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Influence of Growth Temperature on Cyclolipopeptides Production and on Adhesion Behaviour in Environmental Strains of *Pseudomonas fluorescens*

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Summary

The present study deals with the influence of growth temperature on biosurfactant production and the adhesion process in the psychrotrophic species *Pseudomonas fluorescens*. We studied a strain panel composed of nine wild cyclolipopeptide (CLPs) producers and by two biosurfactant mutants. Where cyclolipopeptide production was characterized at either 8°C or 17°C, cyclolipopeptide production was highlighted by hemolytic and tensiometric methods. Their ionic charge was evaluated by a double diffusion test and their identification was made as amphisin- or viscosin- or viscosinamide-like biosurfactants by Reverse Phase- High Performance Liquid Chromatography- Mass Spectroscopy. This categorization was corroborated by the 16S rRNA phylogenetic study. In *Pseudomonas fluorescens*, the number and relative quantity of cyclolipopeptide produced and bacterial adhesion differed with the growth temperature. Seven new cyclolipopeptides were characterized, of which three belong to the viscosinamide family. Biosurfactant secretion is intensive at 17°C and the highest adhesion is obtained at a lower temperature (8°C). Cyclolipopeptides appeared to antagonize the adhesion process. Strain hydrophobicity was wholly independent of growth temperature and could not be correlated with the initial attachment of bacteria, which was thermoregulated. Our study demonstrates that bacterial adhesion is controlled by the growth temperature but not by cyclolipopeptides or cell hydrophobicity.

Keywords: *Pseudomonas fluorescens*; Cyclolipopeptides; Biosurfactant; Growth temperature; Adhesion

Introduction

Surfactants are amphipathic molecules, which are particularly well adapted to distribution at interfaces between solids, liquids or even vapour [1]. These properties were at the origin of their multiple industrial applications in petroleum production, environmental control, food transformation, agriculture, pharmaceuticals and cosmetics [2,3].

Surfactants can be obtained by chemical synthesis but a great variety of microorganisms are also able to produce such molecules designated in this case as biosurfactants. The chemical nature and surface-active properties of biosurfactants are highly variable. Although the competitive advantage of biosurfactant production for microorganisms still remains unanswered, there is now ample evidence that these molecules are essential for survival, host-interactions and growth in many bacterial species [4,5]. These molecules should increase the bioavailability of hydrophobic water-insoluble substrates and improve heavy metal binding. Through this process, biosurfactants are involved in bacterial pathogenesis and quorum sensing [6-7]. As environmental bacteria need to adapt rapidly to variations of their growth conditions [8], the production of biosurfactant is important in these species. This is particularly the case in the *Pseudomonas* genus, which is one of the most ubiquitous bacterial groups. In the present study, we focused on nine environmental *Pseudomonas* strains isolated from plants and the rhizosphere. All these bacteria have been shown to produce cyclolipopeptides (CLPs) as biosurfactants [7-15]. In literature, CLPs are described as being involved in attachment at the surfaces, inert or green, in motility, and in pathogenicity [7-8]. This last point is essential since it appears now that for clinical strain isolates virulence depends on CLP production [7,16-18]. Biosurfactant synthesis is well documented in *Pseudomonas* [7-8], but it is remarkable that, until now, the influences of the growth temperature of *Pseudomonas fluorescens* on biosurfactant production and physico-chemical properties have not been investigated in detail. The production of exoproducts in these species is also a temperature dependent process. 17°C is known as the optimal growth temperature for many exoproducts synthesized by psychrotrophic *Pseudomonas* [19-22]. As amphiphilic exo-products, biosurfactants appear to play a role whenever a microbe encounters an interface, inert or living one. The results of these adaptation mechanisms could be adhesion, biofilm formation, quorum sensing and any response to environmental change [6]. All the knowledge on biosurfactant production in *Pseudomonas* should therefore be carefully reanalyzed taking into consideration this thermal parameter. In the present study, we investigated the influence of growth temperature on biosurfactant production at two key temperatures, 8°C and 17°C. The lower one corresponds to the temperature frequently used in cold storage conditions while the higher one represents the optimal growth temperature for many exoproducts. After sorted out genetically the studied *Pseudomonas fluorescens* strains, the decrease of surface

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tension is verified and reveals biosurfactant production. Their ionic characterization and the hemolytic bacterial behaviour are compared to the RP-HPLC/MS identification and then afford the proposal of a rapid CLP identification. The biosurfactant production could modify the bacterial surfacial properties and, thence, its adaptative potential in their microenvironment. Biosurfactant production are discussed in regard to the consequence of the growth temperature on the adhesive behaviour of *P. fluorescens*.

Materials and Methods

Bacterial strains

P. fluorescens PfA7B is a pathogenic strain of broccoli isolated from inflorescence. *P. fluorescens Pf1vis-* is a Tn5 km^r viscosin mutant of PfA7B [13]. These two strains were provided by Dr G. Braun (Agriculture & Agri-Food, Canada). *P. fluorescens* DSS73, a strain from sugar beet rhizospheric environment, and its km^r *ams*Y::*t*n5 amphisin mutant, *P. fluorescens* DSS73-15C2 [23], were also graciously provided by Dr O. Nybroe (Royal Veterinary & Agricultural University – Thorvaldsensvej, Denmark). Other strains of *P. fluorescens*, namely CTS22, CTS38, CTS50, CTS70, CTS117, CTS193 [11] and DR54 [10] were gifts from Dr T.H. Nielsen (Royal Veterinary & Agricultural University, Denmark). They had been isolated from Danish fallow or sugar beet fields. Both Danish fields have sandy and loamy soils.

Growth conditions and biosurfactant collection

The bacterial cultures were grown at 8°C and 17°C in Davis minimal media (DMM: 30 mmol.L^{-1} K_2HPO_4, 14 mmol.L^{-1} $KHPO_4$, 0.4 mmol.L^{-1} $MgSO_4$, 7.6 mmol.L^{-1} $(NH_4)_2SO_4$, 120 mmol.L^{-1} glucose, and 1 mL of trace element solution per litre (pH 7.3). The trace element solution per litre contained 20 mg of $CoCl_2.6H_2O$, 30 mg of H_3BO_3, 10 mg of $ZnSO_4.7H_2O$, 1 mg of $CuCl_2.2H_2O$, 2 mg of $NiCl_2.6H_2O$, 3 mg of $NaMoO_4.2H_2O$, 10 mg of $FeSO_4.7H_2O$, and 2.6 mg of $MnSO_4.H_2O$). 15g.L^{-1} agar was added to obtain a solid medium DMA. The glucose and trace elements were sterilized separately by filtration (Steriltop, Millipore, France) and aseptically added to the rest of the medium. Plates were inoculated with 40µL of a stock strain suspension. To collect the potential biosurfactant production, the bacterial colonies were carefully scraped off after 4 days incubation at either 8°C or 17°C and resuspended in 15mL natural mineral water. This suspension was centrifuged 18000g at 4°C for 30 min. The rinsing solution corresponded to the obtained supernatant.

Phylogenetic study

The 16S rRNA sequences of the biosurfactant producing strains were sequenced in double stranded by Cogenics Genome Express (Meylan, France). The sequences were deposited in the Genbank database under Accession Numbers indicated in Figure 1 and Table 1.

The 16S rRNA sequences of the *Pseudomonas* type strains used for phylogenetic analyses were retrieved from the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). The 16S rRNA sequences of the *P. fluorescens* SBW25 and Pf0-1 strains were retrieved from the *Pseudomonas* Genome Database (http://v2.*Pseudomonas*.com). All the sequences were aligned using Clustal X version 1.81 with default parameters [26]. The alignment was truncated to the same size corresponding to the shortest sequence (positions 119 to 1368 in the *E. coli* numbering system). All ambiguous positions and positions with gaps were removed. A neighbour-joining tree was inferred using the Kimura-two-parameter correction with MEGA v3.0 [27]. A maximum likelihood analysis was performed using fastDNAml based on the

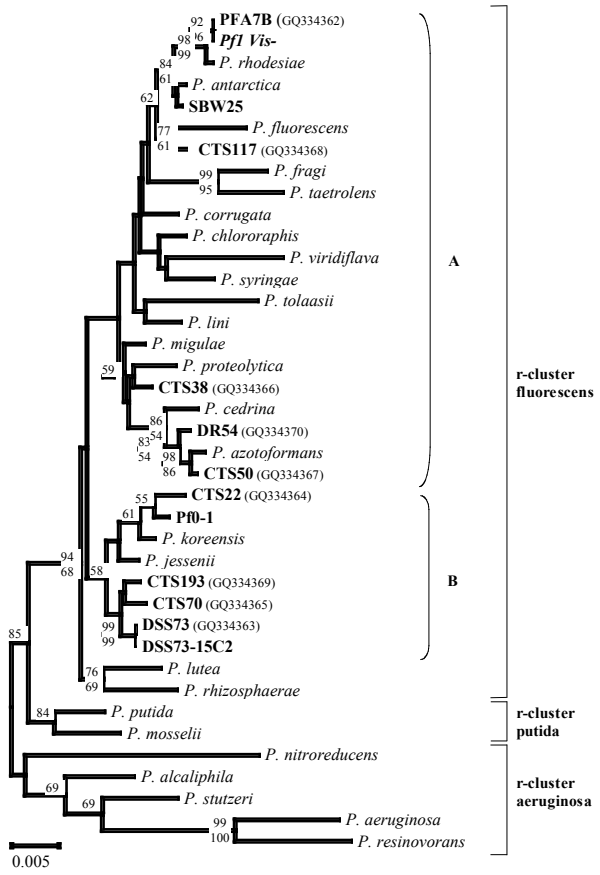

Figure 1: Phylogenetic relationships among 16S ribosomal RNA genes from the panel strain and 28 *Pseudomonas* type strains.
The nine biosurfactant-producing strains, the two biosurfactant negative mutants, SBW25 and Pf0-1 strains are in bold print. The unrooted dendrogram was carried out using the Neighbour-Joining algorithm. Numbers on tree branches report bootstrap results from Neighbour-Joining (above branch) and Maximum Likelihood (below branch) analyses for those branches having ≥50% support. A and B are two distinct phylogenic groups in *P.fluorescens* r-cluster.

HKY model with PhyloWin v2.0 [28]. The degree of statistical support for the branches was determined with 1000 bootstrap replicates for the neighbour-joining analysis or 100 bootstrap replicates for the maximum likelihood analyses.

Hemolysis activity test

Hemolysis has been described as a potential indirect method to demonstrate surfactant production [29]. In this test, each bacterial strain was streaked onto a 2% sheep red blood cell plate and incubated for 48h at 8°C or 17°C. The plates were visually inspected for zones of clearing around the colonies, indicative of biosurfactant production.

Surface tension measurement

Surface tension measurement of a rinsing solution of bacteria cultured on solid agar medium is a direct method for determining global biosurfactant production. In this study, the rinsing solution, obtained as described in paragraph Growth conditions and biosurfactant collection, was filtered through 0.22µm filters (Steritop, Millipore, France) and the fluid containing the biosurfactants was collected. The surface tension of the filtered solution was determined by the direct method of the pendant drop using a G40 goniometer (Krüss, France).

Strains	Access Number	Hemolysis activities onto 2% sheep red blood cell plate and incubated for 48 h		Surface tension+/-STD mN/m		Ionic charge of the rinsing solution	
		Growth at 8°C	Growth at 17°C	Growth at 8°C	Growth at 17°C	Growth at 8°C	Growth at 17°C
PfA7B	GQ334362	β ++	β ++	27.0+/-0.0	26.7+/-0.0	anionic	anionic
Pf1 vis-		-	-	71.2+/-0.1	60.9+/-0.7	non ionic	non ionic
CTS38	GQ334368	β +	β +	63.8+/-1.2	31.1+/-0.3	anionic	anionic
CTS50	GQ334367	β ++	β +	27.5+/-0.1	27.2+/-0.1	anionic	anionic
CTS117	GQ334368	β ++	β ++	35.9+/-0.7	29.6+/-1.1	anionic	anionic
DR54	GQ334370	β +	β +	38.2+/-0.9	34.6+/-1.4	non ionic	non ionic
DSS73	GQ334363	α +	α +	32.1+/-0.9	33.2+/-0.1	anionic	anionic
DSS73-15C2		-	-	69.8 +/-0.9	70.8+/-0.3	non ionic	non ionic
CTS22	GQ334364	α +	α +	57.1+/-2.3	33.2+/-0.2	anionic	anionic
CTS70	GQ334365	α +	α +	31.4+/-0.1	30.1+/-0.0	anionic	anionic
CTS193	GQ334369	α +	α +	37.6+/-1.4	35.2+/-0.1	anionic	anionic

Table 1: Registration number of sequenced 16S rRNA fragments and surfactant production evaluated by hemolytic phenotype, surface tension and ionic charge of the bacterial rinsing solution for the 11 strains.
Hemolysis on plate at 8°C and 17°C was realized as described in materials and methods. α corresponds to opaque lysis areas and β to transparent lysis areas. - : absence of clearing zone; + : lysis area≤1mm; ++: lysis area > 1mm. Surface tension of bacterial rinsing was determined as indicated in materials and methods at either 8°C or 17°C after four days (triplicate). The ionic charge determination was made with the rinsing solution of bacteria grown at either 8°C or 17°C as described in materials and methods.

Strains	Number of HPLC peaks	Molar mass (g.mol-1)	Retention time (min)		
	Growth at 8°C	Growth at 17°C		Growth at 8°C	Growth at 17°C
PfA7B	2	2	1111.7	24.0+/-0.7	24.2+/-0.0
			1125.7	28.5+/-0.9	28.7+/-0.0
Pf1 vis-	0	0	ND	ND	ND
DSS73	1	1	1394.8	16.0+/-0.6	16.0+/-0.6
DSS73-15C2	0	0	ND	ND	ND
CTS22	1	1	1353.8	16.2+/-0.6	16.0+/-0.5
CTS38	2	4	1111.6	ND	22.2+/-0.5
			1125.7	25.1 +/-0.6	26.2+/-0.7
			1151.6	ND	34.8+/-0.4
			1153.7	36.9+/-0.5	38.0+/-0.0
CTS50	3	5	1111.6	22.1+/-0.8	22.0+/-0.5
			1125.6	26.2+/-1.5	26.5+/-0.4
			1139.6	31.0+/-1.0	31.0+/-1.0
			1165.4	ND	38.0+/-0,9
			1167.6	ND	41.1+/-1.1
CTS70	2	2	1394.8	15.7 +/- 0.3	15.7+/-0.3
			1408.5	16.8 +/-0.5	16.8+/-0.5
CTS117	2	2	1111.7	21.2+/-0.6	21.1+/-1.0
			1125.7	24.9+/-0.9	24.5+/-0.8
CTS193	1	1	1408.9	13.8+/-0.8	13.8+/-0.4
DR54	6	6	1110.7	45.5 +/-0.7	45.4+/-0.6
			1124.7	46.9+/-0.4	46.9+/-0.4
			1110.7	47.3+/-0.5	47.3+/-0.4
			1124.7	48.7+/-0.4	48.8+/-0.4
			1150.5	53.7+/-0.5	53.7+/-0.3
			1152.6	57.0 +/- 0.5	56.9+/-0.3

Table 2: Biosurfactant characterization by HPLC for the nine wild strains grown at 8°C and 17°C
HPLC was done for biosurfactants produced by the wild strains as described in materials and methods. ND means not detected. The experiments were done in triplicate.

This method is based on the principle that the drop morphology at the apex, where it is totally independent of any contact, is correlated to the surface tension of the studied liquid. The surface tension can be calculated as the difference of pressure between the inside of the liquid and its environment divided by the sum of the inverse of the radius of the curvature of the drop.

Determining the ionic charge of biosurfactants

The ionic charge of biosurfactants was determined using the agar double diffusion technique adapted from Meylheuc [24]. Briefly, in these studies two regularly spaced rows of wells (10 mm diameter) were made in a low hardness agar Petri dish (1% agar). Wells in the lower row were filled with the rinsing solution. Each well in the upper row was filled with a pure compound of known ionic charge. The reference anionic substance selected was sodium dodecylsulphate (SDS) 20 $mmol.L^{-1}$. The cationic compound was cetyltrimethylammonium bromide (CTAB) 20 $mmol.L^{-1}$. Appearance of a precipitation line between the wells, indicative of the ionic character of the biosurfactant, was monitored for at least one week, at room temperature.

RP-HPLC / MS analysis of biosurfactants

The biosurfactants were analyzed by reverse-phase high

Group	molar mass (g/mol)	CLP	lipidic tail	AA1	AA2	AA3	AA4	AA5	AA6	AA7	AA8	AA9	AA10	AA11
	1110.7	Pseudodesmin B	$C_{10}H_{19}O_3$	L-Leu	D-Gln	D-allo-Thr	D-Val	D-Leu	D-Ser	L-Leu	D-Ser	L-Val		
		Massetolide E	$C_{10}H_{19}O_2$	L-Leu	D-Glu	D-allo-Thr	D-Val	L-Leu	D-Ser	L-Leu	D-Ser	L-Val		
	1111.7	Massetolide I	$C_{10}H_{19}O_3$	x-Leu	x-Glu	x-Thr	x-Abu	x-Leu	x-Ser	x-Leu	x-Ser	x-Ile		
		Massetolide J	$C_{10}H_{19}O_4$	x-Nva	x-Glu	x-Thr	x-Val	x-Leu	x-Ser	x-Leu	x-Ser	x-Ile		
	1124.7	Pseudodesmin A	$C_{10}H_{19}O_2$	L-Leu	D-Gln	D-allo-Thr	D-Val	D-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
		Viscosinamide	$C_{10}H_{19}O_2$	L-Leu	D-Gln	D-allo-Thr	D-Val	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
		Viscosin	$C_{10}H_{19}O_2$	L-Leu	D-Glu	D-allo-Thr	D-Val	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
		Massetolide F	$C_{10}H_{19}O_3$	L-Leu	D-Glu	D-allo-Thr	D-Val	L-Leu	D-Ser	L-Leu	D-Ser	L-Leu		
	1125.7	Massetolide L	$C_{10}H_{19}O_4$	L-Leu	D-Glu	D-allo-Thr	D-Ile	D-Leu	D-Ser	L-Leu	D-Ser	L-Val		
		Massetolide K	$C_{10}H_{19}O_5$	x-cyclopropAla	x-Glu	x-Thr	x-Ile	x-Leu	x-Ser	x-Leu	x-Ser	x-Val		
Viscosin-like CLP		WLIP	$C_{10}H_{19}O_6$	L-Leu	D-Glu	D-allo-Thr	D-Val	D-Leu	D-Ser	L-Leu	D-Ser	L-Leu		
		Massetolide A	$C_{10}H_{19}O_7$	L-Leu	D-Glu	D-allo-Thr	D-allo-Ile	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
	1139.7	Massetolide D	$C_{10}H_{19}O_8$	L-Leu	D-Glu	D-allo-Thr	D-allo-Ile	L-Leu	D-Ser	L-Leu	D-Ser	L-Leu		
		Massetolide G	$C_{10}H_{19}O_9$	L-Leu	D-Glu	D-allo-Thr	D-Val	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
		Pseudophomin A	$C_{10}H_{19}O_{10}$	L-Leu	D-Glu	D-allo-Thr	D-Ile	D-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
	1153.8	Massetolide B	$C_{11}H_{21}O_2$	L-Leu	D-Glu	D-allo-Thr	D-allo-Ile	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
		Massetolide H	$C_{12}H_{23}O_2$	L-Leu	D-Glu	D-allo-Thr	D-Val	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
	1167.6	Massetolide C	$C_{12}H_{23}O_2$	L-Leu	D-Glu	D-allo-Thr	D-allo-Ile	L-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
		Pseudophomin B	$C_{12}H_{23}O_3$	L-Leu	D-Glu	D-allo-Thr	D-allo-Ile	D-Leu	D-Ser	L-Leu	D-Ser	L-Ile		
	1353.8	Lokisin	$C_{10}H_{19}O_2$	D-Leu	D-Asp	D-allo-Thr	D-Leu	D-Leu	D-Ser	L-Leu	D-Ser	L-Leu	L-Ile	L-Asp
Amphisin-like CLP	1394.8	Amphisin	$C_{10}H_{19}O_2$	D-Leu	D-Asp	D-allo-Thr	D-Leu	D-Leu	D-Ser	L-Leu	D-Gln	L-Leu	L-Ile	L-Asp
	1408.8	Tensin	$C_{10}H_{19}O_2$	D-Leu	D-Asp	D-allo-Thr	D-Leu	D-Leu	D-Ser	L-Leu	D-Gln	L-Leu	L-Ile	L-Glu
		Hordersin	$C_{10}H_{19}O_3$	x-Leu	x-Asp	x-Thr	x-Leu	x-Leu	x-Ser	x-Leu	x-Gln	x-Leu	x-Ile	x-Glu

Table 3: Already published CLP structures, adapted from literature [7-8,11,33-35]. A An means amino-acid number n.

performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) using a method adapted from Morin [25]. Before analysis the rinsing solutions were diluted at 50% in a water/acetonitrile/ammonium acetate mixture 10:90:4. An Agilent Technologies Series 1100 vacuum degasser, LC pump and autosampler (Hewlett Packard, Germany) were used. 20µL of the sample were injected into an analytical C18 reverse-phase column (Hypersil ODS, 2.1 x 200 mm, 5 µm). The samples were first submitted for 4 min to an isocratic separation protocol using a water-acetonitrile-ammonium acetate mixture (65:35:4, Solution A), followed by a linear gradient from 0 to 27.3% in 5 min of Solution B consisting of a water-acetonitrile mixture (50:50, v/v). The percentage of Solution B was kept constant for 11 min to allow separation of biosurfactants in isocratic conditions. The column was then rinsed by a gradient of Solution B ranging from 27.3 % to 100% for 35 min. The flow (0.4mL.min^{-1}) was split (1/10) using a micro-splitter valve (Upchurch Scientific, USA) turned towards the detector. The separated compounds were detected by positive-ion electrospray ionization and ion trap mass spectrometry using a Bruker Esquire-LC ESI-MS/MS (Bruker Daltonic, Germany). The relative proportions of CLPs produced at 8°C and 17°C was estimated by peak area divided by the smallest observed area. This was applied for each chromatogram from the different strains of *P. fluorescens*.

The absence of rhamnolipids was verified by parallel HPLC analysis as described by Morin [41].

Adhesion assays

Adhesion assays were adapted from the biofilm formation assay described by O'Toole [30]. The adhesion test corresponded to an incubation period not exceeding one generation time. Thus only the adhesion inherent in the bacterial contact on the inert surface was taken into account without any disruption to the growth phenomenon. In triplicate, for each strain, three independent DMM culture were adjusted to an OD_{580}nm of 0.4. An aliquot of 100µL of each suspension was layered in triplicate in polystyrene micro titration plate. To determine of the adhesive ability, bacteria were incubated in static condition for 24h at 8°C and 4h at 17°C. The negative control consisted in sterile DMM. After removal of the bacterial solution and careful rinsing (x3 in 150µL sterile water) bacteria bound to the wells were stained by incubation for 30 min with 0.1% crystal violet. After rinsing three times with 150µL sterile water, remaining adherent bacteria were lysed by adding 100µL SDS (1% in sterile water). The adherent bacterial population was estimated by direct measurement of absorbance at 595 nm using a microtitration plate reader (Model 680XR, Biorad). To compare of the results, data were normalized as percentage of adhesion ability using as a reference the strain giving the median value in the whole study (i.e. PfA7B grown at 8°C). Results were normalized as percentage of the median value corresponding to the adhesive capability of PfA7B at 8°C. The Mann-Withney test was used to evaluate statistical significance between values.

Evaluation of bacterial surface hydrophobicity

The hydrophobicity of strains was evaluated by the microbial adhesion to solvent (MATS) test. It consisted in evaluating the affinity of the cells to apolar solvents (hexadecane). For the experiments, bacterial cells were carefully scraped off after 4 days at either 8°C or 17°C and resuspended in saline solution, then were harvested by centrifugation at 7000 g for 10 min and resuspended to Abs_{580} nm=0.4 (Abs1) in saline solution. Three rinsing, then centrifugation steps, were done to eliminate any residual CLP before evaluating the cell affinity for hexadecane. This bacterial suspension was mixed with a

solvent at 1:6 (0.4 :2.4 v/v) by vigorous agitation for 1 min in order to form an emulsion. This mixture was then left for at least 15 min until the separation of the two phases. The aqueous phase absorbance was measured (Abs2) and the percentage of adhesion was expressed as: %affinity = $(1\text{-}Abs_2/Abs_1)$ x100.

Results

Phylogenetic classification of the different strains of *P. fluorescens*

The Accession Numbers of the sequenced 16S rRNA genes obtained from the 9 selected environmental strains of *P. fluorescens* and the two mutants, namely PfA7B, *Pf1vis*-, DSS73, DSS73-15C2, CTS22, CTS38, CTS50, CTS70, CTS117, CTS193 and DR54, was compiled in Table 1. A phylogenetic analysis was carried out with the 16S rRNA genes of our 11 studied strains, the sequences of the *Pseudomonas* (*sensu stricto*) type strains close to our sequences and the sequences of the SBW25 and Pf0-1 strains. According to the study of Bodilis [31], it was possible on Figure 1 to distinguish three clusters termed aeruginosa, putida and fluorescens r-clusters. The 11 strains appeared distributed in 2 distinct groups in the fluorescens r-cluster. Six strains, PfA7B, its viscosin mutant *Pf1vis*-, CTS38, CTS50, CTS117 and DR54 were associated with the reference strain of *P. fluorescens* SBW25 in a first group, Group A. The second one, Group B was clearly separated from the others. It was represented by the strains DSS73, its amphisin mutant DSS73-15C2, CTS193, CTS70 and CTS22.

Hemolytic activity of the different strains of *P. fluorescens*

The 11 selected strains of *P. fluorescens* were tested for their hemolytic activity (Table 1). Blood agar lysis was observed with all strains after 4 days of incubation at either 8°C or 17°C except with the viscosin and amphisin mutants *Pf1vis*- and DSS73-15C2. Two types of clearing zones, characterized by opaque or transparent lysis areas corresponding to α- and β- hemolytic activity, respectively, were observed. Hemolytic activity results to pores formation on red blood cell. α-hemolytic activity reveals incomplete lysis, β-hemolysis resulting from complete lysis [18]. It was then possible to classify the strains into three phenotypes: non-hemolytic strains (*Pf1vis*- and DSS73-15C2), α-hemolytic strains (DSS73, CTS22, CTS70 and CTS193) and β-hemolytic strains (PfA7B, CTS38, CTS50, CTS117 and DR54).

Effect of the temperature on the surface tension of the rinsing solution from the culture of the different strains of *P. fluorescens*

We measured the effect of temperature on the surface tension of the rinsing solution from the culture of the different strains of *P. fluorescens*. The surface tension value of poorly mineralized water is 71.7mN.m^{-1} and, as described by Carrillo [32], the limit tension value indicative of the presence of biosurfactant in solution is 40 mN.m^{-1}. For the 11 strains of *P. fluorescens* presently studied, the surface tension of their rinsing solution was compiled in Table 1. When bacteria were grown at 8°C, seven wild bacterial strains, i.e, PfA7B, DSS73, CTS50, CTS70, CTS117, CTS193 and DR54, should be considered as surface-active producers since the surface tension of their rinsing solution was below 40 mN.m^{-1}. As logically expected, the two mutants *Pf1vis*- and DSS73-15C2 appeared negative for biosurfactant production. The two strains, CTS22 and CTS38, were also negative when grown at 8°C, but when the culture conditions were modified to a higher temperature (17°C), the production of biosurfactant was apparently stimulating. Except the mutants *Pf1vis*- and DSS73-15C2 that remained negative, all the wild strains of *P. fluorescens* produced a rinsing solution which showed surface tension under 40 mN.m^{-1}.

Effect of the temperature on the ionic charge of the rinsing solution from the culture of the different strains of *P. fluorescens*

For the 11 strains of *P. fluorescens* presently studied, the ionic charge of their rinsing solution was presented in Table 1. Eight wild strains, i.e. PfA7B, DSS73, CTS22, CTS38, CTS50, CTS70, CTS117 and CTS193, produced an anionic rinsing solution, while DR54 and the two mutants had a non ionic rinsing solution at 8°C and 17°C.

Effect of the temperature on biosurfactant production by the different strains of *P. fluorescens*

Rinsing solutions from the 11 strains of *P. fluorescens* cultured at either 8°C or 17°C were analyzed by HPLC. The chromatogram analysis gave three exploitable data: number of peaks and, for each peak, its molar mass and its retention time. All data were compiled in Table 2. For all of the nine wild strains, the chromatogram revealed at least one peak for each strain, unlike non-biosurfactant producer mutants, *Pf1vis*- and DSS73-15C2. These peaks could be indicative of the biosurfactant presence. After growth at 8°C, the detected compounds produced by the wild strains, with similar molar mass and retention time, were found as after incubation at 17°C, except for two wild strains, CTS38 and CTS50. They respectively produced two and three exoproducts at 8°C instead of four and five after growth at 17°C. The molar mass of these putative biosurfactants ranged from 1110.7 to 1408.5 g.mol-1, as expected for cyclolipopeptides (CLPs), such as viscosin-like, and amphisin-like CLPs. To help in their identification (and according to our current knowledge), we compiled in Table 3 previously published CLPs with similar molar mass. These observed values are related to two CLP families cited above. Table 4 listed, by strain and for each related HPLC peak, its abundance in the rinsing solution obtained at either 8°C or 17°C.

In the rinsing solution obtained at either 8°C or 17°C from PfA7B, two CLPs were detected corresponding to a major peak with a molar mass of 1125.7g.mol^{-1} and 1111.7g.mol^{-1}. The presence of Gln as amino acid in position 2, determined by mass spectroscopie (MS) –m/z: 843.2 and 714.4-, and the anionic charge of the molecule allowed us to identify the major peak as viscosin. The second peak was tentatively identified as massetolide E owing to the existence of CLP isoforms that can skew a precise identification of this molecule.

Strain DSS73 produced a more hydrophilic biosurfactant of 1394.8g.mol^{-1} with amphisin-like characteristics. Determining the anionic charge of this compound and MS spectrum confirmed its identification as amphisin.

Strain CTS22 was also found to produce a biosurfactant that was rapidly characterized as lokisin (1394.8g.mol^{-1}) by its hydrophobicity, mass and anionic charge. In fact, the Asp and Gln residue conferred an anionic charge to amphisin.

As observed with PfA7B, strain CTS38, grown at 8°C, was found to produce two biosurfactant molecules related to the viscosin family. The same molecules are identified at 17°C, plus two new ones, whose structure appeared rather similar. MS analysis of the molecule eluting at 25.1 ± 0.6 min did not make it possible to distinguish between possible viscosin diastereoisomers or very close isomers of a same

mass differing by only one amino-acid inversion between Leu or Ile. This molecule should correspond either to massetolide F or to a new molecule designated 1 with a primary structure of $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile). The second CLP generated at 8°C by CTS38 was identified as massetolide H. This molar mass corresponded to two CLPs: massetolide B or massetolide H, differing in their lipophilic tail (Table 3). The two MS peaks –m/z: 714.5 and 843.4- showed up as massetolide H. CTS38 only produced two CLPs at 8°C, yet 4 different biosurfactants were found at 17°C. In addition to the two previously identified molecules as massetolide F or 1 and massetolide H, a peak corresponding to 2 also detected at 8°C in strains CTS50 and CTS117, was identified in the rinsing solution of CTS38 grown at 17°C. Three CLPs were known with an identical molar mass and they were identified as massetolide E, I and J. Each time only one amino acid differs from viscosin's amino-acid sequence (Table 3). The MS common intense peaks: 829.5, 700.5 and 518.3 eliminated the possibility of massetolide I and J. In addition, massetolide E was already attributed to PfA7B as a co-exoproduct. The structure of 2 may be presumed similar to 1 structure, with the replacement of amino acid 9 Val to Ile/Leu. Moreover, another new CLP named 6 was investigated. The structure study assumed the change in amino-acid 1, Leu, by its cyclic analogue, cyclopropylalanine. The MS peak, m/z 728.4, confirmed the lactone ring described for massetolide H. The putative structure of 6 could be proposed as $C_{12}H_{23}0_2$-cyclopropAla-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile).

The analysis of biosurfactants generated by strain CTS50 was more complex since it was found to produce three forms of CLPs. The first one eluted at 22.1 ± 0.8 min, for a growth temperature of 8°C, and did not correspond to previously identified CLPs. The proposed structure of this molecule designated as 2 is $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Val). Moreover, the second peak eluted at 26.2 ± 1.5 min corresponded to a CLP whose characteristics were very similar to the molecule produced by strain CTS38 and designated as 1 or massetolide F. The last compound, with a more hydrophobic character had a molar mass of 1139.6g.mol^{-1} corresponding potentially

Strains	Molar mass (g.mol^{-1})	CLP	Putative structure	HPLC normalized peak aera	
				Growth at 8°C	Growth at 17°C
PfA7B	1111.7	Massetolide E*	-	199	535
	1125.7	Viscosin	-	1100	2569
DSS73	1394.8	Amphisin	-	141	2
CTS22	1353.8	Lokisin	-	1	72
	1111.6	2 or Massetolide E	For 2 $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Val)	0	26
CTS38	1125.7	1 or Massetolide F	For 1 $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	24	303
	1151.6	6	For 6 $C_{12}H_{23}$O2-cycloproAla-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	0	33
	1153.7	Massetolide H	-	17	288
	1111.6	2 or Massetolide E	For 2 $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Val)	12	37
	1125.6	1 or Massetolide F	For 1 $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	149	214
CTS50	1139.6	Massetolide A or Massetolide G or Pseudophomin A	-	750	556
	1165.4	7	at least the lactone ring (Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	0	5
	1167.6	Massetolide C or Pseudophomin B	-	0	4
CTS70	1394.8	Amphisin	-	2	21
	1408.5	Tensin	-	43	111
CTS117	1111.7	2 or Massetolide E	For 2 $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Val)	18	57
	1125.7	1 or Massetolide F	For 1 $C_{10}H_{19}O_2$-Leu/Ile-Glu-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	21	614
CTS193	1408.9	Hodersin	-	1	2
	1110.7	3 or Pseudodesmin B	For 3 $C_{10}H_{19}O_2$-Leu/Ile-Gln-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Val)	276	184
	1124.7	3 or Pseudodesmin B and Viscosinamide	-	11	49
	1110.7		-	14	41
	1124.7	Viscosinamide	-	1255	2244
DR54	1150.5	4	For 4 $C_{12}H_{23}O_2$-cycloproAla-Gln-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	8	25
	1152.6	5	For 5 $C_{12}H_{23}O_2$-Leu/Ile-Gln-(Thr-Val-Leu/Ile-Ser-Leu/Ile-Ser-Leu/Ile)	14	48
		* tentatively			

Table 4: CLP identification and quantification at 8°C and 17°C
HPLC/MS was made for CLPs produced by the wild strains as described in materials and methods. CLP quantification was evaluated with the help of the chromatogram area under the peaks divided by the smallest observed area, i.e. CTS 22 and CTS193 grown at 8°C.

to massetolide A, D or G or to pseudophomin A as indicated in Table 3. The MS spectrum of this molecule -m/z : 728.4 and 857.5- led to a 3-hydroxy decanoic acid as lipidic tail, incompatible with massetolide G. However, it was not possible to discriminate between massetolide A and D or pseudophomin A.

The number of CLPs produced by strain CTS50 went from 3 at 8°C to 5 at 17°C. The two newly detected molecules were a molecule of 1167.6 g.mol^{-1} that should be massetolide C or pseudophomin B and a new compound called 7 characterized by a molar mass reduction of two units compared to the last product. As proposed for CTS50, it could be a change in amino acid 1 of Leu, by its cyclic analogue, cyclopropylalanine. MS data confirmed a glutamine residue bound to the lactone ring (Thr-Ile-Leu-Ser-Leu-Ser-Ile). -m/z: 714.5 and 843.4-.

Two forms of amphisin-like molecules were detected in the rinsing solution of strain CTS70 grown at 8°C. Comparing them with published CLPs for this strain in different conditions, these molecules were identified as amphisin and tensin.

The analysis of biosurfactants generated by strain CTS117 revealed the presence of two CLPs previously also investigated in strain CTS50, namely the new 2 and the molecule corresponding either to massetolide F or 1.

The biosurfactant secretion profile of strain CTS193 was simpler with only one molecule of a characteristic mass of 1408.5g.mol^{-1} identified as hodersin of the amphisin family.

Conversely, strain DR54 was at the origin of the more complex secretion pattern of biosurfactants with 6 different molecules. For growth at 17°C, the proportions and mass of the corresponding products were respectively 7.13% (1110.7g.mol^{-1}), 1.91% (1124.7g.mol^{-1}), 1.59% (1110.7g.mol^{-1}), 86.54% (1124.7g.mol^{-1}), 0.97% (1150.5g.mol^{-1}) and 1.87% (1152.6g.mol^{-1}). The major peak was clearly identified as viscosinamide [10]. The properties of other secondary compounds, i.e. hydrophobicity, non-ionic character and presence of a glutamine residue, also suggested that they were members of the viscosinamide family, subgroup of non-ionic viscosin-like CLPs. With the help of very few references on viscosinamide-like CLPs [10,35], the exoproduct of 1110.7g.mol^{-1} could be designated like pseudodesmin B, but Sinnaeve [35] identifies this CLP at the same time as pseudodesmin A, which only differs from viscosinamide with respect to the stereochemistry of the Leu at position 5, being D rather than L. Hence an other putative biosurfactant, named 3, could be suggested as a diastereoisomer of pseudodesmin B. The two others molecules left, whose putative structure was deduced from MS analysis, were designated as 4 and 5. MS peaks revealed CLPs containing Glu in amino-acid 2 since m/z: 843.2 and 714.4 instead of 842.5 and 714.4 for Gln, as known for viscosin-like CLPs. For the two residual peaks, their elution overlapped around 47 min and was indicative of a mixture of viscosinamide and, 3 or pseudodesmin B, in minor quantity.

The biosurfactant production from bacteria grown at either 8°C or 17°C was carried out. For the majority of the strains, including PfA7B, DSS73, CTS22, CTS70, CTS117, CTS193 and DR54, growth at 17°C was not associated with changes in CLP pattern production (Table 2 & Table 4). In contrast, two strains, CTS38 and CTS50, showed their panel of CLPs markedly increased with the growth temperature.

Among the 22 different identified CLPs, seven named 1 to 7 are described for the first time, their putative structures are compiled in Table 4. The compounds 1, 2, 6 and 7 are members of viscosin-like CLPs, as the other biosurfactants produced simultaneously by strains CTS50 and CTS117. CLPs 3, 4 and 5 own to the viscosinamid-like CLPs, as their co-produced biosurfactants by DR54. CLPs 3 and 5 present a similar hydrophilic peptidic moiety with, respectively, CLPs 2 and 1 except Glu replaced with Gln.

Effect of the growth temperature on the adhesion ability of the two wild *P. fluorescens* strains, PfA7B and DSS73

With PfA7B and DSS73 and their biosurfactant producer mutants, *Pf1vis*- and DSS73-15C2, adhesion assays were performed at either 8°C or 17°C on DMM on polystyrene microtitration plates. As represented in Figure 2, increasing the growth temperature from 8°C to 17°C was associated with a very significant decrease of the relative adhesion affinity for the both strains. At 8°C and 17°C the wild strains presented reduced adhesion compared to their surfactant mutant (ratio of 1.5 for viscosin mutant and 2 for amphisin mutant at 8°C).

Hydrophobicity

The surfacial hydrophobicity was evaluated for four strains: PfA7B, *Pf1vis*-, DSS73 and DSS73-15C2 by measuring the affinity of the cells towards the apolar solvent hexadecane. A strain with 100% affinity towards hexadecane is highly hydrophobic and a strain with 0% hexadecane affinity is highly hydrophilic. Hexadecane affinity at 8°C was illustrated in Figure 2 by white dots and affinity at 17°C by grey ones. PfA7B and *Pf1vis*- were characterized as highly hydrophilic for both growth temperature of 8°C and 17°C, as their affinity to hexadecane was evaluated less than 10% and appeared rather equal. The growth temperature for DSS73 and for its mutant only slightly modulated their hydrophobicity, at 17°C they were highly hydrophobic (hexadecane affinity greater than 85%) and at 8°C, they became only hydrophobic (hexadecane affinity about 60%).

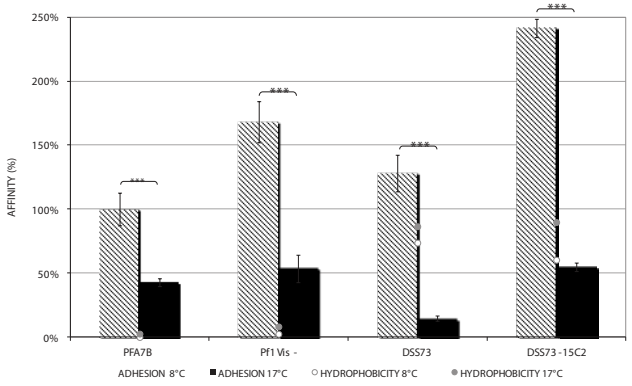

Figure 2: Influence of growth temperature on adhesion of four strains: PfA7B, *Pf1vis*-, DSS73 and DSS73-15C2 and their surface hydrophobicity after incubation at 8°C and 17°C.
Adhesion corresponds to incubation in static condition for 1 day at 8°C (cross-line pattern) and 4h at 17°C (plain black). For each strain, three independent cultures were realized in triplicate. Results were normalized as percentage of the median value corresponding to the adhesion obtained for PfA7B at 8°C. Statistical analyses were performed on these results with the help of Mann-Whitney test. NS means non significant ($p \geq 0.05$); X significant ($p \leq 0.05$); XX: highly significant ($p \leq 0.01$); XXX: extremely significant ($p \leq 0.001$).
Hydrophobicity corresponds to hexadecane affinity evaluated for bacterial cultures after 4 days of incubation at either 8°C (white dot) or 17°C (grey dot). (100%: highly hydrophobic, 0%: highly hydrophilic).

Discussion

The 11 strains of *P. fluorescens* were selected for the present study, since they represent typical environmental microorganisms naturally submit to significant temperature variations. All these strains are included in the "fluorescens r-cluster" defined by Bodilis [31] in the 16S rRNA based phylogenic tree. However the dispersion of these strains is large. In Figure 1 and as previously reported by de Bruijn [14], Group A is close to *P. fluorescens* SBW25 strain from which the genome is sequenced, whereas Group B is located at distance, *i.e.* near the "putida r-cluster", and appears to be close to *P. fluorescens* Pf0-1 strain, another strain from which the genome is sequenced. This situation clearly illustrates the high heterogeneity of the species *P. fluorescens*. Recent taxonomic studies are enabled a separation into distinct species for related bacteria previously associated with *P. fluorescens*, such as P. mosselii [36]. Bacteria still included in the species *P. fluorescens* remain very diverse as it can be noted by analysis of siderophores expression [37]. Nevertheless, for environmental microorganisms, as for clinical strains, the very active process of gene transfer forces us to carefully consider the usually accepted taxonomical boundaries [38] and even the psychrotrophic character of *P. fluorescens* could be discussed since clinical biovar 1 of the species has been shown to grow and adapt at 37°C on rich medium [39].

The two groups presently defined in the phylogenic study on 16S RNA are consistent with the results from the hemolytic activity study, summarized in Table 1. The strains, with β-hemolytic activity are found in Group A. Group A is distinct from Group B where strains characterized by α-hemolytic activity at either 8°C or 17°C are gathered.

Measuring surface tension from a bacterial rinsing solution was used to investigate biosurfactant production. In water, whose surface tension is about 70 $mN.m^{-1}$, if biosurfactants are present in solution, the surface tension falls under 40 $mN.m^{-1}$ [32]. Except for the two mutants *Pf1vis*- and DSS73-15C2 and strains CTS22 and CTS38 grown at 8°C, all other wild strains are positive for biosurfactant production with a surface tension of the rinsing solution between 26.7 and 35.2 $mN.m^{-1}$. The absence of biosurfactant in the rinsing solution of *Pf1vis*- and DSS73-15C2 grown at either 8°C or 17°C suggests that viscosin or amphisin blockage, as reported by Braun [13] and by Koch [23], is not compensated by the release of any other amphiphilic molecules. The case of the strains CTS22 and CTS38 is different since they show low but detectable α or β hemolysis activity at either 8°C and 17°C and have been previously demonstrated as amphisin- and viscosin-like producers, respectively [12]. However, surface tension measurement remains a global technique for the detection of biosurfactant production and even for very active molecules a sufficient amount is necessary to reach the limit value of 40 $mN.m^{-1}$ [32].

To summarize this macroscopic biosurfactant production, the 11 strains are sorted into three classes. The non-biosurfactant producers are *Pf1vis*- and DSS73-15C2 mutants. The second group consists of the α-hemolytic strains able to produce anionic biosurfactants: DSS73, CTS22, CTS70 and CTS193. The third category results from the β-hemolytic strains secreting anionic or non-ionic biosurfactants: PfA7B, CTS38, CTS50, CTS117 and DR54.

To screen rapidly a potential CLPs production by a *P. fluorescens* strain, two tests can be performed: hemolytic activity and ionic nature of the corresponding rinsing solution or culture supernatant. An α-hemolytic phenotype, with anionic rinsing solution, can be assumed to be amphisin-like CLPs producer. An anionic rinsing solution coupled with a β-hemolytic phenomenon is obtained for viscosin-like CLPs producer. Viscosinamid-like producer corresponds to a β-hemolysis with a non-ionic rinsing solution.

The CLP identification is consistent with the 16S ARN groups and the macroscopic biosurfactant evaluation. In fact Group A, in Figure 1, consists in all the viscosin- and viscosinamide-like producers. More precisely, the β-hemolytic strains secreting anionic biosurfactants are in the viscosin-like group. In addition, as a β-hemolytic strain and non-ionic biosurfactant producer, DR54 is characterized as a viscosinamide-like producer. The second category results from the α-hemolytic strains being able to produce anionic biosurfactants: DSS73, CTS22, CTS70 and CTS193. They are related in Group B, shown in Figure 1, and produce amphisin-like CLPs. The 16S rRNA phylogenetic tree corresponds with biosurfactant pattern. Structurally, the peptidic hydrophilic moiety differs amoung the studied CLPs. As shown by shorter retention times in RP-HPLC (Table 2), ampsin-like CLPs are more hydrophilic than viscosin and viscosinamide-like, whose ionic charge is different. Then the hemolysis mechanism could be related to CLP hydrophobicity, but not to their ionic charge. Hydrophilic amphisin-like CLPs help its producer strain to provoke pores formation and partial lysis of red blood cells, while their complete lysis is obtained with *P. fluorescens* producing hydrophobic CLPs (viscosin- or viscosinamide-like).

Over the 22 different CLPs identified in the present study (Table 4), 19 of these molecules have considerably increased production with an increase in the growth temperature of the bacteria. This increase reaches 10 and even 72 times for amphisin-like production by strains CTS70 and CTS22, but, in the vast majority, the CLP quantity detected at 17°C is twice as much as that found at 8°C. A similar temperature dependency has been reported for putisolvin, another CLP secreted by *Pseudomonas* sp. MIS38 [40] or *P. putida* PCL1445 [41] and for other different CLPs in *Bacillus subtilis* ATCC6633 [42]. But three molecules are differently regulated by temperature. In strain CTS50, production of the compound identified as massetolide A, massetolide G or pseudophomin A is reduced by 25.8 % when the bacterium was grown at 17°C. A reduction factor of the same range, -33.3%, is measured for 3 produced in strain DR54. The stronger negative effect of temperature is observed in strain DSS73 that shows its production of amphisin decrease from a mean normalized value of 141 at 8°C to only 2 at 17°C. No general trait about temperature-regulated CLPs production can be ground : the biosurfactant production seems be more strain dependant, than be ruled neither by the bacterial growth temperature neither by the produced CLPs nature. Common environmental pressures could explain such behaviour [1], yet in the present study there is no correlation between the ecology of the strains and their biosurfactant patterns.

As mentioned elsewhere, the role of biosurfactants in bacterial adhesion, and even later in biofilm production, remains to be discussed. Whereas de Bruijn [14,15] showed that in *P. fluorescens* SBW25 viscosin is essential for biofilm formation, as well massetolide A in *P. fluorescens* SS101 other CLPs, putisolvins and arthrofactins, appear to exert a negative effect on the biofilm structure in *Pseudomonas* sp. MIS38 [43] and *P. putida* PCL1445 [44].

In the present study, the adhesion of bacteria was evaluated on two wild strains: PfA7B and DSS73 and their respective CLP mutants: *Pf1vis*- and DSS73-15C2. Moreover these two wild strains produce different CLPs: PfA7B viscosine-like CLPs and DSS73 amphisin. These biosurfactants are all anionics and amphisin is largely more hydrophilic than the viscosin-like CLPs as shown by the great difference in HPLC

retention time compiled in Table 2. The two wild strains are more adherent at 8°C whereas for PfA7B viscosin is more produce at 17°C as described in Figure 2. Moreover, the magnitude of adhesion is lower for the wild strains than for their CLP deficient mutants. Thus it could be assumed that the CLP production reduces the bacterial adhesion process. As reported by Raaijmakers [7] or Palmer [48] or Simoes [49], microbial adhesion to surfaces still remains under discussion. A consensus has appeared about the initial attachment, which is the crucial step of adhesion, even if so little is still understood about it yet. Several factors seem to direct this critical step such as surface conditioning, mass transport or surface topography, but none of these factors is clearly established as dominating. The amphiphilic biosurfactant nature has led them to take part in the conditioning step of the adhesion in modulating the physico-chemical properties of the bacterial microenvironment during its deposition on an inert surface. In our study, whatever their nature, amphisin or viscosin-like, CLPs penalize the cell attachment of their own bacterial producers: the non producing mutant adheres more than the wild strain as shown in Figure 2.

Up to now, no implication of the surfacial cell itself was considered in attachment process. The hydrophobicity of the strains shown in Figure 2 corresponds to cell affinity without any adsorbed biosurfactant at their surface. Hydrophilic strain (PfA7B) and hydrophobic strain (DSS73) have similarly adhesion. Even if adherent cells number at 8°C increases with bacterial hydrophobicity, this parameter seems not dominating in adhesion process. Overall the growth temperature does not greatly modify the cell hydrophobicity.

The present results demonstrate that environmental strains of *P. fluorescens* can synthesize different forms of CLPs from the viscosin, viscosinamide and amphisin family and we propose a method for rapid CLP screening. Biosurfactant presence is determined using two analyses simultaneously: blood agar lysis and tensiometry. If both are positive, the double diffusion test is done. Afterwards the CLP class can be deduced from the hemolytic type and from the biosurfactant charge determination. Precise analysis of the data indicates that in *P. fluorescens* production of CLPs and the crucial adhesion step are both markedly affected by the growth temperature but the two events appear to be independently regulated and not correlated. Indeed, most of the presently studied environmental *P. fluorescens* CLPs producers secrete biosurfactant(s) with a great variety and in larger quantity at the optimal exoproduct secretion temperature, 17°C in our case. These CLPs disturb the bacterial adhesion process. This initial adhesion, a crucial step of biofilm formation, seems to be not controlled by strain hydrophobicity and is increased at growth temperature of 8°C. To define more precisely the origin of this behaviour difference in bacterial adhesion, some more analysis could be done on extracellular polymeric substances (EPS) or on specialized attachment structures, such as flagella or pili, also known to influence the crucial adhesion step [49]. Moreover, increase of flagella length in absence of biosurfactant has been already reported [18].

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