Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of \(N\)-acylhomoserine lactones

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Quorum sensing relies upon the interaction of a diffusible signal molecule with a transcriptional activator protein to couple gene expression with cell population density. In Gram-negative bacteria, such signal molecules are usually \(N\)-acylhomoserine lactones (AHLs) which differ in the structure of their \(N\)-acyl side chains. *Chromobacterium violaceum*, a Gram-negative bacterium commonly found in soil and water, produces the characteristic purple pigment violacein. Previously the authors described a violacein-negative, mini-Tn5 mutant of *C. violaceum* (CV026) in which pigment production can be restored by incubation with supernatants from the wild-type strain. To develop this mutant as a general biosensor for AHLs, the natural *C. violaceum* AHL molecule was first chemically characterized. By using solvent extraction, HPLC and mass spectrometry, a single AHL, \(N\)-hexanoyl-\(L\)-homoserine lactone (HHL), was identified in wild-type *C. violaceum* culture supernatants which was absent from CV026. Since the production of violacein constitutes a simple assay for the detection of AHLs, we explored the ability of CV026 to respond to a series of synthetic AHL and \(N\)-acylhomocysteine thiolactone (AHT) analogues. In CV026, violacein is inducible by all the AHL and AHT compounds evaluated with \(N\)-acyl side chains from \(C_4\) to \(C_9\) in length, with varying degrees of sensitivity. Although AHL compounds with \(N\)-acyl side chains from \(C_{10}\) to \(C_{14}\) are unable to induce violacein production, if an activating AHL (e.g. HHL) is incorporated into the agar, these long-chain AHLs can be detected by their ability to inhibit violacein production. The versatility of CV026 in facilitating detection of AHL mixtures extracted from culture supernatants and separated by thin-layer chromatography is also demonstrated. These simple bioassays employing CV026 thus greatly extend the ability to detect a wide spectrum of AHL signal molecules.

**Keywords**: quorum sensing, \(N\)-acylhomoserine lactones, violacein, *Chromobacterium violaceum*, bioassay

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**INTRODUCTION**

\(N\)-Acyl homoserine lactones (AHLs) were first identified in Gram-negative marine bacteria, where they were discovered to play a central role in the regulation of bioluminescence in bacteria such as *Photobacterium (Vibrio) fischeri* and *Vibrio harveyi* (for reviews see Fuqua et al., 1996; Salmond et al., 1995; Swift et al.,...)

**Abbreviations**: AHL, \(N\)-acylhomoserine lactone; AHT, \(N\)-acylhomocysteine thiolactone (abbreviations for specific AHLs and AHTs are listed in Table 1).

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**METHODS**

**Bacterial strains and growth media.** The *Chromobacterium violaceum* wild-type strain ATCC 31532 was obtained from the American Type Culture Collection (Rockville, MD, USA); *C. violaceum* CV026 is a mini-Tn5 mutant of ATCC 31532 (Throup et al., 1995a; Latifi et al., 1995; Winson et al., 1995). *Yersinia enterocolitica* strain 8/82 was provided by J. Throup (University of Nottingham; Throup et al., 1995a) and *Serratia liquefaciens* strain MG1 by M. Giskov (The Technical University of Denmark; Eberl et al., 1996). Bacteria were routinely grown with shaking in LB broth at 30 °C. For the purification of AHLs, bacteria were grown in M9 minimal medium (1 l) supplemented as follows: for *C. violaceum*, M9 was supplemented with succinate (0.4%, w/v) plus Casamino acids (0.2%, w/v); for *Y. enterocolitica* M9 was supplemented with glucose (0.4%, w/v), Casamino acids (0.2%, w/v), proline 0.001% (w/v) and methionine (0.1 μg ml⁻¹); for *S. liquefaciens* M9 was supplemented as for *Y. enterocolitica* plus thiamin (1 μg ml⁻¹). Where required, antibiotics were incorporated into growth media at the following concentrations: ampicillin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and chloramphenicol (30 μg ml⁻¹).

**AHL reporter plate bioassays.** Five-millilitre volumes of molten semi-solid LB agar (0.3%, w/v) were seeded with 50 μl of an overnight LB culture of *C. violaceum* CV026 and poured immediately over the surface of prewarmed LB agar plates prepared in square Petri dishes (100 mm × 100 mm). When the overlaid agar had solidified, wells were punched in the agar with a sterile cork borer (diameter 6 mm). The wells were filled with the preparation to be assayed [solvent extracts, HPLC fractions or synthetic AHLs (see below)]. Where necessary the final volume per well was adjusted to 50 μl by...
addition of sterile LB broth. Positive [N-(hexanoyl)-l-homoserine lactone: HHL] and negative (sterile LB broth) controls were included in each assay plate. The Petri dishes were incubated in the upright position overnight (30 °C), then examined for the stimulation of violacein synthesis (indicated by blue/purple pigmentation of the bacterial lawn around wells). A modification of this basic assay was also developed to detect molecules which antagonize AHL stimulation of violacein synthesis. In this modified assay, HHL or N-(3-oxohexanoyl)-l-homoserine lactone (OHHL) together with CV026 was added to the semisolid agar (final concentration, 5 μM) and the assay procedure carried out as before. Inhibition of violacein synthesis was defined by the presence of white haloes in a purple background. The limits of detection of activity in the two assays were determined by applying serial dilutions of the compounds (using LB broth as the diluent) to assay plates as described above. End-points were estimated as the lowest quantity of compound giving discernible activation or inhibition of violacein synthesis respectively.

Isolation, purification and chemical characterization of the C. violaceum AHL. Spent supernatants (41) from stationary-phase cultures of C. violaceum ATCC 31532 grown in minimal medium were extracted with dichloromethane (700 ml; 300 supernatant/dichloromethane). Dichloromethane was removed by rotary evaporation and the residue reconstituted in 1 ml acetonitrile. This was then applied to a C18 reverse-phase preparative HPLC column [Kromasil KR100-SC8 (250 mm × 8 mm) column; Hitchrom] eluted with an isotropic mobile phase of 70% (v/v) acetonitrile in water at a flow rate of 2 ml min⁻¹ and monitored at 210 nm. Fractions showing activity in the CV026 violacein induction bioassay were pooled and rechromatographed using 35% (v/v) acetonitrile in water. Active fractions were analysed by mass spectrometry (MS). Mass spectra were obtained on a VG 70-SEQ instrument of EBqQ geometry (Fisons Instruments, VG Analytical). Samples were ionized by positive-ion fast-atom bombardment (FAB). The pseudo-molecular ion ([M + H]⁺ peaks recorded by FAB-MS were further analysed by tandem mass spectrometry (MS-MS) and shown to be identical to the MS-MS spectra of the authentic synthetic AHL.

Thin-layer chromatography (TLC). This was performed on C18 reversed-phase plates using a solvent system of methanol/water (60:40, v/v) essentially as described by Shaw et al. (1997) but using the C. violaceum mutant CV026 rather than A. tumefaciens as the indicator organism. Synthetic AHLs made up as 10 mM solutions in acetonitrile or extracts of culture supernatants (from Y. enterocolitica or S. liquefaciens, extract with dichloromethane as described for C. violaceum) were spotted (2–30 ul) onto glass-backed RP18 reverse-phase TLC plates (BDH) and dried in a stream of cold air. Samples were separated using methanol (60%, v/v) in water as the solvent. Once the solvent front had migrated to within 2 cm of the top of the chromatogram, the plate was removed from the chromatography tank, dried in air and overlaid with a thin film of CV026 seeded in 0-3% (w/v) LB agar without or with OHHL (final concentration 1 μM). After overnight incubation at 30 °C, AHLs were located either as purple spots on a white background or as white spots in a purple background respectively.

Synthesis of AHLs and AHTs. A series of AHLs and AHTs were synthesized as previously described (Table 1; Chhabra et al., 1993). Representative compounds from each series varied in N-acyl chain length from C₄ [N-(3-oxobutanylo)-l-homoserine lactone (OBHL) and N-butanylo-l-homoserine lactone (BHL)], to C₁₄ [N-tetradecanoyl-l-homoserine lactone (tDHL)] in the case of the N-alkanoyl-l-homoserine lactones. In addition, the α-isomer of N-(3-oxohexanoyl)-l-homoserine lactone (α-OHHL), the homocysteine thiolactones N-(3-oxohexanoyl)-l-homocysteine thiolactone (OHHT) and N-hexanoyl-l-homocysteine thiolactone (HHT) were also prepared together with N-benzoylacetyl-l-homoserine lactone (BAHL). The chemical formulae for the compounds used in this paper are given in Table 1. In addition, to facilitate unambiguous identification of the individual compounds a uniform method of abbreviation has been adopted (Table 1; Swift et al., 1996; Winson et al., 1995). Each compound was purified to homogeneity by preparative HPLC and its structure confirmed by MS and proton NMR spectroscopy. Stock solutions (final concentration of compound, 5 mM), were prepared in acetonitrile and stored at -20 °C.

RESULTS AND DISCUSSION

Purification and identification of the C. violaceum AHL

Stationary-phase cell-free supernatants from the C. violaceum wild-type were extracted with dichloromethane and evaporated to dryness as described in Methods. A fraction capable of stimulating violacein production by CV026 was located to a single peak on

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Tandem mass spectrometry (MS-MS) of the active fraction from an extract of C. violaceum culture supernatant. The compound was isolated and purified by preparative HPLC as described in Methods. The MS-MS spectrum obtained for the natural product (a) is indistinguishable from synthetic HHL (b).
Table 1. Structures and relative activities of AHL and AHT compounds in the C. violaceum CV026 agar plate bioassays

<table>
<thead>
<tr>
<th>R</th>
<th>X</th>
<th>Chemical name</th>
<th>Abbreviation</th>
<th>Activation</th>
<th>Inhibition</th>
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</thead>
<tbody>
<tr>
<td>CH₃CH₂CH₂</td>
<td>O</td>
<td>N-Butanoyl-L-homoserine lactone</td>
<td>BHL</td>
<td>0.9</td>
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</tr>
<tr>
<td>CH₃CH₂CH₂</td>
<td>S</td>
<td>N-Butanoyl-L-homocysteine thiolactone</td>
<td>BHT</td>
<td>1.7</td>
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</tr>
<tr>
<td>CH₃COCH₂</td>
<td>O</td>
<td>N-(3-Oxobutanoyl)-L-homoserine lactone</td>
<td>OBHL</td>
<td>3.5</td>
<td>NA</td>
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<tr>
<td>PhCH₂COCH₂</td>
<td>O</td>
<td>N-Benzoylacetamid-L-homoserine lactone</td>
<td>BAHL</td>
<td>1.53</td>
<td>NA</td>
</tr>
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<td>CH₃(CH₂)₄</td>
<td>O</td>
<td>N-Hexanoyl-L-homoserine lactone</td>
<td>HHHL</td>
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<td>NA</td>
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<tr>
<td>CH₃(CH₂)₂</td>
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<td>NA</td>
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<tr>
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<td>(D)OHHL</td>
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<td>CH₃(CH₂)₆COCH₂</td>
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<td>OHL</td>
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<tr>
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<td>OOHL</td>
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<td>tDHL</td>
<td>NA</td>
<td>4.0</td>
</tr>
<tr>
<td>CH₃(CH₂)₁₀COCH₂</td>
<td>O</td>
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<td>tDHL</td>
<td>NA</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Amount of a given compound added to the agar to either induce violacein production (activation) or inhibit HHL-mediated induction of violacein production (inhibition) in CV026. NA, No activity i.e. the compound is unable to either induce or to antagonize HHL-mediated induction of violacein in CV026.

HHL is, however, not unique to Chromobacterium, having previously been isolated from spent culture supernatants of Agrobacterium tumefaciens (Zhang et al., 1993), Yersinia enterocolitica (Throup et al., 1995a), Serratia liquefaciens (Eberl et al., 1996) Pseudomonas aeruginosa (Winson et al., 1995) and Photobacterium fischeri (Kuo et al., 1994). For both P. aeruginosa and S. liquefaciens, HHL is produced only as a minor component, with BHL as the primary AHL synthesized via the LuxI homologues RhlI (VsmI; Winson et al., 1995) and SwrI (Eberl et al., 1996) respectively. The P. fischeri LuxI protein also directs the synthesis of HHL as a minor component alongside OHHL (Kuo et al., 1994; Schaefer et al., 1996b) whereas Yenl from Y. enterocolitica directs the synthesis of HHL and OHHL in a 50:50 ratio in both the homologous and heterologous (E. coli) genetic backgrounds (Throup et al., 1995a). Since HHL in C. violaceum is synthesized via a LuxI homologue (M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data), this LuxI homologue differs from those of Agrobacterium, Yersinia, Pseudomonas, Serratia and Photobacterium in that HHL is the predominant AHL synthesized.

Utility of C. violaceum mutant CV026 as an AHL sensor

Stimulation of violacein synthesis. To evaluate the potential of CV026 as a generic biosensor for AHLs, a series of AHLs and AHTs were synthesized and their
ability to induce violacein production in CV026 examined in an agar plate assay (Fig. 2a, Table 1). The most active agonist in this assay was HHL, the natural C. violaceum AHL. Replacement of the HHl lactone ring oxygen with sulfur to give HHT had little effect on activity. However, OHHL, the 3-oxo derivative, was about sixfold less active than HHL. Furthermore, the d-isomer of OHHL was some 60-fold less active than the L-isomer, indicating the importance of the chiral centre. Shortening the HHL acyl side chain from C8 to C7, to give BHL, reduced the comparative activity by around 30-fold. Whilst the thiolactone BHT had similar activity to BHL, the 3-oxo derivative, OBHL, was very much weaker (Table 1). However, the addition of a hydrophobic phenyl group to give N-benzoyl-L-homoserine lactone (BAHL) generated a compound with similar agonist activity to BHL. Whilst the C8 acyl chain OHl and OOHL were both able to induce violacein synthesis in CV026, none of the longer C10 to C14 compounds synthesized was able to do so (Fig. 2a, Table 1).

Thus the length of the N-linked acyl chain is a key structural feature determining the relative agonist activities of the series of AHL and AHT compounds evaluated in the CV026 violacein induction assay. In C. violaceum, the natural ligand for the Chromobacterium LuxR homologue, CviR (M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data), is HHL; this is, not surprisingly, the most active compound in the violacein induction assay. Conservative changes to the acyl chain or lactone ring of HHL are tolerated and compounds such as OHHL and HHT have significant violacein-inducing activity. Similar structure–activity data have been obtained in bacteria such as P. fischeri (Eberhard et al., 1986; Schaefer et al., 1996a) and E. carotovora (Chhabra et al., 1993), which both employ OHHL, to regulate bioluminescence and carbapenem synthesis respectively. However, as for the induction of violacein, AHLs with acyl chains longer than C8 have little or no agonist activity (Eberhard et al., 1986; Schaefer et al., 1996a). Conversely, compounds with C4 or C8 acyl chains are unable to activate the P. aeruginosa LuxR homologue LasR, the natural ligand for which is the C12 AHL, OaDHL (Passador et al., 1996).

**Inhibition of AHL-mediated violacein synthesis.** Although AHL compounds with N-acyl side chains longer than C8 failed to stimulate violacein production even at high concentrations (Fig. 2a, Table 1), we noted that if placed in an adjacent well to short-chain (C4 or C8 acyl chain) AHLs, compounds such as N-decanoyl-L-homoserine lactone (DHL) antagonized violacein production (Fig. 2a; DHL in well 9 is clearly inhibiting violacein induction by BHL and OHL in adjacent wells 6 and 8 respectively). To explore this further, we seeded OHHL or HHL and CV026 into the overlay agar and placed DHL in the wells. After overnight incubation, we observed white haloes surrounding the well in a purple background (data not shown). These data suggested that inhibition of violacein production could be exploited as an assay for the detection of longer chain AHLs. Fig. 2(b) illustrates that compounds with C10, C12 or C14 acyl chains, with or without an oxygen at the 3 position, could be detected by inhibition of HHL-mediated activation of violacein production. Whilst none of the shorter-chain C4 or C8 acyl chain compounds were active in this assay, it is clear that the C10 compound OHL does inhibit HHL-mediated activation of violacein...
Fig. 3. Thin-layer chromatogram illustrating the CV026 overlay technique for AHL detection. Synthetic AHLs, either as single compounds or as mixtures, were dissolved in acetonitrile and spotted onto a reversed-phase RP18 TLC plate. The chromatogram was developed with 60% (v/v) methanol in water and then overlaid with CV026 as described in Methods. For each AHL, the amount applied to the TLC plate corresponds to the minimum detectable amount. Lane 1, BHL (1.8 x 10^{-9} mol); lane 2, HHL (1 x 10^{-11} mol); lane 3, OHL (4.4 x 10^{-8} mol); lane 4, a mixture of BHL, HHL and OHL; lane 5, OBHL (7.3 x 10^{-8} mol); lane 6, OHHL (1.4 x 10^{-10} mol); lane 7, OOHHL (8.3 x 10^{-10} mol); lane 8, a mixture of OBHL, OHHL and OOHHL. A purple spot is indicative of AHL-mediated activation of violacein production. O marks the position of the origin.

Fig. 4. Thin-layer chromatogram of the AHLs present in cell-free supernatants of Y. enterocolitica and S. liquefaciens detected using a CV026 overlay. Cell-free supernatants from each micro-organism were extracted with dichloromethane and chromatographed as described in Methods. Lane 1, Y. enterocolitica supernatant extract; lane 2, OHHL standard; lane 3, HHL standard; lane 4, BHL standard; lane 5, S. liquefaciens extract. For the OHHL, HHL and BHL standards, 1.8 x 10^{-9}, 1 x 10^{-11} and 8.3 x 10^{-10} mol respectively were applied to the chromatogram. O marks the position of the origin. The filled arrowhead (lane 5) marks the position of the novel S. liquefaciens AHL.

to some extent (Fig. 2b, well 8). Although the 3-oxo derivative of OHL, OOHHL, does not show any inhibition at the concentration tested in Fig. 2(b), some weak inhibition can be observed in plates containing lower concentrations of the activating AHL.

Thus the length of the acyl side chain is again a major structural feature determining the sensitivity of detection of AHLs in this modified assay. This inhibition assay should prove useful for detecting compounds with acyl chains ranging from C_{10} to C_{14}. Using the violacein inhibition assay, we observed inhibitory activity in culture supernatants from the fish pathogen Vibrio (Listonella) anguillarum (Milton et al., 1997). Subsequently chemical analysis led to the identification of the C_{10} acyl chain compound ODHL as the major V. anguillarum quorum-sensing signal molecule (Milton et al., 1997). Furthermore the development of both the direct and modified C. violaceum assays should facilitate the assay of culture supernatants which contain mixtures of long- and short-chain AHLs. The versatility of the C. violaceum assays should therefore prove extremely valuable for the identification of novel AHLs. However, since many bacteria produce antibacterial substances, the inhibition assay should be interpreted with care. The white zone of antagonism observed should be opaque and not transparent, i.e. antibacterial agents will inhibit the growth of CV026, leading to a clear zone of inhibition around the well, whereas a long-chain antagonist will permit growth of the biosensor.

TLC analysis. Since HPLC can be both expensive and time consuming, other techniques such as TLC may offer an inexpensive and rapid route to the tentative identification of AHL signal molecules. Shaw et al. (1997) have recently demonstrated the utility of TLC in 60% (v/v) methanol/water to effectively separate AHLs with acyl chains from C_{4} to C_{12} in length. We therefore examined the utility of the CV026 bioassay strain to detect these molecules on TLC plates as described in Methods. Fig. 3 illustrates the results obtained when a series of AHLs with C_{4}, C_{6} or C_{8} side chains are separated on a C_{18} reversed-phase TLC plate and, after chromatography, overlaid with agar containing CV026. Members of both the N-alkanoyl-L-homoserine lactones series and the N-(3-oxoalkanoyl)-L-homoserine lactones are well separated using 60% (v/v) methanol as the solvent (Shaw et al., 1997) such that tentative identification of a novel AHL can be made by comparison of the R_{f} value of an unknown with that of a synthetic standard. Furthermore, full chemical characterization can be made by running samples streaked across the entire baseline such that a strip can be removed and overlaid with CV026 to aid location of the putative AHL. Once located on the chromatogram, the area containing the putative AHL can be scraped off,
extracted with acetone and the material obtained subjected directly to MS and/or NMR spectroscopy. Using synthetic BHL, HHL and OHHL, we have found that a spot containing as little as 0.1 μg of the respective AHL is sufficient for detection by MS.

To demonstrate the utility of this assay for the preliminary identification of natural AHLs, we extracted spent culture supernatants from Y. enterocolitica and S. liquefaciens, bacteria we have previously shown to make OHHL plus HHL (Throup et al., 1995a) and BHL plus HHL (Eberl et al., 1996) respectively. Fig. 4 shows that the CV026 TLC overlay clearly detects each of these AHLs, which can be tentatively identified by comparison with AHL standards after extracting as little as 5 ml culture supernatant. Intriguingly, in the S. liquefaciens extract, there is a third positive spot, which runs between BHL and HHL (Fig. 4, lane 5). This compound does not migrate with an Rf value corresponding to any of the AHLs available in our compound library and may represent a novel S. liquefaciens AHL.

By incorporating an AHL such as HHL or OHHL into agar seeded with CV026, we have discovered that it is possible to detect AHLS with acyl chains between \( \text{C}_8 \) to \( \text{C}_{14} \) AHLs, either as single compounds or as mixtures, were dissolved in acetonitrile and spotted onto a reverse-phase RP18 TLC plate. The chromatogram was developed with 60% (v/v) methanol in water and then overlaid with CV026 plus OHHL (1 μM) as described in Methods. For each AHL, the amount applied to the TLC plate corresponds to the minimum detectable amount. Lane 1, OHL (4.4 \( \times \) 10^{-10} mol); lane 2, DHL (2.4 \( \times \) 10^{-9} mol); lane 3, dDHL (1.1 \( \times \) 10^{-7} mol); lane 4, a mixture of OHL, DHL and dDHL; lane 5, OHHL (8.3 \( \times \) 10^{-10} mol); lane 6, ODHL (4 \( \times \) 10^{-8} mol); lane 7, OdDHL (4 \( \times \) 10^{-6} mol); lane 8, OdDHL (1.3 \( \times \) 10^{-7} mol); lane 9, a mixture of OOH, ODHL, OdDHL and OtDHL. Active compounds in this assay give white spots in a purple background. O marks the position of the origin. The positions of OOH, ODHL, OdDHL and OtDHL in lane 9 are marked with open arrowheads on the right. OOH in lanes 5 and 9 is observed as the more intense purple spot with a slight tail of inhibition. Open arrowheads in lanes 1 and 4 mark the positions of the OHL spots; the arrowhead in lane 6 marks the position of the ODHL spot.

Conclusions

This study adds C. violaceum to the growing family of Gram-negative bacteria which produce AHLs. In C. violaceum, it is clear that in addition to violacein synthesis, HHL is involved in the regulation of exoprotease, chitinase and cyanide production (Throup et al., 1995b; M. K. Winson, P. Williams & G. S. A. B. Stewart, unpublished data). However, it is the induction or antagonism of AHL-mediated violacein synthesis in HHL-negative mutants such as CV026 which is likely to prove most useful since it offers a convenient tool for the biological assay of a broad range of AHL and AHT analogues. Furthermore, because supernatants from E. coli do not induce violacein activation or inhibition (unpublished data), E. coli is a very useful cloning host for LuxI homologues, since clones can easily be screened using the Chromobacterium assays. In addition, the ease of the end-point detection and the flexibility of the TLC assays underlines the versatility of this technique, which does not require expensive reagents, HPLC equipment or the use of film or luminometry.

ACKNOWLEDGEMENTS

We thank Nigel Bainton for his contribution to the preliminary experiments to isolate the C. violaceum AHL. We also thank Stephen Farrand and Paul Shaw for their helpful comments on the manuscript and for providing us with pre-publication data. This work was supported by grants and a studentship (for A.T.) from the Biotechnology and Biological Sciences.
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Quorum sensing in C. violaceum


Received 24 June 1997; revised 28 August 1997; accepted 1 September 1997.