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Acyl-Homoserine Lactones Can Induce Virus Production in Lysogenic Bacteria: an Alternative Paradigm for Prophage Induction

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Prophage typically are induced to a lytic cycle under stressful environmental conditions or when the host’s survival is threatened. However, stress-independent, spontaneous induction also occurs in nature and may be cell density dependent, but the in vivo signal(s) that can trigger induction is unknown. In the present study, we report that acyl-homoserine lactones (AHL), the essential signaling molecules of quorum sensing in many gram-negative bacteria, can trigger phage production in soil and groundwater bacteria. This phenomenon also was operative in a λ lysogen of *Escherichia coli*. In model coculture systems, we monitored the real-time AHL production from *Pseudomonas aeruginosa* PAO1 using an AHL bioluminescent sensor and demonstrated that λ-prophage induction in *E. coli* is correlated with AHL production. As a working model in *E. coli*, we show that the induction responses of λ with AHL remained unaffected when recA was deleted, suggesting that this mechanism does not involve an SOS response. Instead, studies of λ lysogen we also demonstrated that sdiA, the AHL receptor, and recA, a positive transcriptional regulator of exopolysaccharide synthesis, are involved in the AHL-mediated induction process. These findings relate viral reproduction to chemical signals associated with high host cell abundance, suggesting an alternative paradigm for prophage induction.

The reproductive cycle of bacteriophage may be lytic, resulting in the rapid destruction of host cells, or lysogenic, in which the viral genome instead is stably maintained as a prophage in the host genome and replicated as the host cell grows and divides (1). The lysogenic state can be converted to lytic either by various inducing agents, such as mitomycin C (mitC), UV light, antibiotics, and other chemicals (22, 27), or by subjecting the host to physiological stresses such as amino acid deprivation (33). Most inducing agents as well as physiological stresses evaluated to date result in damage to host cell DNA, and the RecA-mediated molecular mechanism of this induction process is well characterized (27, 28). However, RecA-independent inductions also occur in *E. coli* that do not involve an SOS response (41, 45), but the signal(s) that trigger RecA-independent induction is not known.

In environmental samples, it is not possible to directly measure the portion of bacteria that are lysogenic, nor is it possible to determine the number of lysogenic (temperate) phages relative to that of lytic (virulent) phages. Instead, studies of lysogeny in environmental samples involve an estimation of the lysogenic fraction of bacterial populations based on a comparison of viral production and host cell lysis in induced and control samples (60). Estimates of the lysogenic fraction vary widely in aquatic environments (0.7 to 82%), and temporal variations in the prevalence of lysogeny in heterotrophic bacterial populations have been reported in marine environments (13, 62). Seasonal studies of marine cyanobacteria have shown the inducible fraction to be inversely proportional to host cell abundance (31, 32). In other words, lysogeny seemed to be more prevalent in late winter during periods of low host cell abundance. While studies of soils are rather limited, they suggest that the environmental conditions within the soil ecosystem select for lysogeny as a more prevalent reproductive strategy among soil phages (19).

It is believed that lysogeny is an adaptive reproductive strategy that allows viruses to survive in a quiescent state within the cell during suboptimal physiological conditions of the host, especially during situations when host cell abundance is very low (52). This seems particularly relevant in the harsh environment of the soil ecosystem, where extracellular viruses may be rapidly inactivated before infecting a new host. Therefore, there also may exist chemical signals that initiate the lytic cycle under favorable conditions, especially when host cell abundance is high. Such a signal of high host cell abundance should be an SOS-independent response but may exploit the same molecular switch that determines the SOS-dependent lytic/lysogenic decision in well-characterized lambda-like phages (36, 58). In near-surface aquatic environments, UV irradiation is likely an important factor leading to prophage induction. However, this probably is not the case in soil, since virtually all soil prokaryotes reside in intra- and interaggregate pores and thus are protected from UV exposure even near the soil surface. Therefore, alternative prophage induction mechanisms may exist in terrestrial ecosystems.

Quorum sensing in bacteria is a cell density-dependent phenomenon that regulates the coordinated expression of diverse biological phenotypes, such as motility, biofilm formation, chemiluminescence, and the production of toxins, exopolysaccharides, biosurfactants, and other virulence factors (55).
Thus, quorum sensing is a critical component of the adaptive survival and activity of many bacteria as well as an essential factor in the virulence of bacterial pathogens. Interactions of host bacteria with temperate bacteriophage also may influence microbial processes. Most notable are virulence factors of many pathogenic bacteria, such as exotoxins, that are phase encoded (7, 8). Indirect evidence for a link between quorum sensing and the regulation of the lytic/lysogenic switch appeared recently when quorum sensing was shown to increase Shiga toxin (Stx toxin) production along with the transcription of \( \lambda \)-like phage genes in \( E. coli \) O157:H7 (48). In this particular study it was shown indirectly that Stx expression was induced by an SOS response, and genes involved in the SOS response were regulated by quorum sensing (48). Further evidence for this linkage includes the spontaneous induction of prophage during biofilm development (15, 23, 57), the upregulation of phage-related genes in \( Desulfovibrio vulgaris \) during stationary phase, and the induction of prophage Mu in stationary phase (11, 40). In other instances, the spontaneous induction of prophage has been observed as cultures enter stationary phase and conditions associated with high cell density (10, 12, 40, 52, 57), situations in which quorum-sensing compounds for some bacteria might reach threshold concentrations that are necessary for the induction of cell-density-dependent processes.

These results formed the basis for the present study involving the effect of exogenously added AHL on prophage induction in bacteria extracted directly from soil samples, ground-water samples, and bacterial communities colonizing field-deployed porous beads designed to simulate the highly porous nature of the soil environment (19). An induction response also was observed for bacteria grown under pure culture conditions. In coculture systems, we monitored real-time AHL production from \( Pseudomonas aeruginosa \) PAO1 (i.e., no exogenously added AHL) using an AHL bioluminescent sensor and demonstrated that \( \lambda \)-prophage induction in \( E. coli \) was correlated with AHL production. By using single-gene knockout mutations in an \( E. coli \)-\( \lambda \) system, we establish the molecular basis of this induction mechanism, which suggests that AHL-mediated prophage induction is an SOS-independent process and involves an AHL receptor, SdiA (34, 54), and a transcriptional regulator of exopolysaccharide synthesis, RcsA (29).

### MATERIALS AND METHODS

**Bacteria, plasmids, and phages.** \( E. coli \) strain BW25113 (lac\(^{\text{pro}}\) rpsL15 \( \	ext{msbB}_{1716} \text{msbB} \text{BAD}_{1873} \text{BadBAD}_{1913}, \text{BadBAD}_{1776} \) ) was used as the host for prophage induction experiments, and plasmid pKD46 was used for deletion mutagenesis by the method of Datsenko and Wanner (16). These resources were obtained from the \( E. coli \) Genetic Stock Center (Yale University). \( P. aeruginosa \) strains PAO1 (21) and PAO214 (\( \Delta \text{msl} \) ) (20) were kindly provided by Herbert Schweitzer (Colorado State University, Ft. Collins, CO). \( Agrobacterium tumefaciens \) A136 ( \( pCF218 \) ) (\( pMV26 \) ) (9, 47) was a gift from Pamela Sokol (University of Calgary, Calgary, Canada). \( E. coli \) strain MG1655 (F\(^{\text{+}}\) M) with Pir was used for deletion mutagenesis by the method of Datsenko and Wanner (16), were adopted to construct knockout strains.

**Generation of PCR fragments for constructing knockout mutants of the \( \lambda \) lysogen.** The primer design and the PCR-based single-locus deletion method, as described by Datsenko and Wanner (16), were adopted to construct knockout mutants. Primers used for constructing gene deletions consisted of 50 nucleotides (nt) homologous to the adjacent upstream or downstream flanking region of the target gene, followed by the 20-nt sequence upstream or downstream of the kanamycin resistance gene (kan). The N-terminal primer consisted of the 50-nt upstream region of the target gene including the initiation codon (H1) and the 20 nt upstream of the \( \text{kan} \) gene, \( 5'-\text{ATTCGCGGATCTCGCACGAC-3'} \) (P1), whereas the C-terminal primer consisted of 29 nt of the adjacent downstream region plus the C-terminal 21 nt of the target gene, including the termination codon (H2), followed by the 20 nt of \( \text{kan} \) downstream sequence, \( 5'-\text{TGTAGG CGTGGAGGCTTCG-3'} \) (P2). All extensions for individual genes (sdiA, rcsA, and recA) were cut out in this study. The primers were given in Table 1. PCR were carried out in 50-\( \mu \)l reaction mixtures containing 2.5 U of TaKaRa Ex Taq polymerase, 1 ng pKD13 plasmid DNA, 1.0 \( \mu \)M of each primer, and 200 \( \mu \)M dNTPs. Reactions were run for 30 cycles consisting of 94°C for 30 s, 58°C for 30 s, 72°C for 2 min, plus an additional 2 min extension at 72°C after the final

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cycle. PCR products were digested with DpnI, ethanol precipitated, suspended in 6 μl H2O, and analyzed by 1.5% agarose gel electrophoresis using 0.5× Tris-acetate buffer.

Lysogenization and construction of sdiA, recA, and recA knockout mutants. E. coli K-12 BW25113 was lysogenized with λmr1 as described previously (4). Single-gene knockout mutants of the BW25113 lysogen were constructed using the PCR-based direct deletion method described by Datsonko and Wanner (16), with a slight modification of recommended temperatures in various steps. To suppress the heat induction of λ, all incubations were done at 30°C. Deleted genes were verified by PCR with kanamycin-specific primers k1 and k2 and locus-specific primers U and D, as described previously (16) and summarized above. PCR products were analyzed by agarose gel electrophoresis (1.5%).

In situ demonstration of AHL-dependent prophage induction in cocultures of E. coli and P. aeruginosa PAO1. Single colonies from P. aeruginosa PAO1, PAO214 (Δladd), E. coli BW25113 (λmr1), and sdiA, recA, and recA mutants of the same E. coli strain were picked with a sterile loop and placed in 1 ml TSB medium and vortexed for 30 s. Different combinations of E. coli and Pseudomonas cocultures were prepared by mixing 200 μl of an overnight-grown (24 h) culture of A. tumefaciens A136 biosensor and incubated for up to 18 h at 29°C. Samples were taken every 3 h, including the ts0 sample for bacterial (CFU per milliliter) and viral (PFU per milliliter) enumeration and bioluminescence analysis. Luminescence was determined by taking 100-μl samples in glass tubes and measuring them with a portable luminometer (Femtomax FB14; Zylux Corporation, Huntsville, AL). Viruses (PFU per milliliter) were counted as described in the previous section. After viruses were taken, aliquots of the remaining coculture were plated on Levine cosin methylene blue (EMB) (Becton, Dickinson and Co., Sparks, MD) agar plates to determine the number of CFU (CFUs per milliliter) of E. coli and P. aeruginosa (26). E. coli BW25113 produced dark green colonies that were methyl red-positive lactose fermenters. P. aeruginosa, a non-lactose-fermenting bacterium, produced no acid from fermentation, therefore the colonies were lighter colored and translucent, and they could be differentiated from E. coli colonies. The colony color and morphology of pure E. coli and P. aeruginosa also were determined separately after 18 h for comparisons to the cocultures. In a separate control experiment, it was determined that A. tumefaciens could not produce any viable colonies on EMB agar plates in 18 h.

16S rRNA gene-Phylochip analysis. In experiments involving agricultural soil, phylogenetic analyses using 16S rRNA gene microarrays (Phylochip) were performed (5). Three types of samples were prepared and used as a template for the amplification of 16S rRNA genes: (i) DNA extracted directly from the soil representing the entire prokaryotic community; (ii) viral DNA extracted and induced in the presence of the AHL mix- 0.002, respectively) to mitC was comparable to the response elicited by AHL, but a more significant decrease in bacterial abundance (P = 0.0009) was observed (Fig. 1A and B). This observation suggested that at least some fraction of the soil microbial community was inducible by exposure to AHL. The corresponding induction response in the samples exposed to mitC was comparable to the response elicited by AHL, but a more significant decrease in bacterial abundance (P = 0.0009) was observed (Fig. 1A and B). We isolated a bacterium closely related to Sinorhizobium meliloti (as determined by sequencing 1,484 bp of the 16S rRNA gene) from the same
Tennessee soil discussed previously that showed a significant prophage induction response (P/H11005 < 0.0001) when grown in the presence of AHL (Fig. 1C). The induction response elicited by mitC resulted in a much sharper decline in bacterial abundance relative to those of AHL-induced and control samples (P/H11005 < 0.0043) (Fig. 1C). We also monitored a time-dependent induction response in this bacterium. The cultures were sampled at 4-h intervals, and viral abundance increased over time from 6 to 18 h of incubation (Fig. 1D).

The phylogenetic diversity of the bacteria extracted from the Tennessee soil used in these induction assays also was analyzed with a 16S rRNA gene Phylochip microarray. The analysis revealed a highly diverse community dominated by proteobacterial groups (Fig. 2A). In three previous studies, 16S rRNA genes could be detected by PCR in the viral DNA fraction collected from wastewater (42) and soil (19) communities, and a broad-host-range phage also was detected (5), suggesting that at least generalized transducing phage have the capacity to carry the 16S rRNA gene. Thus, we used this approach as a proxy measure of the phylogenetic diversity of potential host bacteria carrying either mitC- or AHL-inducible prophage, and the viral DNA fractions purified from inducible lysogenic bacteria also were analyzed in a similar fashion. A total of 26 phyla were detected in the virus-free bacterial fraction (Fig. 2A). In the induced prophage fractions fewer phyla were detected, but the AHL- and mitC-induced samples had similar distributions of the major phyla common to both samples (Fig. 2B and C). Interestingly, inducible Proteobacteria constituted a smaller percentage of the total chip intensity than the bacterial community (38 and 50%, respectively), suggesting either that not all proteobacterial lysogens were inducible by AHL or mitC or that not all Proteobacteria in the sample contained prophage. Conversely, the relative abundance of Actinobacteria and Firmicutes was overrepresented in the induced samples.

FIG. 1. Induction response upon exposure to AHL and mitC in two microbial communities Tennessee soil and in Sinorhizobium. Shown are viral and bacterial abundances as viral direct counts (VDC) or bacterial direct counts (BDC) in samples incubated with or without AHL and mitC for 18 h in bacteria extracted from KBS soil (A), Tennessee soil (B), and Sinorhizobium (C). Bars are means from triplicate experiments, and vertical bars represent the standard errors. Data sets with different letter designations were significantly different from one another (P < 0.05). (D) Viral production in AHL-induced cultures of Sinorhizobium taken at 0, 4, 8, and 18 h. The data points represent means from triplicate experiments, and the vertical bars represent one standard error. Statistical analyses of viral and bacterial abundance in all samples were performed separately.
compared to the overall bacterial community, possibly indicating that more species within these phyla are inducible by AHL or mitC (Fig. 2B and C). The apparent AHL induction response in the Actinobacteria and Firmicutes is surprising, since gene expression in gram-positive bacteria is not known to be regulated by AHL. Thus, with the data presented here, it is not possible to determine if these bacteria responded directly to the presence of AHL or if there was an indirect effect brought about by the AHL-mediated release of other inducing agents from AHL-responsive bacteria.

**AHL-dependent prophage induction in groundwater community.** To further evaluate the AHL-mediated prophage induction in environmental samples, we tested the induction response in a bacterial community concentrated from a groundwater aquifer undergoing biostimulation with acetate to enhance uranium reduction (Rifle, CO) (3). The viral abundance increased significantly upon the exposure of the groundwater microbial community to either the AHL mixture ($P = 0.0023$) or mitC ($P = 0.0001$) (Fig. 3A). A corresponding decrease in bacterial abundance also was observed, suggesting that the community included a large population of lysogenic bacteria inducible by AHL ($P = 0.0037$) and mitC ($P = 0.0002$) (Fig. 3A).

**AHL-mediated induction in Bio-Sep beads.** To more accurately assess induction responses under soil conditions, we employed Bio-Sep beads as a novel in situ enrichment matrix. Given their high surface area and porosity, Bio-Sep beads mimic the soil environment and previously have been used in many environmental applications as a cell immobilization matrix and to sample and characterize microbial communities from various environments (14, 37). We recently used them to assess the prevalence of lysogeny within soil bacterial communities (19). Beads that previously had been equilibrated in a yeast extract solution were incubated in the field at the same sites where soil samples were taken for the experiment described above. Recovered beads were rinsed in sterile water and used for induction experiments. Induction assays were performed in a buffered soil extract solution that was prepared by extracting the same soils in which the beads were buried. Triplicate bead samples were immersed in their respective soil extracts containing either mitC or AHL. All samples were incubated statically at room temperature in the dark for 18 h.

![FIG. 2. 16S rRNA gene Phylochip analysis of extracted bacteria from Tennessee soil (A) and the DNA fractions of inducible temperate phage concentrated and purified from mitC-induced (B) or AHL-induced (C) bacteria.](http://aem.asm.org/)

![FIG. 3. Viral and bacterial abundances as viral direct counts (VDC) and bacterial direct counts (BDC) in samples incubated with or without AHL and mitC for 18 h in bacteria extracted from groundwater (A) or from soil-incubated Bio-Sep beads (B). Bars are means from triplicate experiments, and vertical bars represent the standard errors. Data sets with different letter designations were significantly different from one another ($P < 0.05$).](http://aem.asm.org/)
Viral production from bacterial communities associated with beads exposed to AHL was similar to that from mitC-treated beads, and the abundance of viruses was considerably greater in the treated samples compared to that of the controls (P < 0.0001) (Fig. 3B). The bacterial count was significantly lower in the mitC-induced samples (P < 0.0015) relative to that of the controls but was increased in the AHL treatments (P < 0.007). The increase in bacterial abundance in AHL-induced samples most likely was due to a stimulatory effect of AHL on the growth of at least a portion of the bacteria in the samples that exceeded the loss of cells due to viral production (49), whereas mitC had an inhibitory effect on bacterial growth in addition to cell loss through viral lysis (Fig. 3B). This result also indicates that only a portion, but not all, of the bacterial community in the beads was inducible by AHL. Recent work indicates that mitC-inducible lysogens comprise ca. 5 to 40% of natural soil bacterial communities (61) or ca. 80% of the community enriched in Bio-Sep beads (19).

**AHL-mediated induction response in the E. coli-λ system and its mutants.** Motivated by the results from environmental samples, we attempted to demonstrate this quorum-sensing mediated prophage induction in the well-characterized λ system of *E. coli* as a model. We lysogenized wild-type *E. coli* BW25113 with λimm434 and examined the inducibility of the lysogen by adding a mixture of six AHL compounds of various chain lengths to the culture medium. The results showed a significant increase (P < 0.0007) in the abundance of phage in the culture supernatant after 18 h of incubation compared to that of the uninduced control culture without AHL (Fig. 4). The initial and final cell counts (CFU) did not decrease significantly (P < 0.41) in the presence of AHL.

Although *E. coli* cannot produce AHL, it has an AHL receptor encoded by *sdiA* that responds to AHL produced by other microbial species (34, 54). To address more directly the involvement of the quorum-sensing receptor, *sdiA* was deleted by single-gene knockout from the same lysogen and the experiment was repeated. The *sdiA* knockout mutant of *E. coli* lysogen showed no increase in phage production after incubation with AHL for 18 h with respect to the uninduced control lacking AHL (Fig. 4). This result directly demonstrates that the quorum-sensing receptor SdiA is required for the AHL-dependent induction of λimm434. mitC-dependent prophage induction was unaffected by the deletion of *sdiA*. A similar induction response with mitC was observed for both the *sdiA* mutant and the wild-type lysogen (Fig. 4), indicating that RecA-mediated SOS induction does not require SdiA. To confirm this conclusion, we knocked out the key component of the SOS cascade, *recA* (28), from wild-type *E. coli*. A positive induction response was observed when the sample was incubated with AHL, but mitC failed to induce the lysogen (Fig. 4). This indicates that AHL-mediated induction does not involve
any DNA damage-dependent mechanism controlled by recA. Unlike mitC-induced samples, however, the bacterial count in all AHL-dependent inductions did not decrease significantly (P = 0.3 to 0.4) (Fig. 4).

We acknowledge that mitC-induced E. coli should result in at least 100-fold more phage particles than we observed. The probable reason for a low titer is the readsoption of phage by the surviving and growing E. coli population during the 18-h incubation. In a separate time course induction experiment using mitC, we observed that the phage titer increased significantly and peaked during the first 3 h of exposure and subsequently decreased over the next few hours (data not shown). With AHL as the inducing agent provided via coculture with P. aeruginosa PAO1, significant viral production was not observed prior to 6 h of incubation, at which time the phage titer began to increase (Fig. 5). Exogenously added AHL also yields results similar to those of coculture experiments (data not shown). As these experimental conditions were not optimal for mitC induction, we conducted a control experiment under optimal induction conditions in which the cells of the same lysogen were harvested at mid-log phase (optical density at 600 nm = 0.6), washed twice with medium, and incubated with mitC (1 μg ml⁻¹) for 4 h. The supernatants from these incubations produced at least 10³-fold more phage than the uninduced control, whereas the recA mutant of this lysogen did not exhibit an increase in phage production after 4 h of incubation with mitC (data not shown). These results indicate that the model E. coli-λ system was functionally equivalent to other canonical model systems that have been used to investigate RecA-mediated prophage induction.

FIG. 5. Time-dependent prophage induction in E. coli BW25113 λ lysogen (A) or its knockout mutants of recA (B), sdiA (C), and rcsA (D) when cocultured with either Pseudomonas aeruginosa PAO1 or its las knockout mutant, P. aeruginosa PAO214. The production of N-butanoyl-l-homoserine lactone and N-3-oxo-dodecanoyl-homoserine lactone (AHL) was monitored in situ using a bioluminescent reporter strain, A. tumefaciens A136, carrying the traI-luxCDABE plasmid grown in all cocultures with other bacteria (solid red lines). Supernatant of each coculture experiment also was analyzed in vitro for AHL concentration by incubation with the biosensor separately (broken red lines). Strain designations in parentheses in the key indicate the strain of P. aeruginosa cocultured with E. coli. Values plotted are means from triplicate experimental cultures, and the error bars are one standard deviation.
In situ demonstration of AHL-dependent prophage induction in cocultures of *E. coli* and *P. aeruginosa* PAO1. To determine if AHL-mediated prophage induction could occur in the absence of exogenously added AHL but from AHL generated in coculture (i.e., cell-to-cell communication), we conducted batch experiments containing bacterial cul-
tures of *P. aeruginosa* PAO1, which produces N-butanoyl-L-
-homoserine lactone (C\textsubscript{4}-AHL) and N-3-oxo-dodecanoyl-
-homoserine lactone (3-oxo-C\textsubscript{12}-AHL), *E. coli* \(\lambda\textsuperscript{mm34}\) lysogen inducible by AHL, and *A. tumefaciens* A136 (9, 47) containing the *traI*-lucCDABE construct that produces light (lumi-
nescence) at a level that is directly proportional to the AHL concentration. The production of phage and luminescence was monitored every 3 h for 18 h in two coculture systems, one with wild-type *P. aeruginosa* PAO1 that actively produces AHL, *E. coli* \(\lambda\) lysogen, and *A. tumefaciens* A136, and the other with *P. aeruginosa* PAO1 replaced by a *lasI* knock-
-out mutant of PAO1 (PAO214) that could not produce AHL (25, 38). In both systems, the initial cell density of *P. aerugi-
 nosa* was standardized (Fig. 5A). In both cocultures the *E.
coli* \(\lambda\) lysogen was provided from the same stock suspension made by vortexing a single colony of *E. coli* in TSB, and *A.
tumefaciens* was inoculated to the coculture as a 1:500 dilu-
tion from a single overnight culture. The level of the pro-
duction of \(\lambda\) lysogen in the first system was five- to sixfold greater than that in the second system containing the *lasI* mutant and was similar to the induction response observed in the presence of exogenously added AHL (Fig. 4 and 5A). In the first system, AHL synthesis monitored by biolumines-
cence in situ reached a maximum at 6 h, while the second system containing a *lasI* mutant of PAO1 did not produce any light, which is consistent with the inability of this mutant to produce AHL (Fig. 5A). In a control experiment that included only the AHL bioreporter strain and the *lasI* mut-
ant of strain PAO1, no luminescence was detected, con-
firming that neither the biosensor (*A. tumefaciens*) nor *P.
aeruginosa* PAO214 was able to produce any AHL comp-
ounds. The onset of an increase in phage production in the first system coincided with the maximal AHL production (Fig. 5A). In a pair of similar coculture experiments, a *recA* knockout mutant of the same \(\lambda\) lysogen also showed an increase in phage production similar to the increase in luminescence when incubated with *P. aeruginosa* PAO1, but phage abundance did not increase when incubated with the *lasI* mutant of PAO1, and no luminescence was detected (Fig. 5B). In all cases, the increase in viral production oc-
curred between 6 and 15 h (Fig. 5A and 5B). However, in a similar system the *sdiA* knockout mutant of the \(\lambda\) lysogen did not show any increase in phage production with either wild-
type *P. aeruginosa* PAO1 or the *lasI* mutant of *P. aeruginosa* PAO1 (Fig. 5C). In all coculture experiments, the initial cell numbers of *P. aeruginosa*, *E. coli*, and *A. tumefaciens* were standardized, and the initial cell counts (CFU per milliliter) were similar to those of the control cultures (Fig. 5A, B, and C). In all cases, the luminescence decreased significantly after 9 h, probably because *A. tumefaciens* grew more slowly or was inhibited in coculture with *P. aeruginosa* and *E. coli*. Thus, the entire set of bioluminescence measurements was repeated by taking archived cell-free aliquots of culture supernatants and incubating them with *A. tumefaciens* A136 separately. As in the in situ measurements, luminescence (i.e., AHL concentration) peaked at 6 h and declined there-
after but was sustained at a level above 1,000 relative lumi-
nescence units throughout the experiment (Fig. 5A, B, and C). In this relatively simple model system, the results con-
firm that AHL produced by a different species can influence prophage induction in *E. coli* and directly demonstrate the involvement of *sdiA*, the AHL receptor of *Enterobacteria-
ceae* (56).

*P. aeruginosa* PAO1 is known to produce other potential SOS-dependent inducing agents, such as quorum-sensing-con-
trolled toxins, rhamnomipid, cyanide, and pyocyanin (18, 24, 39, 43). However, these cannot be responsible for the induction response we observed in coculture experiments, because the AHL-dependent viral production was unaffected in the *recA* mutant (Fig. 5B). As previously demonstrated with *A. tumefaciens* (2), we observed no effect on the growth of *E. coli* in TSB amended with filtered culture supernatants from *P. aeruginosa* PAO1 relative to that of unamended control cultures (data not shown). This result confirms that the induction of the \(\lambda\) lysogen was not due to a potential SOS response created by toxins but from AHL produced by *P. aeruginosa* PAO1. Furthermore, were this the case, we should have observed a comparable induction response in coculture experiments with the mutant *P. aeruginosa* PAO214 that is compromised only in its ability to produce AHL. No such induction response was observed.

Probable molecular induction mechanism in \(\lambda\) involves *SdiA* and *RcsA*. A positive transcriptional regulator of exopo-
lysaccharide synthesis, *rcsA*, is the only regulator known to be directly involved in SOS-independent spontaneous \(\lambda\) induction in *E. coli* (41). In *Pantoea stewartii*, the expression of *rcsA* was shown to be directly dependent on the AHL concentration (35). These previous findings led to the hypothesis that *rcsA* is also involved in AHL-mediated prophage induction in *E.
coli*. To test this, we constructed an *rcsA* knockout mutant of the \(\lambda\) lysogen used in the previous experiments. When this lysogen was exposed to exogenous AHL (Fig. 4) or cocultured with *P. aeruginosa* PAO1 (Fig. 5D), phage production did not increase compared to that of their respective controls that had either no AHL (Fig. 4) or were cocultured with a *lasI* mutant of *P. aeruginosa* PAO1 (Fig. 5D). The AHL production in the coculture of *rcsA* mutant lysogen and PAO1 was monitored based on luminescence with *A. tumefaciens* A136 biosensor and showed a trend similar to that of the cocultures of wild-type or *recA* mutant lysogens (Fig. 5A, B, and D) but decreased more than that in the cocultures of *sdiA* mutant lysogens (Fig. 5C and D). This result may indicate that when the functional SdiA re-
ceptor is present, it binds AHL, thereby reducing the extracellular AHL concentration and leading to the lower luminescence values. Taken together, these findings directly demonstrate that *rcsA* is an essential component of the quorum-sensing circuit that in-
duces prophage.

Notably, the *rcsA* mutant showed significantly less sponta-
neous induction (no AHL added) than the wild type (Fig. 4), which is consistent with results obtained by Rozanov et al. (41). We did not complement the *rcsA* knockout mutant with an *RcsA*-overexpressing plasmid, since this was performed al-
ready by Rozanov et al. (41) and was shown to increase sponta-
neous prophage induction in a complemented \(\lambda\) lysogen of *E. coli*. 

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In contrast, we noticed a higher spontaneous induction (at least twofold) in the sdiA mutant relative to the wild type. To restore its original phenotype, we complemented the mutation by transforming it with overexpressing sdiA-containing plasmid and monitored induction with AHL. A small but significant decrease in spontaneous induction relative to that of the sdiA mutant was observed (Fig. 6A). However, in our results, the complementation of this mutation did not fully restore the wild phenotype when it was incubated with AHL (Fig. 6A). To determine how AHL-bound SdiA affects RcsA, we monitored RcsA expression during induction by AHL via immunoblotting with polyclonal anti-RcsA. In the wild-type λ lysogen, RcsA expression increased in the presence of AHL (Fig. 6B). From this result we hypothesized that SdiA is a negative regulator of the rcsA promoter (35), as it is in Pantoea stewartii, that becomes derepressed when AHL binds to a SdiA homolog (EsaR) (35). If this hypothesis is correct, an increase in RcsA expression should be expected in the sdiA mutant relative to that in the wild type. However, our analysis showed no difference in RcsA expression (Fig. 6B). Likewise, in the complemented sdiA mutant no difference in RcsA expression was observed relative to that of the sdiA mutant or the wild type. These two results suggest that sdiA does not have any direct negative or positive regulatory role in RcsA expression. However, RcsA expression did not increase in the sdiA knockout mutant when AHL was applied (Fig. 6B), suggesting that SdiA is required for AHL-mediated RcsA expression. Thus, there must be intermediate transducers involved in carrying AHL-SdiA-mediated signal to activate rcsA for prophage induction. From these results a working model could be deduced for E. coli where sdiA and rcsA play the terminal roles in the AHL-mediated signal transduction of RecA-independent prophage induction. It has been demonstrated that SdiA is insoluble unless it is bound to AHL, but it appears to have a regulatory role in many other physiological processes in its insoluble state (64). It is unclear at this point how the insoluble fraction of SdiA, as would be the case in the absence of AHL which cannot be produced by E. coli, is involved in spontaneous prophage induction.

Conclusion. Our findings are the first report of an in vivo chemical signal that can trigger the lytic/lysogenic switch under conditions indicative of high host cell density, thereby maximizing the probability of subsequent infection by progeny viruses. Our hypothesis regarding cell density-dependent prophage induction at first glance appears to be in conflict with the generalized model of homoimmunity in pure bacterial cultures. However, in highly diverse microbial ecosystems such as soil or complex biofilms, a broad host range may be the rule rather than the exception. Nevertheless, our results directly demonstrate the induction of λ lysogen in the presence of an AHL-producing P. aeruginosa strain in the absence of any exogenously added AHL. This finding, combined with the demonstration of AHL-mediated prophage induction in microbial communities from two very different natural ecosystems (i.e., soil and groundwater), suggests that this is a widespread chemical signaling phenomenon governing an important interaction between host bacteria and temperate phage. Our observation illustrates the clever nature with which phage may exploit the chemical communication of their host bacteria by using a system that regulates many vital bacterial processes. These processes are essential for the successful establishment of symbiotic or pathogenic relationships with their respective eukaryotic hosts. For example, it raises questions regarding a possible role of AHL-producing bacteria in the phage-mediated horizontal transfer of genes such as that encoding Stx toxin in E. coli (48), which already was shown to be transferred spontaneously through prophage induction by the simple coculture of lysogenic and nonlysogenic strains nearly 40 years ago (46). With the recent discovery of AHL production by cyanobacteria (44), our results also may have implications for understanding broad-host-range lysogenic cyanophages (51) involved in the horizontal transfer of photosystem II genes (50), which has global importance in the open ocean. We acknowledge that our work is limited to either an in vivo model of E. coli and Pseudomonas or to field-collected soil and groundwater samples assayed in vitro. Additional studies with other phage-host systems and direct in situ measurements will be required to better understand any possible ecological or pathological implications of these findings. For example, understanding how and when prophage are activated to lytic reproduction may lead to better treatment strategies for containing disease agents such as Shigella dysenteriae type 1 and enterohemorrhagic E. coli O157:H7, which possess prophage-encoded toxins.

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