Arch Microbiol (2007) 187:249–256
DOI 10.1007/s00203-006-0186-5

SHORT COMMUNICATION

# *N*-acyl homoserine lactones are degraded via an amidolytic activity in *Comamonas* sp. strain D1

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Received: 8 August 2006 / Revised: 5 October 2006 / Accepted: 9 October 2006 / Published online: 29 November 2006


**Abtsract** *Comamonas* strain D1 enzymatically inactivates quorum-sensing (QS) signal molecules of the *N*-acyl homoserine lactone (*N*-AHSL) family, and exhibits the broadest inactivation range of known bacteria. It degrades *N*-AHSL with acyl-side chains ranging from 4 to 16 carbons, with or without 3-oxo or 3-hydroxy substitutions. *N*-AHSL degradation yields HSL but not N-acyl homoserine: strain D1 therefore harbors an amidohydrolase activity. Strain D1 is the fifth bacterium species in which an *N*-AHSL amidohydrolase is described. Consistent with its *N*-AHSL degradation ability, strain D1 efficiently quenches various QS-dependent functions in other bacteria, such as violacein production by *Chromobacterium violaceum* and pathogenicity and antibiotic production in *Pectobacterium.*

**Keywords** *Comamonas* · Quorum sensing · Quorum quenching · *N*-acyl homoserine lactone · Acylase · *Pectobacterium*

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## Introduction

Quorum-sensing (QS) is a widespread phenomenon which facilitates the coordinated regulation of gene expression in bacteria as a function of cell population density (Winans and Bassler 2002). In gram negative bacteria, the most intensively investigated QS systems employ *N*-acyl homoserine lactone (*N*-AHSL) signal molecules which all incorporate a homoserine lactone ring but differ with respect to the length and C3 substituent of the *N*-linked acyl side chain. *N*-AHSL-based QS regulates a number of key physiological and ecological functions, often those involved in the interaction of the bacterium with its environment (Whitehead et al. 2001; Von Bodman et al. 2003). Because pathogenicity or pathogenicity-related functions are controlled via QS in several pathogenic bacteria (as for instance in the phytopathogen *Pectobacterium sp*.), the QS regulatory mechanism was proposed as a novel target to develop innovative bio-control strategies (Rasmussen and Givskov 2006 and references therein). These may involve the development of chemical inhibitors of the production and/or the perception of QS signals (Castang et al. 2004; Olsen et al. 2002; Smith et al. 2003). Consistent with this, the production of *N*-AHSL natural antagonists has been demonstrated in various eukaryotes (Manefield et al. 1999; Teplitski et al. 2000; Peters et al. 2003). Furthermore, the ability to inactivate *N*-AHSL enzymatically exists and has been demonstrated in a range of bacteria belonging to the α- (Zhang et al. 2002), the β- (Leadbetter and Greenberg 2000; Lin et al. 2003; Uroz et al. 2003), and the γ-proteobacteria (Uroz et al. 2003; Huang et al. 2003; Kang et al. 2004), and the gram positive bacteria (Dong et al. 2000,

2002; Lee et al. 2002; Uroz et al. 2003; Park et al. 2005, 2006). The *N*-AHSL-inactivating enzymes described to date belong to two families: the *N*-AHSL lactone hydrolases (e.g. AiiA, AttM, AiiB, Carlier et al. 2003; Dong et al. 2000, 2002; Lee et al. 2002; Zhang et al. 2002) and the *N*-AHSL acylases/amidohydrolases (AiiD, PvdQ or AhlM, Lin et al. 2003; Huang et al. 2003; Park et al. 2005).

Several attempts to identify effective, non-toxic inhibitors of *N*-AHSL-dependent QS, efficient in vivo have met with limited success (Wu et al. 2004; Persson et al. 2005; Rassmussen et al. 2005). Exploiting wild-type *N*-AHSL bacterial quorum-quenching strains offers a useful alternative means of interfering QS. In this respect, bacteria harbouring a *N*-AHSL amidohydrolase have a greater biotechnological potential since the cleavage of the *N*-AHSL molecule generates homoserine and an acyl chain, which cannot spontaneously re-generate a functional QS signal. On the contrary, the ring-open *N*-acyl homoserine (*N*-AHS) generated by *N*-AHSL lactonases can re-circularize under acidic conditions into a functional *N*-AHSL (Yates et al. 2002). It is therefore crucial to be able to quickly screen wild-type isolates or clone libraries for their *N*-AHSL amidohydrolase activity and quantify their quenching ability.

In this paper, reported data demonstrate that *Comamonas* strain D1 harbors a very efficient *N*-AHSL amidohydrolase activity with the largest chemical spectrum described to date, and excellent QS quenching capabilities using three different quenching assays.

## Materials and methods

### Bacterial strains, growth media, culture conditions and chemicals

The strains used in this study are listed in Table 1. All strains were grown in LBm (5 g/l NaCl) at 25°C except *E. coli* strains, which were grown at 37°C. Growth on *N*-AHSL or homoserine lactone (HSL) as sole carbon, or sole carbon and nitrogen source, was performed in the liquid minimal medium described by Leadbetter and Greenberg (2000) supplemented with 1.5 mM *N*-AHSL or HSL, with or without 0.3 g/l $NH_4Cl$. All culture media were buffered to pH 6.5 with 10 mM phosphate buffer ($KH_2PO_4/K_2HPO_4$), to prevent a rapid alkaline degradation of *N*-AHSL (Yates et al. 2002). Where necessary, growth media were supplemented with *N*-AHSL (25 μM), tetracycline (10 mg/l), ampicillin (100 mg/l), kanamycin (50 mg/l). *N*-AHSL were synthesized as described previously (Chhabra et al. 1993, 2003). HSL, homoserine (HS) and dansyl-chloride were obtained from Sigma (UK).

### *N*-AHSL abbreviations

*N*-AHSL were abbreviated as follows : C6-HSL, *N*-hexanoyl homoserine lactone; C12-HSL, *N*-dodecanoyl homoserine lactone; C16-HSL, *N*-hexadecanoyl homoserine lactone; 3O,C6-HSL, *N*-(3-oxo)-hexanoyl homoserine lactone; 3O,C8-HSL, *N*-(3-oxo)-octanoyl homoserine lactone, 3O,C10-HSL, *N*-(3-oxo)-decanoyl homoserine lactone; 3O,C12-HSL, *N*-(3-oxo)-dodecanoyl homoserine lactone; 3OH,C12-HSL, *N*-(3-hydroxy)-

**Table 1** Bacterial strains

| Strain species and name | Characteristics | References |
|---|---|---|
| *A. tumefaciens* NTL4 (pZLR4) | *N*-AHSL biosensor | Luo et al. (2003) |
| *C. violaceum* CV026 | *N*-AHSL biosensor | McClean et al. (1997) |
| *E. coli* pSB401 | *N*-AHSL short chain biosensor | Winson et al. (1998) |
| *E. coli* pSB1075 | *N*-AHSL long chain biosensor | Winson et al. (1998) |
| *E. coli* pGEM-AiiA$_{soil}$ | pGEMT easy plasmid containing the *aii*A$_{soil}$ PCR product | Carlier et al. (2003) |
| *E. coli* ESS | β-lactam supersensitive, indicator strain | Bainton et al. (1992) |
| *Comamonas* sp. D1 | Wild-type soil isolate. Degrade several *N*-AHSL | Uroz et al. (2003) |
| *Pseudomonas fluorescens* 1855–344 | Rhizosphere isolate, producing and degrading no *N*-AHSL | Laboratory collection |
| *Pectobacterium carotovorum* | | |
| Pcc797 | Tobacco pathogen | Uroz et al. (2003) |
| GS101 | Carbapenem-producing strain | Bainton et al. (1992) |
| PNP22 | *carI* mutant of GS101 unable to produce *N*-AHSL | Bainton et al. (1992) |

dodecanoyl homoserine lactone; 3O,C14-HSL, *N*-(3-oxo)-tetradecanoyl homoserine lactone.

Preparation of *Comamonas* sp. strain D1 resting cells and cell crude extracts

Resting cells (RC) and cell crude extracts (CCE) were prepared as previously described (Uroz et al. 2005) from a 1 l, 24 h-culture of *Comamonas* sp. strain D1 in rich LBm medium ($\sim 5 \times 10^8$ cfu/ml).

Detection and separation of *N*-AHSL

*N*-AHSL were detected using the *lux*-based biosensors, *E. coli* [pSB401] and [pSB1075] and the *tra::lacZ*-based biosensor *Agrobacterium tumefaciens* NTL4 (pZLR4) (Winson et al. 1998; Luo et al. 2003). Bioassays were performed in microtitre plates as described by Reimmann et al. (2002). *N*-AHSL were separated on thin layer chromatography (TLC) plates as described by Shaw et al. (1997). *N*-AHSL and their degradation products for in vitro and in vivo experiments were separated by reverse phase (RP)-HPLC as described by Uroz et al. (2005).

*N*-AHSL inactivation assays

Degradation of each *N*-AHSL by actively growing D1 cells was determined in phosphate-buffered LBm (pH 6.5) after an overnight culture, by assessing the disappearance of *N*-AHSL with the appropriate biosensor strain. Degradation of each *N*-AHSL by RC or CCE was assessed as described by Uroz et al. (2005).

Identification of *N*-AHSL degradation products (Fig. 1)

The formation of the corresponding *N*-acyl-homoserine (*N*-AHS) was investigated using the method described by Yates et al. (2002). HSL released during *N*-AHSL amidohydrolysis was chemically trapped using dansyl chloride as described (Jiang et al. 1998). The resulting dansylated-HSL was identified by HPLC as described in Uroz et al. (2005)

Quenching assays

The ability of strain D1 to interfere with QS was tested in three bacterial systems via: (1) the inhibition of violacein production by *Chromobacterium violaceum* strain CV026, (2) the reduction of pathogenicity of *Pectobacterium carotovorum* strain Pcc797 on potato tuber and (3) the reduction of carbapenem antibiotic and *N*-AHSL production by *P. carotovorum* strains GS101 and its *N*-AHSL-synthesis deficient mutant PNP22. Quenching assays were performed as described by Uroz et al. (2003) for the first two models, and as described by Chhabra et al. (1993) for the third, except that strain D1 was used as a quencher, and *P. fluorescens* strain 1855–344 as negative control. Statistical analyses involved a two-factor (3O,C6-HSL concentration, presence of D1) ANOVA test ($P$=0.0001), and a Bonferroni-Dunn test.

## Results and discussion

Strain D1 enzymatically inactivates both short and long chain *N*-AHSL in vivo

Actively growing cells of strain D1 are able to inactivate all assayed *N*-AHSL, e.g. *N*-AHSL with acyl chains ranging from 4 to 16 carbons, with or without substitution at carbon 3 (Table 2). This extends the range of *N*-AHSL degraded by this strain to C12-HSL, 3O,C12-HSL, 3OH,C12-HSL, 3O,C14-HSL, 3OH,C14:1-HSL and C16-HSL. Compared to know *N*-AHSL degrading organisms, strain D1 exhibits the broadest degradation activity described to date. In contrast to growing cells, RC or CCE degraded all but the assayed unsubstituted *N*-AHSL, i.e. C6-HSL and C12-HSL (Table 2). The *N*-AHSL 3O,C12-HSL was the most efficiently degraded molecule. Shorter and longer chain *N*-AHSL such as 3O,C10-HSL and 3O,C14-HSL were degraded with comparable efficiency by D1 resting cells. Based on assayed molecules, the substitution of a hydroxy on carbon 3 had the same impact on the activity than the increase or decrease of the acyl chain length of two carbon units. Assessed using 3O,C10-HSL, the optimal pH and temperature observed with the CCE were pH 6.5 and 25°C (data not shown). No degradation was observed using the insoluble protein fraction, or boiled CCE confirming the enzymatic nature of the *N*-AHSL degradation activity in strain D1.

All above data indicate that degradation of unsubstituted *N*-AHSL such as C6-HSL and C12-HSL requires actively growing cells. A similar phenomenon was reported recently in *Rhodococcus* (Uroz et al. 2005; Park et al. 2006) for different *N*-AHSL. No obvious explanation accounts for these observations. One can propose that the N-AHSL degradation activity results from the action of different enzymes, one specific for unsubstituted *N*-AHSL being unstable or not active in RC or CCE. This view is partially supported by the identification of three activities targeting *N*-AHSL in *Rhodococcus erythropolis*, one being

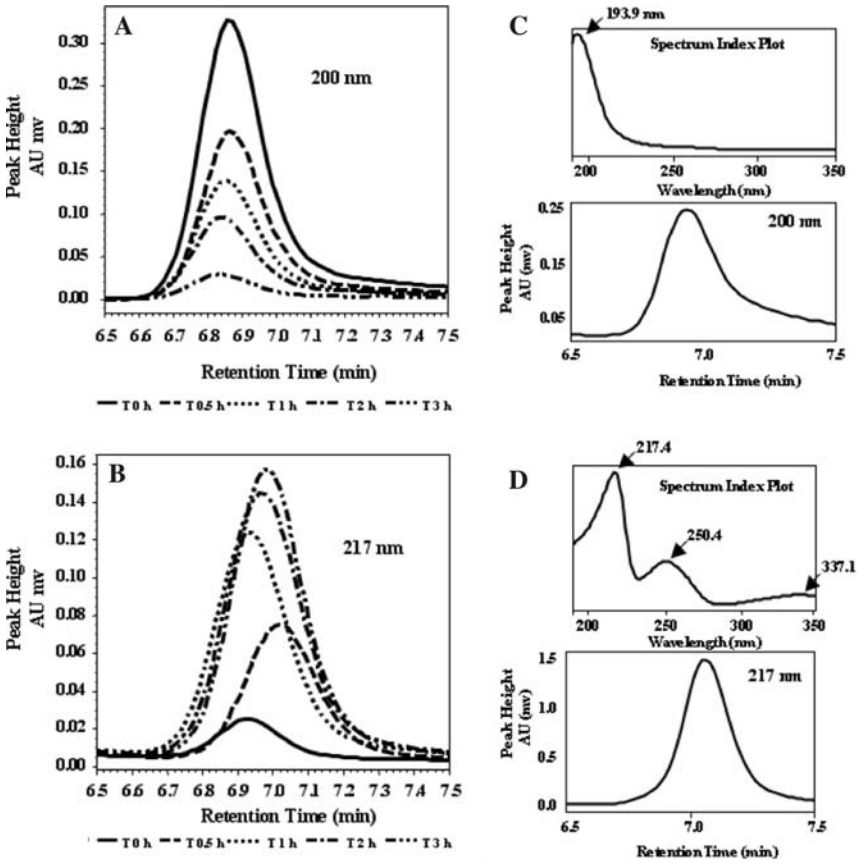


**Fig. 1** HPLC analysis of the dansylated products obtained after incubation of 3O,C10-HSL with the D1 CCE. 3O,C10-HSL (1 mM) was incubated in the presence or absence of D1 CCE. Free amines, including HSL released by degradation of the *N*-AHSL were trapped with dansyl chloride as indicated in Materials and methods. The products obtained after incubation for 0, 0.5, 1.0, 2.0 or 3.0 h were separated by HPLC and their chromatographic and UV/vis. spectral profiles compared with those of synthetic standards for 3O,C10-HSL (**a**, **c**) and dansylated-HSL (**b**, **d**). Under the chromatographic conditions used, 3O,C10-HSL elutes at 6.8 min and dansylated-HSL at 7.0. The spectrum index plots (**c**, **d** top panels) define the UV/vis. spectral profiles of 3O,C10-HSL and dansylated-HSL, respectively. The identity of the product as dansylated HSL is confirmed by the PDA spectrum index plot

**Table 2** *N*-AHSL-degrading activity of actively growing or resting cells of *Comamonas* sp. strain D1

| *N*-AHSL degradation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Acyl chain length (C atoms) | 6 | 6 | 8 | 10 | 12 | 12 | 12 | 14 | 16 |
| Substitution at C3 | none | oxo | oxo | oxo | none | oxo | OH | oxo | none |
| Degradation[a] | + | + | + | + | + | + | + | + | + |
| EA[b] | <0.05 | 4.1 | 4.2 | 5.5 | <0.05 | 8.3 | 5.8 | 3.0 | n.d.[c] |

[a] Degradation, degradation by actively growing D1 cells, in rich LBm media, after 24 h. The symbol + indicates a complete degradation of these compounds, added at 25 μM

[b] EA, estimated activity of D1 resting cells, expressed as pmoles *N*-AHSL degraded $min^{-1}$ $OD600^{-1}$ . Values are given with an estimated standard error of ±5%

[c] Not determined

specific for 3-keto derivatives (Uroz et al. 2005; Park et al. 2006). Alternatively, the enzyme(s) responsible for the degradation of unsubstituted *N*-AHSL may require a rapidly turned-over co-factor, or a co-factor specific for the degradation of the unsubstituted signals.

Finally, the *N*-AHSL degrading ability did not correlate with the ability to grow on *N*-AHSL as sole carbon or carbon and nitrogen source. Indeed, no growth of strain D1 on *N*-AHSL was observed in minimal medium buffered at pH 6.5 and supplemented with C6-HSL, 3O,C6-HSL or homoserine lactone (HSL) as sole carbon sources, or as sole carbon and nitrogen sources (data not shown). This phenomenon has already been observed in different bacteria for other substrates, as well as for *N*-AHSL (Dong et al. 2001; Park et al. 2003). In relation with this, *N*-ASHL degradation may not be the primary function of the degradative enzymes, the coding genes of which being only poorly expressed under most growth conditions. In agreement with this hypothesis, the AttM lactonase of *Agrobacterium*, while degrading *N*-AHSL, is primarily involved

in gamma-butyrolactone degradation, and its encoding gene is not induced by *N*-AHSL (Zhang et al. 2002; Carlier et al. 2003, 2004).

## *N*-AHSL degradation by *Comamonas* sp. strain D1 CCE proceeds via an amidohydrolase activity

No *N*-AHSL could be recovered, even after prolonged acidification of the *N*-AHSL degradation products generated by strain D1, regardless of which *N*-AHSL (3O,C10-HSL, C6-HSL and 3O,C6-HSL ) was used as a substrate for degradation. Under the same conditions, ca. 100% of the input *N*-AHSL was recovered in positive controls that consisted in recycling native *N*-AHS, or the products of the degradation of *N*-AHSL by an *E. coli* strain expressing the *N*-AHSL lactonase gene $aiiA_{soil}$ (Carlier et al. 2003). Therefore, it appears that the degradation of *N*-AHSL by strain D1 did not generate *N*-AHS; hence strain D1 does not harbor demonstrable *N*-AHSL lactonase activity.

To investigate the presence of an amidohydrolase activity, the *N*-AHSL degradation products and controls were trapped using dansyl chloride. No dansylated HSL was detected in the incubation medium at $t_0$ (Fig. 1) whereas the presence of dansylated HSL became readily apparent on HPLC analysis after 30 min of incubation with D1 CCE. The appearance of the dansylated HSL peak at 7 min (when monitored at 217 nm, Fig. 1b, d) correlated with the reduction in the 3O,C10-HSL peak at 6.8 min (monitored at 200 nm; Fig. 1a, c), and demonstrated that *N*-AHSL degradation involved an amide bound cleavage. Therefore, strain D1 inactivates *N*-AHSL via an amidohydrolase activity.

*Comamonas* is the fifth bacterium in which a *N*-AHSL amidohydrolase is demonstrated, following *P. aeruginosa* (encoded by the *pvd*Q gene, Huang et al. 2003), *Ralstonia* (encoded by the *aii*D gene, Lin et al. 2003), *Streptomyces* sp. strain M664 (encoded by the *ahl*M gene, Park et al. 2005) and *Rhodococcus erythropolis* (unknown gene, Uroz et al. 2005). Attempts to PCR-amplify sequence(s) homologous to these amidohydrolase encoding genes, in strain D1 were unsuccessful, even under low stringency conditions (data not shown). The genes encoding the *N*-AHSL amidohydrolase activity of strain D1 could not be identified after the screening of more than 5,000 cosmid clones from a genomic library of this strain (data not shown). It is therefore possible that the genes are not expressed in the *E. coli* host strain used in our screen, e.g. *Escherishia coli* VCS237. Also, although none have been described to date, it is possible that the *N*-AHSL activity may be encoded by two genes located on separate parts of the D1 genome preventing their cloning on a single cosmid. The answer to this question will first require the identification of an appropriate expression system for *Comamonas* genes and an appropriate system to test for the degradation of *N*-AHSL encoded by this gene.

## Strain D1 efficiently quenches QS-regulated phenotypes

Strain D1 was capable of inhibiting the QS-regulated production of violacein by *Chromobacterium violaceum* in a quenching microtitre plate assay starting at $2 \times 10^7$cfu per well (data not shown). The amount of C6-HSL degraded was related linearly with D1 cell population density, within the range $10^7$ to $8 \times 10^7$ cfu per well of D1 cells ($R^2 = 0.96$).

In liquid co-cultures, strain D1 also prevented the accumulation of *N*-AHSL produced by the plant pathogen *P. carotovorum* strain GS101, without interfering with its growth (Fig. 2, filled symbols). After 8 h of incubation, there was a 3-fold reduction in the concentration of 3O,C6-HSL in the medium (Fig. 2, open symbol). No increase in the pH was observed in the medium, excluding the possibility of a spontaneous alkaline hydrolysis of the *N*-AHSL.

Two main functions are regulated by QS in *P. carotovorum*: carbapem antibiotic production and virulence.

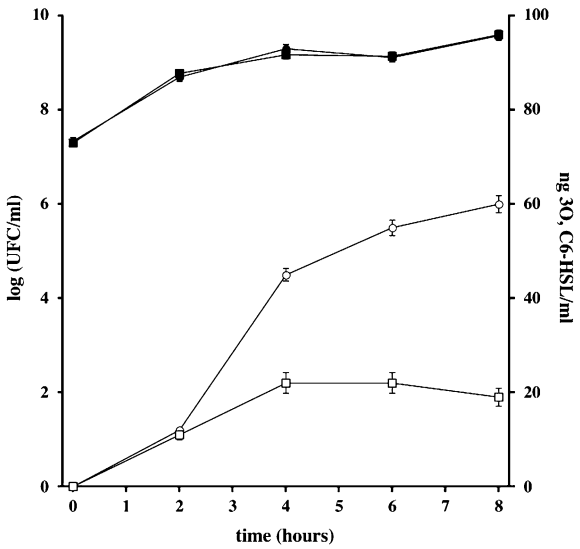


**Fig. 2** Influence of strain D1 on the growth of, and *N*-AHSL production by *P. carotovorum* strain GS101. The cell population densities reached by GS101 grown in the absence (*closed circles*) or presence (*closed squares*) of strain D1 are plotted together with the concentration of *N*-AHSL attained in absence (*open circles*) or presence (*open squares*) of strain D1 (starting ratio 1:1). Experimental errors for the enumeration of growth are smaller than the size of the symbols. For *N*-AHSL concentrations, the errors bars are shown on the graph

No antibiotic was produced by the wild-type strain in presence of strain D1 (not shown). To quantify more precisely the impact of strain D1 on carbapenem production, PNP22, a GS101 mutant responding only to exogenous *N*-AHSL was used (see Materials and methods). In control experiments (i.e. without the quencher strain or with *Pseudomonas* strain 1855.344), growth inhibition of the indicator strain ESS, hence production of carbapenem, was visible for *N*-AHSL concentration as low as 50 ng/well (Fig. 3). Strikingly, at this concentration no growth inhibition zone was detectable in the presence of the quencher D1, revealing that the production of the carbapenem antibiotic was abolished. As a rule, with amounts of 3O,C6-HSL added in the wells ranging from 50 to 500 ng, the antibacterial activity of strain PNP22 was statistically (ANOVA and Bonferroni-Dunn tests) less important or missing when D1 cells were also introduced. Under the growth conditions used in this assay that involves strain PNP22, the concentrations of exogenous *N*-AHSL applied and degraded by strain D1 were much higher than those naturally produced by the wild-type strain GS101, which reached ca. 60 ng/ml (Fig. 2).

Strain D1 was also capable of inhibiting the virulence of *P. carotovorum* strain Pcc797 as evidenced in the potato tuber assay (Uroz et al. 2003). In the absence of strain D1, macerating zones attained average values of $22.5 \pm 1$ to $28 \pm 4$ mm (diameter) upon inoculation of $10^5$ and $10^6$ cells of strain Pcc797 per tuber, respectively. Co-inoculation of strain D1 with the pathogen at a 1:1 ratio totally abolished maceration at both concentrations of inoculum. Reduced maceration zones ($6.5 \pm 2$ mm) appeared when D1 was co-inoculated at a 1:10 ratio with the pathogen. From the above, *Comamonas* strain D1 appeared to exhibit an excellent quenching ability, at least in the three quorum quenching assays used in this study.

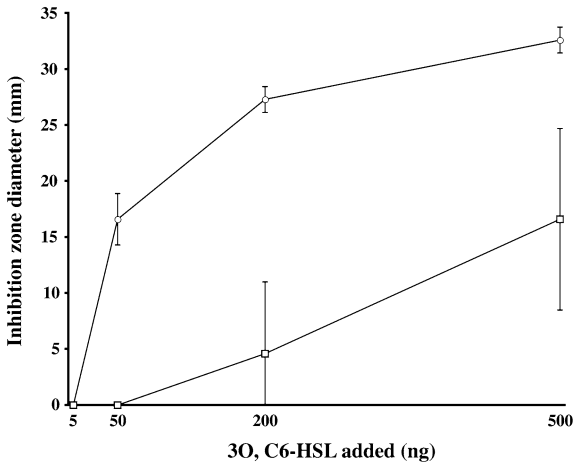


**Fig. 3** Inhibition of carbapenem synthesis in *P. carotovorum* by *Comamonas* strain D1. The carbapenem-mediated inhibition zone (mm) of *E. coli* indicator strain ESS in response to *P. carotovorum* strain PNP22 grown in the absence (*closed diamonds*) or presence (*closed squares*) of D1 is shown as a function of the amount of 3O,C6-HSL added to the wells of the assay medium

The value of the *Chromobacterium* and PNP22 assays is to demonstrate that the ability of strain D1 to degrade *N*-AHSL is the key element responsible for quenching of QS regulated functions. The *C. violaceum* microtiter plate assay is quantitative. Therefore, it can be can be used as a quick and precise estimator of the potential quorum-quenching ability of a novel bacterial isolate in large-scale screening procedures.

Quenching of pathogenicity related functions has been already reported in the literature, either using transgenic plants or microbes producing an *N*-AHSLase enzyme (i.e. lactonase, Dong et al. 2000, 2001; or amidohydrolase, Lin et al. 2003). However, these strategies involved genetically modified organisms, plants or bacteria, an issue that has been and still is heavily debated especially in Europe. In this respect, the use of bacteria harboring no genetic modification is an asset for the development of widely-acceptable biocontrol procedures. The excellent in vivo quenching abilities of strain D1, coupled with the broad range of molecules targeted by its amidohydrolase(s) increase significantly its biotechnological potential.

**Acknowledgments** This work was made possible by grants from the European Union Vth Framework Programme EcoSafe (QLK3-2000-01759) to P.W. and Y.D.; from the European Union Marie Curie programme (QLK1 1999 50 455) to P.W. and S.U. S.U. was also supported by a Fellowship from the French Ministère de la Recherche et de la Technologie, all of them being gratefully acknowledged. The authors also thank Chris Harty (Nottingham) for helpful discussions and comments on the manuscript, Mavis Daykin and Catherine Ortori (Nottingham) for HPLC and LC-MS/MS analysis, and Denis Faure (Gif-sur-Yvette) for his interest in, and helpful comments on this work.

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