

N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities

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The *Rhodococcus erythropolis* strain W2 has been shown previously to degrade the *N*-acylhomoserine lactone (AHL) quorum-sensing signal molecule *N*-hexanoyl-L-homoserine lactone, produced by other bacteria. Data presented here indicate that this Gram-positive bacterium is also capable of using various AHLs as the sole carbon and energy source. The enzymic activities responsible for AHL inactivation were investigated in *R. erythropolis* cell extracts and in whole cells. *R. erythropolis* cells rapidly degraded AHLs with 3-oxo substituents but exhibited relatively poor activity against the corresponding unsubstituted AHLs. Investigation of the mechanism(s) by which *R. erythropolis* cells degraded AHLs revealed that 3-oxo compounds with *N*-acyl side chains ranging from C₈ to C₁₄ were initially converted to their corresponding 3-hydroxy derivatives. This oxidoreductase activity was not specific to 3-oxo-AHLs but also allowed the reduction of compounds such as *N*-(3-oxo-6-phenylhexanoyl)homoserine lactone (which contains an aromatic acyl chain substituent) and 3-oxododecanamide (which lacks the homoserine lactone ring). It also reduced both the D- and L-isomers of *N*-(3-oxododecanoyl)-L-homoserine lactone. A second AHL-degrading activity was observed when *R. erythropolis* cell extracts were incubated with *N*-(3-oxodecanoyl)-L-homoserine lactone (3O,C10-HSL). This activity was both temperature- and pH-dependent and was characterized as an amidolytic activity by HPLC analysis of the reaction mixture treated with dansyl chloride. This revealed the accumulation of dansylated homoserine lactone, indicating that the 3O,C10-HSL amide had been cleaved to yield homoserine lactone. *R. erythropolis* is therefore capable of modifying and degrading AHL signal molecules through both oxidoreductase and amidolytic activities.

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INTRODUCTION

Bacterial cell-to-cell communication (or quorum sensing; QS) facilitates gene expression in a bacterial population as a function of cell population density (Swift *et al.*, 2001;

Winans & Bassler, 2002; Winzer *et al.*, 2002). QS relies upon the production and the perception by the microbial cell population of small diffusible signal molecules. While many chemically distinct families of QS signal molecules have now been described, diverse Gram-negative bacteria employ *N*-acylhomoserine lactones (AHLs; Whitehead *et al.*, 2001). These contain a conserved homoserine lactone (HSL) ring and an amide (*N*-linked acyl side chain). The acyl groups of the naturally occurring AHLs identified to date range from 4 to 18 carbons in length; they may be saturated or unsaturated, and with or without a C-3 substituent (usually hydroxy- or oxo-; Fuqua *et al.*, 2001; Swift *et al.*, 2001). Most AHL-producing bacteria synthesize multiple AHL QS signal molecules via enzymes belonging to the LuxI or LuxM

Abbreviations: AHL, *N*-acylhomoserine lactone(s); CCE, crude cell extract; HSL, homoserine lactone; LBm, LB medium, modified; QS, quorum-sensing; C6-HSL, *N*-hexanoylhomoserine lactone; 3O,C6-HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone; C8-HSL, *N*-octanoyl-L-homoserine lactone; 3O,C10-HSL, *N*-(3-oxodecanoyl)-L-homoserine lactone; 3O,C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; 3O,C14-HSL, *N*-(3-oxotetradecanoyl)-L-homoserine lactone; 3O,C12-NH₂, 3-oxododecanamide; 3O,6Ph,C6-HSL, *N*-(3-oxo-6-phenylhexanoyl)-L-homoserine lactone.

protein families. AHLs control gene expression by interacting directly with a member of the LuxR family of response-regulator proteins (Fuqua *et al.*, 1994; Zhu & Winans, 2001).

AHL-dependent QS is conserved in a broad range of microorganisms living in various environments, and regulates very diverse physiological functions (Fuqua *et al.*, 2001; Swift *et al.*, 2001; Whitehead *et al.*, 2001). Amongst these, several pathogenicity-related functions are controlled in a population-density-dependent fashion in bacterial species pathogenic for plants and animals (e.g. Jones *et al.*, 1993; Milton *et al.*, 1997; Zhang *et al.*, 1993; reviewed by Von Bodman *et al.*, 2003). QS also controls functions responsible for the interaction of the microbe with both its physical and biological environments, including swimming, swarming, biofilm maturation and symbiosis. With respect to the interaction between bacteria and higher organisms, QS is likely to confer a selective advantage which enables the bacterium to express groups of genes with a highly relevant biological impact, considering that (i) the expression is co-ordinated and (ii) the relevant microbial cell population needs to attain a high density before QS-controlled genes are induced (Fuqua *et al.*, 2001; Swift *et al.*, 2001; Whitehead *et al.*, 2001; Winans & Bassler, 2002; Winzer *et al.*, 2002). Interestingly, AHLs are also perceived by higher organisms. A striking example is the attraction of zoospores from the green alga *Enteromorpha* by AHLs (Joint *et al.*, 2002). These zoospores exhibit chemotaxis for AHLs, leading to their enhanced settlement on AHL-producing biofilms. In addition, the legume plant *Medicago truncatula* responds to AHLs, as shown by proteomic analysis as well as activation of tissue-specific reporter gene fusions (Mathesius *et al.*, 2003). Furthermore, long-chain AHLs such as those produced by the opportunistic pathogen *Pseudomonas aeruginosa* have been shown to exert immunomodulatory (Chhabra *et al.*, 2003; Telford *et al.*, 1998) and cardiovascular effects (Gardiner *et al.*, 2001) in mammalian hosts

Evidence is beginning to accumulate indicating that inhibition of QS may be a strategy adopted by eukaryotic organisms to combat potentially pathogenic bacteria. The production of AHL antagonists has been demonstrated for the marine red alga *Delisea pulchra* (Givskov *et al.*, 1996), higher plants (Tepliski *et al.*, 2000) and the bryozoan *Flustra foliacea* (Peters *et al.*, 2003). Under alkaline growth conditions, AHLs are rapidly inactivated by pH-dependent lactonolysis (i.e. opening of the HSL ring) since the corresponding *N*-acylhomoserine cannot activate LuxR-type response-regulator proteins (Byers *et al.*, 2002; Yates *et al.*, 2002). The ability to inactivate AHLs enzymically has also been demonstrated for a range of bacterial genera belonging to the α -Proteobacteria (*Agrobacterium*, Zhang *et al.*, 2002), the β -Proteobacteria (*Variovorax*, Leadbetter & Greenberg, 2000; *Ralstonia*, Lin *et al.*, 2003; and *Comamonas*, Uroz *et al.*, 2003), the γ -Proteobacteria (*Pseudomonas*, Huang *et al.*, 2003; Uroz *et al.*, 2003; *Acinetobacter*, Kang *et al.*, 2004), the low-G + C Gram-positive bacteria (*Bacillus*, Dong *et al.*, 2000, 2002; Fray, 2002; Lee *et al.*, 2002) and the high-G + C

Gram-positive bacteria (*Rhodococcus*, Uroz *et al.*, 2003). AHL-inactivating activity has also been reported in plants (Delalande *et al.*, 2005) and mammalian cells (Chun *et al.*, 2004). The AHL-inactivating enzymes described to date belong to two families: the AHL lactone hydrolases (e.g. AiiA, AttM, AiiB, Carlier *et al.*, 2003; Dong *et al.*, 2000; Fray, 2002; Lee *et al.*, 2002; Zhang *et al.*, 2002) and the AHL acylases (AiiD; Lin *et al.*, 2003). However, the physiological function(s) of these AHL-inactivating enzymes and whether AHLs are their primary substrates have not yet been entirely clarified, although an involvement of AttM from *Agrobacterium*, in γ -butyrolactone degradation, has been proposed recently (Carlier *et al.*, 2004).

Amongst the bacteria exhibiting AHL catabolic activity, *Rhodococcus erythropolis* strain W2 was of special interest because analysis of its degradative properties revealed that it exhibits a broad AHL-degradation spectrum and rapid AHL inactivation kinetics (Uroz *et al.*, 2003). *In planta*, *R. erythropolis* W2 markedly reduced the pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* in potato tubers, indicating its potential as a biocontrol agent. These traits motivated a more detailed examination of the catabolic activities and pathways involved in AHL inactivation. The results reported in this paper demonstrate that strain W2 grows on various AHLs as the sole carbon and energy source, with a preference for short-chain compounds. Two enzyme activities involved in AHL inactivation were identified: an oxidoreductase which converts 3-oxo-AHLs to their corresponding 3-hydroxy derivatives, and an amidolytic activity which cleaves the amide bond linking the acyl chain to the HSL residue.

METHODS

Bacterial strains, growth media and culture conditions. The strains used in this study were *R. erythropolis* W2, isolated from the tobacco rhizosphere (Uroz *et al.*, 2003), *Escherichia coli* DH5 α and the AHL biosensors *E. coli* [pSB401] and *E. coli* [pSB1075], which respectively respond to short- and long-chain AHLs, supplied exogenously, by emitting light (Winson *et al.*, 1998). The non-selective growth medium used was modified Luria-Bertani (LBm, Vaudequin-Dransart *et al.*, 1995). For growth assessment of *R. erythropolis* strain W2, experiments were performed using the minimal medium described by Leadbetter & Greenberg (2000). PBS and all media used in this study (including LBm), were buffered to pH 6.5 by the addition of 100 mM KH₂PO₄/K₂HPO₄ to prevent spontaneous degradation of AHLs. Where necessary, growth media were supplemented with tetracycline (10 mg l⁻¹) and solidified with agar (16 g l⁻¹). *R. erythropolis* strain W2 was grown at 25 °C and *E. coli* strains at 37 °C.

Synthesis of AHLs and related compounds. The AHLs investigated in this study, as well as the analogue 3O,6Ph,C6-HSL (Fig. 1) were synthesized as described previously (Chhabra *et al.*, 1993, 2003). The compound 3O,C12-NH₂ (Fig. 1) was synthesized as described for AHL synthesis. HSL, homoserine and dansyl chloride were obtained from Sigma.

Growth of *R. erythropolis* on AHLs. *R. erythropolis* strain W2 was inoculated into minimal medium devoid of carbon source and incubated for 14 h overnight at 25 °C to starve the cells. Ten

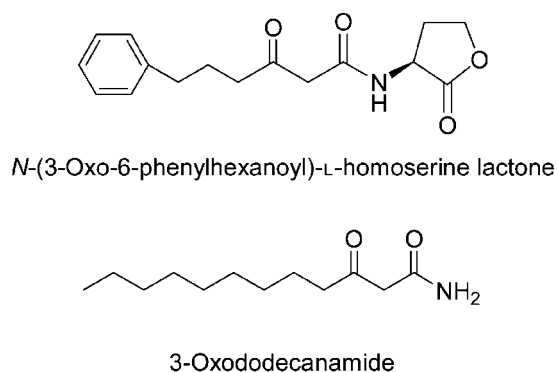


Fig. 1. AHL analogues (3O,6Ph,C6-HSL and 3O,C12-NH₂) used in this study.

microlitres of this suspension was inoculated into 4 ml minimal medium supplemented with 3O,C6-HSL or other AHLs, provided as sole carbon and nitrogen sources (1 mM), and to minimal medium supplemented with or without these AHLs (1 mM) as sole carbon source, in the presence of a nitrogen source (NH₄Cl, 0.3 g l⁻¹). Incubations were performed at 25 °C with shaking. OD₆₀₀ was determined after 0, 2, 7 and 12 days. For each medium, generation times were estimated from the linear portion of the growth curves obtained.

Preparation of resting *R. erythropolis* cells and crude cell extracts. *R. erythropolis* strain W2 cells were harvested after 2 days of culture in rich LBm medium, at approximately 10⁹ c.f.u. ml⁻¹. A 1 l culture was centrifuged for 10 min at 4 °C, 10 000 g. The cells were resuspended in 100 ml PBS and washed twice in PBS. The resulting concentrated cell suspension was used directly as a source of resting cells for *in vivo* AHL inactivation assays or disrupted using a cell disrupter (Constant Systems) under 15 kPa pressure. The lysate obtained was recycled five times through the cell disrupter. Cell debris was removed by centrifugation (120 min, 4 °C, 10 000 g). The resulting supernatant was filtered through a 0.22 µm membrane filter and stored at 4 °C. This crude cell extract (CCE) was the source of enzyme used for the *in vitro* AHL inactivation assays. The protein concentration of the CCE was determined according to the manufacturer's instructions (Sigma protein detection kit), using the Bradford method with bovine serum albumin as the standard.

Separation and analysis of AHLs. AHLs were detected using the *lux*-based biosensors *E. coli*[pSB401] and *E. coli*[pSB1075], for short-chain and long-chain compounds respectively (Winson *et al.*, 1998). Bioassays were performed with the above sensors using a microtitre plate bioassay as described by Reimann *et al.* (2002). Wells containing AHLs were visualized as bright wells in a dark background when viewed with a Luminograph LB980 photon video camera (Berthold). Reverse-phase HPLC analysis of AHLs was performed on a Kromasil C₈ 5µ column, 2.1 × 250 mm (Jones Chromatography), using a Waters 625 HPLC system coupled with a Waters 996 PDA photodiode array detector, and eluted with acetonitrile/water isocratic or gradient combinations as described before (Swift *et al.*, 1996; Yates *et al.*, 2002).

AHL inactivation assays. For the whole-cell assays, aliquots of AHLs in ethyl acetate were dispensed into sterile tubes and the solvent evaporated to dryness under a stream of sterile nitrogen. These tubes were filled with 1 ml of a resting *R. erythropolis* cell suspension obtained as indicated above, rehydrating the AHLs and providing a final AHL concentration of 100 µM. The resting cell suspensions were incubated at 25 °C for up to 360 min. The reactions were

stopped at regular intervals by the addition of ethyl acetate (1 ml), which also served to extract any remaining AHLs. For AHL inactivation assays *in vitro* using CCEs, the reaction mixture contained 0.5 mg ml⁻¹ of bacterial protein and 100 µM AHLs in a final volume of 500 µl. Assays were incubated at 25 °C or 37 °C for up to 360 min and stopped at regular intervals by addition of 3 vols (1.5 ml) ethyl acetate. For both whole-cell and CCE assays, ethyl acetate containing the residual AHL was removed and evaporated to dryness. The solution was reconstituted in acetonitrile (100 µl) and residual AHL concentrations determined using the AHL biosensors and HPLC (see above). The percentage of AHLs inactivated and the specific activity were determined by estimating the amount of AHL (by comparison of the reduction in peak areas for a given retention time) with respect to AHL solutions of known concentration. For both whole-cell and CCE assays, control experiments involving un-inoculated medium or extraction buffer incubated with AHLs, and cells or extracts incubated without AHLs, were always performed. Heat-processed cells and cell crude extracts were also used as negative controls.

Identification of AHL degradation products. To identify the breakdown products generated following the incubation of *R. erythropolis* CCE with AHLs, we used HPLC in conjunction with LC-MS/MS (Waters Micromass Quattro Ultima). Synthetic standards for each assayed molecule were always used as control for HPLC analysis. Free amines released by the action of AHL-inactivating enzymes were 'chemically trapped' by dansylation with dansyl chloride as described by Jiang *et al.* (1998). The formation of the ring-opened AHLs, i.e. the formation of the corresponding *N*-acylhomoserine, was detected using the method described by Yates *et al.* (2002). This is based on the acidification of the reaction mixture with 10 mM HCl to induce lactone recyclization.

***In vitro* determination of the temperature and pH sensitivity of the amidolytic activity of CCEs.** *R. erythropolis* CCEs were incubated for 10, 20 and 30 min at a range of temperatures (25, 50, 75 and 100 °C) in the absence of AHLs. The resulting samples were stored at -20 °C. To evaluate the effect of temperature on AHL-inactivating activity, the CCEs were incubated with AHLs as described above. To examine the influence of pH, the pHs of CCEs in PBS were adjusted from 6.5 to 1.0 with HCl prior to incubation. CCE activity was subsequently determined by incubation with 3O,C10-HSL (100 µM) for 120 min at 25 °C with shaking. The extent of 3O,C10-HSL degradation was monitored by HPLC as described above.

RESULTS

R. erythropolis W2 utilizes a variety of AHLs as sole carbon or carbon and nitrogen sources

The ability of *R. erythropolis* strain W2 to grow on AHLs was investigated by examining growth in a defined minimal medium supplemented with different AHLs as the sole carbon, or carbon and nitrogen source (see Methods). Strain W2 was able to grow in minimal medium supplemented with 3O,C6-, C8-, C10-, 3OH,C10 and 3O,C12-HSL (1 mM) with mean generation times ranging from approximately 36 h in the presence of 3O,C6-HSL to 120 h on 3O,C12-HSL (Fig. 2). When assessed, the presence or absence of NH₄Cl₂ in the medium did not affect the growth capability of the strain (not shown). No growth was observed in a minimal medium devoid of AHLs.

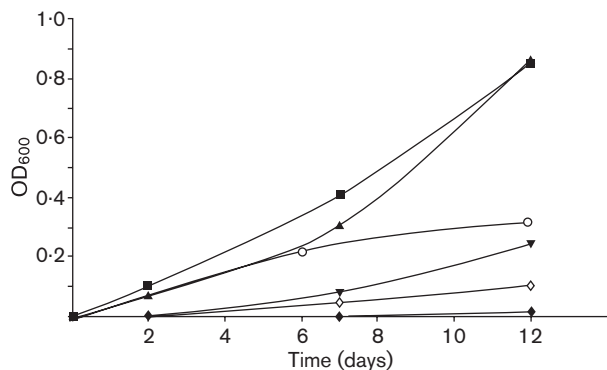


Fig. 2. Growth of *R. erythropolis* strain W2 on AHL as sole carbon and energy source. OD₆₀₀ was determined over 12 days in a minimal medium supplemented with a nitrogen source (ammonium chloride) and with various AHLs: 3O,C6-HSL (■), C8-HSL (▲), C10-HSL (▼), 3OH,C10-HSL (○), and 3O,C12-HSL (◇). Residual growth without carbon source is also shown (◆).

R. erythropolis W2 degrades of a wide range of AHLs

The turnover of AHLs, differing in C-3 substituent and acyl chain length, by resting W2 cells in PBS was investigated as described in Methods, at 25 °C at various times over a 360 min period. In addition, the same assay was used to determine whether W2 cell free culture supernatants contained any AHL-degrading activity. Fig. 3 summarizes the data obtained for whole cells. For example, 3O,C6-HSL was turned over with an estimated activity of 110 pmol AHL min⁻¹ (OD₆₀₀ unit)⁻¹. Increasing the acyl chain by two carbons to 3O,C8-HSL reduced the activity slightly, to 83 pmol AHL min⁻¹ (OD₆₀₀ unit)⁻¹. However, while there were no further reductions in estimated activity when 3O,C10-HSL and 3O,C12-HSL were used as the AHL substrates, extension of the acyl chain to C₁₄ resulted in a substantial reduction in activity [25 pmol AHL min⁻¹ (OD₆₀₀ unit)⁻¹]. Reduction of the 3-oxo substituent of 3O,C12-HSL to give the corresponding 3-hydroxy compound also resulted in a significant decrease in estimated activity. The importance of the C-3 substituent was further highlighted by the relative lack of activity of the resting *R. erythropolis* cells towards C6-, C10- and C12-HSL. For instance, 33 % of the C6-HSL provided was degraded after 360 min incubation, corresponding to an estimated activity of 9 pmol AHL min⁻¹ (OD₆₀₀ unit)⁻¹ whereas C10- and C12-HSL remained intact. The complete turnover of C6-HSL in PBS within the 360 min incubation period could only be achieved by the provision of an energy source (glucose, 1 mM final concentration) to the PBS buffer. In agreement with this observation, all AHLs assayed, whether they were oxo derivatives or not, were degraded in less than 12 h in rich medium (LBM; data not shown). No

AHL	Abbreviation	EA
	C6-HSL	9
	3O,C6-HSL	110
	3O,C8-HSL	83
	C10-HSL	< 1
	3O,C10-HSL	83
	C12-HSL	< 1
	3O,C12-HSL	83
	3OH,C12-HSL	28
	3O,C14-HSL	25

Fig. 3. *In vivo* inactivation of AHLs by *R. erythropolis* W2. EA, estimated activity, expressed as pmol AHL degraded min⁻¹ (OD₆₀₀ unit)⁻¹. Standard errors were within 5 % of the mean values shown.

AHL-degrading activity was found in cell-free culture supernatants (data not shown), indicating that the activity is wholly cell-associated.

Evidence for an AHL oxidoreductase activity

In the experiment described above, the time-course of AHL inactivation was monitored by HPLC. When 3O,C14-HSL was incubated with W2 cells in PBS, the loss of this long-chain AHL from the HPLC spectrum was accompanied by the appearance of a new peak (Fig. 4). This new peak exhibited the same HPLC retention time as 3OH,C14-HSL. LC-MS/MS analysis of this new compound indicated the presence of a molecular ion (*m/z*) 328, two mass units greater than 3O,C14-HSL and with a daughter ion fragmentation pattern identical to that of 3OH,C14-HSL. These data demonstrate that the 3O- substituent of 3O,C14-HSL had been reduced to yield the corresponding 3-hydroxy derivative, 3OH,C14-HSL. Similar observations were made when 3O,C8-HSL, 3O,C10-HSL and 3O,C12-HSL were incubated with W2 whole cells in PBS (data not shown). This activity could be abolished by heat-treating the W2 cells. Interestingly, although 3O,C6-HSL was turned over by W2, no 3OH,C6-HSL could be detected. This suggested that at least one additional enzymic activity is involved in metabolism of 3O,C6-HSL.

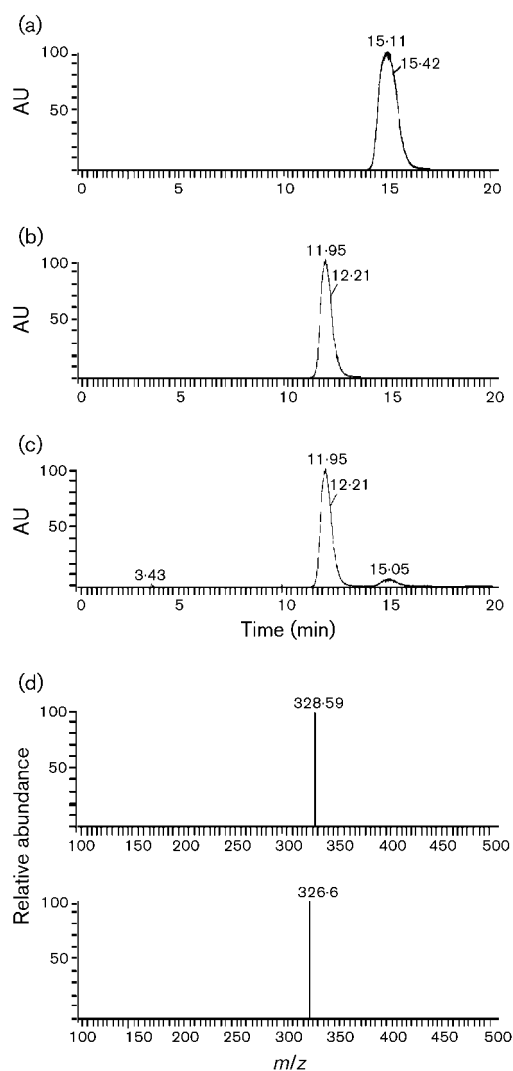


Fig. 4. HPLC and LC-MS analysis of the mechanism of 3-oxo AHL modification by resting whole *R. erythropolis* W2 cells. (a) HPLC profile of 3O,C14-HSL. (b) HPLC profile of synthetic 3OH,C14-HSL. (c) HPLC profile of 3O,C14-HSL after 60 min incubation of W2 cells in PBS. Note that the peak corresponding to 3O,C14-HSL (at 15.05 min) is substantially reduced and a new peak at 11.92 min has appeared with the same HPLC retention time as 3OH,C14-HSL (panel b). AU, arbitrary units. (d) The compound corresponding to the new 11.92 min peak appearing in panel (c) was subjected to LC-MS analysis (top panel) and compared with 3O,C14-HSL (bottom panel) and 3OH,C14-HSL (not shown). The new compound was identified as 3OH,C14-HSL.

Since each of the 3O-AHLs used were L-isomers, the corresponding D-isomer of 3O,C12-HSL was used to determine whether the reduction to the corresponding 3-hydroxy compound was stereospecific. The D-isomer of 3O,C12-HSL was converted to the corresponding 3OH,C12-HSL, indicating that the reaction was not stereospecific (data not

shown). To extend further this structure activity analysis, the requirement for the HSL ring and the influence of an aromatic substituent in the acyl side chain were examined. In these experiments, both 3O,C12-NH₂ and 3O,6Ph,C6-HSL (Fig. 1) were converted to the corresponding 3-hydroxy derivatives. However neither of these 3-hydroxy derivatives nor the D-isomer of 3OH,C12-HSL were degraded further. This contrasted with 3OH,C8-HSL, 3OH,C10-HSL, 3OH,C12-HSL and 3OH,C14-HSL, all of which were subjected to further degradation by resting W2 cells (data not shown).

No oxidoreductase activity was observed with disrupted W2 cells, implying a co-factor requirement. The addition of NADH or NADPH to CCE did not lead to the formation of NAD⁺ or NADP⁺ upon incubation with a 3-oxo-AHL as determined by spectrophotometric analysis (data not shown), suggesting either that alternative cofactors are required, or that the enzyme is sensitive to cellular disruption.

Evidence for pH- and temperature- dependent AHL-inactivating activity in cell extracts of *R. erythropolis* W2

Prolonged incubation of 3-oxo- and 3-hydroxy-substituted and unsubstituted AHLs resulted in the complete removal of these QS signal molecules from the incubation medium. These data suggested the presence of additional AHL-degrading activities. To investigate this possibility, 3O,C10-HSL was incubated with W2 CCE. Under these conditions, the peak corresponding to 3O,C10-HSL in the HPLC spectrum was reduced over time (Fig. 5) but no new peak corresponding to 3OH,C10-HSL was apparent. This degradative activity was investigated in more detail at 25 °C and 37 °C using 3O,C10-HSL as a substrate. After a 120 min incubation period, the apparent activities [expressed as nmol AHL degraded min⁻¹ (mg protein)⁻¹] were 1.17 at 25 °C and 1.18 at 37 °C, with a 5% experimental error. After 180 min incubation, the activities were reduced to 1.0 and 0.8 nmol AHL degraded min⁻¹ (mg protein)⁻¹ at 25 °C and 37 °C respectively.

The thermostability of this AHLase activity was assessed by subjecting the W2 CCE to different temperatures and incubation times as described in Methods prior to incubation with 3O,C10-HSL at 25 °C, after which the residual AHL activity was assayed. The data obtained indicated that the AHL-inactivating activity of *R. erythropolis* W2 CCEs was temperature dependent (Table 1).

The effect of pH on AHL-inactivating activity was also monitored by incubating the CCE with 3O,C10-HSL at 25 °C in PBS, at pHs ranging from 1 to 6.5. PBS was adjusted to a maximum pH of 6.5 because AHLs are readily inactivated under alkaline pH conditions (Yates *et al.*; Byers *et al.*, 2002). The AHL-inactivating activity present in CCEs was maximal at pH 6.5, averaging 1.17 nmol 3O,C10-HSL degraded min⁻¹ (mg protein)⁻¹. It decreased at lower pHs,

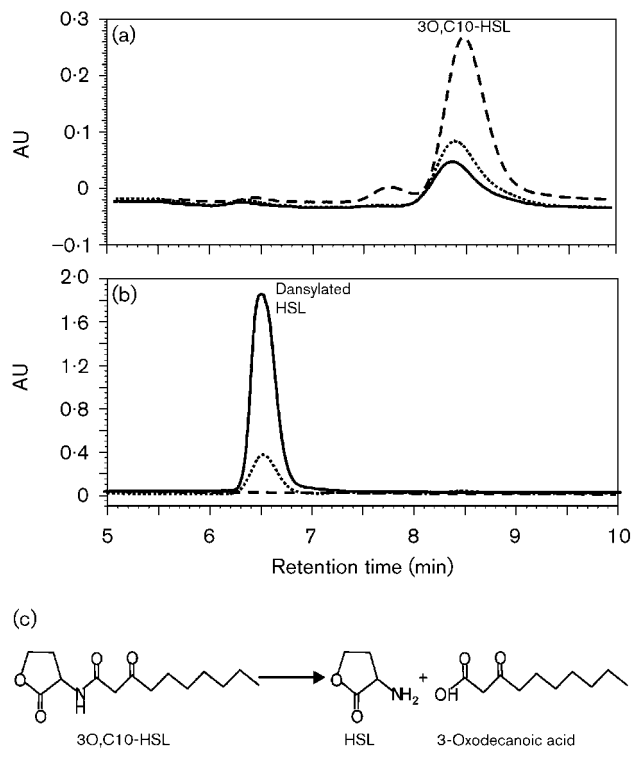


Fig. 5. HPLC analysis and chemical trapping of HSL following incubation of 3O,C10-HSL by *R. erythropolis* W2 CCE. (a) HPLC analysis of 3O,C10-HSL after incubation with CCE from W2 for 0 min (dashed line), 120 min (dotted line) or 180 min (solid line). The trace obtained after 300 min was similar to that after 120 min and for clarity is not shown in the figure. (b) HPLC analysis of the appearance of dansylated HSL following incubation of W2 CCE with 3O,C10-HSL; line symbols as in (a). (c) Amidolytic action of *R. erythropolis* CCE on 3O,C10-HSL, which releases HSL (and probably 3-hydroxydecanoic acid).

the activities at pH 5.0, 4.0 and 3.0 being approximately 90, 75 and 60 %, respectively, of that observed at pH 6.5. No activity could be detected at pHs below 2. In all cases, the amount of 3O,C10-HSL in the controls incubated without CCE remained the same across the pH range and time

period evaluated. These results indicate that W2 CCEs contain enzyme(s) capable of degrading AHLs.

AHL degradation by cell extracts of *R. erythropolis* W2 proceeds via an amidolytic activity

The AHL-inactivating activity of W2 CCEs is likely to be due to either a lactonase or an amidohydrolase. Lactonases convert AHLs to the corresponding *N*-acylhomoserine compound, which can be converted back to the AHL by incubation at acidic pHs (<2.0; Yates *et al.*, 2002). To evaluate this hypothesis, 3O,C10-HSL was incubated with CCE and the reaction mixture acidified prior to extraction with ethyl acetate. The recyclization of the lactone ring was observed for control experiments performed using *N*-acylhomoserine compounds incubated in the absence of CCE and acidified. However, 3O,C10-HSL could not be recovered by acidification after incubation with CCE (data not shown). These data indicated that the AHL-inactivating activity of W2 CCEs was not due to lactonolysis.

To determine whether W2 CCEs contained enzyme(s) capable of cleaving the amide bond linking the acyl side chain to the HSL ring, dansyl chloride was used to chemically trap any HSL released upon incubation of CCE with 3O,C10-HSL. Fig. 5 shows that under the experimental conditions chosen, about 70 % of the 3O,C10-HSL incubated with the CCE was degraded after 120 min and approximately 90 % after 180 min, with an apparent activity of about $1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. Following the addition of dansyl chloride, the presence of dansylated HSL was detected after incubation for 120 and 180 min. After 300 min, the concentration of HSL decreased, and was comparable to that observed at the 120 min time point. These results clearly indicate that HSL is released from 3O,C10-HSL during incubation with *R. erythropolis* W2 CCEs, indicating the presence of an amidolytic activity. They also suggest that the HSL generated via AHL degradation is further degraded by the W2 CCEs. A similar production of HSL was observed using 3O,C6-HSL and the hydroxy-AHL 3OH,C10-HSL as substrates, demonstrating that the W2 CCE contained an amidolytic activity capable of cleaving the amide bond of both short- and long-chain AHLs.

Table 1. Thermostability of the AHL amidolytic activity of *R. erythropolis* strain W2

	Temperature treatment:											
	25 °C			50 °C			75 °C			100 °C		
Duration (min)	10	20	30	10	20	30	10	20	30	10	20	30
EA*	1.15	1.15	1.15	0.95	0.60	0.55	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

*EA, estimated activity, expressed as $\text{nmol 3O,C10-HSL degraded min}^{-1} (\text{mg protein})^{-1}$. Standard errors were within 5% of the mean values shown.

DISCUSSION

R. erythropolis strain W2 was isolated from a tobacco rhizosphere on the basis of its ability to degrade C6-HSL (Uroz *et al.*, 2003). This work extends these observations and clearly demonstrates that W2 is capable of metabolizing a range of AHLs for use as a sole carbon source, or sole carbon and nitrogen source. A preference for short-chain AHLs was observed; this can be explained in part by the C:N ratio of these molecules, which ranges from 10:1 for 3O,C6-HSL to 16:1 for 3O,C12-HSL. The soil bacterium *Rhodococcus* is now the third Gram-positive genus, members of which can degrade AHLs. Comparable AHL-degradative properties have been reported previously for members of the genera *Bacillus* and *Arthrobacter* (Dong *et al.*, 2000, 2002; Lee *et al.*, 2002; Park *et al.*, 2003). During our growth experiments, at least some of the AHLs provided were converted to the corresponding *N*-acylhomoserine (AH) through lactonolysis upon prolonged incubation (Byers *et al.*, 2002; Yates *et al.*, 2002; Delalande *et al.*, 2005). However, the data reported in this study unambiguously demonstrated that AHLs are specifically degraded by strain W2. Firstly, resting cells were shown to degrade AHLs during short-term incubations that did not permit the chemical conversion of AHL to AH. Secondly, the two enzymic activities reported here were found to degrade or modify bona fide AHL molecules, even though oxo-AHLs are not the only substrates of the oxidoreductase activity (see additional discussion below).

In PBS buffer, *R. erythropolis* W2 whole cells metabolized a variety of AHLs with a carbon chain ranging from C₆- to C₁₄. Remarkably, 3-oxo compounds were much more efficiently turned over than their OH-substituted or unsubstituted counterparts. Furthermore, complete degradation of C6-HSL in PBS required the addition of an energy source (glucose) even though C6-HSL could sustain the growth of W2 when supplied as the sole carbon and nitrogen source in the medium described by Leadbetter & Greenberg (2000). The reason(s) for this apparent discrepancy are not clear but it is likely that the lack of a nutrient absent from PBS may be overcome by the provision of glucose. These data did however suggest that *R. erythropolis* possesses multiple mechanisms for metabolizing AHLs.

HPLC analysis and LC-MS/MS revealed that whole W2 cells but not CCEs exhibited a novel oxidoreductase activity which converted 3-oxo-substituted AHLs to the corresponding 3-hydroxy derivatives (Fig. 6), provided that the AHL contained an acyl chain of at least eight carbons (from 3O,C8- to 3O,C14-HSL). W2 cells also reduced 3O,6Ph,C6-HSL and 3O,C12-NH₂ to the corresponding hydroxy compounds, indicating that the oxidoreductase activity observed is not specific for naturally occurring AHLs and does not require the presence of the HSL ring. Furthermore, the reaction is not stereospecific, since the D-isomer of 3O,C12-HSL was converted to 3OH,C12-HSL by resting W2 cells. The loss of oxidoreductase activity on heat treatment of the W2 cells indicates that the reduction of the 3-oxo-AHLs is enzymic. Although we have so far been unable either to purify the enzyme involved or to clone the corresponding gene, a carbonyl reductase has been purified from *R. erythropolis* (Zelinski & Kula, 1994; Zelinski *et al.*, 1994). This enzyme accepts a broad range of aliphatic and aromatic ketones as substrates and for example reduces methyl 3-oxobutanoate and ethyl 4-chloro-3-oxobutanoate to the corresponding hydroxy compounds. Whether this enzyme can reduce 3-oxo-AHLs is not known.

Extended incubation of either C3-substituted or unsubstituted AHLs with growing cells of W2 resulted in the complete disappearance of these QS signal molecules from the incubation medium. This indicates that *R. erythropolis*, as well as the AHL oxidoreductase activity, possesses additional mechanisms for metabolizing AHLs. These are likely to account for the efficient quenching of AHL-dependent QS in *Pectobacterium* by *R. erythropolis* strain W2 (Uroz *et al.*, 2003). Indeed, the observed quenching cannot depend upon the oxidoreductase activity alone because 3O,C6-HSL produced by *Pectobacterium* is not reduced to the corresponding 3-hydroxy compound.

While CCEs prepared from W2 lacked the oxidoreductase activity associated with whole cells, CCEs retained the capacity to inactivate AHLs. The temperature and pH dependency of this activity suggested the involvement of one or more enzymes. Since several different bacteria produce lactonases (Dong *et al.*, 2000; Lee *et al.*, 2002; Park *et al.*, 2003; Zhang *et al.*, 2002), *R. erythropolis* W2 CCEs were

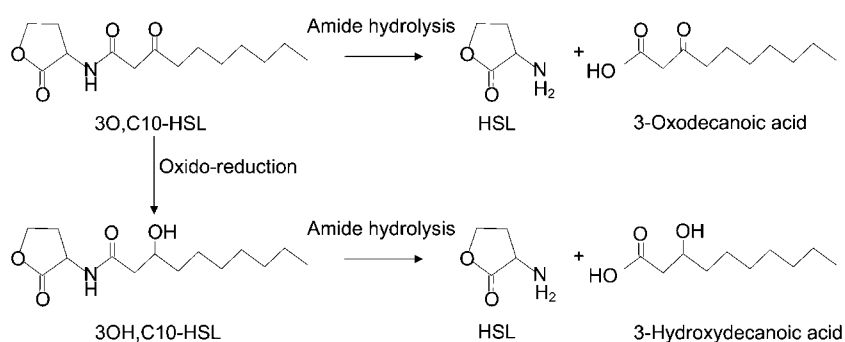


Fig. 6. Proposed enzymic activities involved in long-chain AHL degradation in *R. erythropolis* strain W2. 3-O,C10-HSL can be cleaved by the amidohydrolase to yield HSL and probably 3-oxodecanoic acid which can be further metabolized for growth via fatty acid metabolism. Alternatively, 3-O,C10-HSL can be reduced to the corresponding 3-hydroxy compound and subsequently cleaved by the amidohydrolase.

examined for such an activity. Since no lactonolysis was observed, an alternative mechanism for metabolizing AHLs is the cleavage of the amide bond linking the acyl side chain to the HSL ring. This mechanism of AHL inactivation has previously been observed only in Gram-negative bacteria (Huang *et al.*, 2003; Leadbetter & Greenberg, 2000; Lin *et al.*, 2003). For example, both *Variovorax* and *Ralstonia* species cleave the AHL amide bond during utilization of QS signal molecules as growth nutrients; HSL is released as a product of these reactions and the acyl moiety is further metabolized as an energy source.

To determine whether *R. erythropolis* CCEs inactivated AHLs by cleaving their amide bond, dansyl chloride (Jiang *et al.*, 1998) was used to trap chemically any HSL released. When 3O,C6-, 3OH,C10- or 3O,C10-HSL were incubated with W2 CCEs, these compounds yielded dansylated HSL, indicating that W2 possesses an amidohydrolase activity (Fig. 6). This activity exhibits the characteristics of an enzyme since it is thermosensitive and pH dependent, and HSL is released in a time-dependent manner. In a strain of *Ralstonia*, an amidohydrolase, AiiD, has been characterized and shown to be capable of cleaving AHLs with either long or short acyl side chains (Lin *et al.*, 2003). An AiiD homologue, termed PvdQ because it is located among a cluster of genes required for the synthesis of the siderophore pyoverdinin, is present in *P. aeruginosa* (Huang *et al.*, 2003; Lamont & Martin, 2003). Although PvdQ specifically cleaves long-chain AHLs, e.g. 3O,C12-HSL, *pvdQ* mutants still grow on 3O,C12-HSL as the sole energy source, but produce wild-type levels of 3O,C12-HSL (Huang *et al.*, 2003). The presence of a putative *aiiD*-homologous sequence was investigated in strain W2 using temperature-gradient PCR. However, no homologous sequence was obtained and therefore further work is required to identify the *R. erythropolis* amidohydrolase gene.

The ability to degrade AHLs is a recently identified characteristic of the genus *Rhodococcus* (Uroz *et al.*, 2003), which is well known for its remarkable ability to degrade diverse complex organic compounds (see for example Bock *et al.*, 1996; Chauvaux *et al.*, 2001; Haroune *et al.*, 2002; Sakai *et al.*, 2003; Van der Werf & Boot, 2000; and for a review Warhurst & Fewson, 1994). Two enzymic activities conferring the ability to modify and degrade AHLs upon strain W2 were identified: an AHL oxidoreductase, and an AHL amidolytic activity (Fig. 6). The capacity to reduce 3-oxo AHLs to the corresponding 3-hydroxy compounds has not previously been observed in any bacterium exhibiting AHL-degrading activity. Furthermore, while amidolytic activity towards AHLs has been observed in Gram-negative bacteria including *Ralstonia* (Lin *et al.*, 2003) and *Pseudomonas* (Huang *et al.*, 2003), and postulated for *Variovorax paradoxus* (Leadbetter & Greenberg, 2000), it has not to our knowledge been detected in a Gram-positive bacterium prior to this study.

The AHL inactivation mechanism(s), or at least the mechanism(s) that interfere(s) with cell–cell communication via

AHLs amongst microbial communities, appear(s) to be multiple and widespread within diverse bacterial genera. Whether AHLs are the primary substrates for the relevant enzymic activities in strain W2 as in other AHL-degraders is still unclear, such that the ‘true’ physiological role of these enzyme activities remains to be elucidated. Recent data obtained in *Agrobacterium*, however, indicate that AHL-inactivating lactonases may play another – and possibly a major – role in the intracellular metabolism of lactone compounds such as γ -butyrolactone (Carlier *et al.*, 2004). Clearly, investigation of the substrate specificity and contribution to endogenous metabolic pathways, especially in AHL-nonproducing organisms, will be needed to address the question of the biological function(s) of these AHL-inactivating enzymes. The oxidoreductase activity described in this report appears not to be specific for AHLs, but additional work is required to evaluate this question at the purified protein level. As suggested from earlier work (Uroz *et al.*, 2003), it could, however, be of potential interest to target 3-oxo AHLs within the development of novel biocontrol agents (Fray, 2002) or therapeutic strategies (Cámara *et al.*, 2002) directed at infection control in both plants and animals.

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