LitR Is a Repressor of *syp* Genes and Has a Temperature-Sensitive Regulatory Effect on Biofilm Formation and Colony Morphology in *Vibrio* (*Aliivibrio*) *salmonicida*

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Vibrio (*Aliivibrio*) *salmonicida* is the etiological agent of cold water vibriosis, a disease in farmed Atlantic salmon (*Salmo salar*) that is kept under control due to an effective vaccine. A seawater temperature below 12°C is normally required for disease development. Quorum sensing (QS) is a cell density-regulated communication system that bacteria use to coordinate activities involved in colonization and pathogenesis, and we have previously shown that inactivation of the QS master regulator LitR attenuates the *V. salmonicida* strain LFI1238 in a fish model. We show here that strain LFI1238 and a panel of naturally occurring *V. salmonicida* strains are poor biofilm producers. Inactivation of *litR* in the LFI1238 strain enhances medium- and temperature-dependent adhesion, rugose colony morphology, and biofilm formation. Chemical treatment and electron microscopy of the biofilm identified an extracellular matrix consisting mainly of a fibrous network, proteins, and polysaccharides. Further, by microarray analysis of planktonic and biofilm cells, we identified a number of genes regulated by LitR and, among these, were homologues of the *Vibrio fischeri* symbiosis polysaccharide (*syp*) genes. The *syp* genes were regulated by LitR in both planktonic and biofilm lifestyle analyses. Disruption of *syp* genes in the *V. salmonicida* Δ*litR* mutant alleviated adhesion, rugose colony morphology, and biofilm formation. Hence, LitR is a repressor of *syp* transcription that is necessary for expression of the phenotypes examined. The regulatory effect of LitR on colony morphology and biofilm formation is temperature sensitive and weak or absent at temperatures above the bacterium's upper threshold for pathogenicity.

To protect themselves from environmental stress, bacteria can grow as surface-associated biofilms in their natural environment, as well as on artificial surfaces (reviewed by Karatan and Watnick [1]). The first step in biofilm formation is the attachment of single cells or bacterial aggregates to surfaces. Attachment requires that some adhesive properties to the surface are expressed. The resulting microcolony forms and grows in a self-produced extracellular matrix consisting mainly of secreted polysaccharides, proteins and sometimes also extracellular DNA (eDNA) (2–5). Other environmental factors such as temperature, pH, ion, nutrition, or water current may change the morphology and composition of the biofilm (1, 6).

Members of the vibrios are found in aquatic environments, as planktonic cells or attached to surfaces as bacterial aggregates or biofilms. Some vibrios have evolved to be pathogens, whereas others have developed symbiotic relationships with their hosts (7). Since many *Vibrio* species switch between growth within hosts and aquatic habitats, adaptation to changing environments is critical for their survival and colonization. A key factor for survival is the ability to produce a protective biofilm (8). An example of this is *Vibrio cholerae*, which forms biofilm in the aquatic environment, as well as in the intestines of the infected host (9–12). Similarly, biofilm formation plays a major role in the host colonization of *Vibrio fischeri* (13, 14), and it is also suggested to be an important feature of *Vibrio anguillarum*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* (15–18).

Exopolysaccharides (EPS) are major components of the vibrio biofilm matrix, and their expression is frequently correlated with changes in colony morphology (8). The EPS production in *V. cholerae* depends on two linked polysaccharide loci (*vpsI* and *vpsII*) and the positive regulators VpsT and VpsR (9, 19, 20). For *V. fischeri*, the formation of biofilm aggregates outside the squid light organ is crucial for proper initiation of host colonization. Both biofilm formation and the symbiotic relationship of *V. fischeri* with the squid host depend upon a cluster of 18 polysaccharide biosynthetic genes referred to as the symbiosis polysaccharide (*syp*) locus (14). Regulation of the *syp* locus involves a complex network of regulatory proteins, including the hybrid sensor kinase RscS and the downstream response regulator SypG (21, 22). *V. fischeri* strains overexpressing SypG show a dramatic increase in biofilm formation and have an advantage in colonizing the host (14, 21).

Quorum sensing (QS) is a cell-to-cell communication system that regulates a number of activities in vibrios, including biofilm formation, which is important for successful colonization and survival in environment. The majority of the signal molecules or autoinducers in QS are acylated homoserine lactones (AHLs) (23). A central regulator in QS is the transcription factor LitR,

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which in *V. fischeri* regulates bioluminescence and host colonization (24, 25). Homologs of *V. fischeri* LitR are found in many vibrios and their activities are often species specific (23). For example, the LitR homolog (HapR) in *V. cholerae* is a negative regulator of biofilm formation, colony rugosity, and *vps* expression (19, 26–28), whereas the corresponding homologs in *V. parahaemolyticus* (OpaR) and *V. vulnificus* (SmcR) are positive regulators of colony opacity and biofilm formation (17, 29, 30). Similarly, the *V. anguillarum* LitR homolog (VanT) positively regulates biofilm formation (15).

Vibrio (*Aliivibrio*) *salmonicida* causes cold-water vibriosis in farmed salmonid fish, and the disease occurs mainly in late autumn, winter, and early spring when the water temperature is below 12°C (31–35). The environmental lifestyle and transmission route of *V. salmonicida* is unclear but may be through the seawater either as bacterioplankton or on the surfaces of particles (32). Natural isolates of *V. salmonicida* have been characterized with regard to plasmid profiles and are grouped into at least four plasmid categories (36–38). Although differences in biochemical properties are found between strains, no correlation between biochemical reaction patterns, plasmid profiles, and virulence has been reported (37).

We have recently shown that LitR regulates virulence, as well as motility, cryptic bioluminescence, adhesion, and aggregation, in *V. salmonicida*. By light microscopy we found that LitR regulates negatively the formation of a loosely attached biofilm and speculated that a high content of polysaccharides prohibited quantification using traditional staining methods (39). In the present study, we have optimized the biofilm assay and show that *V. salmonicida* strains isolated at different geographic locations and with different plasmid profiles are poor biofilm producers at the chosen conditions. Inactivation of *litR* in the LFI1238 strain results in formation of a thick biofilm mainly composed of polysaccharides and proteins and to a much lesser extent also eDNA. Furthermore, a correlation between rugose colonies, adhesive properties, and biofilm formation was found, and these phenotypes were more strongly expressed at lower temperatures. Similar to *V. fischeri*, *V. salmonicida* LFI1238 harbors the *syp* locus with all 18 *syp* genes present (VSAL_II0295 to VSAL_II0312), and microarray analyses of cells grown in suspension or biofilm show that LitR is a strong repressor of *syp* transcription. Inactivation of *syp* genes alleviated biofilm, adhesion, and rugose colony morphology in the Δ*litR* mutant.

MATERIALS AND METHODS

Strains and culture conditions. The origins of the bacterial strains are listed in Table 1. Cells from glycerol stocks were streaked on blood agar with 2.5% NaCl (wt/vol) and incubated at 12°C for 4 days. The LFI1238 or the Δ*litR* strains were used as parental strains for mutant construction. The derived *V. salmonicida* mutants were grown in lysogeny broth with 2.5% (wt/vol) NaCl (LB2.5) at 12°C unless otherwise indicated. The *Escherichia coli* strains S17-1 and JM109 were cultured in Luria-Bertani medium with 1% (wt/vol) NaCl at 37°C. The suicide plasmid pNQ705 was propagated in *E. coli* S17-1 cells. For selection of *E. coli* S17-1 transformants or *V. salmonicida* transconjugants, 25 or 2 μg of chloramphenicol/ml was added to the medium, respectively. For selection of *E. coli* JM109 transformants, 100 μg of ampicillin/ml was added.

Two different media were used for the biofilm assays: (i) Leibovitz L15 cell culture medium (Fisher Scientific) supplemented with NaCl to 380 mosM and further modified by the addition of MgCl$_2$ to a final concentration of 10 mM, and the pH was adjusted to 8 (referred to as L15-M). (ii)

A seawater-based (SWT) medium containing 5 g of Bacto peptone (BD), 3 g of yeast extract (Sigma), and 28 g of marine sea salt (Tetra) per liter. Solid medium was prepared by adding 1.5% (wt/vol) agar (Fluka).

Construction of *V. salmonicida* Δ*litR* double mutants. DNA extraction, general recombinant DNA techniques, and transformations were performed according to standard protocols (44). Restriction enzyme digestion, ligation, and PCR product and plasmid purifications were performed as recommended by the manufacturers (NEB Biolabs and OMEGA Bio-Tek). PCR (Phusion, Finnzyme) and BigDye sequencing (Applied Biosystems) were performed with custom-made primers (Sigma and Operon).

Construction of *V. salmonicida* LFI1238 containing a *litR* in-frame deletion (Δ*litR*) and a complemented strain (Δ*litR*$_c$) have been described elsewhere (39). The Δ*litR* double mutants used in the present study were made by chromosomal insertions mainly as described by others (43). Briefly, pNQ705-derived suicidal plasmids (Table 1) were used for chromosomal integration, and these were made by cloning a PCR-amplified part (~250 bp) of the targeted genes into the suicide vector pNQ705. The PCR primers used for amplification are listed in Table 1, and their names correspond to the locus tag of the gene of interest, followed by the suffix "F" or "R" to indicate a forward or reverse primer direction, respectively. The restriction enzyme sites SpeI or XhoI were added to the 5′ end of the forward and reverse primers, respectively, to enable cloning of the PCR product into the corresponding sites in pNQ705. However, before being cloned into pNQ705, A′ overhangs were added to the PCR products and ligated into the pGEM-T Easy vector, followed by transformation into *E. coli* JM109 (Promega). The pGEM-T-derived plasmids were purified and digested with SpeI and XhoI before being electrophoresed in an agarose gel. Finally, the inserts were excised from the agarose gel, purified, and ligated into the corresponding restriction sites of pNQ705 before the transformation of *E. coli* S17-1. The different pNQ705 suicidal constructs were transferred from the S17-1 donor cells to *V. salmonicida* recipient cells by conjugation as described elsewhere (39). It should be noted that chromosomal plasmid insertions are likely to create polar effects of downstream genes in an operon (43).

Identification of the *litR* gene and prediction of σ54 binding sites in *V. salmonicida*. The *litR* open reading frame in the *V. salmonicida* wild-type isolates (Table 1) were amplified by a standard PCR using the LitR-G forward and LitR-H reverse primers, followed by sequencing using BigDye chemistry (39).

Fuzznuc (http://emboss.sourceforge.net/apps/release/6.0/emboss/apps/fuzznuc.html) was used to predict the putative σ54 binding sites within the *syp* locus of *V. salmonicida*. To this end, a region spanning the genes VSAL_II0295 to VSAL_II0312 was extracted (GenBank accession number NC_011313.1) and used as input, together with the σ54 consensus sequence 5′-TGGCACGNNNNTTGCA-3′ (45). Three mismatches were allowed in the analysis.

Static biofilm assay. Precultures of the different strains were made by adding one bacterial colony to 2 ml of medium and grown at 12°C with 200 rpm. After 2 days of incubation, the precultures were diluted 1:20 and incubated overnight. These overnight cultures were then diluted to an optical density at 600 nm (OD$_{600}$) of 1.3 in LB2.5 before being further diluted 1:10 in L15-M or SWT medium. A total volume of 300 μl of each dilution was then added to each well in flat-bottom, non-tissue-culture-treated Falcon 24-well plates (BD Biosciences). The plates were incubated statically at different temperatures (4, 8, 12, 14, and 16°C). Biofilm formation was monitored by light phase-contrast microscopy, and pictures were taken by a photo camera installed with the microscope (Leica DM IRB or Zeiss Primo Vert). To quantify the biomass of the biofilm, the medium was removed, and 300 μl of 0.1% (wt/vol) crystal violet in H$_2$O was added gently, followed by incubation for 30 min. The crystal violet stain was removed, and the wells were washed twice with 0.5 ml of H$_2$O. The wells were air dried, and the biofilm was dissolved in 0.5 ml of 96% ethanol with strong agitation for 1 to 2 days. The dissolved biofilm was diluted 1:10 in 96% ethanol and transferred to a 96-well plate (100 μl/

TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence (5′–3′)[a]	Source or reference
Strains		
V. salmonicida		
LFI1238	Wild type; isolated from Atlantic cod, sequenced strain	40
NCMB2262	Wild type; isolated from Atlantic salmon, type strain	33
VS12	Wild type; isolated from Atlantic salmon, Strønstad (1982)	36
VS26	Wild type; isolated from Atlantic salmon, Seløy (1983)	36
VS103	Wild type; isolated from Atlantic salmon, Valevåg (1987)	36
VS201	Wild type; isolated from Atlantic salmon, Aukra (1987)	36
VS224	Wild type; isolated from Atlantic salmon, Kristiansund (1987)	36
VS295	Wild type; isolated from Atlantic salmon, Trangereid (1987)	36
VS370	Wild type; isolated from Atlantic salmon, Krokelvdalen (1987)	36
VS446	Wild type; isolated from Atlantic salmon, Sjøvegen (1988)	36
VS498	Wild type; isolated from Atlantic cod, Hammerfest (1988)	36
Δ*litR* mutant	LFI1238 containing an in-frame deletion in *litR*	39
Δ*litR*$_c$ mutant	Δ*litR* strain complemented with the wild-type *litR* gene; Cm^r	39
Δ*litR sypC* mutant	Δ*litR* mutant with an insertional disruption in *sypC*; Cm^r	This study
Δ*litR sypP* mutant	Δ*litR* mutant with an insertional disruption in *sypP*; Cm^r	This study
Δ*litR sypQ* mutant	Δ*litR* mutant with an insertional disruption in *sypQ*; Cm^r	This study
Δ*litR tadV* mutant	Δ*litR* mutant with an insertional disruption in *tadV*; Cm^r	This study
Δ*litR rcpC* mutant	Δ*litR* mutant with an insertional disruption in *rcpC*; Cm^r	This study
Δ*litR ptsIIB* mutant	Δ*litR* mutant with an insertional disruption in *ptsIIB*; Cm^r	This study
Δ*litR ptsIIC* mutant	Δ*litR* mutant with an insertional disruption in *ptsIIC*; Cm^r	This study
Δ*litR ptsIIA* mutant	Δ*litR* mutant with an insertional disruption in *ptsIIA*; Cm^r	This study
E. coli		
S17-1	Donor strain for conjugation; λ *pir*	41
JM109	Strain for subcloning pGEM-T Easy constructs	42
Plasmids		
pNQ705	Cm^r; suicide vector with an R6K origin (*pir* requiring)	43
pGEM-T Easy	Amp^r; TA cloning vector, white/blue screening	Promega
pNQ705-*sypC*	pNQ705 containing an internal fragment of VSAL_II0310	This study
pNQ705-*sypP*	pNQ705 containing an internal fragment of VSAL_II0297	This study
pNQ705-*sypQ*	pNQ705 containing an internal fragment of VSAL_II0296	This study
pNQ705-*tadV*	pNQ705 containing an internal fragment of VSAL_II0367	This study
pNQ705-*rcpC*	pNQ705 containing an internal fragment of VSAL_II0368	This study
pNQ705-*ptsIIB*	pNQ705 containing an internal fragment of VSAL_II0823	This study
pNQ705-*ptsIIC*	pNQ705 containing an internal fragment of VSAL_II0824	This study
pNQ705-*ptsIIA*	pNQ705 containing an internal fragment of VSAL_II0825	This study
Primers		
II0823F	CTCGAGAGATTTTAGTGGTATGTGGCA	This study
II0823R	ACTAGTCAGCGTCTTCTAATTGAACG	This study
II0824F	CTCGAGCAGCAATCATGGTAGGTCTT	This study
II0824R	ACTAGTGGAGTGATACGAGCCAATA	This study
II0825F	CTCGAGTTGGTAACGACGGCATCG	This study
II0825R	ACTAGTTGACGCCATTCTTAAACACA	This study
II0296F	CTCGAGATTGCAGTAACCTCTGTCAGT	This study
II0296R	ACTAGTTTACGGTATCGTCGGTACA	This study
II0297F	CTCGAGTCATGTTGTTCAACATCTTTC	This study
II0297R	ACTAGTGATATGGTGTGAATGCACG	This study
II0310F	CTCGAGTCTACTGTCTCAATGGTGCA	This study
II0310R	ACTAGTGGAATCACCATCTGATAATGT	This study
II0367F	CTCGAGTTAGCCGCCATTTCTTATT	This study
II0367R	ACTAGTTTGGGTTAACTGCAATGG	This study
II0368F	CTCGAGTATGGCTTAGGGGAAAGC	This study
II0368R	ACTAGTATCGCTCATTGAAATATAAGTG	This study
LitR-G fwd	ACCAACGGCAGGACTTAGAC	39
LitR-H rev	TTGATAACAATCGAGCAGAGC	39

[a] Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance. For most of the wild-type strains, the source site is indicated, with the year of isolation specified in parentheses. Sequence underlining indicates the SpeI or XhoI restriction site added to the 5′ region of the primer.

well). The absorbance was read at 590 nm (Vmax kinetic microplate reader; Molecular Devices).

Colony morphology assay. The assay was performed mainly as described by others (46) and may allow differentiation of bacteria based on characteristics such shape, size, and color, as well as the texture of the colony. In brief, the different bacterial strains were grown overnight in LB2.5 broth before being diluted 1:10 in SWT medium. Then, 2 μl of each dilution was spotted onto blood agar plates supplemented with 2.5% (wt/vol) NaCl on LB2.5 and on SWT agar plates. The blood agar and LB2.5 plates were incubated at 4°C. The SWT plates were incubated at 4, 8, and 14°C. The colonies were viewed macroscopically and photographed with a Canon D400 digital camera. The colonies were also viewed microscopically (Zeiss Primo Vert) and photographed with AxioCam ERc5s.

Transmission electron microscopy. Bacterial colonies grown at 4°C were removed from the agar plate by a scalpel and fixed in McDowell fixative (4% [wt/vol] formaldehyde and 1% [wt/vol] glutaraldehyde in phosphate buffer with an osmolarity of 320 mosM; pH 7.4) (47). The bacterial cells were postfixed with 1% (wt/vol) OsO_4, dehydrated in graded series of ethanol, and embedded in an Epon substitute (AGAR [AGAR 100, MNA, DDSA, and DMP-30]) with propylene oxide as a transitional solvent. Ultrathin sections were cut using a Leica Ultracut S and a diamond knife (Diatome) and mounted on Formvar-coated copper grids, stained with 5% (wt/vol) uranyl acetate and Reynolds lead citrate (48), before being examined in a JEOL JEM 1010 transmission electron microscope with a Morada camera system (Olympus Soft Imaging System).

Scanning electron microscopy (SEM). The static biofilm assay was performed as described above in SWT medium with Thermanox plastic coverslips with a 13-mm diameter (Nunc) soaked in the wells. The coverslips with the biofilm were removed from the medium at different time points and washed gently in phosphate-buffered saline (PBS) before being fixed and stained in 1.2% (wt/vol) glutaraldehyde, 0.45 M cacodylate buffer (pH 7.3), and 0.1% (wt/vol) ruthenium red. The biofilms were postfixed in 1% (wt/vol) OsO_4, washed in PBS, and dehydrated in a graded series of ethanol. The specimens were dried using hexamethyldisilazane (Sigma-Aldrich) as a drying agent. The samples were mounted on specimen holders and coated with gold-palladium in a Polaron Sputter Coater. Pictures were taken on a JEOL JSM 6300 scanning electron microscope. At day 6, it was impossible to remove the slide without tearing the Δ*litR* biofilm away from the slide. The Δ*litR* biofilm was therefore harvested with a forceps before being fixed and processed as described above.

Biofilm inhibition and detachment assay. In order to characterize the composition of the biofilm proteinase K, DNase I and sodium metaperiodate (NaIO$_4$) were added to the wells, either before (biofilm inhibition assay) or after (detachment assay) biofilm formation. NaIO$_4$ degrades β-1,6-linked polysaccharides. Two different endonucleases were used for degradation of eDNA. For the biofilm inhibition assay, a cold-tolerant and salt-active nuclease (SAN) was used. The SAN enzyme was kept in 20 mM Tris-HCl (pH 7.5), 2 mM MgCl$_2$, 10 mM NaCl, 0.01% (vol/vol) Triton X-100, and 50% (wt/vol) glycerol (SAN buffer). The following reagents were added to the SWT medium to final concentrations of 40 μg of proteinase K/ml, 50 μg of SAN/ml, and 4 mM NaIO$_4$. These concentrations did not inhibit the growth of the bacterium (data not shown). The biofilm was stained and quantified as described above.

The detachment assay was performed mainly as described elsewhere (49). Briefly, the biofilm was allowed to form in SWT for 72 h before being washed twice with PBS and incubated for 24 h at 37°C with 300 μl of (i) 10 mM NaIO$_4$ dissolved in 0.1 M sodium acetate (pH 5.5), (ii) 0.1 mg/ml proteinase K (Sigma) in 20 mM Tris-HCl (pH 7.5)–100 mM NaCl, or (iii) 0.5 mg/ml bovine DNase I (DN25; Sigma) in 5 mM MgCl$_2$. Detachment or dissolution of biofilm was monitored macroscopically by adding 5 μl of 0.1% (wt/vol) crystal violet to each well after incubation.

Microarray analysis. Microarray analysis was performed on RNA isolated from planktonic bacteria grown in suspension and from bacteria grown in biofilm under static conditions as described above. In order to

reveal potential targets for LitR regulation, we chose to grow the cells at low temperatures, when the differences in phenotypes between the wild type and Δ*litR* mutant strains are most prominent. In addition, since the *litR* mutant is prone to aggregation in SWT medium (unpublished observation), as well as in other liquid media when grown at 4°C (39), it was necessary to grow planktonic cells at 8°C to monitor the cell density properly before harvest. Thus, for bacteria in suspension, the wild-type LFI1238 and the Δ*litR* mutant were grown at 8°C in SWT medium with 200 rpm before being harvested at an OD$_{600}$ of 0.8. The biofilms were grown in SWT medium in 72-cm^2 cell culture flasks (Nunc) and harvested by a cell scraper after 72-h static incubation at 4°C. The RNA was extracted using RNA-isol (Fischer Scientific). DNA was removed by using a DNA-free kit (Ambion) and further cleaned up using the RNeasy MinElute Cleanup kit (Qiagen). cDNA was generated from 15 μg of RNA before being labeled by using an aminoallyl cDNA labeling kit (Ambion) and a CyDye postlabeling reactive dye pack (GE Healthcare). The samples were hybridized to "*Vibrio salmonicida* V1.0.1 AROS" slides, washed, and scanned as described elsewhere (50). Each microarray experiment was performed with four biological replicates, including one dye swap. The data were normalized within and between data sets using quantile normalization (51). To identify differentially expressed genes and calculate the fold change log$_2$ values and *P* values, a linear model was implemented using R and the LIMMA package (52).

Accession numbers. The *V. salmonicida litR* gene sequences have been deposited in the GenBank nucleotide sequence database (accession numbers KJ575525 to KJ575534). The microarray data are available at the NCBI Gene Expression Omnibus database (accession numbers GSE42994 and GSE42995).

RESULTS

Biofilm morphology changes with medium composition. We previously showed that LitR in *V. salmonicida* regulates biofilm formation, and by using static conditions at a low temperature (4°C) the Δ*litR* mutant formed a biofilm in the cell culture medium L15. The biofilm formed was viscous and loosely attached to the substratum, and attempts to quantify this biomass were unsuccessful (39). We therefore sought other medium combinations or culture media that could change the properties of the biofilm. Depending on culture media the biofilm showed different morphologies and thicknesses after 48- and 96-h incubations at 4°C. In L15-M the Δ*litR* mutant produced a flat and regular biofilm, whereas in the SWT medium the Δ*litR* mutant produced a much thicker, multilayered biofilm with irregular mushroom-like structures (Fig. 1A). The adhesion of the biofilm to the substratum improved in SWT and L15-M. Although care had to be taken, the biofilms were successfully stained with crystal violet and quantified, and the highest biomass content was found after growth in SWT medium (Fig. 1B).

Naturally occurring *V. salmonicida* strains are poor biofilm producers. To find out whether our reference strain LFI1238, which is an Atlantic cod (*Gadus morhua*) isolate, is typical with regard to biofilm formation, we analyzed a collection of naturally occurring *V. salmonicida* strains isolated from Atlantic salmon (*Salmo salar*) (Table 1). This collection represents isolates from outbreaks at different geographical locations along the coast of Norway, as well as representing isolates with different plasmid profiles (see Table S1 in the supplemental material). Moreover, the isolates encode a complete *litR* open reading frame identical to the one in the LFI1238 strain (data not shown). Since bacterial growth and biofilm formation were more efficient in SWT medium, it was chosen as the standard medium. All wild-type strains produced significantly less biofilm than the Δ*litR* mutant, irre-
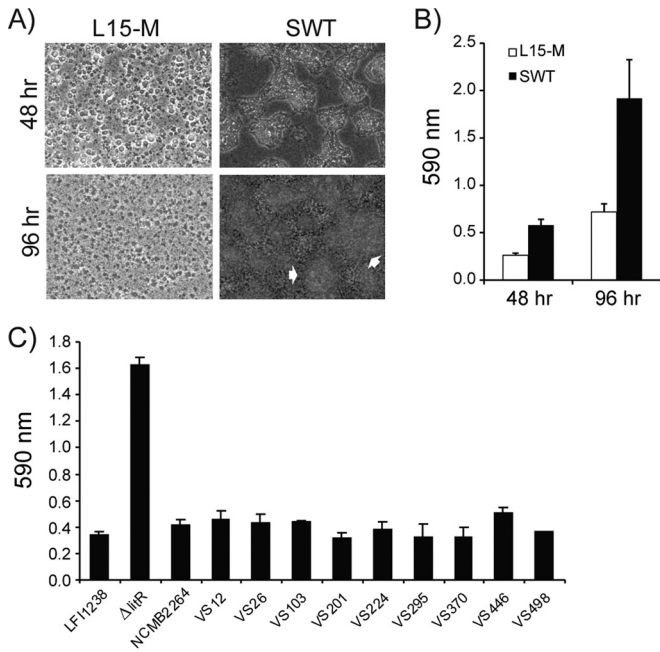
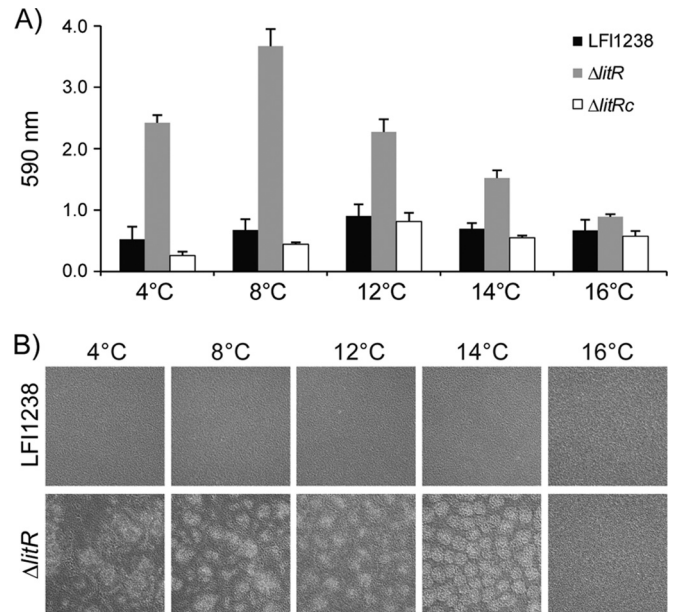FIG 1 Biofilm formation of *V. salmonicida* wild-type strains and the LFI1238-derived Δ*litR* mutant. (A and B) Morphology (A) and quantitation (B) of biofilms formed by the Δ*litR* mutant in L15-M and SWT after 48 and 96 h of static incubation at 4°C. The morphology was monitored by phase-contrast microscopy (Leica), and the white arrows point to irregular mushroom-like structures in the biofilm. (C) Quantification of the biofilm formed by the different wild-type strains and the Δ*litR* mutant in SWT medium after 72 h of incubation at 4°C. The biofilms were stained with crystal violet, and the absorbance was read at 590 nm. The error bars represent the standard deviations of three biological replicates.

FIG 2 Biofilm formation in SWT medium at different temperatures. (A) Quantitation of crystal violet stained biofilms formed by wild-type LFI1238, Δ*litR*, and Δ*litR*c strains at the indicated temperatures. The biofilms were grown statically in the medium for 72 h before being quantified. The error bars represent the standard deviations of three biological replicates. (B) Morphologies of the biofilms formed by wild-type LFI1238 and the Δ*litR* mutant at the indicated temperatures. The morphologies of biofilms were inspected in a Zeiss Primo Vert microscope (10× magnification) and photographed after 72 h of growth.

spective of the geographical location or plasmid profile (Fig. 1C). This is in accordance with former assumptions that strains of *V. salmonicida* are very homogeneous with respect to biochemical reactions and virulence properties (37, 38).

Temperature-sensitive regulation of LitR on biofilm formation. We next analyzed biofilm formation of the LFI1238, Δ*litR*, and Δ*litR*c strains in SWT medium at different temperatures. Increasing the temperature from 4 to 8°C accelerated biofilm formation, and the quantitative differences in biofilm formation between the wild-type LFI1238 strain and the Δ*litR* strain were largest at 4 and 8°C, whereas at 16°C the wild-type and Δ*litR* mutant strains produced similar amounts of biomass (Fig. 2A). Microscopic inspection of the Δ*litR* biofilms revealed that there is a shift from the irregular mushroom-like structures formed at 4, 8, and 12°C to more flat and regular structures at 14°C and, at 16°C, biofilms with mushroom-like structures are no longer formed (Fig. 2B).

***V. salmonicida* LitR regulates medium- and temperature-dependent colony morphology.** The morphology of the colony often correlates with the ability to form biofilm, and biofilm producers usually show a wrinkled or rugose morphology (28, 46). Although the Δ*litR* mutant grew as highly adhesive colonies at 4°C (compared to at 12°C) on blood agar, the colony phenotype seemed otherwise similar to the wild-type LFI1238 when grown from single cells (39). In order to more directly compare the colonies, we performed a spot colony analysis on different agar media. At 4°C, the LFI1238 strain produced smooth colonies on the

three different media, whereas the Δ*litR* mutant produced colonies that had a rough phenotype on blood and SWT agar but not on LB agar (Fig. 3A). The rugose colonies were highly adhesive and impossible to remove from the agar, whereas the smooth colonies had a creamy consistence. Temperature was important for
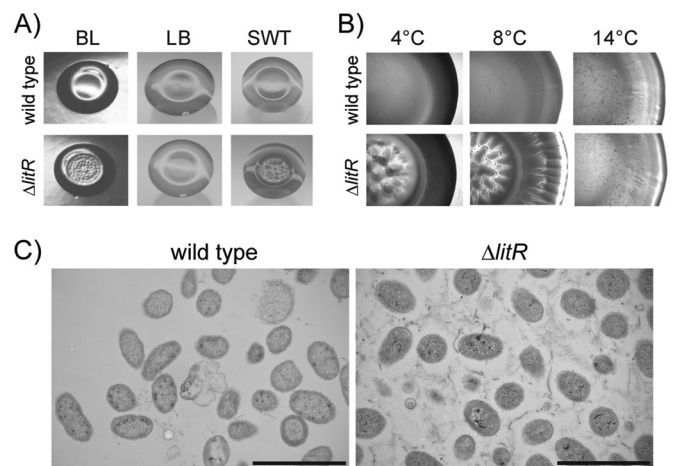
FIG 3 Colony morphology of wild-type LFI1238 and Δ*litR* strains. (A) The colonies were allowed to form on blood agar (BL), SWT, or LB agar plates for 10 days at 4°C before being photographed with a Canon camera. (B) The central and peripheral parts of colonies grown on SWT agar at 4, 8, and 14°C were viewed in a Zeiss Primo Vert microscope at 4× magnification. (C) Thin sections of wild-type and Δ*litR* colonies grown on BL and analyzed by transmission electron microscopy. Scale bar, 2 μm.

the development of the rugose colony morphology; at 14°C the wrinkling of Δ*litR* colonies is absent, and the morphology is similar to the one produced by the wild type (Fig. 3B). The complemented strain (Δ*litR*$_c$) behaves like the wild type in this assay (data not shown).

Thin sections of colonies grown on blood agar plates at 4°C were prepared after the difference in morphology had manifested (8 to 10 days). As shown in Fig. 3C, the bacteria in the Δ*litR* colonies contain an extracellular matrix (with thin filaments) not found in wild-type colonies.

SEM of Δ*litR* biofilm reveals a high content of extracellular matrix. To investigate the formation and architecture of the biofilm produced by the Δ*litR* mutant, we performed SEM analysis. The biofilm was allowed to form on the coverslips for 3 to 6 days in SWT medium before being analyzed. The biofilm formed slower on the slides than to the plastic in the cell culture wells. However, the time course of the biofilm development shows that the biofilm starts as a network of connected microcolonies that progress to a thick biofilm (Fig. 4). Using a higher magnification at day 4, we observe that the Δ*litR* biofilm contained numerous long thin filaments that are likely to mediate the cell-to-cell interactions. Lastly, at day 6 the bacterial cells (in the collapsed biofilm) are completely embedded in a thick matrix. The wild-type strain showed significantly smaller amounts of attached bacteria (see Fig. S1 in the supplemental material).

The major components of the extracellular matrix are polysaccharide and protein. The composition of the extracellular matrix was determined by using a biofilm inhibition assay and a detachment assay. The biofilms were grown for 72 h in SWT media supplemented with SAN, proteinase K, or NaIO$_4$ to analyze the contribution of eDNA, proteins, and polysaccharide in the extracellular matrix. Proteinase K and NaIO$_4$ inhibited the biofilm formation almost completely, whereas biofilms grown in the presence of SAN produced a biofilm mass corresponding to ~55% of the untreated control. The SAN buffer also showed some effect, suggesting that the reduction may not be entirely due to the action of SAN (Fig. 5A).

Similarly, the composition was analyzed by adding proteinase K, bovine DNase I, and NaIO$_4$ after the biofilms had formed. The incubation required in the posttreatment assay lead to detachment of the untreated biofilm, as well as for the treated biofilms. This prohibited standard quantification. However, the effects of the different treatments were visualized macroscopically by adding crystal violet directly to the wells. Dissolution of the biofilm was only detected after addition of NaIO$_4$ and not when treated with bovine DNase I or proteinase K (Fig. 5B). The high quantity of polysaccharides in the matrix may have protected from the action of proteinase K.

LitR regulates *syp* expression in *V. salmonicida*. To identify potential targets of LitR regulation, we performed microarray analyses with RNA isolated from the wild-type LFI1238 and the isogenic Δ*litR* mutant grown in SWT medium either as static biofilm (4°C for 72 h) or in suspension (8°C, 200 rpm, OD$_{600}$ = 0.8). The whole-genome expression data were analyzed, and genes showing a >1.0-fold log$_2$ differential expression and a *P* value below 0.05 were extracted. With these cutoff values, a total of 123 genes were differently expressed in the biofilm experiment, while the number was 87 in the suspension experiment (see Tables S2 to S5 in the supplemental material). Interestingly, ca. 20% (biofilm, 18.7%; suspension, 19.5%) of the differently expressed genes were

located in close proximity on chromosome II (Table 2). Among these were genes within the *syp* locus (VSAL_II0295 to VSAL_II0312) that were significantly upregulated in the Δ*litR* strain compared to LFI1238 in both analyses. Apart from *sypE* (VSAL_II0308), the *syp* genes were more differentially expressed in cells grown as biofilms than in those grown as suspension. Immediately upstream the *syp* locus are two genes found to be highly upregulated in the Δ*litR* mutant, one encoding a putative exported protein and the other a lipoprotein (VSAL_II0313 and VSAL_II0314), followed by a set of genes (VSAL_II0319 to VSAL_II0329) that are downregulated, including the recently described *rpoQ* (53) and a putative anti-sigma factor (Table 2). Moreover, among the upregulated genes in the Δ*litR* mutant after growth in suspension were the phosphoenolpyruvate phosphotransferase system (*pts*) genes VSAL_II0825 and VSAL_II0823 (see Table S2 in the supplemental material). The homologous *pts* genes in *V. cholerae* have been shown to be involved in biofilm formation (54). We detected a fibrous network in the biofilm by SEM analysis, and although not abundant, some genes encoding pili, flagella, and curli were upregulated in the Δ*litR* mutant (see Tables S2 and S4 in the supplemental material).

Disruption of *syp* genes in the Δ*litR* mutant alleviates rugosity and biofilm formation. The organization of the *syp* locus in *V. salmonicida* is similar to the one in *V. fischeri* (14, 40) and contains four operons with putative σ54 binding sites (Fig. 6). To analyze whether expression of the *syp* locus was important for colony morphology and biofilm formation, we disrupted the *sypC*, *sypP*, and *sypQ* genes in the Δ*litR* mutant by insertional inactivation (Fig. 6). We also disrupted the *pts* genes VSAL_II0823 to VSAL_II0825 and the tight adherence (*tad*) genes VSAL_II0367 and VSAL_II0368 since homologues of these have been shown to have an impact on biofilm formation in other species (54, 55). It should be noted that the *tad* genes were not differently expressed in the microarray analyses. Mutations of *tad* or *pts* genes did not show any effect on the colony morphology, and the colonies remained wrinkled and adhesive, similar to its parental Δ*litR* strain, nor were there any significant differences in biofilm formation (Fig. 7A and B). On the other hand, for the Δ*litR syp* double mutants the colony morphology reverted to smooth and nonadhesive, similar to the wild-type LFI1238 morphology (Fig. 7A). Moreover, mutation of the *syp* genes diminished biofilm formation in SWT medium, whereas in L15-M the Δ*litR syp* mutants produced biofilm masses corresponding to wild-type LFI1238 levels (Fig. 7B). The difference in morphology between the Δ*litR* and the Δ*litR syp* mutants suggests that the initial attachment of the biofilm remains intact for the Δ*litR syp* mutants in SWT medium, whereas building up the viscous mushroom-like structure is inhibited (Fig. 7C). Hence, disruption of *syp* expression in the Δ*litR* mutant alleviates rugosity, adhesion and reduces biofilm formation.

DISCUSSION

We have previously shown that inactivation of *litR* reduces the pathogenicity of *V. salmonicida* and enhances motility, adhesion, and biofilm formation *in vitro* (39). It is well established that environmental factors are important for biofilm formation, virulence, and the expression of other phenotypes (1). One of many environmental signals a microbe may encounter is a temperature change which may alter its growth, development, and pathogenesis (56).
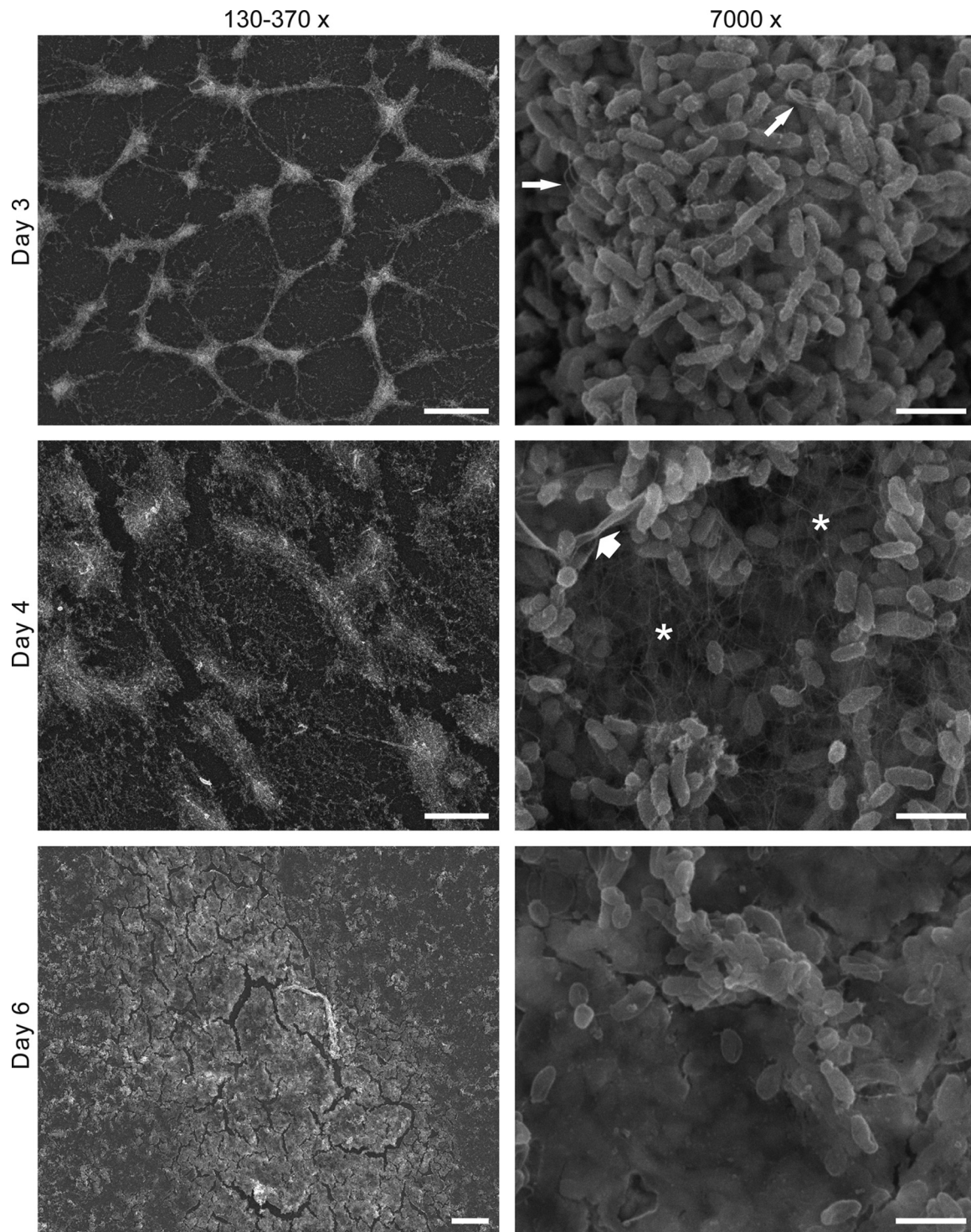
FIG 4 SEM analyses of Δ*litR* biofilms after 3, 4, and 6 days. The biofilms were grown in SWT medium at 4°C before being fixed. The panel to the left shows the biofilms using a low magnification (scale bar, 100 μm for days 3 and 4 and 20 μm for day 6). The panel to the right shows the biofilm at a higher magnification (scale bar, 2 μm). At day 4 a network of fibers (*) is clearly visible, and at day 6 the bacteria are completely encapsulated in the extracellular matrix. The thin arrows point at some flagellum-like structures, and the thick arrow points to a sheet of collapsed extracellular matrix.

V. salmonicida causes disease in the Atlantic salmon when the seawater temperature is below 10 to 12°C (35). In the laboratory, *V. salmonicida* grows at temperatures between 1 and 22°C, with a growth optimum at 12°C in liquid cultures and at 15°C on agar plates (33, 35). Furthermore, iron sequestration has been proposed to be temperature sensitive in *V. salmonicida*, being more efficient at temperatures of ≤10°C compared to at 15°C (57). Here we show that both temperature and medium composition are of
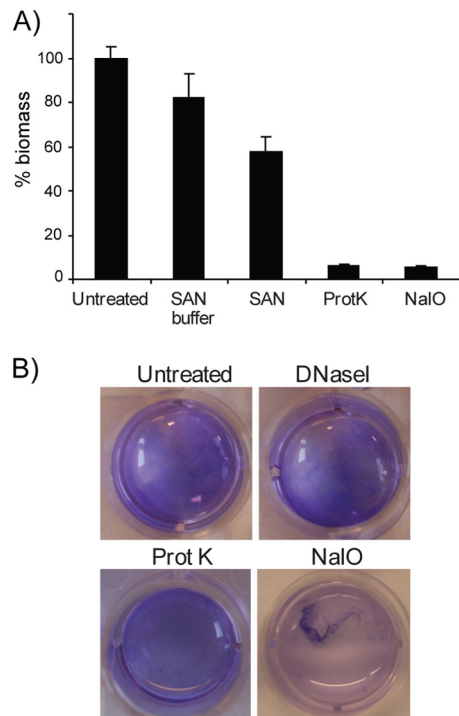
FIG 5 Characterization of extracellular matrix using a biofilm inhibition assay and a biofilm detachment assay. (A) For the inhibition assay, the different treatments (SAN, proteinase K, or NaIO₄) were added directly to the SWT medium before the biofilms were allowed to form. After 3 days of static incubation at 4°C, the biofilms were stained with crystal violet, and the absorbance was read at 590 nm. The inhibition is shown as the percent biomass relative to the untreated control. (B) For the detachment assay, the biofilm was allowed to form for 3 days at 4°C before the medium (SWT) was removed, and the different treatments (bovine DNase I, proteinase K, or NaIO₄) were added. The wells were incubated for 24 h at 37°C before dissolution of the biofilm was visualized by adding a small amount crystal violet into the wells. The error bars represent the standard deviations of three biological replicates.

importance for colony morphology, as well as biofilm formation in *V. salmonicida*. In L15-M medium the morphology of the biofilm was flat and regular biofilm, whereas in artificial seawater-based medium (SWT) thick biofilms with mushroom structures were formed. Thus, the ability of the Δ*litR* mutant to produce biofilm and wrinkled colonies is both temperature and medium dependent. At low temperatures (4 to 8°C) the differences in phenotypes between the wild-type and Δ*litR* strains are pronounced, in which expression of the *litR* inhibits biofilm formation (or activates biofilm dispersal), relieves adhesion, and inhibits the wrinkling of colonies. Clearly, biofilm formation in an artificial growth media may be very different from the natural ocean environment or fish tissues, and caution should be taken when extrapolating *in vitro* result to what may occur *in vivo*. However, the *litR* mutant mimics a low cell density (LCD) phenotype, and we speculate that LCD activities such as adhesion, motility and biofilm formation are important for colonization and survival in the environment. Expression of *litR* is regulated in a cell density-dependent manner (39). Thus, at LCD when *litR* is not expressed, the bacteria show increased adhesion to surfaces, produce an extracellular matrix, and acquire the ability to grow into a biofilm. A cold temperature is favorable for expressing LCD phenotypes and probably in-

creases the ability of the bacteria to colonize a surface. The expression of *litR* is most likely expressed in response to accumulation of QS signals such as acylated homoserine lactones (AHLs), and several AHLs have been identified in the stationary growth phase of *V. salmonicida* (58, 59). Thus, as the cell density increases, the accumulation of AHLs and the expression of *litR* downregulates the biofilm, yielding planktonic cells that are able to escape and colonize new sites. At temperatures clearly above the limit for disease development, the *V. salmonicida* wild type and the Δ*litR* mutant behaved more similarly to each other with regard to the expression of the different phenotypes investigated, and at 16°C neither strain produced biofilms with mushroom-like structures. Hence, this temperature may represent a cue that triggers exit of cells from the biofilm or show that the environment is not favorable for building up a three-dimensional biofilm. These two explanations are not mutually exclusive. If the results obtained at 16°C are due to QS, the lack of LitR regulation or other mechanisms remains to be resolved, and we are currently mapping the production of AHLs at different temperatures. It may be that the production of AHLs itself is subject to temperature regulation.

The ability to produce species-specific EPS and biofilm is probably important in niche selection (8). The Δ*litR* mutant of *V. salmonicida* LFI1238 produced a slimy biofilm that contained an extracellular matrix consisting mainly of proteins and polysaccharides, and the microarray analyses demonstrated clearly that LitR regulates expression of the *syp* locus. Of the 18 *syp* genes, 14 were highly expressed in the biofilms produced by the Δ*litR* mutant compared to similar samples of the wild type, whereas 9 *syp* genes were upregulated after the bacteria were grown in suspension. The difference may be due to time and temperature of growth, in addition to static incubation versus incubation with agitation. However, mutation of three *syp* genes (*sypC*, *sypP*, and *sypQ*) alleviated the wrinkled-colony phenotype and, depending on the medium, reduced or completely alleviated the biofilm formation of the Δ*litR* mutant. Thus, the QS regulator LitR is a negative regulator of EPS in *V. salmonicida*, but the regulation cascade from LitR to the *syp* locus remains to be elucidated. Regulation of EPS expression and biofilm formation in vibrios involves numerous transcriptional regulators, two-component signal transduction, and QS regulators (8). HapR, the LitR homolog in *V. cholerae*, is also known to downregulate biofilm formation, and it does this by repressing the expression of VpsR and VpsT, which are positive regulators of the *vps* genes (19, 28, 60–62). In *V. fischeri* the *syp* locus is the major contributor to biofilm formation and colony wrinkling (14, 21). LitR has, to our knowledge, not been shown to regulate either biofilm or *syp* expression in *V. fischeri*. Instead, SypG is the direct activator of *syp* transcription, which itself is regulated by the sensor kinase RscS (22). *V. salmonicida* encodes homologues of RscS and the regulators SypG, VpsR, and VpsT (40), but none of these genes was found to be differentially expressed in our microarray analyses.

Interestingly, downstream the *syp* locus in *V. salmonicida* follows several genes that are downregulated in the Δ*litR* mutant (VSAL_II0319 to VSAL_II0329). These are annotated as putative lipoproteins, response regulators, a sigma factor, and an anti-sigma factor antagonist (40). The sigma factor RpoQ (VSAL_II0319) was recently described in *V. fischeri* and found to be regulated by QS (53). Of the *Vibrionaceae*, only *V. salmonicida* and *V. fischeri* encode this sigma factor, and in both species LitR positively regulates *rpoQ* expression. Between the *rpoQ* and *syp* locus are two

TABLE 2 Subset of genes differently expressed between *V. salmonicida* wild-type LFI1238 and the Δ*litR* mutant[a]

CDS	Gene	Gene product	Biofilm		Suspension	
			Log$_2$ ratio	P (adjusted)	Log$_2$ ratio	P (adjusted)
VSAL_II0296	*sypQ*	Putative transmembrane glycosyltransferase	−2.45	9.51E−03	−1.82	7.55E−03
VSAL_II0297	*sypP*	Putative glycosyltransferase	−5.17	7.86E−04	−2.54	5.73E−04
VSAL_II0298	*sypO*	Putative membrane protein	−2.88	4.90E−03	−1.62	3.16E−02
VSAL_II0299	*sypN*	Putative glycosyltransferases	−3.37	1.87E−03		
VSAL_II0300	*sypM*	Hypothetical protein	−3.73	7.80E−03		
VSAL_II0301	*sypL*	O-antigen polymerase	−1.01	5.30E−02		
VSAL_II0302	*sypK*	Putative polysaccharide biosynthesis protein	−2.62	6.10E−04	−1.70	4.27E−03
VSAL_II0303	*sypJ*	Putative glycosyltransferase	−2.46	3.57E−03		
VSAL_II0304	*sypI*	Putative glycosyltransferase	−3.69	1.75E−05	−2.13	2.98E−02
VSAL_II0308	*sypE*	Putative response regulator	−1.57	4.42E−02	−1.80	5.44E−03
VSAL_II0309	*sypD*	Putative capsular polysaccharide synthesis protein	−1.71	2.78E−04		
VSAL_II0310	*sypC*	Polysaccharide biosynthesis/export protein	−3.64	3.34E−05	**−1.42**	7.54E−02
VSAL_II0311	*sypB*	Outer membrane protein, OmpA family	−4.15	1.88E−06	−2.05	1.87E−03
VSAL_II0312	*sypA*	Hypothetical protein, putative anti-sigma factor antagonist	−4.34	5.76E−04	−2.66	2.91E−04
VSAL_II0313		Putative exported protein	−4.38	5.87E−03		
VSAL_II0314		Putative lipoprotein	−3.85	2.17E−04		
VSAL_II0316		Response regulator, histidine kinase			**1.24**	7.67E−02
VSAL_II0319	*rpoQ*	*rpoS* RNA polymerase sigma factor	1.34	1.34E−02	2.27	5.34E−03
VSAL_II0320		Putative membrane-associated signaling protein	1.36	2.07E−02		
VSAL_II0321		Putative glycosyltransferase			2.42	7.28E−03
VSAL_II0322		Putative membrane protein	**1.19**	7.05E−02	3.48	2.21E−02
VSAL_II0323		Putative lipoprotein	2.05	3.81E−03	2.80	2.74E−02
VSAL_II0324		Putative lipoprotein			2.77	7.67E−03
VSAL_II0325		Putative exported protein			**1.13**	5.86E−02
VSAL_II0326		Hypothetical protein	**1.17**	6.41E−02	2.50	1.08E−02
VSAL_II0327		Putative nucleotidyltransferase	1.48	1.13E−02	2.13	5.39E−02
VSAL_II0328		spoIIAA putative anti-sigma F factor antagonist	1.10	2.74E−02	2.97	5.32E−03
VSAL_II0329		Putative response regulator	0.99	3.06E−02		
VSAL_II0332		Putative hemolysin-type calcium-binding protein (fragment)	1.55	3.41E−03	1.88	3.11E−02

[a] Positive fold log$_2$ values represent genes downregulated in the Δ*litR* mutant, and negative values represent genes upregulated in the Δ*litR* mutant. The log$_2$ ratios indicated in boldface were originally excluded from the analyses due to P values of >0.05.

genes (VSAL_II0313 and II0314) located that are annotated as a putative export protein and a putative lipoprotein. Both are highly upregulated in the *litR* mutant, and further studies are needed to address the functions or biological activities of these gene products. Interestingly, the genes VSAL_II0317 and VSAL_II0318, as well as VSAL_II0321 to VSAL_II0329, have no apparent orthologues in *V. fischeri* but show similarity to genes of three *Shewanella* species (*S. piezotolerans*, *S. pealeana*, and *S. halifaxensis*) (data not shown). These three species form a distinct clade among the genus *Shewanella* (63). *S. pealeana* is a psychrotolerant species that has been isolated from the nidamental gland of the squid *Loligo peali* (64). Thus, this island of LitR-regulated genes located on chromosome II in *V. salmonicida* (Table 2) shares sequence similarities with *V. fischeri* and *S. pealeana*, both having squids as hosts. This may shed some light on the evolutionary history of *V. salmonicida*.

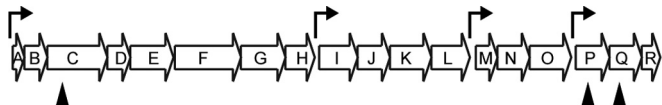The ability to adhere to surfaces and form biofilms also de-

FIG 6 Genetic organization of the *syp* locus in *V. salmonicida* LFI1238. The locus consists of 18 genes (*sypA* to *sypR*) and four operons. Putative σ54 binding sites were predicted using Fuzznuc and are indicated by bent arrows. The *syp* genes that were interrupted by plasmid insertions in the present study are indicated by black arrowheads.

pends upon structural components such as flagella and pili (55, 65, 66). We observed numerous long and thin filaments between the cells in the extracellular matrix of the biofilm and, in addition to flagella, the *V. salmonicida* genome encodes genes for two type IV pili, a Flp-type pilus (Tad pilus) and curli fibers (40). The *tad* locus is present in the genomes of a wide range of bacteria, including *V. cholerae* and *V. vulnificus*, and environmental conditions and QS are known to drastically modulate Flp-pilus expression (55, 67, 68). Since *V. salmonicida* encodes a complete *tad* locus, we inactivated two of the *tad* genes (VSAL_II0367 and VSAL_II0368) in the Δ*litR* mutant. However, interruption of *tad* did not restore the smooth colony morphology, and amounts of biofilm similar to those produced by the parental strain were produced by these two double mutants. Nor did we detect any differential expression of the *tad* locus, suggesting that adhesion and biofilm formation in *V. salmonicida* is dispensable for the *tad* locus under the conditions used here. Lastly, the microarray showed upregulation of some flagella and the curli genes CsgF and CsgE (VSAL_I2856 and VSAL_I2857). Interestingly, the assembly of the *E. coli* curli pili is temperature regulated, and lower temperatures increase the formation of curli (69). However, the contribution of curli fibers in *V. salmonicida* biofilms remains to be verified.

We have shown here that LitR regulates biofilm formation, colony morphology, and *syp* expression. The differences in the phenotypes between wild-type and Δ*litR* strains were more prominent at low temperatures. However, it remains to be demon-
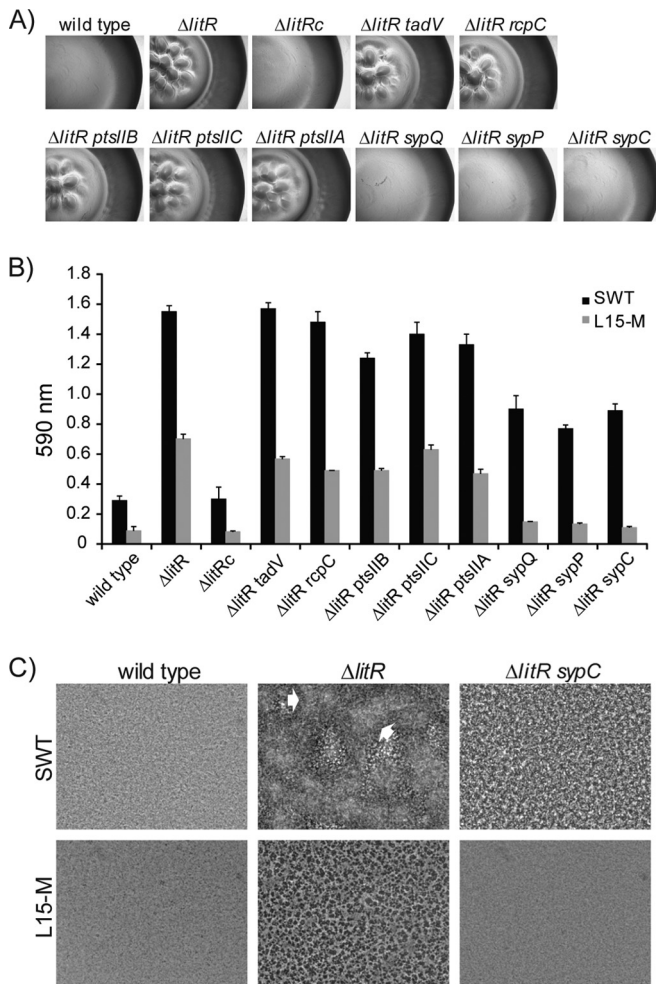
FIG 7 Colony morphology and biofilm formation of *V. salmonicida* wild-type LFI1238 and the different mutants. (A) The colony morphology of the different strains shows that the disruption of *sypC*, *sypP*, and *sypQ* in the Δ*litR* mutant alleviates the rugose appearance. (B) Quantitation of the biofilms formed in L15-M and SWT medium after 3 days of static incubation. The error bars show the standard deviations of three biological replicates. (C) Phase-contrast microscopy (Leica) shows that disruption of *syp* results in a flat and regular biofilm when formed in SWT medium. The white arrows point to irregular mushroom-like structures in the biofilm. All experiments were performed at 4°C.

strated that *litR* expression *per se* is indeed regulated by temperature and in response to AHL accumulation. It also remains uncertain whether LitR regulates *syp* transcription directly or whether other factors are involved. The signaling cascade is likely complex and probably important to fine-tune activities under changing environmental conditions.

ACKNOWLEDGMENTS

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