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Isolation from coastal sea water and characterization of bacterial strains involved in non-ionic surfactant degradation

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Abstract

A bacterial community degrading branched alkylphenol ethoxylate (APE) was selected from coastal sea water intermittently polluted by urban sewage. This community degraded more than 99% of a standard surfactant, TRITON X 100, but I.R. analysis of the remaining compound showed the accumulation of APE_2 (alkylphenol with a two units length ethoxylated chain) which seemed very recalcitrant to further biodegradation. Twenty-five strains were isolated from this community, essentially Gram negative and were related to *Pseudomonas, Oceanospirillum* or *Deleya* genera. Among these strains, only four were able to degrade APE_{9-10} (TRITON X 100). They were related to the *Pseudomonas* genus and were of marine origin. Pure cultures performed with these strains on TRITON X 100 gave APE_5 and APE_4 as end products. These products were further degraded to APE2 by two other strains unable to degrade the initial surfactant.

Introduction

Since many years, the use of non-ionic surfactants has increased for both domestic and industrial applications. Nonionic surfactants are essentially polyethoxylated derivatives of lipophilic molecules. Among these, alkylphenol polyethoxylate (APE) are the most common and in aromatic part, nonyl or octyl phenol is particularly recalcitrant to biodegradation, particularly when the alkyl chain is branched. This is the case with TRITON X 1OO which is a tert-octyl phenol polyethoxylate (Dorn et al. 1993). These molecules are responsible for an important part of the toxicity phenomena related to fauna and flora involving surfactants (Osburn and Benedict, 1966). The biodegradability of the different kinds of non-ionic surfactants has been studied so far mainly in fresh water, and most of the studies were based on activated sludge degradation models, as recommended by O.E.C.D. (Ruiz Cruz & Janer del Valle 1986; Gerike & Jakob 1988). The mechanism of degradation was also extensively studied, using pure cultures of fresh water bacteria (Cain 1981; Mergaert et al. 1992; Maki et al. 1994). Nevertheless, direct and undirect spoiling of the sea by non-ionic surfactants are of considerable importance and contamination of littoral areas is often encountered (Vasquez Una & Nunez Garcia 1983). In marine environment, one can find a microflora adapted to such substrate and able to degrade it (Ekelund et al. 1993). But there is little information in the literature on the role played by each strain belonging to these communities. Fresh water bacteria coming from urban sewage may also take part in the biodegradation of non-ionic surfactants. Therefore, the aim of this study was to select from littoral sea water a bacterial community degrading the non-ionic surfactant, TRITON X100, which is widely used for industrial applications, to characterize the strains and to define their role in the biodegradation process in order to verify the ability of marine bacteria to degrade such a surfactant.

Materials and methods

Bacterial cultures

Culture media

Liquid media were prepared with basal medium (BM)
- NH_4Cl 2 g
- Tris buffer, pH 7.2: trihydroxymethylaminomethane 6.05 g
  4N HCl: 11.05 ml
- 0.1M phosphate buffer, pH 7: 4 ml
- 1 mg of $FeSO_4$. $7H_20$ per ml: 2 ml
- sea water: 1000 ml

The phosphate buffer and the iron sulphate were sterilised separately and added aseptically to the cold medium to prevent phosphate precipitation and iron oxidation. The carbon source tested was added aseptically to the medium at the appropriate concentrations.

For isolations on solid medium, 15 g of agar were added per litre (BMA).

Tryptic soy medium (TSA), used for purification and conservation on agar slants, contained trypcase (7.5 g), soyase (2.5 g), agar (15 g) and sea water (1000 ml). Trypcase and Soyase are trade names from Biomerieux S.A., Marcy l'étoile, France.

Tests for sodium requirement

To obtain satisfactory growth of isolated strains, we modified the basal medium used by Baumann et al. (1971). The medium used was: trypcase (7.5 g), soyase (2.5 g), and half-strength synthetic sea water (1000 ml). Final concentration: NaCl, 0.2 M; $MgSO_4.7\,H_2O$, 0.05 M; KCl, 0.01 M; $CaCl_2.2\,H_2O$, 0.01 M. In this case, the trypcase and soyase NaCl content was negligible: 3 mM, so that this medium was suitable for sodium requirement tests. In order to differentiate the marine strains, NaCl 0.2 M was replaced by KCl at the same molar concentration. NaCl concentrations of up to 30 g/l were reached for differentiation between *Aquaspirillum* and *Oceanospirillum* genus. The tests were carried out in inverted T-shaped tubes, for vigorous shaking and aeration, containing 10 ml of medium. The tubes were placed on an horizontal shaker for 48 hours. The temperature of incubation was maintained at 30°C. Growth was recorded at the end of the experiments by performing optical density measurements at 450 nm.

Selection of the bacterial community

The bacterial community was selected from sea water of Gaou island (Var, France) intermittently polluted by urban sewage.

A culture was grown in a 1 litre Erlenmeyer flask containing 200 ml BM and Triton X100 at a concentration of 100 mg/l. When surfactant degradation reached 90% as verified by residual surfactant titration, a subculture was inoculated by transferring 10 ml of the old culture into a new flask. This process was repeated a second time and then isolation was carried out. The community was conserved by monthly transfers.

Isolation of the bacterial strains

Several bacterial strains were isolated by spreading 0.1 ml of serial dilutions on Petri dishes containing BMA medium with Triton X100, 4-hydroxybenzoate (4HB) or 4-hydroxyphenylacetate (4HPA) as sole carbon source (100 mg/l).

The various colonies were picked and isolated as pure cultures on TSA medium.

Strain characterization

Gram staining, flagellation examination after Rhodes staining (1955), and cytochrome oxidase testing by the Oxy-swab technique (Biological Labs, Austin, Tex.) were performed with each strain. As they were all Gram negative, a numerical profile was determined with API 20NE strips (Api system S.A., La Balme les Grottes, France) in order to characterize and cluster the strains. The API code number alone is not adequate for the identification of marine bacteria and must be used with several other tests. For optimal development of halophilic strains, the API 20NE strips were prepared with 20 g/litre NaCl solution and the basal medium was also supplemented with NaCl so that the API code number was not related to the general code book. The surfactant degradation experiments with isolated strains were carried out in inverted T-shaped tubes with 10 ml of BM containing 100 mg/l or 500 mg/l of carbon source.

The carbon sources used were:
- Triton X100, alkylphenol polyethoxylate (APE) with ethylene oxide chain of 9–10 units length (APE_{9-10}), Triton X114 (APE_{7-8}), Triton X45 (APE_5), 4-(tert-octyl) phenol (AP) which is the commune alkylphenol part of the Triton surfactants used here, and its derivatives with ethylene oxide chain of 1, 2 or 3 units (APE_1, APE_2 and APE_3);

– Polyethylene glycol (PEGs): PEG400, PEG200, Triethylene glycol: TEG, Diethylene glycol: DEG, Monoethylene glycol: MEG.

The putative intermediate products of the different pathways of degradation were also tested (500 mg/l): glycolaldehyde, acetaldehyde, acetate, ethanol, glycollate, glyoxilate, glycerate, malate, oxalo-acetate, pyruvate, citrate, succinate and fumarate. All these products were of analytical grade.

APE_1, APE_2, APE_3 were synthesized in the laboratory. APE_1 was prepared by addition on AP of an epichlorhydrin derivative, in which the alcohol function was protected by addition of dihydro,3,4-pyranne. APE_2 and APE_3 were prepared in the same way by successive additions of epichlorhydrin. N.M.R. and I.R. were used to check the molecular structures obtained.

Analytical methods

Non-ionic surfactant titration

Non-ionic surfactants in culture media were titrated by the colorimetric method described by Baleux (1972) using a iodine reagent: I_2 (1 g), KI (2g), H_2O (100 ml).

Extraction of degradation products

A mixture of 25 ml $MgSO_4$ solution (72.4% of $MgSO_4.7\ H_2O$) and 0.5 ml of H_2SO_4 5N was added to 15 ml of culture medium in a separatory funnel. The total mixture was extracted three times with 25 ml $CHCl_3$. The total organic phase was dried with Na_2SO_4 and evaporated.

The dry residue was dissolved in 5 ml acetonitrile for HPLC analysis or in 0.2 ml of CCl_4 for IR spectroscopy. The HPLC equipment consisted of an LC5A pump (Shimadzu, Kyoto, Japan) and a Pye-Unicam Model LC-UV spectrophotometer (Philips, Cambridge, U.K.). The reverse phase chromatography was run on RP18 column, using acetonitrile – water (50–50) as mobile phase and UV detection at 230 nm for aromatic molecules.

Determination of the EO number by IR spectroscopy (Frazee et al. 1964)

The absorbance ratio ($A_{1120/1250}$) of aliphatic ether bond absorption at 1120 cm^{-1} versus the aromatic ether bond absorption at 1250 cm^{-1} is directly proportional to the ethylene oxide chain length (EO number). The baseline is determined at the 975 cm^{-1} valley. A calibration curve was established using APE_1, APE_2, APE_5, APE_{9-10} as references.

Table 1. Identification and clustering of the isolated strains of the Gaou community using the API 20 NE code numbers in addition to cultural and morphological characters

Characters	Strains	API 20NE Profiles	Na requirement	Genus assigned
Rods	T4	7454745	-	
Gram -	HB2	7454745	-	*Vibrio*
anaerobics	HPA1	7454745	-	or
Polar flagella				*Aeromonas*
Oxidase +				
	HPA8	1000004	-	*Pseudomonas*
Rods	HPA10	1667765	-	*Pseudomonas*
Gram -	T1	0000104	+	marine
aerobics	T2	0000104	+	*Pseudomonas*
Polar flagella	HB4	0000104	+	or
Oxidase +	HPA6	0000104	+	*Alteromonas*
	HPA9	1047765	+	
Rods	GS1	1000762	+	marine
Gram -	GS3	1042762	+	*Pseudomonas*
aerobics				or
Polar flagella				*Alteromonas*
Oxidase -				
Rods,Gram-	HB7	0000004	+	*Deleya*
aerobics				
Peritrichous				
Oxidase +				
Rods,Gram-	HB11	0010004	+	
aerobics	HPA7	1040044	+	
non motile	T6	0467665	-	
Oxidase +	T7	0467665	-	
Curved rods or	T9	1000004	+	
helicoïdal	HB1	0000004	+	
Gram -	HB8	0000004	+	*Oceanospirillum*
aerobics	HB10	0000004	+	
Polar flagella	HPA11	0000004	+	
Oxidase +	T8	1047404	+	
	HB3	1047444	+	*Oceanospirillum*
	HPA4	1047444	+	

Results

Characterization of isolated strains

A total of 25 strains were isolated from enrichment cultures on Triton X100. These strains were classified into 12 clusters on the basis of their morphological characteristics as well as on their biochemical data and sodium requirements (Table 1). All the isolated strains

Table 2. Growth of strains isolated from the Gaou community on various substrates used as sole carbon source

Strain	T4	HB2	HPA1	HPA8	HPA10	T1	T2	HB4	HPA6	HPA9	GS1	GS3	HB7
Triton X100	-	-	-	-	-	+	+	+	+	-	-	-	-
Triton X114	-	-	-	(+)	-	+	+	+	+	-	-	-	-
Triton X45	-	-	-	+	-	+	+	+	+	+	+	+	-
APE3	-	-	-	-	-	-	-	-	-	-	-	+	-
APE2	-	-	-	-	-	-	-	-	-	-	-	-	-
APE1	-	-	-	-	-	-	-	-	-	-	-	-	-
AP	-	-	-	-	-	-	-	-	-	-	-	-	-
PEG 400	-	-	-	-	-	+	-	+	-	-	+	+	-
PEG 200	-	-	-	-	-	+	-	+	-	-	+	+	-
TEG	-	-	-	-	-	+	-	+	-	-	+	+	-
DEG	-	-	-	-	-	-	-	-	-	-	+	+	-
MEG	-	-	-	-	-	-	-	-	-	-	+	+	-
Acetaldehyde	+	+	+	+	-	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol	+	+	-	+	-	-	-	-	-	+	+	+	-
Glycolate	-	-	-	-	+	-	-	-	-	-	+	+	-
Glyoxylate	-	-	-	-	+	-	-	-	-	-	+	+	-
Glycerate	+	+	+	+	+	-	-	-	-	+	+	+	+
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+
Malate	+	+	+	+	+	-	-	-	+	+	+	+	+
Oxaloacetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycolaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	-
Succinate	+	+	+	+	+	-	-	-	-	+	+	+	nd
Citrate	+	+	+	+	+	-	-	-	-	+	+	+	nd
Fumarate	+	+	+	+	+	-	-	-	-	+	+	+	nd

Growth was checked by performing optical density measurements at 450 nm. - the optical density was similar to the control level; (+) the optical density was twice the control level; + the optical density was three times higher or more than the control level.

were Gram negative, 18 were from marine origin and 7 did not require sodium for growth.

On the basis of their sodium requirements, peritrichous bacteria were classified in the genus *Deleya*. Polar flagellated bacteria belonged to two major groups: fermentative or strictly aerobic. Fermentative bacteria showed no sodium requirement and might be classified in the *Vibrio* or *Aeromonas* genera. Strictly aerobic bacteria were classified in the *Pseudomonas* genera (if marine, they were classified as *Alteromonas*) and *Oceanospirillum*. Non motile bacteria remained undetermined.

Metabolic potentialities of the bacterial community strains

All strains were tested for the utilization of Triton X100, previously used to select the community. Only 4 strains (T1, T2, HB4, HPA6) initiated its biodegradation by shortening the EO chain (primary biodegradation). Strains degrading APE_{9-10} were also able to degrade APE_{7-8} and APE_5. This latter product was also degraded by some others strains (HPA7, HPA8, HPA9, GS1, GS3, T6,T7). Only one strain (GS3) degraded APE_3 and none of the strains was able to degrade AP, APE_1 and APE_2.

Ethers can be attacked by the following processes (Cain 1981):

- monooxygenases which affect an oxidative cleavage of the ether and release glycolaldehyde,
- oxidation of the carbon α to the ether and hydrolysis of the ester with production of glycolic acid,
- hydrolysis which releases ethylene glycol or the ethoxyl units will be removed sequentially from the substrate as acetaldehyde,
- a carbon-oxygen lyase effecting a β-eliminative cleavage on the other side of the C–O–C bond would, in the case of polyglycol, also generate ethylene glycol and a PEG, one ethoxyl unit shorter.

Table 3. Growth of strains isolated from the Gaou community on various substrates used as sole carbon source

Strain	HB11	HPA7	T6	T7	T9	HB1	HB8	HB10	HPA11	T8	HB3	HPA4
Triton X100	-	-	-	-	-	-	-	-	-	-	-	-
Triton X114	-	(+)	-	-	nd	-	-	-	-	nd	nd	nd
Triton X45	-	+	+	+	nd	-	-	-	-	nd	nd	nd
APE3	-	-	-	-	-	-	-	-	-	-	-	-
APE2	-	-	-	-	-	-	-	-	-	-	-	-
APE1	-	-	-	-	-	-	-	-	-	-	-	-
AP	-	-	-	-	-	-	-	-	-	-	-	-
PEG 400	-	-	-	-	-	-	-	-	-	-	-	-
PEG 200	-	-	-	-	-	-	-	-	-	-	-	-
TEG	-	-	-	-	-	-	-	-	-	-	-	-
DEG	-	-	-	-	-	-	-	-	-	-	-	-
MEG	-	+	+	+	-	-	-	-	-	-	-	-
Acetaldehyde	-	+	+	+	+	+	+	-	+	+	+	+
Acetate	-	+	+	+	+	+	+	+	+	+	+	+
Ethanol	-	+	+	+	-	+	-	+	-	-	-	-
Glycolate	-	+	-	-	-	-	+	-	-	+	+	+
Glyoxylate	-	+	+	-	-	-	-	-	-	-	+	+
Glycerate	-	-	+	-	-	+	+	+	-	-	+	+
Pyruvate	-	+	+	-	-	+	+	+	+	-	+	+
Malate	-	+	-	-	+	+	+	+	+	+	+	+
Oxaloacetate	-	+	+	-	-	+	+	+	+	-	+	+
Glycolaldehyde	-	-	-	-	-	-	-	-	-	-	–	
Succinate	-	+	+	+	nd	+	+	+	+	+	+	+
Citrate	-	+	+	+	nd	+	+	+	+	-	-	+
Fumarate	-	+	+	+	nd	+	+	+	+	+	+	+

Growth was checked by performing optical density measurements at 450 nm. - the optical density was similar to the control level; (+) the optical density was twice the control level; + the optical density was three times higher or more than the control level.

These four mechanisms would generate *ethylene glycol, glycolaldehyde* or *glycollate* if they acted on the terminal ether bond (Figure 1).

If they cleaved internal ether bonds: *lower molecular weight polyglycols, polyglycol aldehydes* or *carboxylated polyglycols* are produced respectively.

Ethylene glycol, glycolaldehyde and glycollate are oxidized via the glycerate pathway.

In order to investigate the above mentioned mechanisms, all the strains were tested on various PEGs, and on various acids of the glyoxylic cycle. The results are summarized in Tables 2 and 3.

Only four strains degrading PEG were found: strains T1 and HB4 had exactly the same potentialities, and strains GS1 and GS3 differed from precedent strains by the utilization of MEG and DEG. These strains also used all the short molecules of the various pathways described previously with the exception of glycolaldehyde which was not used by any strain.

Even though a great number of pure strains used short molecules, only a few of them such as T1 and T2 degraded PEG or APE. In order to confirm these results, new cultures were performed with the whole community and pure cultures of strains T1 and T2, using BM+Triton X100. The three cultures were followed during one month by titration of the remaining surfactant, extraction of the degradation products and analysis by HPLC and IR. HPLC patterns showed the disappearing of the TRITON peaks and the accumulation of shorter products identified by I.R. The results obtained after 30 days are summarized in Table 4. At this time, the characteristic I.R. alcohol absorption band (1065 cm^{-1}) attributed to the terminal OH of the ethoxylated chain is still unchanged, as well as

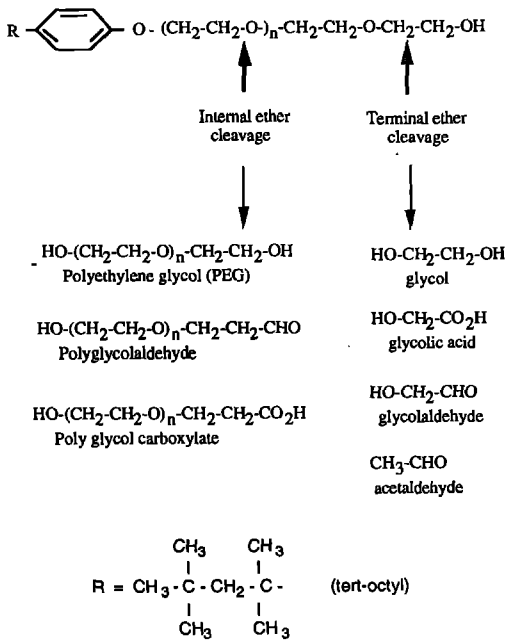

Figure 1. Main cleavage products of the ethylene oxide chain of alkylphenol ethoxylates (adapted from Cain 1981).

Table 4. Comparison of the degradation level of the surfactant Triton X100 by the whole community and pure cultures of strains T1 and T2

	Gaou community	Strain T1	Strain T2
% Triton X 100 remaining after 30 days incubation (*)	0.97	9.60	7.05
$A_{1120/1250}$ day 0	1.85	1.82	1.75
$A_{1120/1250}$ day 30	0.69	1.04	0.92
Number of EO on the residue (**)	2	4	3–4

(*) Titrated by colorimetric method.
(**) Determined by infrared spectroscopy using $A_{1120/1250}$: ratio of infrared absorbance at 1120 cm^{-1} (aliphatic ethers) versus 1250 cm^{-1} (aromatic ethers).

the 2850 cm^{-1} I.R. absorption band (CH_3 groups from tert-octyl chain) identical to the AP spectrum one.

These results were in very good agreement with the capacity of strains T1 and T2 to degrade APE_5 and not APE_3. In the whole community, strains GS1 and GS3 metabolized APE_5 and APE_3 to give APE_2 which is not metabolized by any strain.

Discussion and conclusion

An APE degrading bacterial community was selected from coastal sea water intermittently polluted by urban sewage. After 30 days of incubation, residual APE was only 0.97% of the initial quantity and we observed an accumulation of APE_2 which seemed very recalcitrant to biodegradation. Maki et al. (1994) observed the same phenomenon with a freshwater *Pseudomonas* strain. Our results were also consistent with the work of Patoczka and Pulliam (1990) who showed that recalcitrance of APE increases when the length of the ethoxylated chain decreases. Among the strains isolated from this community, only four (T1, T2, GS1, GS2) were able to degrade APE_{9-10} (TRITON X100) which was used to select the community. These strains were related to the *Pseudomonas* genus and were of marine origin. Pure cultures of these strains were able to degrade APE. After 30 days of incubation with T1 and T2 strains, APE_4 and APE_{3-4} accumulated in the culture medium and the percentage of remaining APE were higher than in the cultures containing the entire community, when cultivated in the same conditions. These strains were also able to metabolize acetaldehyde and acetate. In this case, the mechanism involved in the degradation of the EO chain seemed to be the hydrolysis pathway described by Pearce and Heydeman (1980). This mechanism observed with an *Acinetobacter* strain gave acetaldehyde by piece-wise shortening of the EO chain. Further oxidation of acetaldehyde could give acetate, also metabolized by these strains.

Two other marine strains (GS_1 and GS_3) of the community were able to degrade PEG, an intermediate product which could be liberated by internal cleavage of the ethoxylated chain. They were also able to transform APE_5 and APE_3 to APE_2. However, they were not able to degrade higher APE like APE_{7-8} and APE_{9-10}. These two strains were able to use all the metabolism intermediary products tested except glycolaldehyde and therefore should use the different metabolic pathways described above to degrade short APE or PEG.

The other strains of the community seemed to have little importance in the main degradation phenomena, but their presence indicate that they really participate in the biodegradation of end-of-metabolism products released by the other strains. Due to the absence of methyl groups connected to a carbon containing

a replaceable hydrogen, the alkyl chain of APE's remained undegraded in our experiments.

From an ecological point of view, we can notice that in our bacterial community isolated from coastal water, only marine strains (i.e. strains requiring sodium) play a preponderant role in APE degradation . The role of fresh water strains coming from sewage seems to be negligible in the whole process, but only the repetition of similar studies on various coastal sites can confirm this hypothesis.

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