Gamma-aminobutyric acid acts as a specific virulence regulator in \textit{Pseudomonas aeruginosa}

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Gamma-aminobutyric acid (GABA) is widespread in the environment and can be used by animal and plants as a communication molecule. \textit{Pseudomonas} species, in particular fluorescent ones, synthesize GABA and express GABA-binding proteins. In this study, we investigated the effects of GABA on the virulence of \textit{Pseudomonas aeruginosa}. While exposure to GABA (10 μM) did not modify either the growth kinetics or the motility of the bacterium, its cytotoxicity and virulence were strongly increased. The \textit{Caenorhabditis elegans} ‘fast killing test’ model revealed that GABA acts essentially through an increase in diffusible toxin(s). GABA also modulates the biofilm formation activity and adhesion properties of PAO1. GABA has no effect on cell surface polarity, biosurfactant secretion or on the lipopolysaccharide structure. The production of several exoenzymes, pyoverdin and exotoxin A is not modified by GABA but we observed an increase in cyanogenesis which, by itself, could explain the effect of GABA on \textit{P. aeruginosa} virulence. This mechanism appears to be regulated by quorum sensing. A proteomic analysis revealed that the effect of GABA on cyanogenesis is correlated with a reduction of oxygen accessibility and an over-expression of oxygen-scavenging proteins. GABA also promotes specific changes in the expression of thermostable and unstable elongation factors Tuf/Ts involved in the interaction of the bacterium with the host proteins. Taken together, these results suggest that GABA is a physiological regulator of \textit{P. aeruginosa} virulence.

**INTRODUCTION**

Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid, widespread in the environment (Bouché \textit{et al.}, 2003). Since the 1950s, it has been known that GABA is the major inhibitory neurotransmitter in the nervous system of vertebrates and invertebrates (Owens & Kriegstein, 2002). In other living organisms, such as plants, fungi or bacteria, GABA was considered for a long time to be a stress response factor and/or a nutrient (Higuchi \textit{et al.}, 1997; Shelp \textit{et al.}, 1999). The hypothesis that GABA could also be a communication molecule in these organisms started with the observation that, in plants, GABA controls the guiding of the pollen tube into the female gametophyte (Palanivelu \textit{et al.}, 2003). In plant life, GABA now appears to be a ubiquitous communication molecule produced as a defence against nematodes, insects and also bacterial phytopathogens (Shelp \textit{et al.}, 2006). The role of GABA in communication between plants and bacteria is in line with the identification of GABA-binding proteins in different prokaryotes corresponding not only to transporters such as GabP in \textit{Escherichia coli} or \textit{Bacillus subtilis} (Hu & King, 1998; Brechtel & King, 1998) and Bra in \textit{Agrobacterium tumefaciens} (Chevrot \textit{et al.}, 2006) but also to possible specific receptors (Guthrie & Nicholson-Guthrie, 1989). In addition, many bacteria, including marine micro-organisms (Morse \textit{et al.}, 1980), lactic bacteria (Siragus\textit{a et al.}, 2007), \textit{E. coli} (Richard & Foster, 2003) and \textit{Pseudomonas} (Chou \textit{et al.}, 2008) synthesize GABA, suggesting that, like in eukaryotes, GABA might be a conserved and ubiquitous communication molecule.

That GABA has an effect on the physiology of \textit{Pseudomonas} is supported by several observations. A periplasmic protein...
showing high affinity for GABA and biochemical characteristics similar to a subunit of the mammalian GABA$_A$ receptor was identified in an environmental strain of *P. fluorescens* (Guthrie & Nicholson-Guthrie, 1989; Guthrie et al., 2000). In *P. aeruginosa*, GABA is an intermediate metabolite of organic polycation catabolism and an inducer of the enzymic pathway controlling the intracellular polyanionic levels (Chou et al., 2008). The question of a possible effect of extracellular GABA on *P. aeruginosa* is of medical significance. GABA is present in significant concentrations in the brain matter but also in the cerebrospinal fluid (Tunnicliff & Malatynska, 2003) and, essentially following breaches of the meningeal barrier due to trauma, surgical acts or, more rarely, by diffusion from infected contiguous structures (paranasal sinus or inner ear), *P. aeruginosa* can invade the central nervous system causing life-threatening infections such as brain abscesses, ventriculitis or meningitis (Mesaros et al., 2007). However, GABA is also present in blood (Petty et al., 1999) where it has immunomodulatory functions (Jin et al., 2011) and, as *P. aeruginosa* is a major cause of bloodstream infections (van Delden, 2007), the bacterium is exposed to GABA during bacteraemia or septicaemia. With the emergence of multidrug-resistant strains, it is essential to identify the hosts and communication factors capable of modulating the virulence of *P. aeruginosa*; these should represent new strategies for treatment (Fothergill et al., 2012).

In the present study, we investigated the effect of GABA on *P. aeruginosa* through a multi-phenotypical study. We show that GABA stimulates *P. aeruginosa* virulence through variation of the production of a quorum-sensing-regulated factor and modulation of enzymes involved in the oxidative stress response.

**METHODS**

**Bacterial strains and culture conditions.** *P. aeruginosa* PAO1 was obtained from an international collection (University of Washington). Except for specific tests, as indicated below, bacteria were grown in Nutrient Broth (NB, Merck). The effect of GABA was studied at 0.01 mM to avoid possible direct metabolic and pH effects and since it is the mean value of the concentrations in the synaptic cleft of neurons (1.5–3 mM, Mozrzymas and since it is the mean value of the concentrations in the synaptic studied at 0.01 mM to avoid possible direct metabolic and pH effects were grown in Nutrient Broth (NB, Merck). The effect of GABA was obtained from an international collection (University of vegetal (0.1 mM, Shelp et al., 2001) and the nematode *Caenorhabditis elegans*, a metazoan model used to assess the general virulence of human pathogens and environmental bacteria (Duclairoir Poc et al., 2011). Preliminary controls showed that exposure of *P. aeruginosa* PAO1 to GABA (0.01 mM) during the whole growth phase, or at the beginning of the stationary phase, did not modify the growth kinetics of the bacterium. For the cytotoxic activity, *P. aeruginosa* PAO1 grown in NB was inoculated at OD$_{580}$ 0.2 in 25 ml NB containing GABA (0.01 mM) or no GABA, and cultured at 37 °C with agitation (180 r.p.m.) to early stationary phase. Bacteria were harvested by centrifugation in an Eppendorf centrifuge tube (6000 r.p.m., 4 min, 20 °C), rinsed three times with NB and resuspended at a cell density of 10$^8$ C.F.U. ml$^{-1}$ in gial cell culture medium without antibiotics and antimycotics. The bacterial viability and density, and the absence of contamination were controlled by plating. Glial cells were incubated for 4 h with *P. aeruginosa* and gial cell death was monitored using the lactate dehydrogenase (LDH) assay release as described previously (Picot et al., 2003, 2004) using the Cytotox 96 Enzymic Assay (Promega). Controls realized using bacteria alone showed that in our experimental conditions *P. aeruginosa* PAO1 does not produce molecules that interfere with this assay.

The global virulence of *P. aeruginosa* PAO1 was studied using the wild-type N2 strain of *C. elegans* kindly provided by the *Caenorhabditis Genetics Center* (Minneapolis, MN, USA). Worms were grown at 22 °C on *E. coli* OP50 cultures. Synchronous cultures of worms were obtained as described previously (Bier et al., 2011). Two types of tests: ‘fast-killing’ and ‘slow-killing’ were used. In the ‘fast-killing’ test, worms are killed by bacterial diffusable toxins whose secretion is stimulated by use of a high-osmolarity medium (Aballay & Ausubel, 2002). In the ‘slow-killing’ test, worms are killed after ingestion of the bacteria (Duclairoir Poc et al., 2011). Pathogen lawns used for *C. elegans* survival assays were prepared by spreading 50 μl control or GABA-treated *P. aeruginosa* PAO1 (OD$_{580}$ 1) (obtained as previously described) on 35 mm pettone-glucose-sorbitol (PGS: 1% Bacto-Peptone; 1% NaCl, 1% glucose, 0.15 M sorbitol, 1.7% Bacto-Agar) conditioned Petri dishes for ‘fast-killing’ evaluation or on 35 mm nematode growth medium (NGM: 3 g NaCl, 2.5 g peptone, 17 g agar, 5 mg cholesterol, 1 ml 1 M CaCl$_2$, 1 ml 1 M MgSO$_4$, 25 ml 1 M KH$_2$PO$_4$, H$_2$O to 1 l) conditioned Petri dishes supplemented with 0.05 mg 5-fluoro-2'-deoxyuridine ml$^{-1}$ for ‘slow-killing’ experiments. The plates were incubated overnight at 37 °C and then placed at room temperature for 4 h. Twenty L4 synchronized worms were harvested with M9 solution (3 g KH$_2$PO$_4$, 6 g NaHPO$_4$, 5 g NaCl, 1 ml 1 M MgSO$_4$, H$_2$O to 1 l) conditioned Petri dishes supplemented with 0.05 mg 5-fluoro-2'-deoxyuridine ml$^{-1}$ for ‘slow-killing’ experiments. The plates were incubated overnight at 37 °C and then placed at room temperature for 4 h. Twenty L4 synchronized worms were harvested with M9 solution (3 g KH$_2$PO$_4$, 6 g NaHPO$_4$, 5 g NaCl, 1 ml 1 M MgSO$_4$, H$_2$O to 1 l), placed on the 35 mm assay Petri dishes and incubated at 22 °C. Worm survival was scored at 1 h, 24 h and each subsequent day, using an Axiovert S100 optical microscope (Zeiss) equipped with a Nikon digital Camera DXM 1200F (Nikon Instruments). The worms were considered dead when they remained static without grinder movements for 20 s. The results are expressed as the percentage of living worms.

**Biofilm formation.** The biofilm formation activity of *P. aeruginosa* PAO1 was studied using a technique adapted from O’Toole & Kolter (1998). An aliquot of 100 μl of a bacterial culture adjusted to OD$_{580}$ 0.4 was layered in a PVC microtitration plate and incubated for 24 or 48 h. In the first set of experiments, bacteria were grown in the absence of GABA but were exposed to the molecule during biofilm formation. In a second set of experiments, bacteria were grown in the presence of GABA but GABA was not added to the medium during the biofilm formation. In a third set of experiments, bacteria were grown with GABA as previously, but the medium in PVC microtitre plates was supplemented with GABA to maintain the treatment during the 24–48 h of the biofilm formation test. After removing the bacterial suspension and rinsing, bacteria and matrix bound to the wells were stained with crystal violet (0.1%, 30 min). After rinsing, the dye was recovered by adding 150 μl of a solution of 3% H$_2$SO$_4$ and 1% glacial acetic acid, in deionized water. The absorbance at 590 nm (OD$_{590}$) was measured using a microplate reader (Bio-Tek). Results were normalized as a percentage of the mean value corresponding to the mature biofilm formation activity of CTS117 at 8 °C.
Cell surface properties. The binding index of *P. aeruginosa* PAO1 on biological (cell) surfaces was determined using the gentamicin exclusion test (Mezghani-Abdelmoula et al., 2004).

The binding index of bacteria on glass slides was determined by direct counting. Glass slides were cleaned with ethanol and TDF&4 detergent (4% in 5°C water) to remove any trace of lipids. A bacterial suspension (10^6 c.f.u. ml^{-1} in sterile water (SPW) NaCl 0.9%) was layered on each glass slide. Bacteria were allowed to adhere for 2 h at 37°C. Non-adherent bacteria were removed by rinsing and the remaining adherent bacteria were stained with acridine orange (0.01% in sterile water). The slides were observed using an epifluorescence microscope Zeiss Axiocam. The binding index was determined by counting a minimum of 20 homologous fields.

The surface polarity of *P. aeruginosa* PAO1 was determined using the microbial adhesion to solvent (MATS) technique (Bellon-Fontaine et al., 1996). Bacterial cultures in early stationary phase and treated or not with GABA were harvested by centrifugation at 10,000 g. Pellets were rinsed twice with 0.9% NaCl in water and diluted to OD_{600} 0.8. An aliquot of bacterial suspension (2.4 ml) was mixed with 0.4 ml hexadecane. The tubes were vigorously hand-shaken and the OD_{400} of the aqueous phase was measured after 15 min of decantation. The percentage of affinity for hexadecane was calculated as follows: ([OD_{control}−OD_{test}]/OD_{control}) × 100.

Lipopolysaccharide (LPS) structure. LPS was purified from *P. aeruginosa* PAO1 as described by Darveau & Hancock (1983) and was analysed by MALDI-TOF MS using an Autoflex III mass spectrometer (Bruker Daltonik). For analysis, a 0.5 μl aliquot of purified LPS was spotted onto a steel target plate. An aliquot (0.5 μl) of a solution of x-cyano-4-hydroxycinnamic acid matrix (10 mg ml^{-1} in acetonitrile/ 0.2% TFA, v/v) was added to each spot and these were dried at room temperature. The mass spectrometer was equipped with a pulsed YAG 200 Hz laser and was run in the positive mode. Instrument calibration was achieved by using calibration standards (Care, Bruker Daltonics) spotted on the same target plate. Each spectrum was established over 200 laser shots.

Biosurfactant production assays. The kinetics of biosurfactant production were monitored over 48 h by measuring the surface tension of the rinsing solution (SPW) of colonies of *P. aeruginosa* grown on solid NB medium, using the Wilhelmy plate technique (Hienz & Lagowski, 1977). Rhamnolipids were further extracted from 3 ml culture supernatant of bacteria grown for 24 h, and analysed by HPLC/MS using an Agilent Technologies 1100 system from 3 ml culture supernatant of bacteria grown for 24 h, and analysed by HPLC/MS using an Agilent Technologies 1100 system. A 2.4 ml bacterial suspension was mixed with 0.4 ml Tris buffer (0.1 M, pH 7.2, containing 20 mg elastin/Congo red (Sigma)). The tubes were vigorously hand-shaken and the OD_{400} of the supernatant after removal of the bacteria by centrifugation (5 min, 10,000 g).

Exotoxin A was assayed in the culture supernatants of *P. aeruginosa* PAO1 grown in NB using an ELISA sandwich method on 96-well microtitre plates as described by Davies et al. (1985). Each well was coated with goat anti-exotoxin A antibody (0.25 mg ml^{-1} in 100 mM Na$_2$HCO$_3$) and treated with BSA to block non-specific binding sites. A standard curve was made by using serial dilutions of purified exotoxin A (Sigma-Aldrich). Assays were made on 100 μl culture medium. After 1 h incubation at room temperature, the plates were washed and incubated with rabbit–anti-exotoxin A. The plates were then washed and treated with goat–anti-rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich). The reaction was visualised by adding a substrate solution (Pierce Biotechnology) and was stopped after 30 min by adding H$_2$SO$_4$ (2 M). The absorbance was read at OD$_{450}$ using an ELISA plate reader (Bio-Tek Instruments). The values are expressed as the ratio of Exotoxin A (in pg ml^{-1}) versus the bacterial culture density (at 580 nm).

The HCN/CN$^-$ production by *P. aeruginosa* was determined by using a polarographic method recently developed in our laboratory (Blier et al., 2012). Voltametric measures were made using a Metrohm 757 VA Computrace. Analyses were carried out in a three-electrode configuration using a saturated Ag/AgCl reference electrode, a platinum wire as a counter electrode and a Multi-Mode Mercury Electrode as a working electrode. All experiments were performed at room temperature. Bacterial cultures were centrifuged and filtered on a 0.22 μm filter to remove cell fragments. This medium was then diluted in 0.2 mol borate electrolyte l^{-1} (pH 10.2). The solution was purged for 3 min with N$_2$ to remove dissolved oxygen and then for 20 s more between each cyanide addition. A potential scan was carried out in a negative direction from −0.1 to −0.5 V with a sweep rate of 10 mV s$^{-1}$. The pulse amplitude was 0.05 V with pulse duration of 0.04 s. The cyanide peak was measured at −200 mV in a differential pulse mode and the cyanide concentration was determined by adding triplicate successive (n=4) amounts of 10 mg potassium cyanide standard l^{-1}.

Total proteome analysis. Cultures of *P. aeruginosa* PAO1 grown to early stationary phase were centrifuged (20 min, 12,000 g). Pellets were washed three times in 50 mM Tris/HCl, pH 8. Cells were disrupted by three freeze/thaw cycles and sonication. Unbroken cells were removed by centrifugation. The supernatants were supplemented with benzonase and MgCl$_2$ and incubated for 30 min at 37°C. Proteins were precipitated with cold acetone (v/v) and harvested by centrifugation at 13,000 g for 10 min at 4°C, and resuspended in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS (Bio-Rad), 0.5% IPG buffer 3-10 (Bio-Rad), 70 mM DTT and 5 mM TCEP [Tris(2-carboxyethyl)phosphine hydrochloride] to a final volume of 300 μl. The protein concentration was controlled using the Bio-Rad protein assay (Bio-Rad) and BSA as standard.

The protein extract was then separated by 2D gel electrophoresis and in-gel trypsin digestion. For the first dimension electrophoresis, protein samples were separated by IEF using pH 4–7 IPG strips (immobilized linear gradient, 17 cm, Bio-Rad) as described by Barbev et al. (2012). After IEF, the IPG strips were reduced in an equilibration buffer [6 M urea, 50 mM Trisma base, 2% (w/v) SDS, 20% (v/v) glycerol, a few grams of bromphenol blue and 2% (w/v) DTT for reduction or 2.5% (w/v) iodoacetamide for alkylation]. The second dimension SDS-PAGE was realigned on 12% polyacrylamide (37.5:1 acrylamide/bisacrylamide, Bio-Rad) gels at 50 mA for 4 h. Proteins on gels were visualized with Coomassie brilliant blue R-250 and gel images were captured using a GS-800 densitometer (Bio-Rad). Variations in spot intensity and distribution were studied using the Bio-Rad PDQuest 2D analysis software. In-gel digestion was performed as previously (Barbev et al., 2012). In-gel trypsin digestion...
products were then analysed using MALDI-TOF/TOF (Autoflex III, Bruker Daltonics) in positive/reflector mode. Instrument calibration was achieved using peptide calibration standards (Care, Bruker Daltonics). Samples were spotted onto MTP 384 ground steel target, using freshly prepared matrix solution composed of 2,5-dihydroxybenzoic acid (20 mg ml\(^{-1}\)) in a solution of 0.1% TFA and 50% acetonitrile. Peptide mass fingerprints obtained from MS analysis and MS/MS spectra were used to search the NCBI no redundant database by using online MASCOT software (http://www.matrixscience.com). Search parameters were set as follows: taxonomy was set on bacteria; trypsin was given as the digestion enzyme and a maximum of one missed cleavage site was allowed; carbamidomethylation of cysteine and methionine oxidation were selected as fixed and variable modifications, respectively; mass values were set to monoisotopic. Searches were performed setting a peptide mass tolerance of 0.05 Da. The Student\(\text{-}\)t-test was used to compare the means within the same group, and survival differences were tested for significance by using the Kaplan–Meier analysis. In addition, control experiments realized by direct treatment of glial cells with GABA (0.01 mM) in the absence of bacteria showed that this molecule had no direct effect on glial cell viability (data not shown).

The effect of GABA on *P. aeruginosa* virulence was tested in a second model, the nematode *C. elegans*, which is

**RESULTS**

**Effect of GABA on *P. aeruginosa* virulence**

Preliminary controls showed that exposure of *P. aeruginosa* PAO1 to GABA (0.01 mM) during the whole growth phase, or at the beginning of the stationary phase, did not modify the growth kinetics of the bacterium. When grown in the presence of GABA, *P. aeruginosa* displayed a strong increase of its cytotoxic potential on glial cells (88.6 ± 8.6%, *P*<0.001), as revealed by the LDH release assay (Fig. 1a). Since the rat glial cells might be sensitive to GABA themselves (Angulo \textit{et al.}, 2008), bacteria were carefully rinsed to remove any trace of free GABA prior glial cells infection. In addition, control experiments realized by direct treatment of glial cells with GABA (0.01 mM) in the absence of bacteria showed that this molecule had no direct effect on glial cell viability (data not shown).

![Fig. 1. Effect of GABA (0.01 mM) on the cytotoxicity and virulence of *P. aeruginosa* PAO1. The cytotoxic activity of the bacteria was evaluated on glial cells (a). The global virulence was tested on *C. elegans* using the ‘fast killing’ test, where the worms are killed by bacterial diffusible toxins (b) and using the ‘slow-killing’ test, in which the worms are killed through contact-mediated interactions after ingestion of the bacteria (c). **P<0.001. Grey, control PAO1; black, GABA-treated PAO1. Error bars indicate SEM.](image-url)
considered to be an equivalent of the acute mouse toxicity test (Williams et al., 2000) and has been adapted to compare the virulence of environmental bacteria (Duclairoi poc et al., 2011). In addition, whereas the ‘fast-killing’ test gives information on the contribution of diffusible virulence factors in the lethal activity of the bacterium, the ‘slow-killing’ test reveals global variations of virulence, including those that are due to direct contact with the bacterial surface (Aballay & Ausubel, 2002). The two tests confirmed that GABA increases the virulence of P. aeruginosa: the ‘fast-killing’ test clearly shows that GABA-treated bacteria provoked a sharp decrease in the number of surviving worms after 24 and 48 h (−46.3±5.2 and −75.2±4.4 %, respectively) (Fig. 1b). While the effect of GABA was less visible in the ‘slow-killing’ test (Fig. 1c), the log–rank test analysis of the data demonstrated that GABA treated P. aeruginosa were also significantly more virulent (P=0.0252; n=12).

Effect of GABA on biofilm formation

As the virulence of P. aeruginosa, particularly in the lung, is generally associated with biofilm formation (Høiby et al., 2010), the effect of GABA on the biofilm formation activity was studied in vitro using different protocols to take into consideration a possible metabolization of GABA during biofilm formation. Bacteria in NB medium were exposed to GABA as they were forming a biofilm or were pre-treated with GABA and were then allowed to form a biofilm in the absence or presence of GABA. When PAO1 was exposed to GABA only during the biofilm formation period, no change was observed. Conversely, when the bacteria were exposed to GABA during the growth phase, a reduction in biofilm formation was observed after 24 h (−13±3 and −15±4 %) and 48 h (−10±6 and −18±3 %) (Fig. 2). The reduction observed after 48 h in medium without GABA was not significant, probably because of the instability of mature biofilms.

Effect of GABA on the surface properties of P. aeruginosa

In order to determine more precisely the effect of GABA on the surface properties of P. aeruginosa, the adhesion potential and the surface polarity of the bacteria were investigated. The effect of GABA on bacterial adhesion was studied on living surfaces (glial cells) and on glass; pre-treatment of bacteria with GABA was associated with an increase in the binding index of 136 ± 21 and 138.7 ± 8.6 % on glial cells and glass, respectively. However, since the global variation of adhesion is limited (it appeared only really detectable on a homogeneous surface such as glass) the increase measured on glial cells should not be significant. As bacterial adhesion depends on the surface polarity, we measured the affinity of the bacteria to hexadecane using the MATS test. Considering that the threshold for the affinity to hexadecane of polar (hydrophilic) bacteria is 20 % (Bellon-Fontaine et al., 1996), in our experimental conditions P. aeruginosa PAO1 behaved as a highly polar micro-organism (3.5 ± 0.2 % affinity to hexadecane). The surface properties of P. aeruginosa were not significantly modified by GABA.

Effect of GABA on contact-dependent virulence factors production

Since our results showed that GABA modulates P. aeruginosa virulence by acting both on surface-dependent and diffusible virulence factors, we decided to investigate the effect of GABA on the structure of the LPS, the main constituent of the outer membrane of Gram-negative bacteria. The LPS extracted from P. aeruginosa PAO1 was analysed by MALDI-TOF MS. Multiple peaks, essentially of m/z ratio < 3000, probably generated by fragments of lipid A (Veron et al., 2007), were detected (Fig. 3a). Secondary peaks with a higher m/z ratio and considered to be compounds associated with oligosaccharide cores with different O-antigen repeating units (Veron et al., 2007)

![Fig. 2. Effect of GABA (0.01 mM) on the biofilm formation activity of P. aeruginosa PAO1, measured after 24 or 48 h of incubation. Bacteria were exposed to GABA only during the growth phase (pre-treatment), only during the biofilm formation period (during biofilm) or continuously during growth and biofilm formation (pre-treatment + during biofilm). The biofilm formation activity is expressed as a percentage of the control value. NS, non-significant; *P<0.05; **P<0.01; ***P<0.001. Error bars show SEM.](http://mic.sgmjournals.org)
were present in lower amounts. The mass spectrum of LPS extracted from GABA-treated bacteria was remarkably preserved. As noted in Fig. 3(b), the positions and relative intensities of all the peaks were the same in LPS extracted from control and GABA-treated *P. aeruginosa*, demonstrating that the LPS structure was not altered in response to GABA treatment. The surface properties of bacteria can be also influenced by the production of surfactants which modulate the surface tension between the bacterium and its immediate environment. A rapid method to check the bacterial production of surfactant involves collecting colonies on solid agar medium, cleaning them with pure water and measuring their surface tension (Carrillo et al., 1996).

*P. aeruginosa* PAO1 colonies showed a surface tension of $39.3 \pm 1 \text{ mN m}^{-1}$, indicating the presence of surfactants in solution (the mean surface tension of poorly mineralized water is $71.7 \text{ mN m}^{-1}$; Hiemenz & Lagowski, 1977). Interestingly, the surface tension of the extract of bacterial colonies grown in the presence of GABA was unchanged ($39.3 \pm 1 \text{ mN m}^{-1}$) but when this was measured on diluted extract, we observed a shift in critical micellar concentration for control and GABA-treated bacteria, suggesting a difference in the composition of the biosurfactants (data not shown). Biosurfactants from control and GABA-treated *P. aeruginosa* were then extracted and analysed by HPLC-ESI-MS/MS. A total of 10 different types of rhamnolipids were identified (Table 1). The same molecules were found in

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**Fig. 3.** MALDI-TOF analysis of LPS extracted from control (a) and GABA-treated (b) *P. aeruginosa* PAO1. Each figure is representative of three independent analyses.
Table 1. Rhamnolipids identified and quantified by ESI-MS analysis in cultures of P. aeruginosa PAO1 grown in the absence or presence of 0.01 mM GABA

Data are means ± SEM.

<table>
<thead>
<tr>
<th>Rhamnolipids</th>
<th>Intensity×10^5 (arbitrary units)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control PAO1</td>
</tr>
<tr>
<td>Rha-Rha-C8–C10/Rha-Rha-C10–C8</td>
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</tr>
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<td>Rha-C10–C10</td>
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</tr>
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<td>4.3 ± 1.5</td>
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<tr>
<td>Rha-Rha-C12–C12</td>
<td>2.3 ± 1.3</td>
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the extracts of P. aeruginosa exposed or not to GABA and their relatives proportions were identical, suggesting that the variations of surface tension observed in dilutions were due to other soluble factors such as proteins.

**Effect of GABA on P. aeruginosa diffusible virulence factors**

The effect of GABA on the major exoenzymic activities produced by P. aeruginosa PAO1, i.e. caseinase, esterase, haemolytic, amylase, lecithinase and elastase activities, was tested. None of these was altered in bacteria grown in the presence of GABA (data not shown). We investigated the effect of GABA on the principal secondary metabolites produced by P. aeruginosa, starting with pyoverdine, the production of which depends on iron availability in the environment and thus of the culture medium composition. We therefore studied the effect of GABA in two media known to promote pyoverdine secretion. As shown in Fig. 4(a and b), GABA has no effect on pyoverdine production in any of the tested media.

Exotoxin A is the most toxic secreted virulence factor of P. aeruginosa in terms of mass/activity ratio (Wolf & Elsässer-Beile, 2009). The production of exotoxin A by P. aeruginosa was studied in NB after 10 and 24 h of treatment with GABA. Exotoxin A was detected in the medium using a highly sensitive ELISA. As shown in Fig. 4(c), the secretion pattern of exotoxin A was unchanged in control and GABA-treated bacteria.

P. aeruginosa is also known to produce non-peptidic toxins such as HCN. We have recently developed an original HCN polarographic assay whose sensitivity is below 1 μg l⁻¹ (Blier et al., 2012). The mean concentration of HCN in the medium of control cultures of P. aeruginosa after 24 h of incubation was 1.024 ± 0.015 mg l⁻¹ (Fig. 4d). The concentration of HCN in the medium of GABA-treated bacteria was significantly increased (+35 %, P<0.05), reaching 1.383 ± 0.015 mg l⁻¹.

**Effect of GABA on the protein pattern expression of P. aeruginosa**

The cell-associated proteome was investigated by analysing pellets of P. aeruginosa PAO1 grown in the absence or presence of GABA. The 2-D electrophoresis assays on total protein extracts showed limited differences between the proteome of control (Fig. 5a) and GABA-treated (Fig. 5b) bacteria. Two proteins (spots 1 and 2) were downregulated whereas the expression of five other proteins (spots 3–7) was clearly increased (Fig. 5c). Each spot was excised, digested in-gel with trypsin and analysed by MALDI-TOF/TOF. The seven spots corresponded to P. aeruginosa proteins identified in the NCBI database (Table 2). Spots 1 and 3 corresponded to the same thermo-unstable elongation factor (Tuf), as confirmed by TOF/TOF fragmentation, but the neutral form present in spot 1 was only detected in control bacteria; after treatment with GABA it was replaced as an acidic form identified as spot 3. Another protein, the thermostable elongation factor (Ts), found in spot 6, appeared to be upregulated in the presence of GABA. Spot 2, not present in bacteria exposed to GABA, was identified as an ornithine carbamoyltransferase. The other spots 4, 5 and 7 corresponded to a peroxidase, and alkyl hydroperoxide reductase and a putative USP-like protein, respectively.

**Effect of GABA on P. aeruginosa quorum sensing factors**

Since cyanogenesis is regulated by the quorum-sensing system, we investigated the effect of GABA on 3oxoC12-HSL, C4-HSL and PQS production by P. aeruginosa. The kinetics of 3oxoC12- and C4-HSL secretion were studied over 24 h. In the presence of GABA, the peak of
production of 3-oxoC₁₂-HSL was higher than in control studies (Fig. 6a). In addition, this peak was observed 8 h after the beginning of the experiment, whereas it appeared after 12 h in control bacteria. The production of C₄-HSL was also stimulated in GABA-treated bacteria but the effect was of lower amplitude and statistically non-significant (Fig. 6b). The effect of GABA on PQS production was studied by using two complementary techniques: TLC, the classical method employed to detect PQS, and HPLC, which provides a higher resolution. PQS production was measured after 24 h to allow sufficient accumulation of the molecule in the culture medium. Both techniques clearly

![Graphs showing OD₄₀₀/OD₅₈₀ ratio over time for control and GABA-treated bacteria.](image)

**Fig. 4.** Effect of GABA (0.01 mM) on pyoverdine (a, b), exotoxin A (c) and hydrogen cyanide (d) production by *P. aeruginosa* PAO1. Pyoverdine production, studied in King B (a) and CAA (b) medium, is expressed as the ratio of pyoverdine absorption (OD₄₀₀) versus the bacterial density (OD₅₈₀). Exotoxin A is expressed as the concentration measured in the medium normalized to the value of the OD₅₈₀ of the bacterial culture. HCN concentration was determined by polarographic analysis of bacterial culture supernatant. *P* < 0.05. Grey, control PAO1; black, GABA-treated PAO1. Error bars indicate SEM.

![2D electrophoresis analysis of the total proteome.](image)

**Fig. 5.** 2D electrophoresis analysis of the total proteome of control (a) and GABA-treated (b) *P. aeruginosa* PAO1. Seven spots were modified after exposure of the bacteria to GABA (c). The proteins corresponding to these spots are presented in Table 2.
showed that the production of PQS by P. aeruginosa PAO1 was not modified by GABA (Fig. 7).

**DISCUSSION**

GABA is a non-protein amino acid that is conserved in bacteria and eukaryotes (see Bouché et al., 2003 for a review). In plants, GABA is secreted as a defence signal against bacterial phytopathogens (Chevrot et al., 2006). In the case of P. aeruginosa, we observed that GABA did not affect the growth and motility of the bacterium but did increase its cytotoxicity and virulence. The C. elegans model gave a first indication of the mechanisms involved in the response of P. aeruginosa to GABA. Indeed, GABA had a strong effect on bacterial virulence in the ‘fast killing test’. In this test, the secretion of bacterial diffusible molecules is stimulated by the use of a high-osmolarity medium, suggesting that soluble toxins have a principal role in the increase in virulence. In agreement with this hypothesis, the virulence of P. aeruginosa in the ‘slow killing test’ appeared to be only marginally increased. However, the biofilm formation experiments revealed that the action of GABA should be more complex. When bacteria were exposed to GABA only during the biofilm formation period, GABA had no effect. Only bacteria pretreated during the growth phase showed a generally significant reduction in biofilm formation, suggesting that only the early phases of bacterial adhesion are affected by GABA.

In order to clarify the mechanism of action of GABA on P. aeruginosa, we focused in a first step on surface associated factors. GABA increased bacterial cell adhesion but this effect was not significant. In contrast, we observed a significant increase in the adhesion on glass of GABA-treated Pseudomonas. Glass is a polar surface whereas PVC, on which the biofilm formation tests were done, is hydrophobic. This is in agreement with the opposite effects of GABA on bacterial adhesion on these two surfaces. However, the MATS technique did not reveal any variation of surface polarity suggesting that GABA was acting on a specific adhesin(s). LPS, the principal component of the outer membrane of Gram-negative bacteria, was unchanged in GABA-treated Pseudomonas. Glass is a polar surface whereas PVC, on which the biofilm formation tests were done, is hydrophobic. This is in agreement with the opposite effects of GABA on bacterial adhesion on these two surfaces. However, the MATS technique did not reveal any variation of surface polarity suggesting that GABA was acting on a specific adhesin(s). LPS, the principal component of the outer membrane of Gram-negative bacteria, was unchanged in GABA-treated bacteria. The surface tension of P. aeruginosa extracts was consistent with the presence of biosurfactants, and, since biosurfactants can interfere with the binding properties and biofilm formation activities of Pseudomonas (de Bruijn et al., 2007), these molecules were analysed but, here also, the structure and concentration of rhamnolipids expressed by PAO1 were not modified in bacteria exposed to GABA.

The effect of GABA on diffusible toxins and on the proteome of P. aeruginosa was then investigated. The cytotoxic activity of P. aeruginosa, measured by LDH release from eukaryotic cells when the cytoplasmic membrane is disrupted, can be considered as an equivalent of haemolysis and it is known that this is essentially
induced by a type III secretion system (Goure et al., 2004) or phospholipase C (Ostroff et al., 1989). The tests done on blood-supplemented agar plates showed no variation in bacterial haemolytic activity. All other exoenzymic activities studied also remained unchanged. Pyoverdine is essential for *P. aeruginosa* virulence (Meyer et al., 1996) so the production of this siderophore in GABA-treated bacteria was monitored but it appeared clearly that pyoverdine production is not modulated by GABA. Exotoxin A is the most toxic secreted virulence factor of *P. aeruginosa* in terms of mass/activity ratio (Wolf & Elsässer-Beile, 2009). Its secretion was studied in the same medium used to test the effect of GABA on *P. aeruginosa* virulence (NB). As it is a low-iron-inducible toxin, in our experimental conditions limited concentrations of exotoxin A were detected and its production was not affected by GABA. The arsenal of *P. aeruginosa* is not exclusively composed of peptidic molecules and it is interesting to note that hydrogen cyanide (HCN) is considered to be the principal diffusible lethal toxin acting on *C. elegans* (Gallagher & Manoil, 2001). In the present study, we used a polarographic assay of HCN/CN⁻ based on the redox properties of cyanide ions recently developed in our laboratory (Blier et al., 2012). High quantities of HCN were measured in the growth medium of *P. aeruginosa* PAO1 and GABA induced a significant increase of HCN production. By itself, this increase of HCN (35 %) could explain the virulence of GABA-treated *P. aeruginosa* but this is not sufficient to understand the variations of surfaces properties observed in parallel. The total proteome of GABA-treated *P. aeruginosa* was then analysed and compared with that of control bacteria. The differences of expression were limited to six proteins. A first novel result was the observation of two spots (1 and 3) corresponding to the same protein but showing very different pI values. This protein, identified as the thermo-unstable elongation factor Tuf, is a GTP/GDP and ribosome binding protein, but it is also capable of binding the thermostable elongation factor Ts (Wittinghofer et al., 1983), detected as spot 6 and markedly overexpressed in GABA-treated *Pseudomonas*. The existence of free or GDP+Pi tightly bound Tuf could explain the shift in pI of the molecule between control and GABA-treated bacteria. However, we cannot exclude that GABA itself could bind Tuf, and because of its acidic character decrease the pI of the

![Fig. 6. Effect of GABA (0.01 mM) on the production kinetics of 3-oxo-C12-HSL and C4-HSL by *P. aeruginosa* PAO1. HSL production is expressed as the concentration measured in the medium normalized to the value of the OD₅₈₀ of the bacterial culture. *P < 0.05. Grey, control PAO1; black, GABA-treated PAO1. Error bars indicate SD.](image)

![Fig. 7. Effect of GABA (0.01 mM) on the production of PQS by *P. aeruginosa* PAO1. PQS production was measured by TLC (a) (lanes: 1, synthetic PQS; 2, PQS from control PAO1; 3, PQS from GABA-treated PAO1) and RP-HPLC (b).](image)
protein. This binding should also explain the long-term effect of GABA. In agreement with this hypothesis, which deserves further study, it has been shown that the elongation factor Tuf can be associated with the membrane of *P. aeruginosa* and it acts as a binding protein for the human plasma regulators factor H and plasminogen (Kunert *et al.*, 2007). The change of Tuf charge could also explain the specific effects of GABA on *P. aeruginosa* surface properties independently of variations in polarity, LPS or biosurfactant production. The four other proteins modulated by GABA have in common their role in oxidative metabolism. Ornithine carbamoyltransferase, identified as spot 2, is a key enzyme of the arginine deiminase pathway involved in the anaerobic metabolism of arginine (Vander Wauven *et al.*, 1984). In GABA-treated bacteria, the ornithine carbamoyltransferase was markedly repressed, whereas that of the three other proteins, a putative peroxidase, an alkyl hydroperoxide reductase and a USP-like protein, were upregulated. As for other enzymes of this family, the putative peroxidase over-expressed in the presence of GABA (spot 4) should be implicated in the removal of toxic peroxides. The alkyl hydroperoxide reductase, identified as spot 5, is essential for optimal resistance of *P. aeruginosa* to organic hydroperoxides and has an important protective role against oxidative stress (Ochsner *et al.*, 2000). The hypothetical USP-like protein PA3309 is overproduced during pyruvate fermentation and in anaerobic/aerobic stationary phases (Schreiber *et al.*, 2006). An effect of GABA on the oxidative metabolism of *P. aeruginosa* could also explain the increase in HCN observed in the present study, since cyanogenesis requires a reduced level of free oxygen (Castric *et al.*, 1981).

In *P. aeruginosa*, cyanogenesis is regulated by quorum sensing. The *hcnABC* genes encoding HCN synthases are controlled by LasR and RhlR, respectively, which are involved in the regulation of 3oxoC12-HSL and C4-HSL synthesis (Schuster & Greenberg, 2006). In agreement with this, in GABA-treated *P. aeruginosa* we observed a significant increase in 3oxoC12-HSL formation and a marginal variation in the C4-HSL kinetics. Conversely, the production of PQS, whose synthesis is also under the control of the two acylhomoserine lactones (Schuster & Greenberg, 2006), was not affected by GABA. This is also in agreement with the absence of any effect of GABA on pyoverdin production, which, through PqsE, is regulated by PQS (Diggle *et al.*, 2006). The action of GABA on *P. aeruginosa* is then fundamentally different from other Gram-negative bacteria such as *Agrobacterium tumefaciens*, where GABA provokes a decrease in acylhomoserine lactone synthesis and a reduction in virulence (Chevrot *et al.*, 2006).

To our knowledge, this is the first demonstration that GABA can regulate *P. aeruginosa* virulence. We show that a low concentration of exogenous GABA triggers an increase in virulence of *P. aeruginosa* due to a stimulation of cyanogenesis associated with a reduction in oxygen accessibility and an overexpression of oxygen-scavenging proteins. This mechanism is regulated by quorum sensing. GABA also promotes specific changes in the expression of thermostable and unstable elongation factors involved in the interaction of the bacterium with the host proteins. As GABA is a widespread molecule, these observations should be taken into consideration in agricultural, veterinary and medical practices.

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