substrate convertion. Acetone mobile polar lipids in bacteria mainly include glycolipids. Glycolipids are composed of short chain of mono-, di- and tri- saccharides linked glycolsidically to glyceride lipid moities. The role of glycolipids as surface-active agents is well described (Singer and Finnerty 1990). According to Williams and Fletcher studies (1996), membrane lipopolysaccharides with short sugar chains (such as glycolipids) would facilitate bacteria attachment to hydrophobic surface mainly occuring through lipid moities. Thus, glycolipids would contribute to the adherence properties of *Pseudomonas nautica* in addition to wax esters during alkane growth.

Alkane-grown cultures exhibited an increase of total extracellular proteins, carbohydrates and lipids when compared to acetate-grown cultures. This increase was related to the emulsifying properties and to solubilizing activity of the *Pseodomonas nautica* 617 supernatants which both enhanced on eicosane substrate. Solubilizing activity estimations show

that solubilizing agents were more active in the eicosane cultures than acetate cultures. Further separation (Reddy *et. al*., 1983; Cameotra and Singh, 1990; Goswami and Singh, 1991) are required to enable the identification of the biochemical compounds entering in the composition of the solubilizing agent.

CONCLUSION

The three modes of transfer known to be involved in insoluble substrate metabolism by bacteria were evidenced in *Pseudomonas nautica* culture on n-alkane. Adherence, emulsifying capacity and solubilizing activity were observed. In such a case, adherence and emulsifying activity, solubilizing activity would represent alternative processes to enable the degradation of eicosane. Thus, the relative importance of adherence properties, emulsifying capacities and solubilizing activities of the cultures seems effective enough to maintain *Pseudomonas nautica* 617 growth on eicosane.

The protection of the marine environment depends on the development of bioremediation processes of petroleum hydrocarbons (Prince, 1993; Bragg, 1994; Pritchard, 1991; Rosenberg, *et. al*., 1992). Such processes involve the selection if strains able to degrade hydrocarbon substrate with strong efficiency. It requires the understanding of the fundamental mechanisms which enable bacteria to degrade the different contituents of petroleum.

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