IMPACT OF GASEOUS NO_2 ON *P. FLUORESCENS* STRAIN IN THE MEMBRANE ADAPTATION AND VIRULENCE

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ABSTRACT

Nowadays air pollution is increasing due to anthropogenic activity. Among all air pollutants, nitrogen oxides (NOx) such as NO_2 are predominant. It is well known that those compounds exhibit direct toxic effects on human health. However, microorganisms are also exposed to them, but the effect of NOx on the virulence of air microbiota is still poorly understood. In this study, we evaluated the impact of NO_2 on the adaptability and virulence of an airborne strain of *P. fluorescens*, MFA76a, by exposition of this strain to 45 ppm of NO_2. The growth kinetics and cultivability were analysed. A decrease of cultivability coupled with an increase of the lag phase was observed suggesting a potential toxicity of NO_2. Since NOx particularly target lipids, the membrane permeability was assessed thanks to Live Dead tests and confocal microscopy. A significant alteration of membrane permeability was observed. Furthermore, more abundant bacterial aggregates were detected compared to the control. Thus, a lipidomic study was performed using MALDI-TOF MS Imaging coupled to HPTLC. Interestingly, bacteria exposed to NO_2 were lacking one putative glycerophospholipid molecule. In agreement with a previous study from Kondakova et al., these data demonstrate the adaptation potential of *P. fluorescens* MFAF76a to an air pollutant such as NO_2.

Keywords: air pollution, antibiotic resistance, membrane, adaptation, P. fluorescens, toxicity.

1 INTRODUCTION

Air is a complex environment contaminated by particle matter, nitrogen oxides, ozone and hydrocarbons. It is also a biotic environment since microorganisms, mostly bacteria, are found [1]. Even if this environment is unstable, several studies highlight the presence of members of *Pseudomonas* strain in air microbiota [2, 3]. During the past decades, due to the increase of anthropogenic activities, the level of atmospheric pollutants is rising and the control of air quality remains a topic of major interest [4, 5]. Among all air pollutants, nitrogen oxides species, including nitrogen oxide (NO) and nitrogen dioxide (NO_2), are predominant [4]. It is well known that these compounds are toxic to human health, leading to cardiovascular and respiratory pathologies (allergy, asthma, bronchitis) [4, 5]. Thus, threshold values were set up by World Health Organization (WHO) to prevent the potential risks on human health. Regarding the increase of respiratory pathologies, a potential impact of NO_2 could be presumed on air microbiota. NO_2 induces cells damages, directly by interacting with proteins, DNA and lipids or either via formation of reactive nitrogen species interfering with metabolism, respiration functions and thus homeostasis [6–8]. Bacterial stress is generally leading to the expression of virulence factors; thus, NO_2 may have an impact on bacterial virulence, which could consequently alter human health. Infection by opportunistic pathogens represents a big threat for immune-compromised or cystic fibrosis patients as they are more sensitive. Indeed, this pathogens could alter the healthy microbiota and its homeostasis [9–11].

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This dysbiosis and the pathogen itself could lead to an acute infection with potential lethal outcome [10]. Breathing NO_2-polluted air could potentiate this mechanism. Since *P. fluorescens* are widespread in the environment [12], including in the air [13], and had already been isolated from sputum of patients suffering from pneumonia, it seems particularly interesting to focus on this species [14]. An airborne strain *P. fluorescens* MFAF76a was previously isolated and characterized with virulent traits [13, 14]. Thus, MFAF76a seems to be the perfect candidate to test the bacterial response to NO_2 stress. Its membrane adaptability and virulence response to NO_2 contamination remain to be resolved. Therefore, several approaches were implemented in this study such as physiological tests and lipodomic analysis.

2 MATERIAL AND METHOD

2.1 Bacterial strain and growth condition

In this study, the wild-type *P. fluorescens* MFAF76a strain was used [13]. Overnight cultures in Luria Bertani medium (LB, AES) were grown at 28°C under limited agitation (180 rpm). Then cultures were diluted (A_{580} = 0.08) in Davis Medium Broth (DMB), a minimal medium with 2.16 g/L glucose as carbon source. The incubation was done at 28°C under agitation for 16 h to reach the stationary phase. Bacterial cultures were transferred on cellulose nitrate membrane filters (0.45 µm, pore size 0.2 µm, diameter 47 mm, Sartorius Biolab Products) and incubated on DMB agar plates at 28°C for 4 h to obtain a monolayer of bacteria. Then the membranes covered by bacteria were laid on agar one-well dishes (size 127.8 × 85.5 mm, Thermo Scientific Nunc), which were directly transferred into the gas delivery device [15].

2.2 Exposition to nitrogen dioxide

As previously described by Kondakova et al., an exposure system was developed in order to mimic the environmental exposition [15]. Briefly bacterial bed was exposed during 2 h at 28°C with a constant gas stream of 2 L/min, in two separate exposure chambers. A control was obtained using synthetic air. The NO_2-exposed bacteria were laid in contact with a mixture of N_2/O_2 8/2 (v/v) complemented with 45 ppm of NO_2 (Air Liquide GMP Europe). After exposure, bacteria were resuspended in sterile saline solution.

2.3 Cultivability and growth kinetics

Cultivability tests were performed by serial dilution in saline solution from the bacterial suspension to reach dilution of 10^{-7}. 100 µL of dilution range 10^{-4} to 10^{-7} were spotted onto LB agar plates in triplicate for both conditions. After incubation at 28°C for 24 h, viable colony forming unit were numerated.

After adjustment of the bacterial concentration to A_{580} = 0.08 in DMB, growth kinetics were done for both conditions in triplicate in a 96-well test plate (Nunc™). The bacterial population was evaluated every 15 min by turbidimetry at A_{580} during 24 h.

2.4 Lipid identification

2.4.1 Phospholipids

As previously described by Kondakova et al. [16], the lipids were extracted from lyophilized bacteria according to the method of Bligh and Dyer [17]. Then phospholipids (PLs) were first separated by HPTLC [18]. Retention factors (R_f) were calculated using the Sweday JustTLC

software (v. 4.0.3) after visualizing spots at 365 nm thanks to staining with primuline dye spray. A DHB matrix, allowing the positive ion spectra of lipids, was selected [16]. MALDI TOF mass spectra were acquired using an Autoflex III (Bruker Daltonics) and all MS spectra were obtained in reflector positive ion mode using TLC MALDI software (v. 1.1.7.0). Post-Source Decay spectra were acquired as previously described using FlexControl software (Bruker Daltonics) for each spot related to one specific lipid [16]. Lipids were identified using the LIPID MAPS database. Thanks to previous results, the MS Imaging of lipid spots was obtained using the FlexImaging software (v. 2.1.; Bruker Daltonics). According to the m/z, individual lipidic spots were labelled by a specific colour code.

2.4.2 Fatty acids

The fatty acids (FAs) methyl esters (FAME) were obtained by incubation for 15 min at 95°C in a boron trifluoride (BF_3, 140 g/L) in methanol and extracted by hexane [19]. They were separated and analysed by gas chromatography (GC) coupled to flame ionization detection using an Agilent Technology, 6890 Network GC System. The apparatus was equipped with a CP-Sil 88 capillary column (Chrompack; length, 50 m; inner diameter, 0.25 mm; 0.25 mm film). All experiments were performed in triplicate. The degree of FAs saturation was determined as the ratio between the saturated FAs and the unsaturated FAs [20].

2.5 Microbial adhesion to solvent

The hydrophobicity of *P. fluorescens* MFAF76a strain was evaluated by the microbial adhesion to solvent (MATS) test [21]. It consisted of assessing the affinity of the cells to two solvent duos composed of a monopolar solvent and an apolar solvent. In each set, both solvents have similar surface tension, but the monopolar one was acidic (electron accepting, e.g. chloroform) or basic (electron donor, e.g. ethyl acetate). The apolar solvents were hexadecane and decane, respectively. For the experiments, bacterial cells were resuspended in saline solution to $A_{400} = 0.8$ (Abs_1). This bacterial suspension was mixed with each solvent at 1/6 (v/v) by vigorous agitation for 1 min to form an emulsion. After at least 15 min of delay, the separation of the two phases of the mixture occurred. The aqueous phase absorbance (Abs_2) was measured and the percentage of adhesion was expressed as in eqn (1):

$$\%_{affinity} = 1 - \frac{Abs_1}{Abs_2} \times 100 \quad (1)$$

2.6 Membrane permeability assays

After exposure, 1 mL of bacterial suspension was stained for 15 min with SYTO9 (5.01 nM) and propidium iodide (PI, 30 nM) from the Live/Dead BacLight kit (L-7012, Thermofisher). Prolong diamond (P36965, Thermofisher) was added as anti-fading agent before observation using a confocal laser scanning microscope (CLSM 710, ZEISS). Thence, with an immersion objective × 63, damaged and total bacteria were respectively observed as PI and SYTO9 positive.

2.7 Statistical analysis

All experiments were carried out at least three times. Significances of differences between mean values were assessed using the Mann-Whitney test with significance set at $p<0.05$ (*), <0.01 (**), and <0.001 (***).

3 RESULTS AND DISCUSSION

To investigate the response of air microbiota on the NO_2 air pollutant, the bacterial strain *P. fluorescens* MFAF76a was exposed to 45 ppm of NO_2 for 2 h. After exposure, several physiological tests including cultivability and growth kinetics, as well as membrane permeability and lipidomic studies were done. The results were compared with those of a control samples, coming from the same bacterial culture exposed similarly to synthetic air. In NO_2-treated bacteria, a decrease of one log in cultivability of *P. fluorescens* MFAF76a strain was observed (Fig. 1a). Moreover, the exposure promoted an increase of half an hour of the lag phase compared with the control and no significant variation of generation time (Fig. 1b). Altogether these results suggested a diminution of the culturable bacterial density after exposure. The A_{580} do not reflect the culturable bacterial concentration. Two hypotheses could be proposed to explain such a phenomenon. The exposure to NO_2 seems to enhance the apparition of viable but non-culturable bacteria and/or the bacterial death. Moreover the treatment to NO_2 could also impact the bacterial physiology since they need to detoxify this compound first. Thence all those factors could promote the delay previously discussed. However, the generation time was not significantly impacted. This data suggest no great alteration in metabolic pathway implicated in cell division. An exposure at 45 ppm of NO_2 clearly impacts the physiological behaviour of *P. fluorescens* MFAF76a strain.

Since the membrane is the first barrier protecting bacteria from their environment, a macroscopic study by MATS technique was realized to assess the surface polarity and thence potential membrane alteration of exposed bacteria. The bacterial affinity to chloroform and hexadecane indicated no significant change in Lewis base character after exposure to NO_2 (Fig. 2). This parameter is related to the presence of lone pair usually found in unsaturations or around heteroatoms such as constituting proteins or polar head of glycerophospholipids. As no drastic modification in hydrophobicity was observed, the proteins should not be implicated. However, a decrease in acidity of Lewis (affinity relative to decane and ethyl acetate) from bacteria exposed to NO_2 was noticed and could suggest fewer electron pair acceptors

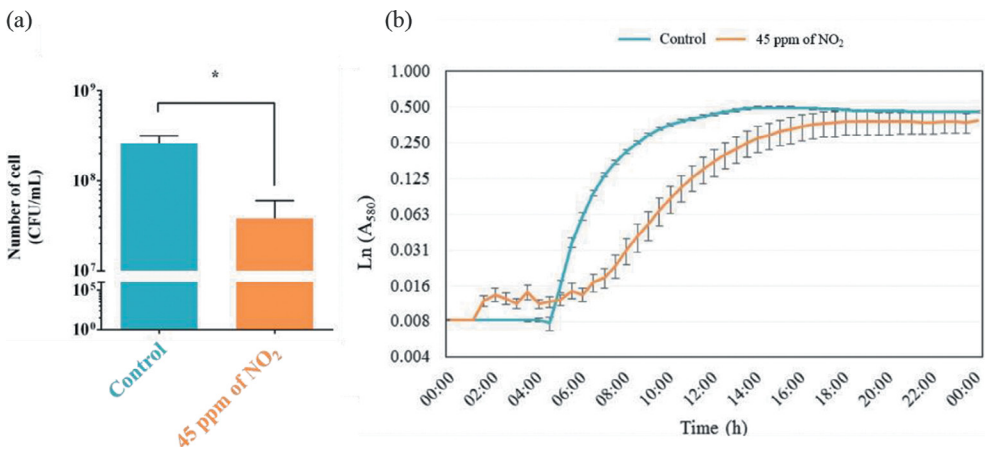


Figure 1: Physiological response of *P. fluorescens* MFAF76a strain after two hours' exposure at 45 ppm of NO_2. (a) Bacterial cultivability. (b) Bacterial growth kinetics in DMB, a minimal medium. For each exposure condition, three independent experiments were done in three replicates.

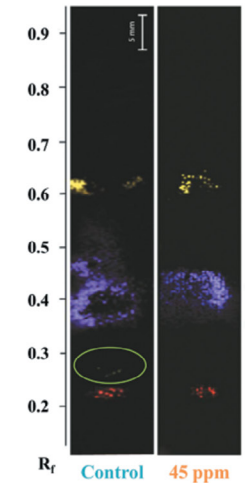
NO_2 concentration (ppm)	MATS : solvent affinity (%)			
	Chloroform	Hexadecane	Decane	Ethyl acetate
0	62 ± 10	10 ± 8	4 ± 3	24 ± 10
45	41 ± 10	0 ± 6	4 ± 4	1 ± 1

Figure 2: Microbial adhesion to solvent tests of *P. fluorescens* MFAF76a strain after exposed or not to NO_2. For each exposure condition, three independent experiments were done in three replicates.

outwards the membrane. These alterations reveal slight changes within the bacterial membrane, which could be limited in composition, but more surely in structure. A combination of both may also be possible. For instance, alkyl chains may swivel outwards burying electron pair acceptors inside membrane.

To better understand the nature of that membrane alteration, lipids extraction and identification using HPTLC-MALDI TOF MS were performed after exposure (Fig. 3). Indeed phosphatidylglycerol (PG), phosphatidylethanolamin (PE) and phosphatidylcholin (PC) were found in both extracts (Figs 3a and 3b). Those results are coherent with previous data [16]. Interestingly, a putative glycerophospholipid named 'unknown glycerophospholipid' (UGP) was identified in this study. This putative PL was only observed in control bacteria exposed to synthetic air and was absent in NO_2-treated cells, indicating its probable sensitivity to NO_2 and/or membrane

(a) (b)



Retention factor (R_f)	GP class	Species	Molar mass (g/mol)	m/z	Assignement	NO_2 level (ppm) 0	45
0.62 ± 0.03	PG	PG [16:0 + 16:1]	720.5	743.5	$[PG_1+Na]^+$		
				765.5	$[PG_1+2Na-H]^+$		
		PG [16:0 + 18:1]	746.5	769.5	$[PG_2+Na]^+$		
				791.6	$[PG_2+2Na-H]^+$		
0.43 ± 0.02	PE	PE [16:0 + 16:1]	689.5	712.5	$[PE_1+Na]^+$		
				734.5	$[PE_1+2Na-H]^+$		
		PE [16:0 + 17:1]	703.5	726.5	$[PE_2+Na]^+$		
				748.5	$[PE_2+2Na-H]^+$		
		PE [16:0 + 18:1]	715.5	738.5	$[PE_3+Na]^+$		
				760.5	$[PE_3+2Na-H]^+$		
0.26 ± 0.02	UGP	UGP [16:0 + 17:0]	904.5	927.5	$[UGP_1+Na]^+$		
		UGP [16:0 + 18:0]	918.5	941.5	$[UGP_2+Na]^+$		
		UGP [17:1 + 18:0]	930.5	953.5	$[UGP_3+Na]^+$		
0.24 ± 0.01	PC	PC [16:0 + 16:1]	731.5	732.6	$[PC_1+H]^+$		
		PC [16:0 + 16:1]	731.5	754.5	$[PC_1+Na]^+$		
		PC [16:0 + 18:1]	759.6	782.6	$[PC_2+Na]^+$		

Figure 3**:** Lipidome of P. fluorescens MFAF76a strain after exposure or not to NO2. (a) HPTLC MALDI-TOF MS Image of their glycerophospholipids. (b) List of glycerophospholipids identified. PG: Phosphatidylglycerol (yellow spot); PE: Phosphatidylethanolamin (purple spot), UGP: Unknown glycerophospholipid (green spot, circle in green), PC: Phosphatidylcholin (red spot), R_f: Retention factor. For each exposure condition, three independent experiments were done in three replicates.

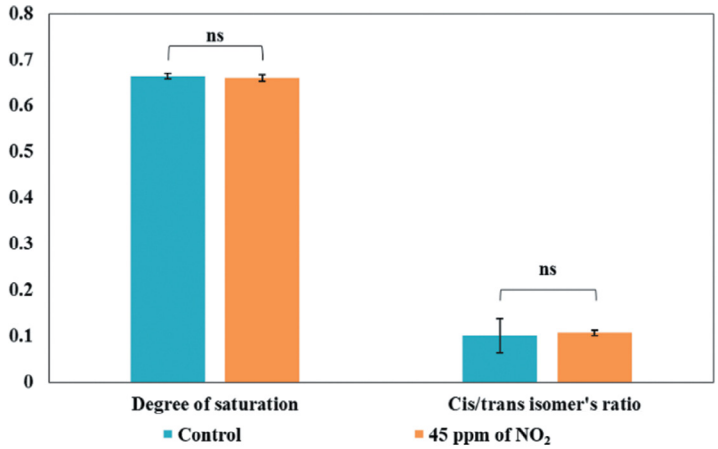


Figure 4**:** Fatty acid methyl esterification of *P. fluorescens* MFAF76a strain after exposition to NO_2 or synthetic air (control). For each exposure condition, three independent experiments were done in three replicates; ns: not significant.

adaptation of MFAF76A to this stress, by exclusion of this PL from the cell wall. We noted that the long FA chains of this UGP (C_{16}, C_{17} and C_{18}) were mostly saturated. Such saturated lipids favour the membrane fluidity. Thence the absence of UGP in exposed bacteria is suggesting a decrease of membrane fluidity. However, after FAME analysis, no significant alteration in the degree of FA saturation and cis/trans isomers ratio was detected (Fig. 4). We hypothesized that bacteria, in response to the lack of UGP after exposure to NO_2, reorganizes the membrane to compensate this loss, suggesting a potential membrane adaptation to NO_2 stress.

In order to investigate potential changes of membrane permeability, Live Dead tests using SYTO9 (green) and IP (red) labels were performed and analysed using CLSM. Interestingly, the red labelling was predominant after exposure to NO_2 unlike the control (Fig. 5). IP dye entered largely in bacterial cells revealing membrane permeabilization. NO_2 alters polyunsaturated lipids through production of nitro alkyl radicals in eukaryotic cells but no literature deals on NO_2 effect on bacterial lipids [8].

An increase of cell aggregation was also observed and it is interesting to note that deletion of the main outer membrane porin OprF, which induces a parietal stress, is known to promote a similar effect in *P. aeruginosa* H103 [21]. Then the bacterial 'community behaviour' was investigated through biofilm formation using CLSM and a CFP-labelled *P. fluorescens* MFAF76a strain. Exposed bacteria form a thicker biofilm with some heterogeneities certainly due to the presence of bacterial amasses unlike the control [15]. This phenomenon was also observed with the *oprF* mutant in *P. aeruginosa* H103 [22]. Thus, the exposure to NO_2 may lead to membrane modification causing a parietal stress to *P. fluorescens* MFAF76a. Subsequently, the aggregation of the bacterial cells could promote the biofilm formation and so the bacterial survival.

Biofilms represent a threat in clinic, as their elimination is more and more challenging. Interestingly, a previous study evaluated the impact of NO_2 on antibiotic resistance using ciprofloxacin and chloramphenicol [15]. Such antibiotics are usually prescribed respectively for treatment of first and last stage of cystic fibrosis to eradicate *Pseudomonas* infection [23]. For the NO_2-treated bacteria a two-fold increase in MICs of both antibiotics was noted, indicating that in response to NO_2, *P. fluorescens* exhibits an increase in resistance against

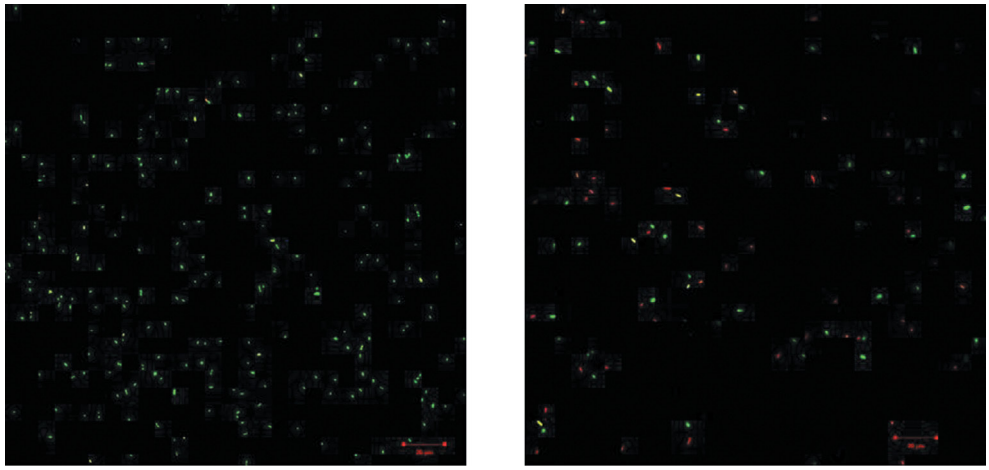


Figure 5: Membrane permeability using Live Dead test and CLSM. Green and red labels signify penetration in bacteria of SYTO9 and Propidium Iodide, respectively. For each exposure condition, three independent experiments were done in three replicates.

chloramphenicol and ciprofloxacin [15]. Surprisingly, a sub-inhibitory concentration of chloramphenicol (25 µg/mL) significantly increased the bacterial growth between 2 and 10 h of culture compared to the control. Thence a protective effect of NO_2 is conserved for 10 h after exposure. On the contrary, the growth kinetics of the exposed strain was unchanged in presence of a sub-inhibitory concentration of ciprofloxacin (3.125 µg/mL) [15]. Various stresses can lead to the modulation of bacterial resistance to antibiotics [24, 25]. Astonishingly, new therapeutic strategies are focusing nowadays on the use of gaseous nitrogen oxide as a pretreatment to decrease the antibiotic resistance encountered in cystic fibrosis patients [26].

In correlation with previous data, a transcriptomic study on genes encoding efflux pump *mexEF-oprN* revealed a significant increase in the expression for those genes [15]. Indeed, in comparison to the controls, the expression of *mexE, mexF* and *oprN* genes reached almost 14-, 3.5- and 4.6-fold, respectively. These results are consistent with the antibiotic tests realized previously since the MexEF-OprN efflux pump is involved in the resistance against fluoroquinolones (such as ciprofloxacin). Moreover chloramphenicol, a nitroaromatic antimicrobial, is also a substrate for MexEF-OprN [27, 28].

As this efflux pump is a macromolecular complex enchased into the plasma membrane, MexEF-OprN could replace UGP to preserve the membrane integrity. Moreover, this efflux pump implements the entry of proton (H^+), which may alter the membrane potential. Indeed H^+ could be accumulated and, consequently, increase the positive charges within cells. Thence the alteration of the potential equilibrium may favour the increase of membrane potential. Thus, the bacterial physiology may be disturbed causing dysfunction of membrane proteins operating through this membrane potential. Indeed a decrease in membrane potential was linked with the susceptibility of *P. aeruginosa* to aminoglycosides antibiotics such as tobramycin [29]. Moreover, this H^+ gradient could lead to a reorganization of PLs, such as a swivelling of alkyl chains outwards, which could justify the Lewis base alteration.

4 CONCLUSION

The present study provides further information concerning the adaptation of an airborne strain of *P. fluorescens* strain, with opportunistic pathogen potential behaviour, to an air pollutant such as NO_2. The bacterial stress caused by this preoccupant air pollutant induces modifications in membrane permeability certainly because of chemical and/or structural variations of cell wall constituents. Thus physiological parameters such as cultivability and growth are negatively impacted. However, the ability of the bacterium to form biofilm increases, certainly favoured by the observed bacterial amasses promoted by the parietal stress. Unfortunately, resistance to fluoroquinolone and phenicol antibiotics increases after exposure to NO_2. Those latter parameters are quiet worrying since they are implicated in bacterial virulence. However, 45 ppm of NO_2 is considered as a high concentration leading to irreversible effects on human health and does not totally reflect usual environmental concentrations measured in chronic pollution rates [29]. The next step of this project will be to focus on a lower concentration of NO_2 close to the threshold alert value [30]. Nevertheless, NO_2 has clearly a drastic impact on the physiology of *P. fluorescens* MFAF76a and these results support the hypothesis that air pollutants could promote the pathogenicity of airborne bacteria.

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