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Lead Precipitation by *Vibrio harveyi*: Evidence for Novel Quorum-Sensing Interactions

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Three pleiotropic, quorum sensing-defective *Vibrio harveyi* mutants were observed to precipitate soluble Pb^{2+} as an insoluble compound. The compound was purified and subjected to X-ray diffraction and elemental analyses. These assays identified the precipitated compound as $Pb_9(PO_4)_6$, an unusual and complex lead phosphate salt that is produced synthetically at temperatures of ca. 200°C. Regulation of the precipitation phenotype was also examined. Introduction of a *luxO*::*kan* allele into one of the mutants abolished lead precipitation, indicating that the well-characterized autoinducer 1 (AI1)-AI2 quorum-sensing system can block lead precipitation in dense cell populations. Interestingly, the *V. harveyi* D1 mutant, a strain defective for secretion of both AI1 and AI2, was shown to be an effective *trans* inhibitor of lead precipitation. This suggests that a previously undescribed *V. harveyi* autoinducer, referred to as AI3, can also negatively regulate lead precipitation. Experiments with heterologous bacterial populations demonstrated that many different species are capable of *trans* regulating the *V. harveyi* lead precipitation phenotype. Moreover, one of the *V. harveyi* mutants in this study exhibited little or no response to intercellular signals from other *V. harveyi* inocula but was quite responsive to some of the heterologous bacteria. Based on these observations, we propose that *V. harveyi* carries at least one quorum sensor that is specifically dedicated to receiving cross-species communication.

Studies of prokaryotic tolerance and resistance to soluble lead have revealed two general mechanisms. The first of these mechanisms is an active efflux mechanism (14, 37, 43), a frequent resistance strategy employed by bacteria against heavy metals (for a review, see reference 44). The second mechanism appears to be precipitation of the lead in an insoluble form. A number of studies have shown that various bacterial strains sequester lead intracellularly (2, 4, 5, 6, 30, 36, 38, 52), and some of these studies have demonstrated that the lead is localized to the cell membrane or the cell surface (4, 5, 30, 38, 52). Both phosphate-replete and phosphate-starved *Pseudomonas* cultures generate an insoluble material containing both lead and phosphorus, although phosphate-replete cultures are apparently more efficient at expelling the material (6). One study in which *Staphylococcus aureus* was examined demonstrated that lead precipitation occurred in both lead-sensitive and lead-resistant strains; however the resistant strains were more effective at expulsion (30). Formation of the precipitate does not seem to be associated with a decrease in the growth rate (2).

Detailed analyses of microbially precipitated lead compounds are rare. The precipitate produced by *Pseudomonas fluorescens* is known to contain lead and phosphorus, but the stoichiometry has not been reported (6). Aickin et al. (4) identified the precipitate produced by a *Citrobacter* sp. as $PbHPO_4$, whereas Levinson et al. (30) suggested that $Pb_3(PO_4)_2$ is the precipitate produced by *S. aureus*. The one instance in which PbS precipitation has been suggested was a *Klebsiella* strain cultured in phosphate-limited medium (5). The picture that emerges is that a variety of bacteria precipitate lead, most typically as a lead phosphate in the few cases in which the compound has been examined. Depending on the strain and the growth conditions, the precipitate can either accumulate at the cell membranes or be expelled.

Experiments described in this paper revealed that the coastal marine bacterium *Vibrio harveyi* is capable of precipitating lead as an unusual phosphate compound, $Pb_9(PO_4)_6$, and that the process is regulated at least in part by quorum sensing. The quorum-sensing system in *V. harveyi* has been studied extensively (for recent reviews see references 7, 32, and 40). *V. harveyi* is known to secrete two signaling molecules, autoinducer 1 (AI1) and AI2 (9). AI1 is a homoserine lactone (HSL) molecule synthesized by the protein LuxM (9, 15). AI2 is a furanone, very unlike AI1, and is the product of LuxS (41, 49). The levels of AI1 and AI2 in the environment are detected by the LuxN and LuxPQ sensors, respectively (9, 10). In populations in which the cell density is low, the LuxN and LuxQ proteins function as kinases for the LuxU phosphorelay protein (16, 17). In turn, LuxU transfers its phosphoryl groups to the LuxO response regulator (16). Phospho-LuxO is responsible for regulating a number of phenotypes, including colony morphology, bioluminescence inhibition, and siderophore production (11, 31). In populations with high cell densities, the concentrations of AI1 and AI2 are sufficiently high to induce the phosphatase activities of the LuxN and LuxQ sensor proteins

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TABLE 1. *V. harveyi* strains used in this study

Strain	Relevant genotype and/or features[a]	Reference and/or source
BB120	Wild type	8
D1	Derivative of *V. harveyi* strain B392; defective for production of AI1 and AI2	15
RVH3	Spontaneous Zn^r $EDTA^r$ (class I mutant) derivative of BB120	Our laboratory; Tourjee et al., unpublished
RVH4	Spontaneous Zn^r $EDTA^r$ (class I mutant) derivative of BB120	Our laboratory; Tourjee et al., unpublished
RVH6	Spontaneous Zn^r $EDTA^r$ (class I mutant) derivative of BB120	Our laboratory; Tourjee et al., unpublished
RVH221	Rif^r *luxO*::*kan* derivative of BB120	Our laboratory; Tourjee et al., unpublished
RVH233	Rif^r *luxO*::*kan* derivative of RVH4	Our laboratory; Tourjee et al., unpublished

[a] Abbreviations: Zn^r, resistance to Zn^{2+}; $EDTA^r$, resistance to EDTA; Rif^r, resistance to rifampin.

(16, 17). As a result, the levels of the phosphorylated forms of the LuxU and LuxO proteins decline. The consequences of this shift include a decrease in siderophore production and activation of the *luxCDABEGH* luminescence operon (17, 31).

As will be described elsewhere, a collection of *V. harveyi* mutants resistant to extracellular Zn^{2+} (Zn^r) has been isolated (J. A. Tourjee, C. E. Mire, W. F. O'Brien, N. Budnik, and G. B. Hecht, unpublished data). A subset of three of these strains, designated class I mutants, are pleiotropic. In addition to being Zn^r, these strains are also resistant to metal-limited conditions and exhibit altered luminescence phenotypes, and there is evidence that at least some of these phenotypes arise from alterations in the AI1-AI2 quorum-sensing system (Tourjee et al., unpublished). In this paper, we present evidence that all three of the class I mutants hyperprecipitate lead phosphate. Furthermore, data presented here show that *V. harveyi* lead precipitation is under control of LuxO and therefore is subject to regulation by the AI1-AI2 quorum-sensing system. Additional observations suggest that cell-to-cell communication independent of AI1 and AI2 can also influence lead precipitation by *V. harveyi*. We show that these quorum-sensing interactions include a novel *V. harveyi* autoinducer, as well as responses to exclusively heterologous communication molecules.

MATERIALS AND METHODS

Bacterial strains and media. The *V. harveyi* strains used and their relevant properties are shown in Table 1. Sources of all other bacterial strains are indicated below (see Table 3).

V. harveyi strains were typically grown at 30°C in Luria marine (LM) medium containing (per liter) 20 g of NaCl, 10 g of Bacto Tryptone (Difco Laboratories), and 5 g of yeast extract (BBL). When solid medium (LMA) was employed, 15 g of agar per liter was added prior to sterilization. Lead nitrate supplements were added to the medium following sterilization and a substantial period of cooling; in the case of solid media, these supplements were added immediately prior to pouring.

Other bacterial strains were propagated in Luria-Bertani (LB) broth (10 g of NaCl per liter, 10 g of Bacto Tryptone per liter, 5 g of yeast extract per liter), on LB agar plates (LB broth plus 15 g of agar per liter), in nutrient broth (3 g of beef extract per liter, 5 g of Bacto Tryptone per liter), or on nutrient agar (nutrient broth plus 15 g of agar per liter). All plate-based lead precipitation and quorum-sensing assays were carried out on LMA supplemented with 2.5 mM lead nitrate, except as noted below (see Table 3).

The *luxO*::*kan* allele was constructed by inserting a kanamycin resistance cassette (33) into the *luxO* open reading frame by standard in vitro molecular techniques (39). Strains RVH221 and RVH233 were constructed by introducing the *luxO*::*kan* allele into BB120 and RVH4, respectively, by established protocols (45). The details of these constructions will be presented elsewhere (Tourjee et al., unpublished). We assume that the kanamycin resistance cassette has a polar effect on the *luxOU* operon, resulting in the loss of not just *luxO* but also *luxU* expression.

Preparation of lead phosphate precipitate. A flask containing 1 liter of LM broth supplemented with 2.5 mM lead nitrate was inoculated with 1 ml of an overnight LM broth culture of *V. harveyi* strain RVH6. To ensure maximum production of the brown lead precipitate, the flask was incubated with aeration at room temperature for 2 weeks. Following incubation, cells were harvested by centrifugation. During centrifugation, the cells were collected in such a way that 500 ml of culture was collected in two plastic centrifuge bottles, each of which was subjected to the following procedure. The cell pellets were frozen overnight at −20°C and then lysed at room temperature by using 100 ml of 10% sodium dodecyl sulfate (SDS). Each lysate was centrifuged at 5,000 rpm in a Sorvall SLA1500 rotor for 10 min at room temperature. Because the brown precipitate was insoluble, it was located in the pellet fraction; no significant amount of a brown solid was present in the supernatant. To ensure that there was adequate removal of the cellular debris, each pellet was resuspended and centrifuged in the same way by using 100 ml of 5% SDS, 100 ml of 1% SDS, and 100 ml of 0.5% SDS. At this point, the precipitate was washed six times with distilled water to remove the SDS. During these washes, each pellet was vigorously resuspended by first vortexing it and then horizontally clamping the tightly sealed centrifuge bottle onto a platform shaker rotating at 300 rpm for ≥30 min. Between washes, the precipitate was concentrated by centrifugation at 5,000 rpm in an SLA1500 rotor for 20 min at room temperature; the increased centrifugation time was necessary because the pellets during these stages were less compact than the pellets during the early stages of preparation. Finally, the precipitate was washed two times in distilled and deionized water. During these washes, the pellets were resuspended by strong vortexing only. Centrifugation between washes was carried out by the same procedure that was used for the distilled water washes. After the final centrifugation, the supernatants were decanted, and the pellets were gently washed off the walls of the centrifuge bottles with distilled and deionized water and decanted into two clean, empty plastic petri dishes. To dry the brown solid, the dishes were placed with their lids ajar in a 27°C incubator with strong air circulation for 48 h. The entire procedure yielded a total of 0.3 g of a dark brown powder with some transparent crystalline material that superficially resembled small curved shards of glass.

Analysis and identification of the lead phosphate precipitate. Samples for X-ray diffraction analysis were prepared by smearing well-ground powder on a glass slide precoated with an ultrathin layer of petroleum jelly. Powder X-ray diffraction data were collected with a Scintag X3 diffractometer by using Cu Kα radiation and an Li-drifted germanium detector. Data were collected at a speed of 2 degrees/min. Phase identification was carried out with the library supplied by the Joint Commission on Powder Diffraction Software (JCPDS). Elemental analysis was carried out by Quantitative Technologies, Inc. (Whitehouse, N.J.).

X-ray diffraction of the glass-like fragments that were found in the $Pb_9(PO_4)_6$ preparation described above produced a pattern identical to the pattern obtained for the brown powder.

RESULTS AND DISCUSSION

Class I mutants precipitate $Pb_9(PO_4)_6$. When the class I mutants were tested for resistance to various divalent metal cations (Tourjee et al., unpublished), it was observed that all three strains produced an insoluble brown compound when they were grown for ≥24 h at 30°C on LMA supplemented with 2.5 mM lead nitrate (Fig. 1 and Table 2). Growth of wild-type *V. harveyi* strain BB120 does not appear to be significantly inhibited by these conditions (W.F.O. and G.B.H., unpublished observations), but this strain does not efficiently produce the brown compound (Fig. 1). *V. harveyi* cultures growing on LMA supplemented with sodium nitrate did not

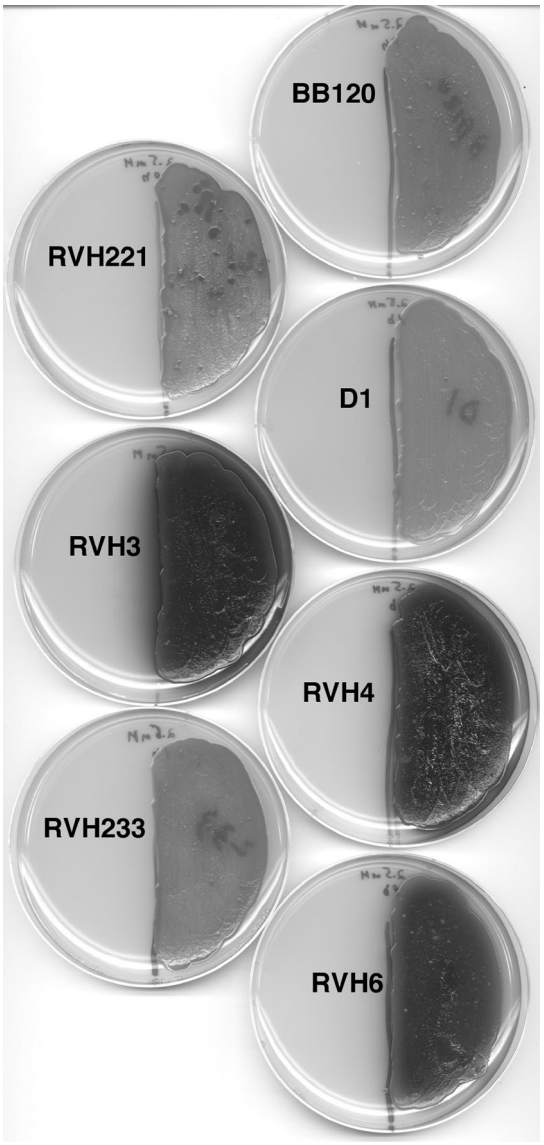

FIG. 1. Lead precipitation phenotypes of *V. harveyi* strains used in this study. Bacteria were inoculated onto one-half of a petri plate containing LMA supplemented with 2.5 mM $Pb(NO_3)_2$. The plates were incubated for 24 h at 30°C and then for 24 h at room temperature.

produce the dark compound, confirming that the presence of Pb^{2+} in the medium was necessary for appearance of the brown compound (data not shown). Observation of LM broth cultures supplemented with lead nitrate showed that the brown material was neither soluble nor free floating in the liquid culture and was instead contained inside the cells.

The brown precipitate was purified as described in Materials and Methods. The X-ray diffraction pattern of the brown precipitate, shown in Fig. 2, was identified by using the JCPDS library of compounds. The close match between the pattern reported for $Pb_9(PO_4)_6$ (JCPDS card no. 33-0768) and our compound gave a preliminary indication that the major crystalline phase in the precipitate is a complex lead phosphate. In order to further confirm the identity of this phase, we carried out elemental analysis for the Pb and phosphorus in the brown precipitate. Within the experimental errors and in view of the variations in the Pb/P ratio reported for this phase (23), the elemental analysis seemed to corroborate the identity of the compound. $Pb_9(PO_4)_6$ is a rare form of lead phosphate and generally is prepared with the aid of hydrothermal synthetic methods at temperatures in the vicinity of 200°C (12, 13). The crystal structure of this compound is reported to be apatite-like and is stable only at temperatures above 170°C (23). The reason for the unusual stability of $Pb_4(PO_4)_9$ observed in the present study is not clear at present. However, it is tempting to attribute the stability to the synthetic route employed here, which is quite different than traditional high-temperature methods. Nevertheless, it is quite fascinating that the mutants provide a facile pathway for the production of complex lead phosphate at temperatures much lower than those required by classical laboratory techniques.

We believe that the *V. harveyi* class I mutants analyzed in this study are the most efficient prokaryotic lead precipitators identified to date, and this facilitated a level of chemical analysis generally not available in previous prokaryotic lead tolerance studies. We observed different degrees of brown precipitate formation by plate cultures of several different eubacteria (G.B.H., unpublished observations), suggesting that $Pb_9(PO_4)_6$ precipitation is a common, albeit not universal, property of bacteria. It is worth noting that at least one previously published comparison of lead-sensitive and lead-resistant bacteria indicated that both types of bacteria precipitate lead as a brown compound (29) and that there does not seem to be a consistent correlation between enzymatic phosphatase activity and generation of a lead phosphate precipitate (3, 29). Taken together, these observations raise the issue of whether lead precipitation confers lead resistance or is simply a chemical consequence of high intracellular lead concentrations. The hyperprecipitating strains described here should be helpful in determining whether lead precipitation is in fact a detoxification mechanism.

Evidence that the *luxOU* operon regulates lead phosphate precipitation. As will be described elsewhere (Tourjee et al., unpublished), we observed that at least some phenotypes of

TABLE 2. Efficiency of lead precipitation by *V. harveyi* strains in the presence of previously inoculated *V. harveyi* strains

Donor strain (primary inoculum)[a]	Amt of lead precipitation with indicator strain (secondary inoculum)[b]:						
	BB120	RVH221	D1	RVH3	RVH4	RVH233	RVH6
None	+/−	+/−	−	+++	+++	+/−	++
BB120	−	−	−	++	+/−	−	+
D1	−	−	−	++	−	−	+/−
RVH3	+/−	+/−	−	++	++	−	++
RVH4	−	−	−	++	+/−	−	+/−
RVH6	−	−	−	++	+/−	−	+/−
RVH221	−	−	−	++	+/−	−	+
RVH233	−	−	−	++	+/−	−	+/−

[a] Tests were carried out by inoculating one half of a sectored LMA–2.5 mM $Pb(NO_3)_2$ plate with the primary inoculum strain and incubating the plate for 24 h at 30°C.

[b] Indicator strains were added to the second half of a preinoculated plate. Data were collected following 48 h of incubation (30°C for the first 24 h and then room temperature for 24 h). The amount of lead precipitation was qualitatively scored by using a scale from +++ (extremely dark brown) to − (no brown color observed).

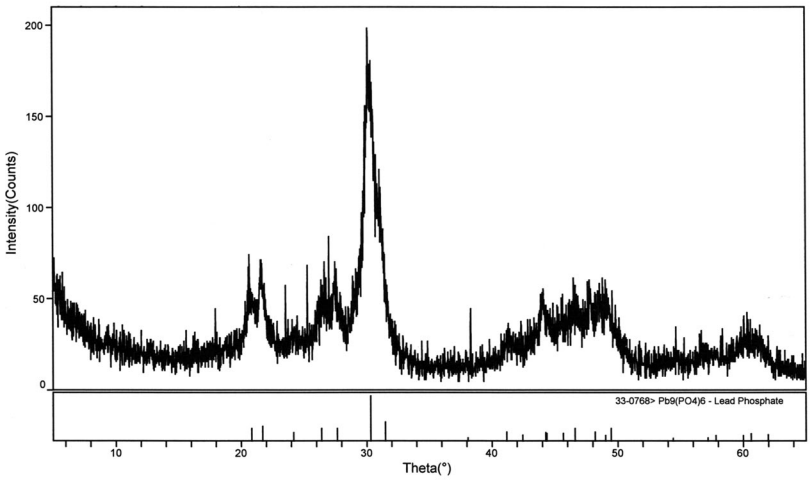

FIG. 2. Powder X-ray diffraction pattern of the brown precipitate purified from a *V. harveyi* batch culture superimposed with vertical drop-down lines corresponding to the expected peak positions of $Pb_9(PO_4)_6$ (JCPDS pattern 33-0768).

the class I mutants are influenced by the *V. harveyi* quorum-sensing system. To determine whether quorum sensing influences the lead precipitation phenotype of these mutants, LMA plates supplemented with 2.5 mM $Pb(NO_3)_2$ were divided in half; one half was inoculated with an autoinducer donor and incubated at 30°C, and 24 h later the second half was inoculated with an autoinducer indicator strain. Following an additional 24 h of incubation at 30°C, the indicator strains were assayed qualitatively for lead phosphate production. The results of these experiments are shown in Table 2 and Fig. 3.

Strain RVH4 was markedly more efficient at lead precipitation when it was grown by itself than when it was grown in the presence of a BB120 donor inoculum (Fig. 3A and Table 2), suggesting that autoinducers from BB120 were inhibitory for this phenotype. RVH6 also exhibited a discernible response to the BB120 inoculum (Table 2), but it was somewhat less responsive than the RVH4 strain. As shown in Table 2, RVH4 and RVH6 were also observed to *trans* inhibit lead precipitation. Importantly, efficient lead precipitation on one half of a petri dish did not reduce the potential for observable precipitation on the other half of the same plate (see below and also the results of the RVH3→RVH3 experiment shown in Fig. 3B and in Table 2).

Because AI1 and AI2 act through the LuxU phosphotransferase and the LuxO response regulator, we also performed a test to determine whether the *luxOU* operon is required for $Pb_9(PO_4)_6$ precipitation. To do this, the lead precipitation phenotypes of a BB120 *luxO*::*kan* derivative (RVH221) and an RVH4 *luxO*::*kan* derivative (RVH233) were assayed. When plated onto LMA supplemented with lead nitrate, both strains were tan rather than dark brown (Fig. 1).

If the $Pb_9(PO_4)_6$ precipitation observed in our experiments was in fact a detoxification activity specifically directed towards Pb^{2+}, then individual wild-type *V. harveyi* cells in a dense population might be predicted to invest fewer resources in Pb^{2+} detoxification than cells whose population density is low invest. The experiments reported here clearly demonstrate that wild-type strain BB120 is capable of *trans* inhibiting lead phosphate precipitation by RVH4 and RVH6. Because the *luxO*::*kan* mutant RVH233 exhibited significantly less precipitation than the parental RVH4 strain, we concluded that the *V. harveyi* AI1-AI2 dual-autoinducer system plays a role in regulating this process and that lead precipitation is normally an activity that is preferentially performed by cells in low-density populations.

Plate-based quorum-sensing experiments also showed that RVH221's ability to *trans* inhibit lead precipitation by RVH4 or RVH6 is apparently identical to that of its parent, BB120 (Table 2). Similarly, the abilities of RVH4 and RVH233 to *trans* inhibit lead precipitation are identical. These observations indicate that the *luxOU* operon is not required for generation of the intercellular signals observed.

Evidence that AI1 and AI2 are not required to *trans* inhibit lead precipitation. To further verify that AI1 and/or AI2 was responsible for inhibiting lead precipitation by RVH4 and RVH6, plate-based quorum-sensing experiments were carried out by using *V. harveyi* strain D1 as the donor inoculum (Table 2). Although the precise nature of the mutations in D1 are not known, the strain was specifically isolated as a *V. harveyi* mutant that was not capable of producing either AI1 or AI2 (15); subsequent experiments independently verified this property of D1 (8). Surprisingly, D1 was quite effective at blocking the lead precipitation phenotype of RVH4 and RVH6 (Fig. 3A and Table 2). Furthermore, if AI1 and/or AI2 were absolutely required for blocking $Pb_9(PO_4)_6$ precipitation, one would expect that the D1 strain itself would produce a dark color when it was plated onto medium supplemented with lead nitrate. To the contrary, D1 appeared to be incapable of precipitating lead in our assays (Fig. 1 and Table 2).

To explain the *trans* signal generated by D1, we considered a model postulating that D1 secretes very low but functionally significant levels of AI1 or AI2. This model would explain the ability of D1 to inhibit lead precipitation in RVH4 and RVH6 by virtue of low levels of residual AI1 and/or AI2 secretion. Indeed, an autoinducer has been detected in spent culture fluid from D1 batch cultures, albeit at levels that are less than 1% of the wild-type *V. harveyi* level (15). However, careful examination of Fig. 3A shows that D1 is in fact slightly more effective

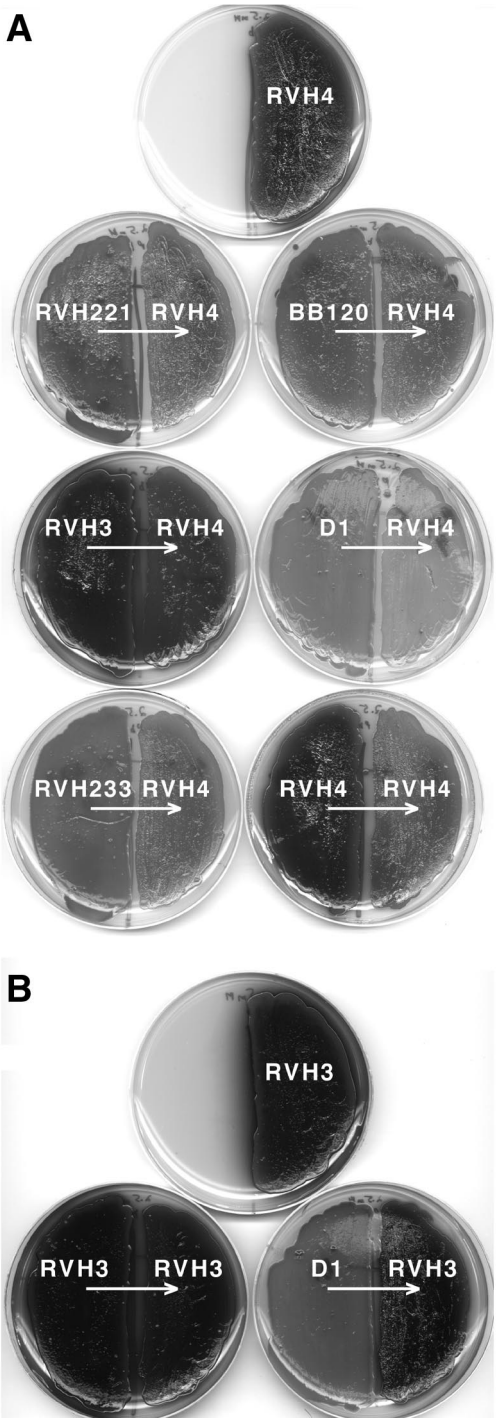

FIG. 3. Responses of lead precipitation mutants to the presence of other *V. harveyi* inocula. In each plate the strain on the left is the primary inoculum and the strain on the right is the indicator strain. All plates contained LMA supplemented with 2.5 mM $Pb(NO_3)_2$. The primary inocula were added and incubated for 24 h at 30°C, after which the indicator strains were added and incubated as described in the legend to Fig. 1. (A) Responses of RVH4 to other *V. harveyi* inocula. (B) Responses of RVH3 to other *V. harveyi* inocula.

at inhibiting lead precipitation than BB120 is. If AI1 or AI2 is the mediator of the D1-generated signal, then it is not obvious how D1 can be a better *trans* inhibitor than BB120 and still exhibit a dark phenotype. Although it is theoretically possible that qualitative plate-based lead precipitation assays may be a more sensitive indicator for AI1 and AI2 than quantitative liquid-based luminescence assays are, this by itself does not appear to provide an obvious explanation for why the *trans* signal generated by D1 is stronger than the signal generated by BB120.

We propose that the simplest explanation for the set of observations reported here is that *V. harveyi* normally secretes a minimum of three autoinducers which act through two distinct quorum-sensing systems. Our data are consistent with the conclusion that the AI1 and AI2 convergent pathways, in addition to regulating luminescence and other previously described phenotypes (9, 31), can also negatively regulate lead phosphate precipitation. To explain the D1 phenotypes described here, we suggest that *V. harveyi* is capable of secreting a third autoinducer, AI3, which is capable of inhibiting lead phosphate precipitation independent of AI1 or AI2. We further propose that although D1 is defective for the production of AI1 and AI2, this strain is still capable of secreting AI3. The D1 phenotype (nonluminescent yet still capable of *trans* inhibiting lead precipitation) suggests that the AI3 signal regulates neither luminescence nor the LuxO regulon. If this model is correct, it seems reasonable to conclude that BB120 is also capable of secreting AI3, although none of the experiments described here directly verified this.

As will be reported elsewhere (Tourjee et al., unpublished), the *V. harveyi* class I mutants appear to behave constitutively as if the population density is low. The observations reported here are also consistent with this observation since lead precipitation appears to be an activity preferentially performed by low-density populations (see above). To explain the RVH4 phenotype, we propose that this strain has a lower rate of autoinducer secretion than either BB120 or D1 has. Because RVH4 does exhibit some *trans* inhibition, it is not entirely unable to secrete an autoinducer(s). However, an RVH4 inoculum grown in isolation (as shown in Fig. 1) contains visible quantities of a lead phosphate precipitate before it is able to produce enough autoinducer to inhibit the process. In our RVH4→RVH4 experiment (Fig. 3A) the situation was different, presumably because the 24 h of incubation used for the primary inoculum permitted accumulation of enough autoinducer to affect the phenotype of the indicator strain. RVH4 is also nonluminescent (Tourjee et al., unpublished), a phenotype that is predicted by this model. RVH6 displays phenotypes that are largely similar to those of RVH4 (Table 2), and we propose a similar mechanism to explain the lead precipitation phenotype of this strain.

Analysis of the lead precipitation phenotype of RVH3. The ability of RVH3 to participate in cell-cell communication was also examined (Table 2 and Fig. 3). The use of RVH3 as a primary inoculum resulted in some inhibition of lead precipitation by the RVH4 and RVH6 indicators (Table 2 and Fig. 3A), although the change was relatively minor. The ability of RVH3 to inhibit lead precipitation was greatly enhanced by incubating the RVH3 primary inoculum for an extra day (data not shown). When RVH3 was used as an indicator strain, it was

observed that lead precipitation by this strain was not significantly inhibited by a preinoculum consisting of BB120, D1, or any other *V. harveyi* strain (Table 2 and Fig. 3B). Thus, RVH3 behaved as if it was poorly responsive to all three of the *V. harveyi* autoinducers that control lead precipitation, suggesting that RVH3 has defects in both the AI1-AI2 response system and the putative AI3 response system. RVH3 was able to *trans* inhibit lead precipitation to some degree and must therefore produce low levels of at least one autoinducer.

Some heterologous species can transmit a signal to RVH4 but not to RVH3. Previous studies (8, 22, 48) have shown that quorum-sensing signals produced by other species have the potential to regulate light production by *V. harveyi*. The ability of heterologous autoinducers to inhibit lead precipitation by RVH4 and RVH3 was tested by using the plate-based assay described above. A few representative results are shown in Fig. 4, and all of the results are summarized in Table 3.

Indeed, it was observed that a wide variety of other bacteria are capable of *trans* inhibiting $Pb_9(PO_4)_6$ precipitation by RVH4. Interestingly, *Vibrio fischeri* strains ATCC 7744 and ES114 exhibited some ability to *trans* inhibit precipitation by RVH4. Some *trans* inhibition was also observed for *V. fischeri* EM17, although the effect was less pronounced than the effect of the other two *V. fischeri* strains (Fig. 4A and Table 3). Consistent with the proposal that RVH3 is a poor responder to intercellular signals, this strain responded weakly or not at all to a preinoculum consisting of any of the three *V. fischeri* strains (Table 3).

Table 3 shows that additional heterologous species were able to *trans* inhibit lead precipitation by RVH4 but not lead precipitation by RVH3. The best examples of this pattern of heterologous signaling included *Escherichia coli* ATCC 43895, *Salmonella enterica* serovar Typhimurium, *Enterobacter aerogenes*, *Bacillus subtilis*, and *Micrococcus luteus*.

The quorum-sensing defects of RVH4 have not been characterized at the molecular level. Thus, it is not known which of the heterologous autoinducers shown in Table 3 mimic AI1, AI2, or the putative AI3. HSL autoinducers seem to be fairly common among the eubacteria and are most often synthesized by members of the *V. fischeri* LuxI protein family (reviewed in references 18, 19, and 53). In contrast, *V. harveyi*'s AI1 HSL is synthesized by a member of a different protein family, LuxM (9). Previously described experiments showed that HSL molecules synthesized by LuxI homologs apparently cannot serve as an alternate to the *V. harveyi* AI1 HSL in liquid culture-based luminescence assays (8, 22). This led to the suggestion that AI1 may serve primarily as a species-specific quorum indicator (7, 32). On the other hand, a wide variety of bacteria carry homologs to LuxS, the enzyme responsible for synthesis of the AI2 furanone autoinducer (41, 49). It has been demonstrated that a heterologous autoinducer from a variety of bacteria carrying *luxS* can induce luminescence in *V. harveyi* (8, 48; for reviews see references 32, 40, and 53). For this reason, it has been suggested that the AI2 furanone autoinducer system may serve in part as a non-species-specific quorum indicator (7, 32, 41).

The data presented here are particularly interesting because cell-free fluid from *V. fischeri* cultures has not been shown to induce luminescence in *V. harveyi* (8, 22). We believe that this report is the first to suggest that intercellular communication may be possible between *V. harveyi* and *V. fischeri*. Consistent with this suggestion is the fact that *V. fischeri* carries *ainS*, a homolog of *luxM* (20). It is important to note that previous *V. fischeri*→*V. harveyi* communication experiments were carried out in liquid medium and in the absence of a toxic metal

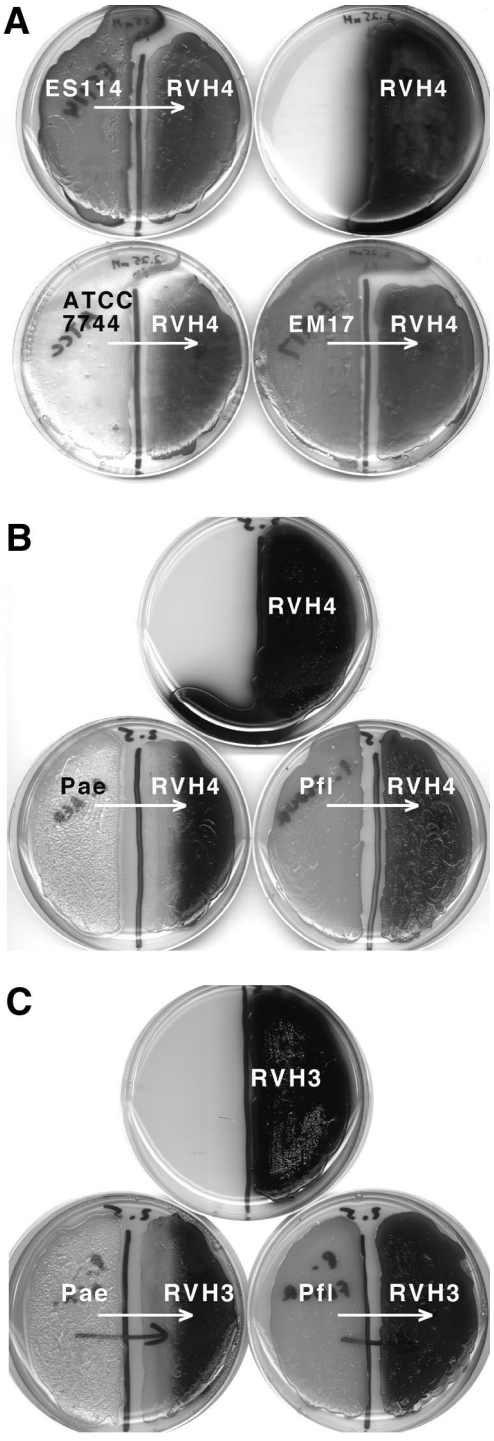

FIG. 4. Responses of lead precipitation mutants to the presence of heterologous bacteria. Experiments were carried out exactly as described in the legend to Fig. 3. (A) Responses of RVH4 to *V. fischeri* strains ATCC 7744, EM17, and ES114. (B) Responses of RVH4 to *P. aeruginosa* and *P. fluorescens*. (C) Responses of RVH3 to *P. aeruginosa* and *P. fluorescens.* Abbreviations: Pae, *P. aeruginosa*; Pfl, *P. fluorescens*.

TABLE 3. Efficiency of lead precipitation by *V. harveyi* strains in the presence of heterologous bacterial species

Primary inoculum[a]		Lead precipitation phenotypes of indicator strain[b]	
Strain	Reported autoinducer system[c]	RVH4	RVH3
None		+++	+++
None[d]		++/+++	+++
Vibrio harveyi BB120	HSL-M, LuxS	+/−	++/+++
Vibrio fischeri strains	HSL-I, HSL-M		
ATCC 7744[d]		+/− and +[e]	++ and +++[e]
EM17[d]		++	+++
ES114[d]		+/− and +[e]	++/+++
Escherichia coli strains	X, LuxS		
ATCC 43895 (O157:H7) (= CDC EDL 932)		+/−	++
DH5α[f]		+++	+++
CC118		++/+++	+++
HB101		+++	+++
MC1061		+++	+++
Salmonella enterica serovar Typhimurium ATCC 49416	X, LuxS	−	+++
Bacillus subtilis ATCC 6633 (= NRS 231)	LuxS, peptide	+	++/+++
Bacillus coagulans ATCC 7050 (= NRS 609)		++	+++
Micrococcus luteus strains	DKP observed for a marine *Micrococcus* sp. but no previous reports of intercellular signaling[g]		
ATCC 272[d]		+	+++
ATCC 4698[d]		+	+++
Proteus vulgaris ATCC 13315	X (LuxS and DKP in *Proteus mirabilis*)[h]	+/−	+/++
Klebsiella pneumoniae ATCC 13883 (= NCTC 9633)	X, LuxS	+/++	+/++
Enterobacter aerogenes ATCC 13048	HSL-I and DKP in *Enterobacter agglomerans*	+	++
Serratia marcescens ATCC 14756 (= PCI 1107)	HSL-I	−[i] and +[e]	− and ++[e]
Serratia liquefaciens ATCC 27592	HSL-I; also shown to respond to exogenous DKP[j]	− and +[e]	++
Citrobacter freundii ATCC 8090	HSL? in *Citrobacter* spp.; DKP	+/−	+
Pseudomonas aeruginosa ATCC 27853	HSL-I, DKP	−[i] and +++[e]	− and +++[e]
Pseudomonas fluorescens ATCC 13525	HSL-I, DKP	+	+/++
Streptomyces griseus ATCC 10137[d]	γBL	+	+
Bacillus sphaericus ATCC 14577[d]		+/++	++
Bacillus megaterium ATCC 14581		++	+++
Bacillus thuringiensis ATCC 10792	Peptide	+++	+++
Bacillus cereus ATCC 14579		++/+++	+++
Staphylococcus aureus ATCC 27661[d]	LuxS, peptide	++	+++
Staphylococcus epidermidis strains	Peptide		
ATCC 146[d]		++/+++	+++
ATCC 155[d,k]		++[l]	+++
Sporosarcina ureae ATCC 13881 (= BS 860)[d]		++	++
Mycobacterium smegmatis ATCC 14468 (= W-113)[d]	LuxS in *Mycobacterium tuberculosis*	+++	++/+++

[a] Unless otherwise specified, all tests were carried out by inoculating one half of a sectored plate with the primary inoculum strain and incubating it for 24 h [2.50 mM $Pb(NO_3)_2$] or for 48 h [2.25 mM Pb $(NO_3)_2$] at 30°C. All strains used as primary inocula were obtained from either Ward's Scientific (Rochester, N.Y.) or Carolina Biological Supply Company (Burlington, N.C.), except as noted in Table 1 or as follows: *V. harveyi* BB120, gift from B. Bassler; *V. harveyi* D1, gift from E. Meighen; *V. fischeri* ES114, *V. fischeri* EM17, and *E. coli* CC118, gift from K. Visick and E. Ruby; *E. coli* HB101, gift from F. Eiserling; *E. coli* MC1061 and *E. coli* DH5α, gift from A. Newton; and *E. coli* ATCC 43895, gift from C. W. Caspar.

[b] Indicator strains were added to the second half of a preinoculated plate. Data were collected following 48 h of incubation (30°C for the first 24 h and then room temperature for 24 h). The amount of lead precipitation was qualitatively scored by using a scale from +++ (extremely dark brown) to − (no brown color observed).

[c] Except where noted otherwise, information was obtained from recent reviews and reports (18, 19, 21, 25, 28, 34, 35, 40, 50, 51) and the University of Nottingham Quorum Sensing web site (http://www.nottingham.ac.uk/quorum/index.htm). Abbreviations: HSL-I, species has been demonstrated or is believed to carry at least one homolog of the *V. fischeri luxI* gene; HSL-M, species has been demonstrated or is believed to carry a homolog of the *V. harveyi luxM* gene; HSL?, species is believed to secrete an HSL of some type, but a homolog of *luxI* or *luxM* has not been reported (this may actually be an observation of diketopiperazine secretion [see reference 24]); X, species has been reported to be negative for secretion of any HSL molecules; DKP, diketopiperazine secretion observed (24); LuxS, species has been demonstrated or is believed to carry a homolog of the *V. harveyi luxS* gene; Peptide, species is known or believed to use a secreted peptide(s) as an autoinducer; γBL, species has been demonstrated to use a γ-butyrolactone molecule(s) as an autoinducer. For some species, the presence of a particular quorum-sensing system is not universal for all isolates. It is important to note that the strains used in this study are not necessarily the same strains used to generate all of the quorum-sensing information cited.

[d] The experiment was carried out on LMA supplemented with 2.25 mM $Pb(NO_3)_2$. Whenever possible, the experiments were carried out on LMA supplemented with 2.5 mM $Pb(NO_3)_2$. However, in some cases, the primary inoculum strain exhibited a growth deficiency in the presence of 2.50 mM $Pb(NO_3)_2$.

[e] Inhibition of lead precipitation by the indicator inoculum was particularly prominent in the region that was nearest the primary inoculum; other areas of the indicator inoculum showed significantly greater lead precipitation, and the demarcation between the two regions of the indicator inoculum was particularly pronounced (see Fig. 4B and C).

[f] Strain known to carry a nonfunctional *luxS* gene (49).

[g] See reference 47.

[h] See reference 42.

[i] Induction of luminescence was exhibited also by the *V. harveyi* indicator inoculum in the region nearest the primary inoculum.

[j] See reference 24.

[k] The primary inoculum was incubated for 24 h at 37°C; the indicator strain was incubated at 30°C and at room temperature as described above.

[l] The strain displayed less lead precipitation when the experiment was carried out with 2.5 mM $Pb(NO_3)_2$ even though the primary inoculum grew more poorly (data not shown).

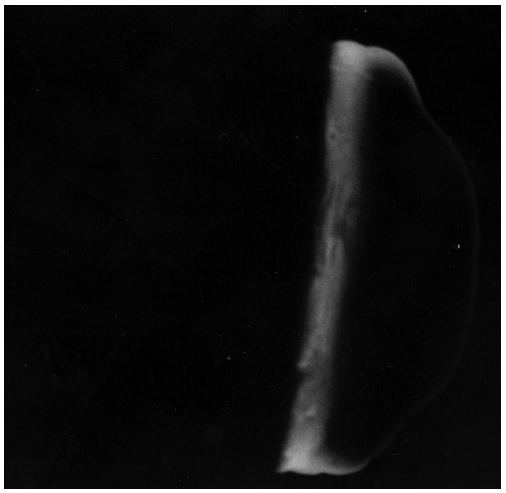

FIG. 5. Induction of luminescence in RVH4 by preinoculum of *P. aeruginosa*. The plate is the same as the Pae → RVH4 plate shown in Fig. 4B.

stressor. The conditions under which our experiments were conducted may have induced different levels of autoinducer secretion by *V. fischeri* or may have induced the production of autoinducers not previously observed for *V. fischeri*.

Similarly, a few cases have been documented in which *luxS*$^+$ bacteria do not induce luminescence in *V. harveyi*. For instance, cell-free fluid from cultures of *Bacillus subtilis* 168 has been reported to not induce luminescence in *V. harveyi* (8). Upon the discovery that *B. subtilis* 168 carries a *luxS* homolog, it was suggested that the inability of this strain to induce *V. harveyi* luminescence may be because the growth conditions used to grow strain 168 were not conducive to production of the AI2-like autoinducer (49). Table 3 shows that *B. subtilis* strain ATCC 6633 was capable of *trans* inhibiting lead precipitation by RVH4. Again, our positive results with *B. subtilis* may have been the consequence of different growth conditions. Alternatively, it may be that the ATCC 6633 strain secretes higher levels of the autoinducer than strain 168 secretes.

Some heterologous species can transmit a signal to both RVH3 and RVH4. Significantly, another group of bacteria were able to *trans* inhibit lead precipitation in both RVH4 and RVH3. Although the level of responsiveness by RVH3 was dependent upon the species employed as the preinoculum, this strain did exhibit a readily observable response a variety of species, including *Serratia marcescens*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *P. fluorescens*, and *Streptomyces griseus* (Table 3 and Fig. 4B and C). This is surprising because RVH3 exhibited little response to autoinducers from other *Vibrio* strains (Tables 2 and 3 and Fig. 3B). This observation may be an indication that *V. harveyi* employs a sensor(s) dedicated to the detection of at least one group of signals that it does not secrete, thereby expanding the range of autoinducer molecules to which it can respond. If this is correct, then RVH3 is apparently defective only in its response to *V. harveyi* autoinducers and can still produce auxiliary sensors devoted exclusively to the heterologous signals detected here. Examples of other bacteria that respond to exclusively heterologous signals have been reported (e.g., *E. coli* and *S. enterica* serovar Typhimurium) (1, 26, 27, 46). Some of the implications of interspecies cross talk have been discussed elsewhere (32, 40, 53).

Several heterologous species exhibited signaling to neither RVH4 nor RVH3. Some examples included *Bacillus megaterium*, *Bacillus cereus*, *Bacillus thuringiensis*, *S. aureus*, *Staphylococcus epidermidis*, *Sporosarcina ureae*, *Mycobacterium smegmatis*, and several domesticated strains of *E. coli* (Table 3). Although most of the nonsignaling strains are gram positive, it is important to note that several gram-positive species did transmit an intercellular signal to *V. harveyi*. Moreover, any correlation between intercellular signaling and phylogeny in Table 3 may simply have been due to the selection of strains used in this study.

Virtually none of the heterologous combinations listed in Table 3 stimulated light induction in RVH4. The only exceptions were *P. aeruginosa* (Fig. 5) and, on a less consistent basis, *S. marcescens* (data not shown). The *P. aeruginosa* induction of luminescence correlated very well with the inhibition of lead precipitation. The plate shown in Fig. 5 is the same as the Pae plate shown in Fig. 4B; the region of the plate that is strongly luminescent corresponds precisely to the region of the RVH4 inoculum in Fig. 4B where lead precipitation was inhibited. None of the heterologous donors listed in Table 3, including *P. aeruginosa* and *S. marcescens*, were able to induce luminescence in RVH3. All of these observations are in agreement with the models which we have proposed for the RVH4 and RVH3 phenotypes.

Summary and model for the regulation of lead precipitation in *V. harveyi*. We have reported that certain quorum sensing-

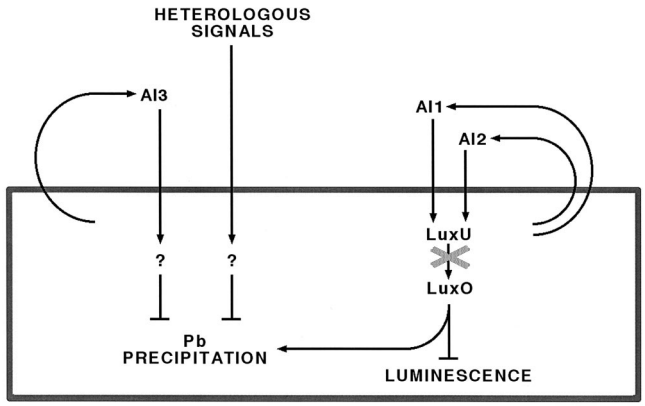

FIG. 6. Model describing the three different types of intercellular communication capable of regulating lead precipitation by *V. harveyi*. The arrows indicate positive regulation, and the vertical lines with horizontal lines at the ends indicate negative regulation. The LuxO response regulator is shown as inhibiting luminescence and positively regulating lead precipitation in response to LuxU phosphotransferase activity. At a high cell density, AI1 and AI2 negatively regulate the LuxU-LuxO system (stippled X). For simplicity, not all components of the LuxU-LuxO regulatory system are shown. In addition to AI1 and AI2, another autoinducer, AI3, is shown. This putative autoinducer negatively regulates lead precipitation but does not influence bioluminescence. Finally, signaling molecules from heterologous species are also shown as negative regulators of lead precipitation. The molecules responsible for receiving the AI3 and heterologous signals are not known and are indicated by question marks. Although the AI3 and heterologous signals are depicted as traversing separate signal transduction pathways, it is possible that the two systems share some components.

defective mutants of *V. harveyi* display a hyperactive lead precipitation phenotype. X-ray diffraction analysis and elemental analysis indicated that the precipitated compound is $Pb_9(PO_4)_6$, a complex form of lead phosphate that has previously been prepared synthetically only at temperatures well outside the range suitable for mesophilic biological processes.

We also investigated the regulation of the lead precipitation phenotype, and a model summarizing our conclusions is shown in Fig. 6. Figure 6 shows three different autoinducer response systems that are capable of regulating *V. harveyi*'s lead precipitation phenotype.

The first of these systems is the well-described AI1-AI2 quorum-sensing circuit that includes the LuxO response regulator. At a low cell density, LuxO is phosphorylated and is responsible for negatively regulating bioluminescence (11, 31). Because disruption of the *luxO* gene blocks lead precipitation, we also believe that phosphorylated LuxO positively regulates lead precipitation. AI1 and AI2 stimulate the dephosphorylation of LuxO, thereby permitting the cell to luminesce and also blocking further lead precipitation. The second response system shown in Fig. 6 is activated by a novel third autoinducer that we designated AI3. AI3 appears to be produced by *V. harveyi* strain D1, but it does not induce luminescence in this strain. Thus, we depict the AI3 response pathway as being independent of luminescence regulation. Finally, to account for the fact that lead precipitation by RVH3 is responsive only to a distinct set of heterologous bacteria, we included a third response system in Fig. 6 that is specific for an autoinducer type(s) that is not produced by *V. harveyi*. We acknowledge that other models consistent with our data may be constructed, but we suggest that Fig. 6 represents the simplest explanation for all of the observations reported here.

The precise mechanism by which quorum sensing regulates lead precipitation is not clear at the present time. In principle, it may be that quorum sensing regulates the availability of inorganic phosphate or controls the synthesis of a structure that serves as a complexing agent. Examination of the mechanism of lead precipitation and of the molecular nature of the quorum-sensing defects of RVH3, RVH4, and RVH6 should be instructive.

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REFERENCES

1. **Ahmer, B. M. M., J. van Reeuwijk, C. D. Timmers, P. J. Valentine, and F. Heffron.** 1998. *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. J. Bacteriol. **180:**1185–1193.
2. **Aickin, R. M., and A. C. R. Dean.** 1977. Lead accumulation by microorganisms. Microbios Lett. **5:**129–133.
3. **Aickin, R. M., and A. C. R. Dean.** 1979. Lead accumulation by *Pseudomonas fluorescens* and a *Citrobacter* sp. Microbios Lett. **9:**55–66.
4. **Aickin, R. M., A. C. R. Dean, A. K. Cheetham, and A. J. Skarnulis.** 1979. Electron microscope studies on the uptake of lead by a *Citrobacter* species. Microbios Lett. **9:**7–15.
5. **Aiking, H., H. Govers, and J. van't Riet.** 1985. Detoxification of mercury, cadmium, and lead in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. Appl. Environ. Microbiol. **50**:1262–1267.
6. **Al-Aoukaty, A., V. D. Appanna, and J. Huang.** 1991. Exocellular and intracellular accumulation of lead in *Pseudomonas fluorescens* ATCC 13525 is mediated by the phosphate content of the growth medium. FEMS Microbiol. Lett. **83:**283–290.
7. **Bassler, B. L.** 1999. A multichannel two-component signaling relay controls quorum sensing in *Vibrio harveyi*, p. 259–273. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
8. **Bassler, B. L., E. P. Greenberg, and A. M. Stevens.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi.* J. Bacteriol. **179**:4043–4045.
9. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Mol. Microbiol. **9:**773–786.
10. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Multiple signalling systems controlling luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:**273–286.
11. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi.* Mol. Microbiol. **12:**403–412.
12. **Bhatnagar, V. M.** 1968. The mineral lead apatites. Bull. Soc. Fr. Mineral. Cristallogr. **91:**479–486.
13. **Bhatnagar, V. M.** 1971. Lead fluorapatite, $Pb_{10}(PO_4)_6F_2$. Mater. Res. Bull. **6:**1–3.
14. **Borremans, B., J. L. Hobman, A. Provoost, N. L. Brown, and D. van der Lelie.** 2001. Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34. J. Bacteriol. **183:**5651–5658.
15. **Cao, J.-G., and E. A. Meighen.** 1993. Biosynthesis and stereochemistry of the autoinducer controlling luminescence in *Vibrio harveyi.* J. Bacteriol. **175:** 3856–3862.
16. **Freeman, J. A., and B. L. Bassler.** 1999. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi.* J. Bacteriol. **181:**899–906.
17. **Freeman, J. A., B. N. Lilley, and B. L. Bassler.** 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi.* Mol. Microbiol. **35:**139–149.
18. **Fuqua, C., and A. Eberhard.** 1999. Signal generation in autoinduction systems: synthesis of acylated homoserine lactones by LuxI-type proteins, p. 211–242. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
19. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. **35:**439–468.
20. **Gilson, L., A. Kuo, and P. V. Dunlap.** 1995. AinS and a new family of autoinducer proteins. J. Bacteriol. **177:**6946–6951.
21. **Gominet, M., L. Slamti, N. Gilois, M. Rose, and D. Lereclus.** 2001. Oligopeptide permease is required for expression of the *Bacillus thuringiensis plcR* regulon and for virulence. Mol Microbiol. **10:**963–975.
22. **Greenberg, E. P., J. W. Hastings, and S. Ulitzer.** 1979. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Arch. Microbiol. **120:**87–91.
23. **Hata, M., F. Marumo, S. Iwai, and H. Aoki.** 1980. Structure of a lead apatite. $Pb_9(PO_4)_6$. Acta Crystallograph. Sect. B Struct. Crystallogr. Cryst. Chem. **36:**2128–2130.
24. **Holden, M. T., S. RamChhabra, R. de Nys, P. Stead, N. J. Bainton, P. J. Hill, M. Manefield, N. Kumar, M. Labatte, D. England, S. Rice, M. Givskov, G. P. Salmond, G. S. Stewart, B. W. Bycroft, S. Kjelleberg, and P. Williams.** 1999. Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. Mol. Microbiol. **33:**1254–1266.
25. **Horinouchi, S.** 1999. γ-Butyrolactones that control secondary metabolism and cell differentiation in *Streptomyces*, p. 193–207. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
26. **Kanamaru, K., K. Kanamaru, I. Tatsuno, T. Tobe, and C. Sasakawa.** 2000. SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **38:**805–816.
27. **Kanamaru, K., K. Kanamaru, I. Tatsuno, T. Tobe, and C. Sasakawa.** 2000. Regulation of virulence factors of enterohemorrhagic *Escherichia coli* O157:H7 by self-produced extracellular factors. Biosci. Biotechnol. Biochem. **64:**2508–2511.
28. **Leonard, B. A. B., and A. Podbielski.** 1999. Emerging density-dependent control systems in gram-positive cocci, p. 315–331. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
29. **Levinson, H. S., and I. Mahler.** 1998. Phosphatase activity and lead resistance in *Citrobacter freundii* and *Staphylococcus aureus.* FEMS Microbiol. Lett. **161:**135–138.
30. **Levinson, H. S., I. Mahler, P. Blackwelder, and T. Hood.** 1996. Lead resis-

tance and sensitivity in *Staphylococcus aureus.* FEMS Microbiol. Lett. **145:** 421–425.
31. **Lilley, B. N., and B. L. Bassler.** 2000. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol. Microbiol. **36:**940–954.
32. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165–199.
33. **Norioka, N., M.-Y. Hsu, S. Inouye, and M. Inouye.** 1995. Two *recA* genes in *Myxococcus xanthus.* J. Bacteriol. **177:**4179–4182.
34. **Novick, R. P.** 1999. Regulation of pathogenicity in *Staphylococcus aureus* by a peptide-based density-sensing system, p. 129–146. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
35. **Perego, M.** 1999. Self-signaling by Phr peptides modulates *Bacillus subtilis* development, p. 243–258. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
36. **Rani, D. B. R., and A. Mahadevan.** 1993. Patterns of heavy metal resistance in marine *Pseudomonas* MR1. Indian J. Exp. Biol. **31:**682–686.
37. **Rensing, C., Y. Sun, B. Mitra, and B. P. Rosen.** 1998. Pb(II)-translocating P-type ATPases. J. Biol. Chem. **273:**32614–32617.
38. **Roane, T. M.** 1999. Lead resistance in two bacterial isolates from heavy metal-contaminated soils. Microb. Ecol. **37:**218–224.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Schauder, S., and B. L. Bassler.** 2001. The languages of bacteria. Genes Dev. **15:**1468–1480.
41. **Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler.** 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. **41:**463–476.
42. **Schneider, R., C. V. Lockatell, D. Johnson, and R. Belas.** 2002. Detection and mutation of a *luxS*-encoded autoinducer in *Proteus mirabilis.* Microbiology **148:**773–782.
43. **Sharma, R., C. Rensing, B. P. Rosen, and B. Mitra.** 2000. The ATP hydrolytic activity of purified ZntA, a Pb(II)/Cd(II)/Zn(II)-translocating ATPase from *Escherichia coli.* J. Biol. Chem. **275:**3873–3878.
44. **Silver, S.** 1996. Bacterial resistances to toxic metal ions—a review. Gene **179:**9–19.
45. **Silverman, M., R. Showalter, and L. McCarter.** 1991. Genetic analysis in *Vibrio.* Methods Enzymol. **204:**515–536.
46. **Sitnikov, D. M., J. B. Schineller, and T. O. Baldwin.** 1996. Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction. Proc. Natl. Acad. Sci. **93:**336–341.
47. **Stierle, A. C., J. H. Cardellina 2nd, and F. L. Singleton.** 1988. A marine *Micrococcus* produces metabolites ascribed to the sponge *Tedania ignis.* Experientia **44**: 1021.
48. **Surette, M. G., and B. L. Bassler.** 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium.* Proc. Natl. Acad. Sci. **95:**7046–7050.
49. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. **96:** 1639–1644.
50. **Swift, S., P. Williams, and G. S. A. B. Stewart.** 1999. N-acylhomoserine lactones and quorum sensing in proteobacteria, p. 291–313. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. American Society for Microbiology, Washington, D.C.
51. **Swift, S., M. K. Winson, P. F. Chan, N. J. Bainton, M. Birdsall, P. J. Reeves, C. E. Rees, S. R. Chhabra, P. J. Hill, J. P. Throup, B. W. Bycroft, G. P. C. Salmond, P. Williams, and G. S. A. B. Stewart.** 1993. A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. Mol Microbiol. **10:**511–520.
52. **Tornabene, T. G., and H. W. Edwards.** 1972. Microbial uptake of lead. Science **176:**1334–1335.
53. **Whitehead, N. A., A. M. L. Barnard, H. Slater, N. J. L. Simpson, and G. P. C. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365–404.