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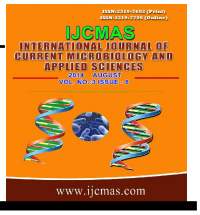

Original Research Article

Airborne fluorescent pseudomonads : What potential for virulence?

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K e y w o r d s

Fluorescent pseudomonads; *Pseudomonas fluorescens*; *Pseudomonas putida*; air; airborne; bio-contamination, virulence; exoproducts

A B S T R A C T

The presence of human pathogens in the environment is a key concern, therefore the air needs to be evaluated as a potential source of bio-contamination. This study dealt with the characterization of fluorescent pseudomonads strains isolated from air in order to evaluate their factors of virulence. 19 strains were identified by API® strips, by MALDI-Biotyper and by 16S rDNA gene sequencing. Their growth at 30°C and 37°C, biosurfactant and biofilm production, motility and production of exoproducts were tested. A *Pseudomonas fluorescens* clinical strain was used as reference. By comparison with the virulence factors of this climical strain, most of these strains isolated from air did not produced highly virulent factors. However a bacterial couple : *P. fluorescens* MFAF76a and *P. putida* MFAF88, was selected thanks to their observed characteristics linked to virulence quite similar with traits of the clinical reference. The cytotoxicity of their culture supernatant was investigated toward human epithelial pulmonary cells. Results revealed that these airborne fluorescent *Pseudomonas* strains secreted exoproducts, such as enzymes, surfactants and siderophores, highly virulent against the studied pneumocytes.

Introduction

For environmental policies, air quality is generally related to the presence of chemicals and particulate matter and its pollution is correlated with uncontroversial health impact. According to a survey in European intensive care units, 68% of sepsis are lung infections and *Pseudomonadaceae* are the second most common organisms (14%), but the only ones that induce rising mortality rates (Vincent et al., 2006).

Gram-negative *Pseudomonadaceae* bacteria

present a great adaptive ability related to their large genome (Stover et al., 2000). *P. aeruginosa* is well known as prevalent pathogen in acute and chronic infections (Clifton & Peckham, 2010; Fernstrom & Goldblatt, 2013), although other fluorescent *Pseudomonas* species are ubiquitous. In fact, *Pseudomonas* are widespread Gram-negative bacteria present in various ecological niches: soil, water (Rajmohan et al., 2002), care units (Vincent et al., 2006), humans (Chapalain et al., 2008; Donnarumma et al., 2010) and air (AFSSET, 2010; Morin et al., 2013).

Moreover, some strains, isolated from a clinical environment, are able to grow at or above 37°C (Chapalain et al., 2008). *P. fluorescens* MFN1032, a clinical strain, was recently isolated from a patient with a lung infection (Chapalain et al., 2008) and induces cytotoxic responses (Rossignol et al., 2008; Madi et al., 2010; Sperandio et al., 2010; Sperandio et al., 2012). The pathogenicity of *Pseudomonas* bacteria is correlated with their enzymatic secretion (Gessner & Mortensen, 1990 ; Rossignol et al., 2008 ; Strateva & Mitov, 2011).

The purpose here was, after identification, to assess the potential for virulence of 19 airborne fluorescent pseudomonads strains, selected from Gram-negative oxidase-negative strictly aerobic rods collected during a previous study (AFSSET, 2010; Duclairoir Poc et al., 2011a ; Morin et al., 2013). For each of them, physiological characterization and determination of virulence factors were confronted. Two airborne bacteria appeared to have great similarities with the clinical standard, *P. fluorescens* MFN1032.

To compare the cytotoxicity of their secreted factors, A-549 human pulmonary type II-like epithelial cell line was implemented in order to assess their potential virulence toward human by airways. Those cells play a critical role in coordinating both innate defence and inflammatory responses (Hawdon et al., 2010) and make it suitable to study virulence in conditions fairly close to *in vivo*.

Materials and Methods

19 airborne fluorescent *Pseudomonas* strains

In a previous study (AFSSET, 2010; Morin et al., 2013), more than 3000 bacteria were collected in air samples between June 2008 and September 2009 in 3 areas : in peri-suburban Evreux (Normandy, France), in the suburbs of Rouen (Normandy, France) and in dust clouds generated during crop ship loading in Rouen harbor installations (Normandy, France). About 3000 bacterial isolates were stored and then frozen at -80°C. Morphological characters, Gram staining and biochemical tests used to separate this bacterial population into 8 groups, which included a Gram-negative oxidase-positive strictly aerobic rods group containing *Pseudomonas* spp.

This group appeared among the most predominantly collected groups of airborne Gram-negative bacteria on harbor installations. Proportionally to their relative sample representation, 19 fluorescent *Pseudomonas* strains were randomly selected.

Clinical standard strain, MFN1032

P. fluorescens MFN1032 is a clinical strain isolated after a lung infection, related to biovar I of *P. fluorescens* species (Chapalain et al., 2008).

Identification of the 19 airborne strains API® identification

The 19 representative strains were submitted to metabolic characterization using API® 20NE strips. API® kits were operated according to the manufacturer's instructions (BioMérieux, France).

Mass spectrometric MALDI-Biotyper bacterial identification

These isolates were submitted to bacterial identification by MALDI mass spectrometry (MS) based on the total proteome screening analyzed using an algorithmic method to identify bacterium. The bacterial proteomes were obtained using an Autoflex III Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight mass spectrometer (MALDI-TOF) (Bruker, Germany) coupled to the MALDI-Biotyper 3.0 algorithmic system for microbial identification (Hillion et al., 2013). Before MS analysis, the bacterial material was spotted onto a MALDI target plate and overlaid by matrix ($10g.L^{-1}$ α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid provided by Sigma-Aldrich, France).

The software generated scores evaluating the probability of correct identification of the microorganism. The species identification is considered acceptable for score values over 2.0 and, between 1.7 and 2.0, the genus identification is assumed confident. (Hillion et al., 2013)

16S rDNA gene sequencing and identification

The same isolates were identified by 16S ribosomal DNA gene sequencing. For amplification of the complete 16S RNA gene, universal primers UNI_OL (AGAGTGTA GCGGTGAAATGCG) and UNI_OR (ACGGGCGGTGTGTACAA) were used as already described (Duclairoir Poc et al., 2011a). The nucleotide sequences were registred on GenBank, NCBI (http://www.ncbi.nlm.nih.gov/genbank/submit/) and were compiled in Table 1.

Partial 16S rRNA gene sequences were aligned with reference sequences using BLAST data bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

The minimum similarity among all members of the *Pseudomonas* genus is assumed at 77% (Balet et al., 2010). Over 95% of similarity, the species is considered surely identified.

Physiological characterization of airborne bacteria: Growth conditions

The bacteria were cultured in Luria Bertani medium (LB) (AES Chemunex, France) under shaking (180 rpm) at 30°C, close to optimum growth temperatures of *Pseudomonas fluorescens* species, i.e. 28°C (Merieau et al., 1993), and at 37°C, i.e. human body temperature. The bacterial density was determined by measuring optical density (OD) at 580 nm (Helios ε, Thermo Spectronic,USA) during at least 120h. Thanks to these growth kinetiks curves, the maximum growth rate, μ_{max}, was calculated for each strain.

Determination of virulence factors of airborne bacteria

Bacterial enzymatic characterization

Several methods were used to screen enzymatic activities. Each test was done at 30°C and 37°C.

Proteolytic activity was determined on Trypticase soy agar (TSA) (AES Chemunex, France) with skimmed milk 20%. Esterase was detected by growth on TSA (AES Chemunex, France) containing Tween 80 1% colored with Phenol red (1%).

Lecithinase and lipoproteolytic activities were analyzed by growth on TSA (AES Chemunex, France) with egg yolk 50% (VWR, Germany). Bacteria hydrolyse lecithin contrasting medium next to the inoculated streak. On the contrary a lighter colored zone shows lipoproteolytic activity.
In the secreted hemolysis test, bacterial strains were streaked onto a 2% sheep red blood cell plates, whose were visually inspected the zones of clearing around the colonies. The pyoverdine production was observed by growth on King B Agar plates (AES Chemunex, France). After 48h incubation, the plates were observed with Ultra-Violet light at 365nm (VL-6LM, Vilber Lourmat, France), as the produced pyoverdine induces fluorescence.

Biosurfactant production

Surface tension measurement of a rinsing solution of bacteria cultured on solid agar medium is a direct method for determining global biosurfactant production, in ideal conditions for biosurfactant production and was done as previously described (Duclairoir Poc et al., 2011b).

Bacterial motility

Motility characters were observed through the evaluation of swim, swarm, twitch deplacements after 40h at 30°C and 37°C. These motilities were obtained thanks to specific agar concentrations: 0.3% for the swim deplacement, 0.5% for the swarm and 1% for the twitch. Initially, the plates were inoculated with overnight culture in LB at 30°C and 37°C (Rossignol et al., 2008).

Adhesion assays on abiotic surface

The bacterial adhesion was evaluated in triplicate in polystyrene microtitration plate at 30°C and 37°C incubated in LB during 24h and 48h. The adherent bacterial population was estimated by direct measurement (quantification) of absorbance at 595 nm after 0.1% crystal violet coloration and after cell lysis by sodium dodecylsulfate 1% (Duclairoir Poc et al., 2011b).

Evaluation of supernatant virulence using the A549 pneumocyte model

The cytotoxicity of *P. fluorescens* MFN1032, MFAF76a and MFAF88 culture supernatants, in LB at 30°C and 37°C, was evaluated toward A-549 human pulmonary type II-like epithelial cell line as described elsewhere (Pimenta et al., 2006).

The percentage (%) of total lysis was calculated as follows:
$\%LDH = 100 \times (OD_{sample} - OD_{0\%}) / (OD_{100\%} - OD_{0\%})$

A percentage as lower than 20% could be assumed linked to an avirulent strain. Four other classes were defined : 20-40%: weak virulence, 40-60%: virulence, 60-80%: highly virulent and greater than 80%: extremely virulent.

Results and Discussion

Identification of airborne fluorescent pseudomonads : preponderance of

***Pseudomonas fluorescens* cluster**

The *Pseudomonas* genus is splitted into three well-supported clusters in the 16S rRNA phylogeny named aeruginosa, putida and fluorescens r-clusters (Bodilis et al., 2012). Different approaches : API/ MALDI

–Biotyper/ 16S rRNA gene sequencing, were done to identify the 19 airborne selected strains. According to the classification stated by Bodilis (Bodilis et al., 2012), a putative identification was proposed for each strain based on the more reliable identification by 16S rRNA sequencing (homology>95%) and /or by MALDI-Biotyper (score>2). The three strains : MFAE20, MFAB75 and MFAK14, were stated as *Pseudomonas* spp., their genus was surely identified, but their species could not confidently assumed. The majority of the 19 airborne strains are identified as *Pseudomonas fluorescens*.

The preponderance of *Pseudomonas fluorescens* species among the airborne Gram-negative bacteria was no surprise, due to their ubiquitous character created by their large genome (Stover et al., 2000). Morever *P. fluorescens* is observed as the most common *Pseudomonas* species (at least 42%) in outdoor air, as already reported by Nevalainen (Nevalainen et al., 1990).

Although the pathogenicity of *P. aeruginosa* is largely described and cause several human diseases, specially in lung infections (Clifton & Peckham, 2010), the literature on other fluorescent *Pseudomonas* is poorly documented in airways transmitting route, even if such bacteria are able to induce pulmonary pathologies such as *P. fluorescens* strains (Chapalain et al., 2008).

This study aimed to contribute on a better knowledge of the impact of fluorescent pseudomonads on the safety of the air, that we breathe. To evaluate the potential of virulence on this panel of 19 airborne strains, two contamination situations were explored. Firstly, conventional airway infections at 37°C, corresponded to an internal infection such as pulmonary, and, at 30°C, temperature of an external infection such as burned dermal injuries (Church et al., 2006) or as contamination of medical devices (Gershman et al., 2008).

Physiology of the 19 airborne bacteria : overall able to growth at 37°C

The growth rate for each of 19 airborne *Pseudomonas* strains, compiled in Table 1, was evaluated in LB medium at 30 and 37°C. Most of them were able to grow at 37°C, except MFAH4a and MFAD21c. Twelve other strains had an higher grow rate at 30°C, similar to the optimal growth temperature for *P. fluorescens* species, 28°C (Merieau et al., 1993). The first 5 strains, namely, MFAF76a, MFAF49a, MFAF88, MFAO2 and MFAF80b, presented an equivalent or increasing growth rate between 30 and 37°C. In addition to survival, they were able to multiply at human physiological temperature, which could facilitate the infectious processes.

Characterization of bacterial extracellular virulence factors : all the airborne strains produce several factors of virulence

MFN1032 was introduced in the panel to have a standard of virulence with a clinical origin that assures its pathogenic potential (Chapalain et al., 2008, Rossignol et al., 2008; Rossignol et al., 2009; Madi et al., 2010; Sperandio et al., 2010; Sperandio et al., 2012) and then makes a possible comparison of studied airborne *Pseudomonas* spp.

The 19 airborne *Pseudomonas* strains showed different patterns of secretion, as tabulated in Table 2. All strains synthesized lipoproteolytic enzymes and, except for MFAE48, induced clearing zones on sheep red blood cell plates revealing secreted hemolysis *a minima* incomplete for at least

one growth temperature. According to conventional microbiological nomenclature, the secreted hemolysis could be categorized as complete (β), incomplete (α) or no hemolysis (Luo et al., 2001). In any case, hemolytic phenomenon could result from synergy between activity of several hydrolysases, such as proteases, lecithinases, lipoproteases, and biosurfactants (Rossignol et al., 2008; Zhang et al., 2009). The hemolysis was complete for MFAF49a, MFAF80b and MFAD21c at 30°C and for MFAO2, MFAO39, MFAB75 and MFAH4a at 37°C.

Some virulence factors could be, on the one hand, strain-dependant: no protease and no lecithinase for MFAF49a, MFAF80b and for several strains less adapted at 37°C, no esterase for MFAF88 and MFAO39, no pyoverdine production for MFAF80b, MFAE88, MFAE20 and MFAH106a. On the another hand, some virulent exoproducts were strain- and temperature-dependant in the case of MFAF88 and the most of the strains were more adapted at 30°C than at 37°C.

Surprisingly, both strains, MFAH4a and MFAD21c, were not able to grow at 37°C in broth, but presented virulence factors at 37°C. To stimulate enzymatic secretion, bacterial growth was done on agar medium, promoting sessile lifestyle (i.e. in a surface adherent community). Such conditions seemed to facilitate the growth of MFAH4a and MFAD21c, contrary to more planktonic and less protective cell growth in broth. An other common character for this couple was their secretion of biosurfactant, as for MFN1032, MFAF88, MFAO2, MFAA66a, and MFAK14. This surfactive production was established through a surface tension lowered above 45mN/m (Carillo et al., 1996). Biosurfactants favor bacterial displacement, but may be affecting adhesion, even colonisation of the host (37°C) or of a medical device or even burn injuries (30°C) (Van Hamme et al., 2006).

To conclude, only MFAF76a, MFAO2 and MFAO40 exhibit activity for all the tested enzymes like the clinical strain *P. fluorescens* MFN1032, but, among them, only MFAO2 produces biosurfactant as MFN1032.

Bacterial motility : some strains swim, all swarm and none twitch

Infection deals in part with bacterial motility to allow their colonisation in the host.

The 3 motile types : twitch, swim and swarm, were tested at 30 and 37°C.

No twitching, but swarming motility was noted for any strain at either temperature, as shown in Table 3. The swarming motility is a collective microbial motility resulting from, at least, one functional flagella, often completed by biosurfactant production (Kearns, 2010).

Although all strains were able to swim at 30°C, only 6 of them maintained the swimming mode at 37°C. This displacement allows bacteria to individually move towards the host with the help of functional flagella.

The flagella dependant motilities, i.e. swim and swarm, are involved in the development of biofilms (O'Toole & Kolter, 1998). For instance, functional flagella are needed for swarming mobility, which is related to the surface movement in the case of bacterial groups. This mobility is favored by biosurfactive production or by lipopolysaccharides, outer membrane components, for some Gram-negative bacteria (Kearns, 2010).

Bacterial adhesion on abiotic surface: different behaviours

As noted in Table 3, thicker biofilms were observed at 30°C than at 37°C. For MFAF76a, MFAF88, MFAE48 and MFAE8b, their biofilm were observed at 24h and 48h at either temperature. When the biofilm evolved between 24h and 48h, most of the time it increased, the mature structure was not yet reached, the biofilm seemed still under edification. However, for both temperatures, MFN1032 had a lowest ability to form biofilm at 48h than at 24h, this phenomenon was already reported about *P. fluorescens* biosurfactant production (Duclairoir Poc et al., 2011b). This biofilm reduction was noted at 30°C for MFAF88, MFAF80b and MFAO29 and at 37°C for MFAH4a.

Concerning contamination, the bacterial presence on biofilm is stabilized in the host or on a surface, and even protects from hostile microenvironment behind its shelf-shielding extracellular polymeric substances. Nevertheless, a biofilm could reduce over the time and persists slimmer; the lacking cells could be assumed in dispersing and colonising elsewhere in host (McDougald et al., 2012).

Selection of two airborne strains: *P.fluorescens* MFAF76A and *P. putida* MFAF88

In *P. aeruginosa* infections, tissue damage is due to the production of several extracellular and cell-associated virulence factors (Strateva & Mitov, 2011), similar exoproducts are produced by all *Pseudomonas* species and also known as factors of virulence.

Through the obtained results, two airborne strains, MFA76a and MFAF88, are of particular interest: they are able to grow at 37°C and possibly better than at 30°C. They swim and swarm, thanks to their flagella and completed for MFAF88 by its biosurfactant production, as shown in Table 2. They present some virulent factors and form biofilm at either temperature. *P. fluorescens* MFA76a is very close to MFN1032 except for biosurfactant production and *P. putida* MFAF88 had an enzymatic pattern very different from MFN1032, but produced biosurfactant, as MFN1032.

Virulence of culture supernatant of MFN1032, MFAF76a, MFAF88 toward A-549 pneumocytes : strain-dependant virulence

Exoproducts such as lipases and proteases produced by *P. fluorescens* are known for their virulence (Rossignol et al., 2008; Zhang et al., 2009). Thus testing the growth supernatant could have a promising outcome. The need of an adequate cytotoxic model was crucial at this point and had to be ideally close to *in vivo* contamination by airway or pulmonary infection mode. The evaluation of virulence toward pneumocytes, such as cellular line A549 was assayed. The cytotoxic mechanism involved only the action of exoproducts, such as enzymes, siderophores or biosurfactants. All the supernatants, resulting from bacterial growth at 30°C and 37°C, induced lysis of the pulmonary cells by contact for 12h. Supernatant of MFAF88 induced a strong virulent response, in similar range (around 80%) at 30°C and 37°C. Furthermore, surpernatants of MFN1032 and MFAF76a had significantly changed their virulence towards epithelial cells for both growth temperatures. MFN1032 was extremely virulent at 30°C and MFAF76a at 37°C and their virulence was less severe, i.e. only highly virulent, at 37°C for MFN1032, and at 30°C for MFAF76a, respectively.

Table.1 Characterization of the 19 airborne fluorescent *Pseudomonas* spp. strains

Strain	Sampling localisation & season	API® 20NE identification	MALDI Biotyper: total proteomic comparison (score)	Identity percentage of 16S ribosomial RNA sequence <GenBank accession numbers>	Putative identification	μ_{max} at 30°C (h^{-1})	μ_{max} at 37°C (h^{-1})
MFAF76a	Rouen Harbor, Summer	*P. fluorescens* (excellent)	*P. koreensis* (2.122)	*P. koreensis* strain AGB-1 (96%) <KJ470785>	fluorescens r-cluster	1	1.08
MFAF49a	Rouen Harbor, Summer	*P. fluorescens* (excellent)	*P. abietaniphila* (2.094)	*P. lundensis* (85%) <KJ470784>	fluorescens r-cluster	0.79	1.05
MFAF88	Rouen Harbor, Summer	*Bulkholderia pseudomallei* (very good)	*Pseudomonas* spp. (1.982)	*P. putida* strain CG29 (99%) <KJ470787>	putida r-cluster	0.47	1.01
MFAO2	Rouen Harbor,Winter	*P. fluorescens* (excellent)	*P. koreensis* (2.001)	*Pseudomonas* sp. strain E1 (86%) <KJ470791>	fluorescens r-cluster	0.93	0.88
MFAF80b	Rouen Harbor, Summer	*P. fluorescens* (excellent)	*P. abietaniphila* (2.152)	*P. abietaniphila* strain HMGU118 (99%) <KJ470786>	fluorescens r-cluster	0.71	0.77
MFAE88	Rouen Harbor, Summer	*P. oryzihabitans* (very good)	*Pseudomonas* spp. (1.866)	*P. rhizosphaerae* strain BKB1(95%) <KJ470782>	fluorescens r-cluster	0.83	0.38
MFAO39	Rouen Harbor,Winter	*P. fluorescens* (excellent)	*Pseudomonas* spp. (1.962)	*P. putida* strain bD1 (100%) <KJ470793>	putida r-cluster	0.84	0.35
MFAA66a	Surburban Rouen, Summer	*P. oryzihabitans* (good)	*P. congelans* (2.164)	*P. syringae pv. Syringae* strain XJLX-2-2 (99%) <KJ470777>	fluorescens r-cluster	0.68	0.32
MFAO40	Rouen Harbor,Winter	*P. fluorescens* (excellent)	*P. koreensis* (2.149)	*P. fluorescens* strain TCA33 (99%) <KJ470794>	fluorescens r-cluster	0.85	0.3
MFAE20	Rouen Harbor, Summer	*P. oryzihabitans* (very good)	*Pseudomonas* spp. (1.917)	*P. graminis strain 8B2* (84%) <KJ470780>	*Pseudomonas* spp.	0.64	0.26
MFAE48	Rouen Harbor, Summer	*P. oryzihabitans* (very good)	*P. graminis* (2.403)	*P. graminis* strain R5SpM3P2C1 (99%) <KJ470781>	fluorescens r-cluster	0.59	0.22
MFAB75	Surburban Rouen, Summer	*P. oryzihabitans* (very good)	*Pseudomonas spp.* (1.728)	*P. plecoglossicida* strain ETLB-3 (78%) <KJ470778>	*Pseudomonas* spp.	0.8	0.22

MFAE8b	Rouen Harbor, Summer	*P. fluorescens* (excellent)	*Pseudomonas* spp. (1.856)	*P. rhizosphaerae* strain - Y12 (79%) <KJ470783>	fluorescens r-cluster	0.82	0.21
MFAO48	Rouen Harbor,Winter	*P. oryzihabitans* (very good)	*P. savastanoi* (2.098)	*P. syringae* CC1557 (99%) <KJ470795>	fluorescens r-cluster	0.6	0.19
MFAK14	Surburban Rouen, Winter	*P. fluorescens* (good)	*Pseudomonas spp.*(1.923)	*P. putida strain SCR2* (78%) <KJ470790>	*Pseudomonas* spp.	0.62	0.17
MFAO29	Rouen Harbor,Winter	*P. fluorescens* (good)	*P. chlororaphis* (2.006)	*P. thivervalensis* strain BD2-26 (99%) <KJ470792>	fluorescens r-cluster	0.6	0.15
MFAH106a	Rouen Harbor, Summer	*P. oryzihabitans* (excellent)	*Pseudomonas* spp. (1.821)	*P. rhizosphaerae* strain R2-255 (99%) <KJ470788>	fluorescens r-cluster	0.64	0.14
MFAH4a	Rouen Harbor, Summer	*P. fluorescens* (excellent)	*P. poae* (2.255)	*P. fluorescens strain RK2* (83%) <KJ470789>	fluorescens r-cluster	0.79	No growth
MFAD21c	Residential Evreux, Summer	*P. fluorescens* (very good)	*P. poae* (2.147)	*P. fluorescens strain RK2* (86%) <KJ470779>	fluorescens r-cluster	0.78	No growth

Score >1.700 : probable genus
Score >2.000 : probable species, secure genus

Air sample localisation and season; bacterial identification by API®20NE strips, by MALDI-Biotyper and by16S rDNA gene sequencing after matching with NCBI data bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome); and maximum specific growth rate (μ_{max}) at 30 and 37°C in LB medium, 180rpm, classified by decreasing μ_{max} at 37°C.

Table.2 Extracellular virulence factors, at 30°C and 37°C, and biosurfactant production for MFN1032 (clinical strain) and for each airborne strain

The observed characters were proteolytic, esterase lecithinase, lipoproteinase and hemolytic activities, after 5 days, or pyoverdine production, after 2 days. The biosurfactant production was observed on Davis Minimum medium after 5 days. Experiments done in three independant assays.

Activity	Proteolytic		Esterase		Lecithinase		Lipoproteolytic		Secreted hemolytic		Pyoverdin production		Biosurfactant
Incubation temperature (°C)	30	37	30	37	30	37	30	37	30	37	30	37	production
MFN1032	++	++	++	++	++	++	+	+	α	α	++	++	+
MFAF76a	++	++	++	++	++	++	++	++	α	α	++	++	-
MFAF49a	-	-	++	++	-	-	++	+	β	α	++	++	-
MFAF88	-	+	-	-	-	++	+	++	α	α	++	+	+
MFAO2	++	++	++	++	++	++	++	+	α	β	++	++	+
MFAF80b	-	-	++	++	-	-	++	++	β	α	-	-	-
MFAE88	-	-	-	++	-	-	++	++	α	-	-	-	-
MFAO39	-	+	-	-	++	-	+	++	β	β	++	-	-
MFAA66a	+	+	+	-	-	-	+	++	β	α	++	++	+
MFAO40	++	++	++	++	++	++	++	++	α	α	++	++	-
MFAE20	++	-	++	++	-	-	++	++	α	α	-	-	-
MFAB75	-	-	+	-	-	-	+	++	β	β	++	++	-
MFAE48	-	V	++	V	-	V	++	V	-	-	-	+	-
MFAE8b	-	-	++	++	-	-	++	+	α	α	++	-	-
MFAO48	-	-	++	++	-	V	++	V	-	α	++	-	-
MFAK14	-	+	++	-	-	-	+	++	α	α	+	-	+
MFAO29	+	V	++	V	-	V	++	V	β	α	++	-	-
MFAH106a	++	++	++	++	-	+	++	++	-	α	-	-	-
MFAH4a	++	++	++	++	-	-	+	+	β	β	++	++	+
MFAD21c	++	++	++	++	-	-	++	++	β	α	++	++	+

++: important activity
+: light activity
V: weak and variable
-: no activity

α: partial hemolysis
β: complete hemolysis

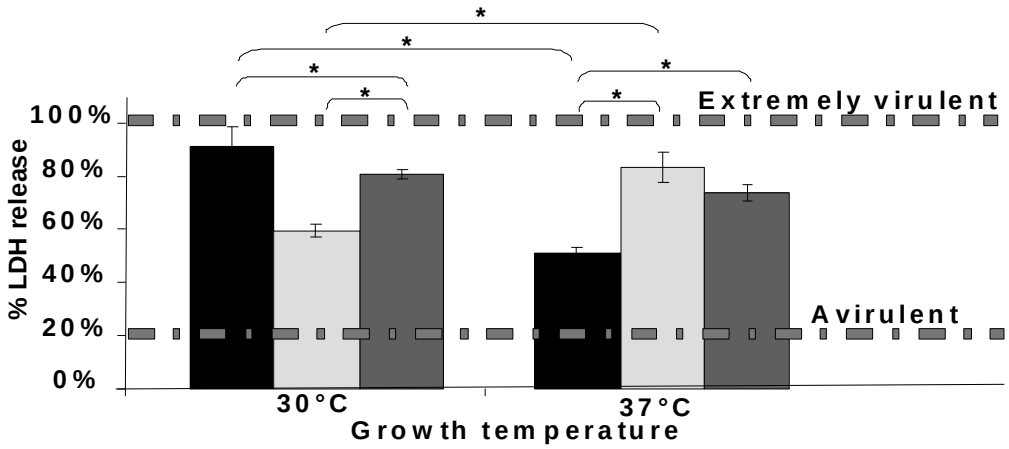
Table.3 Motility and biofilm ability, at 30°C and 37°C, for MFN1032 (clinical strain) and for each airborne strain. The observed motility characters were swim, swarm, twitch, after 40h. The biofilm formation was observed after 24h and 48h

Activity	**Swim**		**Swarm**		**Twitch**		**Biofilm formation after 24h**		**Biofilm formation after 48h**	
Incubation temperature (°C)	**30**	**37**	**30**	**37**	**30**	**37**	**30**	**37**	**30**	**37**
MFN1032	++	++	+	+	-	-	++	+	+	-
MFAF76a	++	++	+	+	-	-	++	+	++	+
MFAF49a	+	-	+	+	-	-	-	-	+	+
MFAF88	++	++	+	+	-	-	++	+	+	+
MFAO2	++	+	+	+	-	-	+	-	+	-
MFAF80b	+	-	+	+	-	-	++	-	+	-
MFAE88	+	-	+	+	-	-	+	-	+	-
MFAO39	+	-	+	+	-	-	++	-	++	-
MFAA66a	+	-	+	+	-	-	-	-	-	-
MFAO40	+	-	+	+	-	-	++	-	++	-
MFAE20	+	-	+	+	-	-	+	-	+	+
MFAB75	+	-	+	+	-	-	-	-	+	-
MFAE48	+	-	+	+	-	-	+	+	+	+
MFAE8b	++	+	+	+	-	-	++	+	++	+
MFAO48	+	-	+	+	-	-	-	-	-	-
MFAK14	+	+	+	+	-	-	-	-	-	-
MFAO29	+	-	+	+	-	-	+	-	-	-
MFAH106a	+	-	+	+	-	-	+	-	+	-
MFAH4a	**V**	-	+	+	-	-	-	+	+	-
MFAD21c	**V**	-	+	+	-	-	-	-	-	-

++: deplacement upper than 30mm
+: deplacement upper than 10mm
=: deplacement lower than 10mm
V: weak and variable

++: OD595 higher than 0.200
+: OD595 higher than 0.050
-: OD595 lower than 0.050

Figure.1 Virulence towards A549 pneumocytes exposed to culture supernatants obtained at 30°C and 37°C



The virulent pattern is strain- and temperature-dependant. In fact, *P. putida* MFAF88 shows a constant virulence, its secretion varies nevertheless with temperature. Virulence could then be attributed to the common production at 30 and 37°C, i.e. lipoproteases, siderophores, and may be completed by biosurfactants, even if their quantities could be secreted quite variably at the two temperatures.

This variability in intensity of secretion could also explain the decrease in virulence for MFN1032. As shown in Table 2, all the enzymes and pyoverdine were expressed at 30°C as well as at 37°C, but no evidence was given about quantity. Rossignol and cowokers rightly note, at 37°C, a weaker production of phospholipase C, than at 28°C (Rossignol et al., 2008). This enzyme is known as a major factor of virulence.

Like MFN1032, the same families of exoproducts are secreted by *P. fluorescens* MFAF76a at both temperatures. However a slightly significative increase in virulence is noted at 37°C and again might be due in the exoproducts secretion modulation in concentration or in nature.

In any case, airborne *P. fluorescens* MFAF76a and *P. putida* MFAF88 may induce by their secretion, especially at 37°C, cytotoxic responses from A549 airway epithelial cells. Thus the biohazard, that they create as biocontamination, must be identified and not ignored. Moreover the virulence of these airborne strains and of the clinical standard, *P. fluorescens* MFN1032 operates on different ways from each other. To better understand these virulent mechanisms, an exoproteomic study would create insight.

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