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SPECIAL REPORT

The *Pseudomonas aeruginosa* quorum-sensing signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone, inhibits porcine arterial smooth muscle contraction

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The *Pseudomonas aeruginosa* quorum sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) has been shown to suppress cytokine production in macrophages. We have examined the effect of OdDHL and related compounds on constrictor tone of porcine blood vessels. OdDHL (1–30 μM) caused a concentration-dependent inhibition of U46619-induced contractions of the coronary artery through a largely endothelium-independent mechanism, but was markedly less effective in the pulmonary artery. Quantitively similar effects to those produced by OdDHL were observed with N-(3-oxododecanoyl)-L-homocysteine thiolactone, a thiolactone derivative, while N-3-oxododecanamide, a lactone-free acyl analogue, possessed 1/3rd the potency as a vasorelaxant. Neither N-butanoyl-L-homoserine lactone nor L-homoserine lactone (up to 30 μM) were active. Our findings indicate that OdDHL inhibits vasoconstrictor tone of both pulmonary and coronary blood vessels from the pig. The vasorelaxant action of OdDHL appears to be primarily determined by the N-acyl chain length, with a minor contribution by the homoserine lactone moiety.

Keywords: N-acyl homoserine lactone; N-(3-oxododecanoyl)-L-homoserine lactone; vascular smooth muscle; quorum-sensing molecules; coronary artery

Abbreviations: AHLs, N-acyl homoserine lactones; BHL, N-butanoyl-L-homoserine lactone; OdDHL, N-(3-oxododecanoyl)-L-homoserine lactone; TNF, tumour necrosis factor; LPS, lipopolysaccharide; $OdDNH_2$, N-3-oxododecanamide; OdDhCysTL, N-(3-oxododecanoyl)-L-homocysteine thiolactone

Introduction It is now recognized that bacteria release a number of diffusible, low molecular weight molecules that allow individual cells to sense population density. This phenomenon, termed 'quorum sensing', is essentially an intercellular signalling mechanism that is integrated with other environmental stimuli, e.g. pH, temperature and oxygen tension, to regulate the phenotype of bacterial animal and plant pathogens (Swift *et al*., 1996). For many gram-negative bacteria, the most investigated quorum-sensing molecules are the N-acyl homoserine lactones (AHLs) which activate transcriptional regulator proteins and so influence gene expression (Winson *et al*., 1995). In the case of *Pseudomonas aeruginosa*, an opportunistic pathogen of immunocompromised individuals, the two principal quorum-sensing molecules, N-butanoyl-L-homoserine lactone (BHL) and N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) are involved in regulating diverse virulence determinants including exotoxin A, elastase, alkaline protease and haemolysin (Winson *et al*., 1995; Williams *et al*., 1996). As such, it has been suggested that the action of quorum sensing molecules represent a potential target for treating gram-negative infection (Finch *et al*., 1988; Hartman & Wise, 1998).

A number of recent observations suggest that the biological action of AHLs may not be restricted to prokaryotic cells. High concentrations of OdDHL (>30 μM) have been reported to stimulate interleukin-8 production in pulmonary epithelial cells (Dimango *et al*., 1995). On the other hand, lower concentrations of OdDHL (<10 μM) inhibited interleukin-12 and tumour necrosis factor (TNF) production in lipopolysaccharide (LPS)-treated murine macrophages and reduced concanavalin-stimulated proliferation of spleen cells (Telford *et al*., 1998). Interestingly, these activities of OdDHL were not shared by N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), a minor, short chain AHL also produced by *P. aeruginosa* and other Gram-negative bacteria (Bainton *et al*., 1992; Swift *et al*., 1996). Since the inhibitory effects of OdDHL on cytokine production were observed with concentrations similar to those found in the media of *in vitro* cultures of *P. aeruginosa* (5 μM; Pearson *et al*., 1995), this quorum sensing molecule may also operate *in vivo* to limit LPS-induced activation of the immune system. Taken together, these observations raise the possibility that other eukaryotic cells may be influenced by AHLs for the benefit of the microorganism. It is generally recognized that expansion of a bacterial colony is critically dependent upon the adequate supply of nutrients. Thus, the vasculature of the host would appear to be a possible target for AHLs.

In the present study we have compared the effect of OdDHL and BHL against vasoconstrictor tone of porcine isolated coronary and pulmonary arteries. In addition, the importance of the lactone ring and the alkyl chain for the vascular activity of quorum-sensing molecules has been assessed independently by evaluating the effects of L-homoserine lactone, N-3-oxododecanamide ($OdDNH_2$), and a thiolactone derivative, N-(3-oxododecanoyl)-L-homocysteine thiolactone (OdDhCysTL), in the coronary artery.

Methods Hearts and lungs from male or female pigs were obtained from a local abattoir within 10 min of the death of the animal and immediately immersed in ice-cold modified

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Krebs–Henseleit saline, previously gassed with 95% O_2/5% CO_2. The organs were then transported to the laboratory. Five to six cm segments (4–5 mm internal diameter) of either the left coronary artery or pulmonary artery were dissected and refrigerated overnight at 4°C in modified Krebs–Henseleit solution. The solution had been previously gassed with 95% O_2/5% CO_2 and contained 2% ficoll to prevent osmotic swelling of the vessel. This storage procedure has been shown to have negligible effect on constrictor and dilator function in isolated blood vessels (Lot & Wilson, 1994).

The following day vessels were cleaned of connective tissue and divided into 5 mm ring segments. Stainless steel wire (0.2 or 0.4 mm thick) supports were then inserted into the lumen and each segment suspended in a 5 ml isolated organ bath containing modified Krebs–Henseleit solution maintained at 37°C and gassed with 95% O_2/5% CO_2. The composition of the modified Krebs–Henseleit saline was (mM) NaCl 118.4, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose. The lower support was fixed, and the upper support connected to a Grass FT-03 transducer which in turn was linked to an AD Instruments Quad Bridge pre-amplifier unit coupled to a Maclab 4e unit running Chart 3.5.4. The results were displayed on a Macintosh LCII computer. After 30 min equilibration, an initial resting tension of 10 g was slowly applied to the coronary artery segments and the tissues allowed to relax. Sixty minutes later the resting tension was finally re-adjusted to 6–7 g. For the pulmonary artery segments, an initial resting tension of 4 g. was applied to each segment 30 min after equilibration, which levelled off to 1.5 to 2 g after a further 30 min.

Each preparation was then exposed to 60 mM KCl and the response allowed to reach maximum. This was repeated on two further occasions until the responses were reproducible. In some experiments using the coronary artery, the endothelium was removed by gently rubbing the lumen of the vessel with a fine pair of forceps. Confirmation of the success of the manoeuvre was based upon the failure of 10 nM substance P to relax submaximal contractions to the thromboxane-mimetic, (1,5), S-hydroxy-11a,9a-(epoxymethano)-prosta-5Z,13E-dienoic acid (U-46619). Following washout of both agents, and an equilibration period of 30 min, each preparation was again stimulated with 5–20 nM U46619 to produce a contraction equivalent to 50–70% of the response to 60 mM KCl. In the case of the coronary artery, OdDHL, L-homoserine lactone HCl, BHL, $OdDNH_2$ or OdDhCysTL (1–30 μM) was then added cumulatively at 30 min intervals or until the response reached equilibrium. Only one drug was used per segment. For the pulmonary artery segments, preliminary experiments indicated that the U46619-induced contractions were less stable than those of the coronary artery and each segment was exposed to a single concentration of OdDHL and the effect determined after a minimum of 20 min.

The effect of the drugs have been calculated as a percentage of the U46619-induced tone, and are expressed as the mean $\pm$ s.e.mean of observations in tissues from different animals. The potency (pIC_{50}) of the agents have been determined as the negative logarithm of the concentration required to produce a 50% reduction of the vasoconstrictor tone. Where necessary, a paired Student's *t*-test was used to assess whether drug-induced effects were statistically significant ($P<0.05$).

The drugs used were: U46619 (Upjohn), substance P (Bachem), Ficoll 70,000 (Sigma) and L-homoserine lactone HCl (Sigma). BHL, OdDHL, N-3-oxododecanamide ($OdDNH_2$) and N-(3-oxododecanoyl)-L-homocysteine thiolactone (OdDhCysTL) were synthesized as described by Chhabra *et al.* (1993). While L-homoserine lactone and BHL, were dissolved in distilled water, all the other AHLs were dissolved in acetonitrile at a concentration of 10 mM and kept on ice during the course of the experiment. The drugs were added to organ baths in a volume of 20 μl or less and the concentration of the vehicle never exceeded 0.3% v v^{-1}.

Results Unless otherwise stated, all experiments were conducted on unrubbed, endothelium intact segments of the pulmonary and coronary arteries. OdDHL (3–30 μM) produced a concentration-dependent relaxation of U46619-induced contractions of the porcine isolated coronary artery. Typically, these responses were characterized by a slow onset, often requiring 30–40 min to reach equilibrium (Figure 1).

As shown in Figure 2a, the relaxation caused by these concentrations of OdDHL was significantly greater than that observed in the vehicle preparations. The maximum response to OdDHL (30 μM) was 86.9 $\pm$ 9.2% with a pIC_{50} of 5.13 $\pm$ 0.14 ($n=7$). OdDHL (30 μM) also produced a slow-developing relaxation of U46619-induced contractions of the pulmonary artery, but the maximum response (41.0 $\pm$ 7.2%, $n=6$) was significantly smaller than that observed in the coronary artery. Furthermore, when compared with the vehicle preparation of the pulmonary artery, neither 3 μM nor 10 μM OdDHL caused a significant response. In view of the more pronounced effects observed in the coronary artery, no further experiments were conducted on the pulmonary artery. Figure 2b shows that the reduction in U46619-induced contraction produced by BHL (3–30 μM) was not significantly different from the vehicle preparation. In this separate series of experiments, OdDHL again produced a concentration-dependent relaxation of U46619-induced contractions of the coronary artery, a response which was reduced slightly, but significantly ($P<0.05$), by endothelium-denudation.

In order to determine the relative importance of the alkyl chain and the lactone ring in the vasorelaxant action of OdDHL, we compared the effect of L-homoserine lactone HCl, $OdDNH_2$, OdDhCysTL and OdDHL against U46619-induced contractions of the coronary artery. L-Homoserine lactone (1–30 μM) did not significantly affect U46619-induced contractions (Figure 3a), but both $OdDNH_2$ and OdDhCysTL caused concentration-dependent relaxations (Figure 3b). Although both agents (at 30 μM) produced greater than 90% inhibition of U46619-induced contractions, $OdDNH_2$ (pIC_{50} 4.98 $\pm$ 0.06, $n=10$) was approximately 1/3rd as potent as either OdDhCysTL (pIC_{50} 5.50 $\pm$ 0.11, $n=6$) or OdDHL (pIC_{50} 5.40 $\pm$ 0.08, $n=10$).

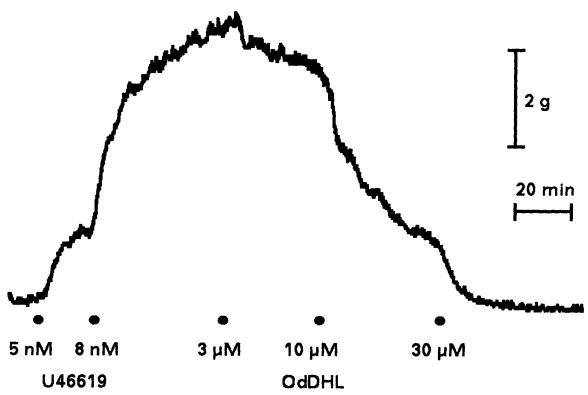

Figure 1 Representative digitized recordings of the effect of OdDHL on U46619-induced contractions of the porcine isolated coronary artery.

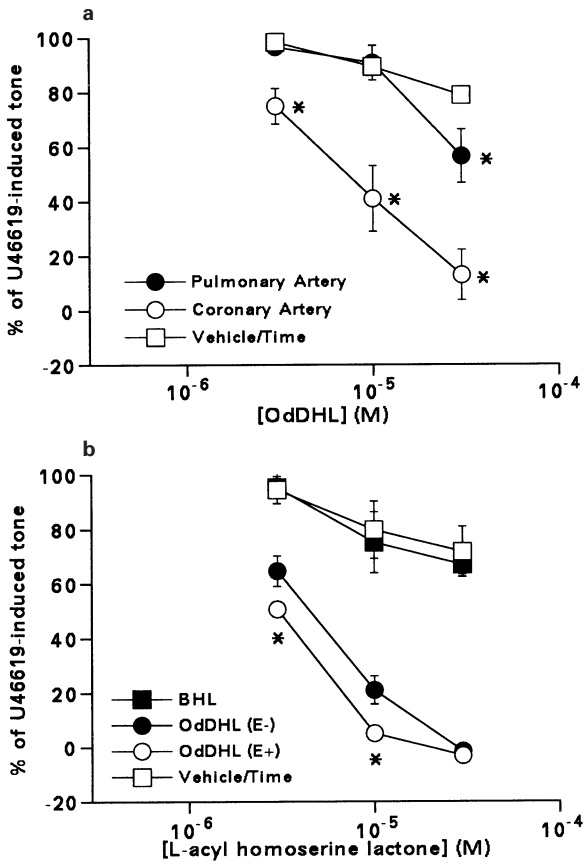

Figure 2 (a) A comparison of the effect of OdDHL on U46619-induced contractions of the porcine isolated pulmonary and coronary artery. * - Denotes a statistically significant difference from the time/vehicle control. (b) A comparison of the effect of OdDHL against U46619-induced contractions in endothelium-intact and endothelium-denuded segments of the porcine isolated coronary and the effect of BHL in endothelium-intact segments. All responses have been calculated as a percentage of the U46619-induced tone in the absence of OdDHL and have been expressed as the mean ± s.e. mean of 5–7 (a) and 6 (b) separate experiments. Also shown is the change in vascular tone over the period of observation associated with the addition of the vehicle.

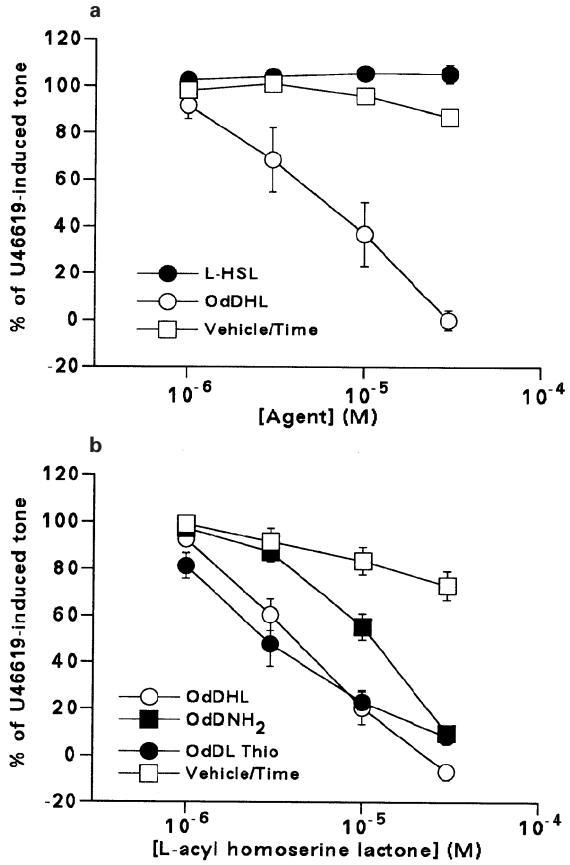

Figure 3 (a) A comparison of the effect of L-homoserine lactone and OdDHL against U46619-induced contractions of the porcine isolated coronary artery. (b) A comparison of the effect of OdDHL, $OdDNH_2$ and OdDhCysTL on U46619-induced contractions of the porcine isolated coronary artery. All responses have been calculated as a percentage of the U46619-induced tone in the absence of OdDHL or related analogue and have been expressed as the mean ± s.e.mean of 4 (a) or 6–10 (b) separate experiments. Also shown is the change in vascular tone over the period of observation associated with the addition of the vehicle.

Discussion We have demonstrated that OdDHL, a quorum-sensing signalling molecule produced by *P. aeruginosa*, possesses vasorelaxant activity in both pulmonary and coronary arteries from the pig. In the latter blood vessel, this effect was observed with concentrations of OdDHL that have been detected in the medium of *in vitro* grown cultures of *P. aeruginosa* at stationary phase, approximately 5 μM (see: Pearson *et al*., 1994), which raises the possibility that it contributes to the ability of the organism to maintain the supply of key nutrients *in vivo*, by increasing local blood flow. Since this action was not shared by either BHL or L-homoserine lactone, it would appear that the length of the N-acyl chain is the primary determinant of the vasorelaxant activity. However, two observations from experiments with synthetic derivatives of OdDHL indicate that the N-acyl chain alone is not sufficient to account for all the inhibitory activity on vascular smooth muscle. First, $OdDNH_2$ exhibited approximately 1/3rd the activity of OdDHL against U46619-induced contractions of the coronary artery. Secondly, OdDhCysTL, a sulphur-containing AHL with quorum-sensing activity (Chhabra *et al*., 1993; McLean *et al*., 1997), was 3 fold more potent than $OdDNH_2$, indicating that the substitution of oxygen by sulphur in the lactone ring does not lead to a loss of vasorelaxant activity. The potential importance of the lactone ring and the keto-substitution on the alkyl chain, is further underlined by the report that palmitic acid (a C_{16} saturated fatty acid) did not affect prostaglandin $F_{2\alpha}$-induced vasoconstrictor tone of the porcine isolated coronary artery (Pomposiello *et al*., 1998). Furthermore, while capric acid (a C_{10} saturated fatty acid) has been shown to inhibit vasoconstrictor tone in the cat basilar artery (White *et al*., 1991), the IC_{50} was approximately 10 fold greater than that for OdDHL in the present study.

OdDHL appears to exert its inhibitory effect principally at the level of the smooth muscle, although a minor contribution of an endothelium-derived vasodilator factor(s) remains a possibility. While we have not determined whether the effect of OdDHL is reversible, in preliminary experiments, ouabain, an inhibitor of Na^+/K^+-ATPase (Kuriyama *et al*., 1995), was found to elicit large contractions in the continued presence of U46619 and OdDHL (unpublished observations). Interestingly, activation of Na^+/K^+-ATPase, and subsequent hyperpolarization of vascular smooth muscle, has been proposed as the mechanism responsible for the vasorelaxant effect of linoleic acid (a C_{18} polyunsaturated fatty acid) on porcine coronary arteries (Pomposiello *et al*., 1998). While vascular

Na^+/K^+-ATPase is a potential site of action for OdDHL, further experiments are also warranted to examine the possible contribution of endothelial or smooth muscle eicosanoids to the vasodilator action. The finding that OdDHL was a less potent vasorelaxant of pulmonary arterial smooth muscle is perhaps surprising, since the lungs of cystic fibrosis patients are a major site of infection for *P. aeruginosa*, but raises the possibility that other vascular beds may exhibit differential sensitivity to quorum sensing molecules. Indeed, a better indicator of the significance of the vasodilator action of OdDHL may be provided by an assessment of its effects against resistance arteries and small veins, particularly those of human origin, rather than conduit arteries.

The biological activity of OdDHL, but not BHL, in vascular smooth muscle is qualitatively similar to the report of Telford *et al.* (1998), who showed that OdDHL, but not OHHL, inhibited LPS-induced TNF production in macrophages. Taken together, these observations suggest that long chain AHL molecules play a critical role in the early stages of infection by *P. aeruginosa*. This may involve not only the regulation of virulence determinants for prokaryotic cells, but also the orchestration of eukaryotic cells to maximise the provision of nutrients *via* the blood without significant activation of the host's defence system. As such, these quorum sensing molecules join a growing list of bacterial products that appear to influence both the immune and cardiovascular systems (Henderson & Wilson, 1996). The inhibitory effects of OdDHL on vascular and immune cells may also have implications for the use of LPS in experimental models of endotoxic shock. Presently, most studies simply involve the use of bolus injections of LPS and an endpoint several hours later (see: Deitch, 1998). However, if Gram negative pathogens implicated in endotoxic shock also produce vasoactive quorum-sensing molecules, the cardiovascular changes in the early stages of this condition may be importantly influenced by the effect(s) of two prokaryotic-derived molecules on vascular and immune cells, rather than the action of LPS alone. Moreover, it is likely that the relative contribution of these two effects will vary during the progression of the condition.

In conclusion, we have demonstrated that OdDHL, a quorum-sensing molecule produced by *P. aeruginosa*, inhibits vasoconstrictor tone of both pulmonary and coronary blood vessels from the pig. This action of OdDHL on vascular smooth muscle appears to be primarily determined by the N-acyl chain length, with a lesser contribution by the homoserine lactone moiety.

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References

BAINTON, N.J., BYCROFT, B.W., CHHABRA, S.R., STEAD, P., GLEDHILL, L., HILL, P.J., REES, C.E.D., WINSON, M.K., SALMOND, G.P.C., STEWART, G.S.A.B. & WILLIAMS, P. (1992). A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic synthesis in *Erwinia*. *Gene.,* **116,** 87–91.

CHHABRA, S.R., STEAD, P., BAINTON, N.J., SALMOND, G.P.C., STEWART, G.S.A.B., WILLIAMS, P. & BYCROFT, B.W. (1993). Autoregulation of carbapenem biosynthesis in Erwinia carotovora ATCC 39048 by analogues of N-(3-oxohexanoyl)-L-homoserine lactone. *J. Antibiotic,* **46,** 441–454.

DEITCH, E.A. (1998). Animal models of sepsis and shock: a review and lessons learned. *Shock* **9,** 1–11.

DIMANGO, E., ZAR, H.J., BRYAN, R. & PRINCE, A. (1995). *Diverse Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J. Clin Invest.,* **96,** 2204–2210.

FINCH, R.G., PRITCHARD, D.I., BYCROFT, B.W., WILLIAMS, P. & STEWART, G.S.A.B. (1998). Quorum sensing–a novel target for anti-infective therapy. *J. Antimicrobial Chemotherapy,* **42,** 569–571.

HARTMAN, G. & WISE, R. (1998). Quorum sensing: a potential means of treating gram-negative infections? *Lancet,* **351,** 848–849.

HENDERSON, B. & WILSON, M. (1996). Cytokine induction by bacteria: beyond lipopolysaccharide. *Cytokine* **8,** 269–282.

KURIYAMA, H., KITAMURA, K. & NABATA, H. (1995). Pharmacological and physiological significance of ion channels and factors that modulate them in vascular tissue. *Pharmacol. Rev.,* **47,** 387–573.

LOT, T.Y. & WILSON, V.G. (1994). Overnight storage of the porcine isolated splenic artery enhances endothelium-dependent contractions to N^G-nitro-L-arginine methyl ester without impairing endothelium-dependent dilator function. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **349,** 95–100.

MCLEAN, K.H., WINSON, M.K., FISH, A., TAYLOR, A., CHHABRA, S.R., CAMERA, M., DAYKIN, M., SWIFT, S., LAMB, J., BYCROFT, B.W., STEWART, G.S.A.B. & WILLIAMS, P. (1997). Quorum sensing in *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology,* **143,** 3703–3711.

POMPOSIELLO, S.I., ALVA, M., WILDE, D.W. & CARRETERO, O.A. (1998). Linoleic acid induces relaxation and hyperpolarization of the pig coronary artery. *Hypertension,* **31,** 615–620.

PEARSON, J.P., GRAY, K.M., PASSADOR, L., TUCKER, K.D., EBERHARD, A., IGLWESKI, B.H. & GRENNBERG, E.P. (1995). A second N-acyl homoserine lactone signal produced by *Pseudomonas aeroginosa*. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 1490–1494.

SWIFT, S., THROUP, J.P., WILLIAMS, P., SALMOND, G.P.C. & STEWART, G.S.A.B. (1996). Quorum sensing: a population-density component of bacterial phenotype. *Trends Biochem. Sci.,* **21,** 214–219.

TELFORD, G., WHEELER, D., WILLIAMS, P., TOMKINS, P.T., APPLEBY, P., SEWELL, H., STEWART, G.S.A.B, BYCROFT, B.W. & PRITCHARD, D. (1998). The *Pseudomonas aeruginosa* quorum-sensing signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone, has immunomodulatory activity. *Infection and Immunity* **66,** 36–42.

WHITE, R.P., EL-BAUMONY, A.M. & WOOD, W.B. (1991). Capric acid as a potent dilator of canine vessels *in vitro* and *in vivo*. *Gen. Pharmacol.,* **22,** 741–748.

WILLIAMS, P., STEWART, G.S.A.B., CAMERA, M., WINSON, M.K., CHHABRA, S.R., SALMOND, G.P. & BYCROFT, B.W. (1996). Signal transduction through quorum sensing in *Pseudomonas aeruginosa*. In: T. Nakazawa, K., Furukawa, D., Haas & S. Silver (eds). *Pseudomonas: Molecular Biology and Biotechnology*. American Society for Microbiology: Washington D.C. pp. 195–206.

WINSON, M.K., CAMERA, M., LATFI, A., FOGLINO, M., CHHABRA, S.R., DAYKIN, M., BALLY, M., CHAPON. V., SALMOND, G.P.C., BYCROFT, B., LAZDUNSKI, A., STEWART, G.S.A.B. & WILLIAMS, P. (1995). Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 9427–9431.

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