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Regulation of the *xcp* secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*

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Summary

The virulence of the opportunistic pathogen *Pseudomonas aeruginosa* is largely dependent upon the extracellular production of a number of secreted proteins with toxic or degradative activities. The synthesis of several exoenzymes is controlled in a cell-density-dependent manner by two interlinked quorum-sensing systems. Their secretion across the outer membrane occurs through the Xcp translocation machinery. The *xcp* locus located at 40 min on the chromosome consists of two divergently transcribed operons, namely *xcpPQ* and *xcpR* to *xcpZ*. In this study, transcriptional fusions were constructed between the *xcpP* and *xcpR* genes and the *lacZ* reporter. Transcriptional activation of the *xcpP* and *xcpR* genes in *P. aeruginosa* is growth-phase dependent and the *lasR–lasI* autoinduction system is required for this control. In the heterologous host *Escherichia coli*, the *lasR* gene product, together with its cognate autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), activates both the *xcpP–lacZ* and the *xcpR–lacZ* gene fusion. The second *P. aeruginosa* quorum-sensing modulon *rhlR–rhlI* (*vsmR–vsmI*) is also involved in the control of the *xcp* genes. Expression of the *lacZ* fusions is strongly reduced in PANO67, a pleiotropic mutant defective in the production of *N*-acyl-homoserine lactones responsible for the activation of RhlR. Furthermore, introduction of the *lasR* mutation in PANO67 results in additional diminution of *xcpR* transcription, indicating that the two systems can regulate their target genes independently. These data demonstrate that expression of the *xcp* secretion system depends on a complex regulatory network involving cell–cell signalling which controls production and secretion of virulence-associated factors.

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Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen responsible for a diverse range of infections in patients compromised by defects in host defences. This Gram-negative bacterium is the major pathogen associated with persistent pulmonary infections in patients with cystic fibrosis. A wide variety of extracellular enzymes and toxins contribute to the virulence of *P. aeruginosa* (Liu, 1974). To be released from the cell, these exoproteins must cross the double membrane that surrounds this Gram-negative bacterium. The majority, including exotoxin A, the LasA and LasB proteases, lipase and phospholipase C are secreted via a two-step secretion pathway (Tommassen *et al*., 1992; Wretlind and Pavlovskis, 1984). The amino-terminal signal sequences found in precursors direct these proteins across the inner membrane in the same way as periplasmic proteins. The second step across the outer membrane requires the products of at least 12 *xcp* secretion genes. Homologous secretion systems have been found in several other Gram-negative pathogens, including *Erwinia* species (Reeves *et al*., 1993), *Vibrio cholerae* (Overbye *et al*., 1993) and *Aeromonas hydrophila* (Howard *et al*., 1993). This conserved two-step secretion mechanism has become known as the general secretory pathway (GSP) (Pugsley, 1993). In most bacterial species, the genetic organization of the secretion genes is similar, consisting of large operon structures of 12 or 13 genes. However, genetic analysis of the *xcp* cluster located at 40 min on the chromosome of *P. aeruginosa* revealed a different genetic organization. Nine *xcp* genes, *xcpR* to *xcpZ*, are arranged in a single operon structure, whereas *xcpP* and *xcpQ* are co-transcribed divergently with respect to the *xcpR* to *xcpZ* operon (Akrim *et al*., 1993; Bally *et al*., 1992; Filloux *et al*., 1990).

Like other pathogens, *P. aeruginosa* has evolved regulatory mechanisms governing the expression of virulence-associated genes in response to multiple environmental parameters (Mekalanos, 1992). For example, iron or phosphate limitation influences the production of several

extracellular proteins (Vasil *et al*., 1985). Phospholipase production is induced by osmoprotective compounds derived from the lung surfactant phosphatidylcholine (Shortridge *et al*., 1992). Growth phase also appears to regulate expression of several exoproducts in *P. aeruginosa* (Bjorn *et al*., 1979; Whooley *et al*., 1983). The common occurrence of growth-phase-dependent regulation of virulence determinants in diverse bacterial pathogens suggests that it may have an important role during the infection processes (Chen *et al*., 1995; Pirhonen *et al*., 1993). It has recently become apparent that a signalling mechanism called 'quorum sensing' is involved in the transcriptional activation of genes in concert with cell density (Fuqua *et al*., 1994; Salmond *et al*., 1995). Quorum sensing relies on the accumulation of autoinducers (*N*-acyl-substituted homoserine lactones, AHLs). The synthesis of AHLs is directed by two regulatory genes: the signal generator, or *luxI* homologue, and the response regulator, or *luxR* homologue. In the marine symbiont *Photobacterium fischeri*, LuxI is responsible for the production of the *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) which is thought to interact with the regulator LuxR (Meighen, 1991). The LuxR–OHHL complex will in turn activate the transcription of *luxI* and the bioluminescence structural genes. This activation occurs only under conditions in which the diffusible autoinducer signal molecule has accumulated to a critical concentration in the surrounding environment. In *P. aeruginosa*, two separate quorum-sensing systems have been identified. The *lasR–lasI* system co-ordinately regulates genes encoding elastase (*lasB*) (Passador *et al*., 1993), LasA protease (*lasA*) (Toder *et al*., 1991), and alkaline protease (*aprA*) and increases expression of the exotoxin A (*toxA*) gene (Gambello *et al*., 1993). LasR is activated by *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), the synthesis of which depends on the LasI protein (Pearson *et al*., 1994). A second AHL-dependent circuit, containing the *rhlR–rhlI* (*vsmR–vsmI*) genes, is also involved in the regulation of virulence-associated exoproducts. RhlI directs the synthesis of two AHL molecules, predominantly *N*-butanoyl-L-homoserine lactone (BHL) and a small amount of *N*-hexanoyl-L-homoserine lactone (HHL) (Winson *et al*., 1995). RhlR positively regulates a considerable range of virulence factors, including elastase, alkaline protease, chitinase, rhamnolipids, cyanide and pyocyanin production (Latifi *et al*., 1995; Ochsner and Reiser, 1995). Thus, two AHL-dependent regulons both regulate several common target genes in response to cell density. Such regulatory circuits are assumed to facilitate a primitive form of intercellular communication that enables *P. aeruginosa* to launch a concerted attack upon the host (Passador *et al*., 1993).

In this report, we have studied the regulation of the *xcpPQ* and *xcpR* to *xcpZ* secretion operons. The analysis of *xcpP* and *xcpR* gene expression in *P. aeruginosa*, using *lacZ* transcriptional fusions as reporters, clearly shows growth-phase regulation. Futhermore, our results demonstrate that *xcp* gene control involves autoinduction circuits, thus adding secretion to the rapidly growing number of cellular processes regulated through quorum sensing. In *P. aeruginosa*, the genes encoding the GSP appear to be integrated into a complex network that co-ordinates the synthesis of virulence factors and the expression of the machinery responsible for their extracellular localization.

Results

Growth-phase regulation of xcp *genes*

To provide a sensitive assay of *xcpPQ* and *xcpR* to *xcpZ* operon expression, we constructed two transcriptional fusions with the *lacZ* reporter gene (see the *Experimental procedures*). As the two *xcp* operons are transcribed in opposite directions (Akrim *et al*., 1993), a 0.4 kb DNA fragment encompassing the translational starts of *xcpP* and *xcpR* genes as well as the 219 bp intergenic sequence was cloned in both orientations into the promoter-probe plasmid pMP220 (Spaink *et al*., 1987) (Fig. 1A). This plasmid contains a promoterless *lacZ* gene enabling the promoter activity of fragment cloned upstream to be monitored. The activities of *xcpP–lacZ* and *xcpR–lacZ* fusions were measured during the growth of *P. aeruginosa* PAO1 in a rich medium (tryptic soy broth, TSB). The curves presented in Fig. 1B show that the β-galactosidase activity from both fusions carried on pMPP (*xcpP–lacZ*) and pMPR (*xcpR–lacZ*) increased during the log-to-stationary phase transition. During the exponential phase, *xcpP* and *xcpR* are expressed at low levels, in good agreement with previous results obtained by quantification of *xcp* mRNAs (Akrim *et al*., 1993). The rate of expression of the *xcpP–lacZ* fusion is increased by a factor of 3–3.5 between the end of the exponential phase and the early stationary phase (OD_{600} values of 3–9 absorbance units). The *xcpR–lacZ* expression is induced by a slightly higher factor (about fourfold induction) during the same period of growth. Thus, the expression patterns of *xcpP* and *xcpR* are both growth-phase dependent, exhibiting a marked increase as the cell growth rate begins to slow down, i.e. during the transition into stationary phase.

To confirm that the entry of cell growth into stationary phase is accompanied by an increase in the intracellular concentration of the *xcp* gene products, we monitored the synthesis of XcpR protein by Western blot analysis (Fig. 1C). *P. aeruginosa* PAO1 was grown in TSB medium and sampled periodically along the growth curve. Immunoblotting of total cellular proteins from a constant number of cells shows that XcpR significantly increased at the onset of the stationary phase.

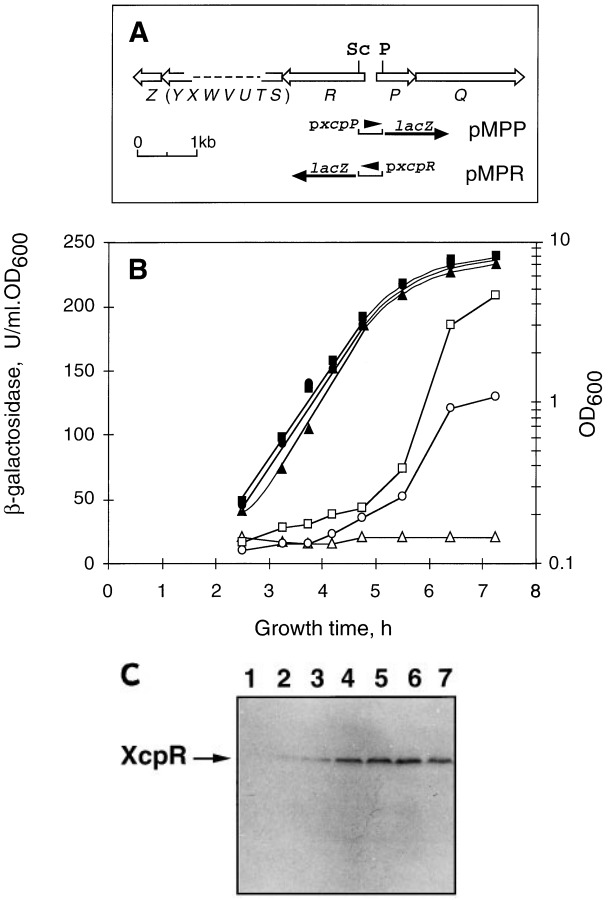

Fig. 1. A. Genetic organization of the *xcp* gene cluster at 40 min. The fragment transcriptionally fused to the *lacZ* gene in pMPP and pMPR plasmids is shown. Sc, *Sca*I; P, *Pst*I.
B. Growth-dependent expression of *xcpP* and *xcpR* genes. *P. aeruginosa* strain PAO1 containing plasmid-borne *xcpP–lacZ* fusion (circles) or *xcpR–lacZ* fusion (squares) was grown in TSB medium at 37°C. The β-galactosidase activities (open symbols) were determined at different phases of the cell growth monitored by measuring the optical density at 600 nm (OD_{600}; closed symbols). The vector plasmid pMP220 (Spaink *et al*., 1987) was used as a negative control (triangles).
C. Growth-phase dependency of the XcpR protein level in PAO1 strain. Cells were grown in TSB medium and harvested at the following optical densities: lane 1, 0.28; lane 2, 0.84; lane 3, 2.2; lane 4, 3.2; lane 5, 4.2; lane 6, 5.5; lane 7, 6.4. Aliquots of lysates corresponding to a constant number of cells were examined by Western blot analysis with anti-XcpR antibodies.

xcp *gene regulation involves diffusible molecules*

The production of several *P. aeruginosa* exoenzymes is regulated such that maximal protein synthesis occurs during the late-exponential growth phase. It has recently become apparent that the regulation of several of these factors involves diffusible chemical molecules released in the medium during the bacterial growth (Passador *et al*., 1993; Winson *et al*., 1995). To determine whether the regulation of the *xcp* genes involved such diffusible factors, the cell-free supernatant of the PAO1 wild-type strain grown to stationary phase was added prior to the inoculation of cultures of PAO1/pMPP and PAO1/pMPR. The results shown in Fig. 2 indicate a marked stimulatory effect of aged PAO1 supernatant on *xcp* gene expression. The addition of 90% (v/v) of supernatant in fresh broth resulted in elevated levels of β-galactosidase activity during the early exponential phase. In such a conditioned medium, expression of the *xcpP–lacZ* and *xcpR–lacZ* fusions was induced at an OD_{600} value of 0.2–0.3 (in comparison to the appearance of significant activity at OD values of 1.2–1.5 in normal growth conditions in TSB). This effect is not due to a different growth rate in the conditioned medium since the doubling times of cells grown either in the presence or in the absence of spent culture supernatant were identical until an OD_{600} of 1 ± 0.3 was reached (not shown). Moreover, an *Escherichia coli* TG1 supernatant used as a control does not induce *xcp* gene expression (Fig. 2). These results suggest that diffusible signalling molecules may be involved in the *xcp* gene control.

Effect of a lasR *mutation on* xcp *expression*

In *P. aeruginosa*, cell-density-dependent expression of elastase, LasA protease and alkaline protease is under the control of *lasR* (Passador *et al*., 1993). Transcription of the corresponding *lasB*, *lasA* and *aprA* genes occurs when the LasR protein is activated by its cognate autoinducer, OdDHL (Pearson *et al*., 1994). To examine whether the stimulatory effect of spent supernatant reflects a role of the *lasR* regulatory system in the control of *xcp* gene expression, we monitored the activities of the two

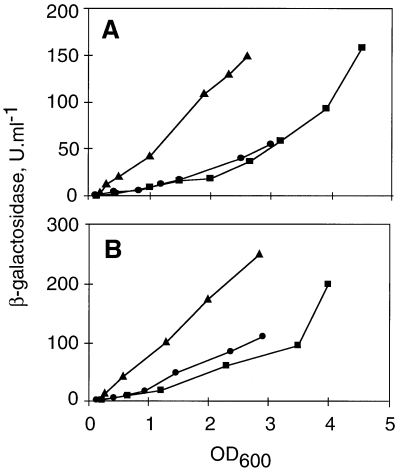

Fig. 2. Stimulation of *xcpP* and *xcpR* gene expression in the presence of spent culture supernatants. The β-galactosidase activities of the *xcpP–lacZ* (A) or *xcpR–lacZ* (B) fusions were determined during growth of PAO1 cultivated either in TSB medium (squares) or in TSB containing cell-free culture supernatant from PAO1 (triangles) or *E. coli* TG1 (circles).

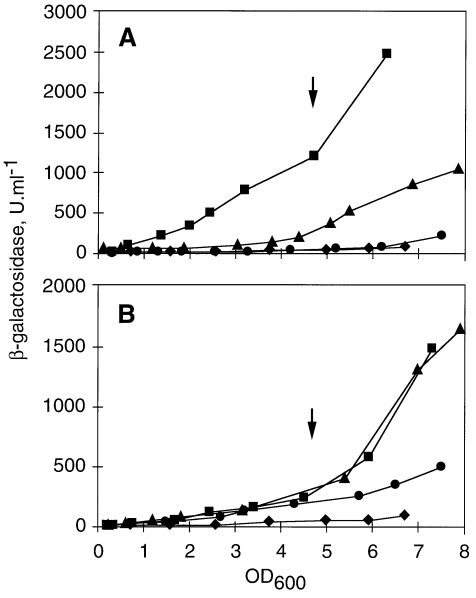

Fig. 3. Effect of *lasR* mutation on *xcpP* and *xcpR* transcription.
A. *P. aeruginosa* strain PAO1 carrying pMPP (*xcpP–lacZ*) (triangles) or pMP220 vector (diamonds), and PAOR strain containing pMPP (*xcpP–lacZ*) (circles) or pMPP.R (*xcpP–lacZ lasR*) (squares).
B. Strain PAO1 carrying pMPR (*xcpR–lacZ*) (triangles) or pMP220 (diamonds), and strain PAOR with pMPR (*xcpR–lacZ*) (circles) or pMPR.R (*xcpR–lacZ lasR*) (squares). Beta-galactosidase activities were assayed as a function of the growth in TSB medium. The transition between exponential and stationary phases of growth is indicated by an arrow.

transcriptional fusions *xcpP–lacZ* and *xcpR–lacZ* in a *lasR* genetic background. To this end, the pMPP and pMPR constructions were separately mobilized into the PAOR strain, which lacks a functional *lasR* gene (Latifi *et al*., 1996). Figure 3 demonstrates that the β-galactosidase activities were reduced in PAOR. In the case of the *xcpR–lacZ* fusion (Fig. 3B), the production of β-galactosidase in early stationary phase was reduced by approx. threefold relative to PAO1. Nevertheless low-level induction was still visible in the late period of growth, suggesting that additional regulatory control mechanisms operate in the absence of LasR. Inactivation of the *lasR* gene has no discernible effect on expression of the fusion during the exponential phase of growth. Introduction *in trans* of a wild-type *lasR* gene (by direct cloning of *lasR* into the pMPR fusion plasmid, giving pMPR.R; Table 1) restored the activity to a level similar to that of the parental strain, PAO1. The *lasR* mutation affects *xcpP* expression more severely than *xcpR*, as the activity of the *xcpP–lacZ* fusion was essentially abolished in PAOR (Fig. 3A). However, when *lasR* was supplied *in trans* (on pMPP.R, see Table 1), there was a marked increase in β-galactosidase activity above the level observed in the wild-type strain. These results indicate that the *xcpP* gene requires a functional *lasR* for efficient transcription. Introduction of multiple copies of *lasR* in PAOR results in growth-phase-independent expression of the fusion; this is perhaps due to an

Table 1. Bacterial strains and plasmids.

Strain/Plasmid	Relevant characteristics	Reference/Origin
Strain		
P. aeruginosa		
PAO1	Wild type, prototroph	Holloway collection
PAOR	PAO1 *lasR*	Latifi *et al.* (1996)
PANO67	PAO1 derivative, elastase negative	Jones *et al.* (1993)
PANO67 *lasR*	*lasR* mutant derived from PANO67	This work
E. coli		
TG1	*supE* Δ(*lac–proAB*) *thi hsdR*Δ*5* (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}ZΔ*M15*)	Maniatis *et al.* (1982)
Plasmid		
pUC19	Ap^R, cloning vector	Laboratory collection
pRK2013	ColE1, Tra$^+$ Mob$^+$, Km^R	Figurski and Helinski (1979)
pMP220	IncP, Tc^R, vector for *lacZ* transcriptional fusions	Spaink *et al.* (1987)
pMPP	0.4 kb *Sca*I–*Pst*I DNA fragment containing the *xcpP* promoter cloned in pMP220	This work
pMPR	0.4 kb *Pst*I–*Sca*I DNA fragment containing the *xcpR* promoter cloned in pMP220	This work
pAL17	1.7 kb *Eco*RI–*Bam*HI *lasR* insert cloned in pLAFR3	Latifi *et al.* (1995)
pAL13	1.3 kb *Pst*I–*Bam*HI *rhlR* insert cloned in pMMB206	Latifi *et al.* (1996)
pMPP.R	pMPP (*xcpP–lacZ*) containing the *lasR* gene on a 1.7 kb *Eco*RI–*Bam*HI fragment from pAL17	This work
pMPR.R	pMPR (*xcpR–lacZ*) containing the *lasR* gene on a 1.7 kb *Eco*RI–*Bam*HI fragment from pAL17	This work
pMW47.1	2 kb *Pst*I DNA fragment containing *rhlRI* genes cloned in pUCP18	Latifi *et al.* (1995)

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tc^R, tetracycline resistance.

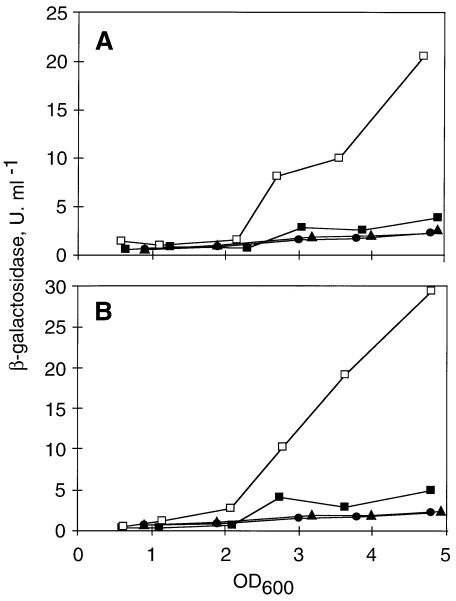

Fig. 4. Effects of *lasR* and OdDHL on *xcpP–lacZ* and *xcpR–lacZ* expression in *E. coli* strain TG1.
A. TG1 harbouring pMPP (*xcpP–lacZ*) (circles), and pMPP.R (*xcpP–lacZ lasR*) in the absence (closed squares) or in the presence (open squares) of synthetic OdDHL.
B. TG1 containing pMPR (*xcpR–lacZ*) (circles), and pMPR.R (*xcpR–lacZ lasR*) in the absence (closed squares) or in the presence (open squares) of OdDHL. The vector pMP220 (triangles) was taken as the negative control for each experiment. Synthetic OdDHL was added to the LB culture medium at a concentration of 30 μM.

autoinducer-independent activation of *xcpP* transcription by LasR since it has been reported that the homologous regulator LuxR can function to some extent in the absence of autoinducer when supplied on a multicopy plasmid (Choi and Greenberg, 1992). Taken together, these results demonstrate the requirement for *lasR* in the control of *xcp* expression, with a maximal effect on *xcpP* transcription.

Expression of xcpP *and* xcpR *is* lasR*-dependent in* E. coli

To evaluate whether *lasR* plays a direct role in the regulation of *xcpP* and *xcpR*, we tested the expression of the *lacZ* fusions in the heterologous host *E. coli*. The plasmids pMPP and pMPR containing the *xcpP–lacZ* and *xcpR–lacZ* fusions, and their derivatives pMPP.R and pMPR.R carrying the *lasR* gene, were introduced in *E. coli* strain TG1. Beta-galactosidase production was monitored throughout the growth in Luria–Bertani (LB) medium, in the absence or in the presence of the LasR autoinducer, OdDHL. We draw two conclusions from the curves shown in Fig. 4: (i) the *xcp* fusions on pMPP and pMPR are not significantly expressed in the *E. coli* background, and (ii) *lasR* alone has no effect on *xcp* gene expression. In contrast, addition of synthetic OdDHL to the growth medium caused significant induction of β-galactosidase in TG1/pMPP.R and TG1/pMPR.R. As observed in *P. aeruginosa*, the rate of expression of the *xcpR–lacZ* fusion was greater than that of the *xcpP–lacZ* fusion (1.5-fold factor). The OdDHL-dependent induction was only visible after the cells reached mid-log phase. Presumably the LasR level is limiting during the earlier growth phase, since expression *in trans* of a *lasR* gene cloned under the exogenous *lac* promoter in TG1/pMPP and TG1/pMPR abolished this effect (not shown).

To quantify the response to OdDHL, the production of β-galactosidase in the presence of *lasR* (pMPP.R and pMPR.R) was examined for a range of autoinducer concentrations. The dose–response curves in Fig. 5 demonstrate the dependence of *xcpR–lacZ* and *xcpP–lacZ* on OdDHL concentration in the *E. coli* background. The autoinducer concentration required for half-maximal expression of the two fusions in the heterologous system was similar (roughly 400–500 nM), suggesting that LasR may have the same level of affinity for both the *xcpP* and the *xcpR* promoter. Thus, LasR and OdDHL are sufficient to activate *xcpP* and *xcpR* expression in *E. coli* and, although the magnitude of activation differs between the two *xcp* genes, their sensitivity to autoinducer appears to be similar.

The rhlRI *circuit participates in regulation of the* xcp *genes*

The *rhR–rhlI* (*vsmR–vsmI*) genes are homologous to *lasR–lasI*, and are involved in the regulation of several exoproteins. PANO67 is a PAO1-derived pleiotropic mutant unable to express the *rhlR–rhlI* circuit (Jones *et al*., 1993; Winson *et al*., 1995) but in which *lasR* expression and OdDHL production are unaffected (Latifi *et al*., 1996;

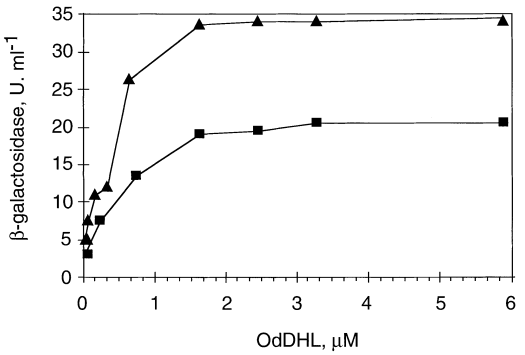

Fig. 5. Comparison of the dose–response expression for *xcpP–lacZ* (squares) and *xcpR–lacZ* fusions (triangles) in *E. coli* TG1. Cells were grown to a density of 5 OD_{600} units in LB medium containing a range of synthetic OdDHL concentrations. Beta-galactosidase activity was plotted against autoinducer concentration.

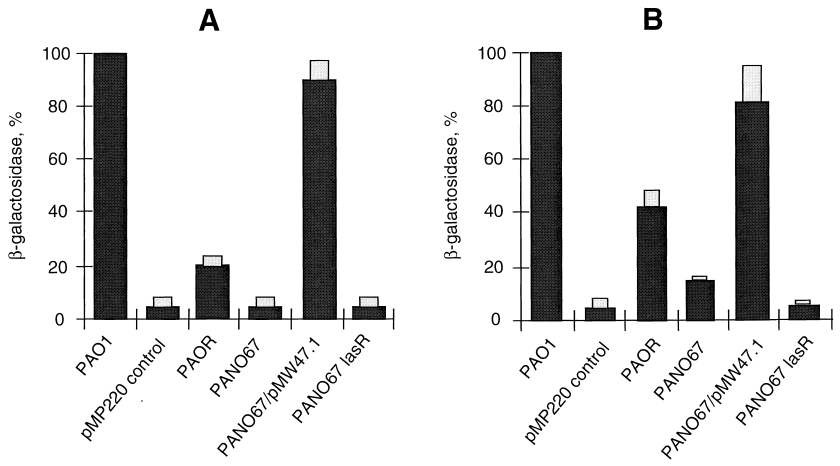

Fig. 6. Expression of the *xcpP* and *xcpR* transcriptional fusions in the *P. aeruginosa* PANO67 strain and analysis of the cumulative effect of a *lasR* mutation. Strains were grown for 8 h in TSB medium, and β-galactosidase assays were performed. Each bar represents the resulting activities for the *xcpP–lacZ* fusion on pMPP (A) or the *xcpR–lacZ* fusion on pMPR (B), expressed as a percentage of the value obtained for PAO1. The plasmid pMW47.1 carries *rhlR* and *rhlI* genes, and allows phenotypic complementation of the PANO67 mutant strain (Latifi *et al*., 1995). Beta-galactosidase activity of PAO1 containing the pMP220 vector is shown as a control in (A) and (B). Mean values are shown, with rectangles at the top showing standard error.

Winson *et al*., 1995). The deficiency in exoprotein production displayed by the PANO67 mutant (Latifi *et al*., 1995) encouraged us to examine the expression of the *xcp* secretion genes in this strain. For this purpose, the *xcpP–lacZ* and *xcpR–lacZ* fusions on pMPP and pMPR were transferred to PANO67. The resulting conjugants were grown in TSB medium, and β-galactosidase activity was quantified. The values obtained demonstrated that the *xcpP–lacZ*-directed β-galactosidase activity was indistinguishable from that of the control vector (Fig. 6A), while the expression of the *xcpR–lacZ* fusion was diminushed by approx. sixfold in comparison to the level observed in the parental strain, PAO1 (Fig. 6B). In the same assay, the β-galactosidase expression in the *lasR* mutant PAOR was reduced by 5- and 2.5-fold for the *xcpP* and *xcpR* fusions, respectively. The introduction of *rhlRI* genes *in trans* into PANO67/pMPP and PANO67/pMPR restored at least 80% of the activity detected in the wild type. These results show that the *rhlR–rhlI* system also positively regulates the transcription of *xcpP* and *xcpR* genes, having a more pronounced effect on *xcpP*. To determine whether RhlR contributes directly to activation of the *xcp* promoters, plasmid pMW47.1 carrying *rhlRI* (Latifi *et al*., 1995) was introduced *in trans* of pMPP and pMPR in *E. coli* TG1. In another set of experiments, we introduced *rhlR* alone cloned under p*tac* control (pAL13; Latifi *et al*., 1996) *in trans* of the *lacZ* fusions, and we measured their activities in the presence of BHL. In both cases, *rhlR* had no effect on the activity of either the *xcpP* or the *xcpR* promoter (results not shown).

As mutations in PAOR or in PANO67 did not abolish expression of the *xcpR–lacZ* fusion, we sought to determine whether *lasR* still regulates the *xcp* genes in a PANO67 background. This is of particular interest because recent studies have revealed that the *lasR–lasI* and *rhlR–rhlI* regulatory circuits are interdependent (Latifi *et al*., 1996). The *lasR* gene in PANO67 was inactivated by the same gene-disruption procedure used to construct the PAOR mutant. In the double mutant PANO67 *lasR*, the expression level of the *xcpR–lacZ* fusion was slightly lower than that in PANO67 (Fig. 6B), indicating that the effect of the two mutations was cumulative.

Discussion

In *P. aeruginosa*, the two quorum-sensing circuits, *lasR–lasI* and *rhlR–rhlI* (*vsmR–vsmI*), are involved in the synthesis of multiple exoproducts, and their regulatory activities clearly overlap. In particular, the expression of elastase and alkaline protease is influenced by both the *lasR–lasI* and *rhlR–rhlI* circuits (Gambello *et al*., 1993; Latifi *et al*., 1995; Passador *et al*., 1993). Here, we demonstrate that the *xcp* genes, encoding the GSP in this organism, are also regulated by the two autoinducer-dependent regulatory systems. In both the *lasR* mutant, PAOR, and in the *rhlRI*-negative background of PANO67, the transcription of the *xcpP* and *xcpR* genes was reduced. Moreover, the expression studies carried out in *E. coli* strongly indicate that LasR, activated by the presence of OdDHL, functions as a transcriptional activator of *xcpP* and *xcpR*.

The *lasR–lasI* and *rhlR–rhlI* circuits are connected via a hierarchical cascade (Latifi *et al*., 1996). The *rhlR* gene is not expressed in a *lasR* mutant and its induction depends directly on LasR–OdDHL. The mutation in PANO67 abolished *xcpP–lacZ* expression and reduced the activity of the *xcpR–lacZ* fusion with a greater magnitude than the *lasR* mutation, even though *lasR* expression and OdDHL production appear unaffected in this strain (Latifi *et al*., 1996). Similarly, the *lasB* gene has been shown to be directly activated by LasR–OdDHL in *E. coli* (Pearson *et al*., 1994), but *lasR* does not allow the expression of *lasB* in the *rhlRI*-deficient background of PANO67 (Latifi *et al*., 1995) or in *rhlRI* mutants (Brint and Ohman, 1995; Ochsner and Reiser, 1995). As the mutation of PANO67

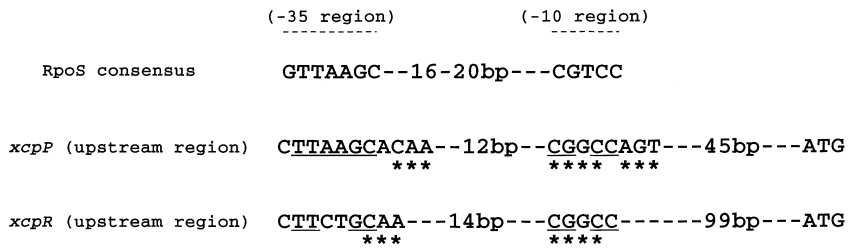

Fig. 7. Comparison of nucleotide sequences in the *xcpP–xcpR* intergenic region and the consensus for RpoS-dependent and gearbox promoters. Conserved −10 and −35 sequences and the gearbox sequence are taken from Lange and Hengge-Aronis (1991). Nucleotides homologous to the RpoS consensus in the upstream region of *xcpP* and *xcpR* are underlined, and the distance to the translation start codon is indicated in base pairs (bp). Homology to the gearbox (CGGCNAGTA) is represented by asterisks.

is not in the *rhlRI* locus, it has been proposed that the defect in this strain might be in an unidentified regulatory gene that influences expression of the *rhlRI* genes (Latifi *et al*., 1996). It can be hypothesized that this regulatory factor is also involved in activation of the *xcp* genes and might directly or indirectly interact with LasR or the *xcp* promoters to regulate their activity. This implies that the *rhlR–rhlI* circuit may regulate the *xcp* genes only indirectly, and may explain the inability of RhlR–BHL to activate the *xcp–lacZ* fusions in *E. coli* although the activation by RhlR–BHL of two target genes (i.e. *rhlI* and *rpoS*) has been demonstrated in the same genetic background (Latifi *et al*., 1996).

The situation becomes even more complex with respect to the expression pattern in the PANO67 *lasR* double mutant. The level of *lasR*-dependent activation in the absence of the second autoinducer regulatory system differs between the two *xcp* promoters. The residual activity of the *xcpR* promoter in PANO67 is essentially abolished by the *lasR* mutation, while the *xcpP* promoter has no detectable activity in a PANO67 background. Although data obtained in *E. coli* indicate that the affinity of LasR for the two promoters could be similar, the requirements for their transcriptional activation might differ in *P. aeruginosa.* A better knowledge of the interplay between the components involved in the multiple quorum-sensing systems of *P. aeruginosa* will certainly be a prerequisite for obtaining a clearer picture of the factors influencing *xcp* expression.

The activation of target promoters by LuxR homologues appears to involve a DNA region of dyad symmetry, termed the *lux* box, that could act as a binding site for the regulatory protein (Fuqua *et al*., 1994). Sequences that resemble the canonical *lux* box have been shown to be of importance for transcription of the *lasB* gene (Rust *et al*., 1996) and to interact with LasR–OdDHL *in vitro* (You *et al*., 1996). A similar motif has been detected upstream of the *lasA* protease gene (Toder *et al*., 1991). Therefore, this suggests that a similar activator site might be recognized by LasR in the promoter sequences of *xcpP* and *xcpR*. However, there is no recognizable *lux* box in the region between the translational start sites of *xcpP* and *xcpR*. This finding indicates that LasR may activate gene transcription in the absence of the putative *cis*-acting element. Such a lack of consensus sequence also exists for *lasI* and *rhlR*, both of which depend on LasR–OdDHL for expression. Determination of the precise operator sequences recognized by LasR in these genes will await DNA-footprinting experiments. Interestingly, the analysis of the *xcpP–xcpR* intergenic region led to the identification of sequences shearing features related to growth-phase-regulated promoters (Fig. 7). The sequence upstream of *xcpP* exhibits good homology to the consensus for the stationary-phase sigma factor RpoS, and also contains a 'gearbox' motif (Lange and Hengge-Aronis, 1991). Similar elements (but conserved to a lesser extent) are also detectable upstream of *xcpR*. These findings could be of particular significance in the context of the recently established link between the *lasR–lasI/rhlR–rhlI* autoinduction systems and *rpoS* gene expression (Latifi *et al*., 1996), and it is tempting to speculate that RpoS exerts a level of regulation that may interface with the AHL-dependent direct control of the *xcp* promoters by LasR. The possibility of an *rpoS*-mediated control of *xcp* gene expression is currently being investigated in our laboratory. Determination of transcriptional start sites and promoter deletion analysis will be required to assess the role of these sequences.

Several exoprotein genes are controlled in response to environmental conditions of growth. For example, iron and zinc have been shown to regulate the expression of elastase (Brumlik and Storey, 1992). Iron concentration also regulates the transcription of *toxA*, the structural gene of exotoxin A. Two other extracellular exoproteins secreted by the Xcp pathway, phospholipase C and alkaline phosphatase, are produced in low-phosphate conditions (Vasil *et al*., 1985). These multiple forms of regulation modulate exoprotein synthesis independently of the cellular density, and suggest that the *xcp* genes might have to be subject to additional control mechanisms in order to respond to such signals. Preliminary investigations, however, did not uncover any effect of environmental factors such as iron, phosphate or ionic strength. Conversely, as *xcp* expression is not strictly coupled to entry into the stationary phase but is also significant during earlier stages of growth cycle, the secretion capacity may be sufficient in conditions in which the quorum-sensing activation will

not operate. We have previously observed that elastase produced from a multicopy plasmid is secreted during the exponential growth phase (Akrim *et al*., 1993). Toder *et al.* (1994) reported that LasA protease is secreted by PAO1 in the absence of a functional *lasR* gene. The exotoxin A produced in the natural *lasR*-negative strain PA103 is also efficiently secreted into the extracellular medium (Lory *et al*., 1983), while the level of *xcp* expression in this strain is similar to that observed in the *lasR*-mutant PAO1 strain (M. Akrim, unpublished). These data indicate that the basal expression of the *xcp* genes may be a safety feature allowing the bacteria to modulate the exoprotein production in response to cell-density-independent stimuli.

The exoenzyme production by the plant pathogen *Erwinia carotovora* is regulated in a growth-phase-dependent manner by the *exp* locus (Jones *et al*., 1993; Pirhonen *et al*., 1993). The *expR–expI* genes co-ordinately control the synthesis of the plant cell wall-degrading enzymes by an AHL-mediated signal pathway similar to the *lasR–lasI* and *rhlR–rhlI* autoinduction systems of *P. aeruginosa* (Salmond *et al*., 1995). These enzymes are secreted through the Out secretion system, which is homologous to the Xcp pathway (Reeves *et al*., 1993). These similarities lead to the suggestion that the *E. carotovora outC* to *outO* secretion operon might also be regulated via the *expR–expI* autoinduction system. However, to our knowledge, such AHL-dependent co-ordinate regulation has not been demonstrated in this organism.

In the recent years, the spectrum of physiological traits controlled by quorum sensing has expanded rapidly (Fuqua *et al*., 1994; Salmond *et al*., 1995). Here, we report the first demonstration that genes encoding the GSP in *P. aeruginosa* are regulated via AHL-dependent circuits. Our data strengthen the concept of quorum sensing as a key regulatory process involved in the adaptation of *P. aeruginosa* as a pathogen. Such a balanced expression of virulence determinants and secretion genes at high cell density may contribute to a rapid elevation of exoenzyme levels capable of overwhelming the host defences.

Experimental procedures

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this work are described in Table 1. Cells were grown at 37°C in TSB (Difco) for *P. aeruginosa* and LB medium for *E. coli*. Plasmids were mobilized in *P. aeruginosa* strains by using the conjugative properties of pRK2013 (Figurski and Helinski, 1979). Where required for plasmid maintenance, antibiotics were added at the following concentrations: tetracycline (Tc), 15 $\mu g\,ml^{-1}$ and kanamycin (Km), 25 $\mu g\,ml^{-1}$ for *E. coli*; Tc, 200 $\mu g\,ml^{-1}$ and carbenicillin (Cb), 300 $\mu g\,ml^{-1}$ for *P. aeruginosa*. Synthetic OdDHL and BHL were synthesized as described before (Winson *et al*., 1995) and added to the growth medium, where required, prior to inoculation.

Construction of the xcpP–lacZ *and* xcpR–lacZ *fusions*

The 0.4 kb *Sca*I–*Pst*I fragment encompassing the translational start codons of *xcpR* and *xcpP* divergent genes (Akrim *et al*., 1993) was cloned upstream of *'lacZ* in vector pMP220 (Spaink *et al*., 1987). Intermediate cloning steps in plasmid pUC19 were performed to obtain the fragment inserted in the two possible orientations into pMP220 digested by *Eco*RI and *Pst*I. For the experiments carried out in *E. coli* and the complementation of the *lasR* mutant strain PAOR, the *lasR* gene contained on a 1.7 kb DNA fragment from pAL17 (Latifi *et al*., 1995) was cloned at the unique *Eco*RI site of pMPP and pMPR, to generate pMPP.R and pMPR.R. For DNA manipulations such as plasmid preparation, restriction digests, DNA electrophoresis, ligation, and transformation, standard procedures were followed (Maniatis *et al*., 1982).

Preparation and use of cell-free supernatants

Cultures of *P. aeruginosa* PAO1 or *E. coli* TG1 were grown in TSB medium for 18 h at 37°C. Cell-free supernatants were prepared by centrifugation (6000 × *g* for 15 min), filter-sterilized through 0.2-μm-pore-size Nalgene filtration units, and their pH values adjusted to 7.5. To support growth, 10% (v/v) of fresh TSB medium was added to sterile supernatant. Strain PAO1 containing the *xcpP–lacZ* or *xcpR–lacZ* fusion was at first grown in TSB to an OD_{600} of 0.2 before the cells were centrifuged and used to inoculate the conditioned medium.

Insertional inactivation of lasR *in PANO67*

The 0.4 kb internal *Pst*I fragment from the *lasR* gene in pAL17 (Latifi *et al*., 1995) was cloned into pUC19. As pUC19 cannot replicate autonomously in *P. aeruginosa*, its maintenance depends on its ability to integrate into the chromosome by homologous recombination. The resulting plasmid was introduced into PANO67 by electroporation and the integration events were selected on plates containing Cb (300 $\mu g\,ml^{-1}$). Chromosomal DNA was prepared from putative insertion mutants, and correct integration of the suicide plasmid at the *lasR* locus was verified by Southern blot analysis (not shown).

Beta-galactosidase assays

Overnight cultures of *P. aeruginosa* or *E. coli* TG1 harbouring the *xcp–lacZ* fusions or the control vector pMP220 were diluted to 100-fold in fresh medium containing the appropriate antibiotics. Culture samples were harvested during growth for determination of OD_{600} and β-galactosidase activity. Within the range of growth time studied, OD_{600} values are proportional to living cell number ($\approx 5 \times 10^8$ cells ml^{-1} per OD_{600} unit) as determined by plating aliquots diluted in 0.9% NaCl onto LB agar plates. Cells were centrifuged in microtubes and β-galactosidase activities were measured on SDS/chloroform-treated cells. Briefly, 10–100 μl of cell extract was added to Z buffer (Miller, 1972) to a final volume of 200 μl in a microtitre well. A 40 μl aliquot of *o*-nitrophenyl galactoside (ONPG; 4 mg ml^{-1} solution in phosphate buffer, 0.1 M, pH 7.5) was added and a hydrolysis reaction performed at 28°C for 1–15 min. Reactions were stopped by the addition of 100 μl of

Na_2CO_3 (1 M), before the determination of A_{414} values of blanks and assays on a microtitre plate reader (Ardvison *et al*., 1991). One β-galactosidase unit (U) corresponds to the enzyme activity liberating 10^{-9} mol ONP min^{-1} at 28°C (with $\epsilon = 4.5 \times 10^3\ M^{-1}$). All experiments were repeated at least three times and the data from a representative experiment were plotted.

SDS–PAGE and immunoblotting

Samples were treated as described before (Bally *et al*., 1992) prior to electrophoresis on 11% acrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose membranes before immunodetection (Akrim *et al*., 1993). Reactions with an antiserum directed against XcpR were visualized by means of the ECL chemiluminescence system (Amersham).

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