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A *Serratia marcescens* OxyR Homolog Mediates Surface Attachment and Biofilm Formation[▽]

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OxyR is a conserved bacterial transcription factor with a regulatory role in oxidative stress response. From a genetic screen for genes that modulate biofilm formation in the opportunistic pathogen *Serratia marcescens*, mutations in an *oxyR* homolog and predicted fimbria structural genes were identified. *S. marcescens oxyR* mutants were severely impaired in biofilm formation, in contrast to the hyperbiofilm phenotype exhibited by *oxyR* mutants of *Escherichia coli* and *Burkholderia pseudomallei*. Further analysis revealed that OxyR plays a role in the primary attachment of cells to a surface. Similar to what is observed in other bacterial species, *S. marcescens* OxyR is required for oxidative stress resistance. Mutations in *oxyR* and type I fimbrial genes resulted in severe defects in fimbria-associated phenotypes, revealing roles in cell-cell and cell-biotic surface interactions. Transmission electron microscopy revealed the absence of fimbria-like surface structures on an OxyR-deficient strain and an enhanced fimbrial phenotype in strains bearing *oxyR* on a multicopy plasmid. The hyperfimbriated phenotype conferred by the multicopy *oxyR* plasmid was absent in a type I fimbrial mutant background. Real-time reverse transcriptase PCR indicated an absence of transcripts from a fimbrial operon in an *oxyR* mutant that were present in the wild type and a complemented *oxyR* mutant strain. Lastly, chromosomal P_{lac}-mediated expression of *fimABCD* was sufficient to restore wild-type levels of yeast agglutination and biofilm formation to an *oxyR* mutant. Together, these data support a model in which OxyR contributes to early stages of *S. marcescens* biofilm formation by influencing fimbrial gene expression.

Serratia marcescens is the etiological agent of white pox in elkhorn coral and cucurbit yellow vine disease in plants (47, 48). *S. marcescens* is also an emerging opportunistic pathogen noted for causing uropathogenic, respiratory, bloodstream, and ocular infections (1, 23). *S. marcescens* is one of the most common bacterial species that causes keratitis and is frequently found contaminating contact lenses and lens cases (3, 8, 23, 54). Biofilm formation, the adherence of microbes to surfaces such as contact lenses, likely plays a role in *S. marcescens* pathogenesis (23). Growth in biofilms enables bacterial populations to survive better in inhospitable conditions (e.g., in the presence of antibiotics or a hostile immune system) (18, 28).

Recent studies of *S. marcescens* biofilms have implicated a number of genes as having an important role in attachment to abiotic surfaces and biotic surfaces in a quorum sensing-dependent or -independent manner (30, 31). The genes important for attachment to hydrophilic abiotic surfaces include the *luxI* homolog *swrI*, the two-component regulator genes *rssA* and *lipB*, a type I secretion system gene, and two quorum-sensing-controlled genes that are important for biofilm structure and exopolysaccharide production, *bsmA* and *bsmB* (30,

31). The same study found a role for certain genes, including *bsmA*, *bsmB*, and the type I fimbrial adhesin gene *fimA*, in the attachment of *S. marcescens* MG1 to immortalized corneal epithelial cells (31). Other studies have revealed the importance of quorum sensing regulation in biofilm maturation and biofilm-dependent protection against predation by protozoans (30, 50, 51). While these reports have provided important insights into *S. marcescens* biofilm formation and have defined some of the genes required for wild-type biofilm formation, it is likely that other important biofilm factors await discovery.

OxyR is reported to play a role in the oxidative stress response and pathogenicity in several genera of bacteria (4, 15, 19, 25, 26, 32, 34, 45, 52). There is substantial evidence that OxyR also responds to the thiol-disulfide status in *Escherichia coli* (46, 60). In *E. coli*, OxyR is posttranscriptionally modified through the formation of a disulfide bond that leads to an altered transcriptional profile. Also in *E. coli*, fimbria assembly-associated disulfide bridge formation can cause a net reduction of the cell, leading to OxyR-dependent inhibition of the surface-associated antigen 43 protein (55).

Antigen 43 (encoded by the *flu* gene) in *E. coli* creates a Velcro-like adhesive protein that mediates cell-cell interactions and is a positive factor in biofilm formation (13, 58). In *E. coli*, OxyR is a negative regulator of *flu*, such that *oxyR* mutants become hyper-biofilm formers and exhibit elevated levels of autoaggregation (13, 58). OxyR is also a negative regulator of biofilm formation in *Burkholderia pseudomallei*, but it appears to be a positive regulator of biofilm formation in *Neisseria*

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Serratia marcescens strains		
WT-D	*S. marcescens* D (pigmented)	Presque Isle Cultures
RSS1	WT-D *rpsL* mutation, Str^r	R. M. Q. Shanks, unpublished
50A3	WT-D with *oxyR*::Tn-mariner, *oxyR-1*	This study
RSS3	WT-D with *oxyR-2*, *oxyR*::pRMQS133	This study
RSS4	RSS3 with restored wild-type *oxyR*	This study
E30B5	WT-D with *oxyR*::Tn-mariner, *oxyR-3*	This study
50E2	WT-D with *fimC*::Tn-marniner	This study
59F12	WT-D with *fimB*::Tn-mariner	This study
Escherichia coli strains		
SM10 λ-pir	*thi-1 thr leu tonA lacY supE recA*::RP4–2-Tc::Mu *pir*	36
S17-1 λ-pir	*thi pro hsdR hsdM$^+$ ΔrecA* RP4–2::TcMu-Km::Tn7 *pir*	36
Saccharomyces cerevisiae InVSc1	*MATa/MATa leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 his3-Δ1/his3-Δ1*	Invitrogen
Plasmids		
pBT20	Mariner delivery vector	29
pMQ118	oriR6K, suicide/allelic replacement vector	R. M. Q. Shanks and G. A. O'Toole, unpublished
pMQ125	p15a-yeast shuttle vector with P_{BAD}	R. M. Q. Shanks and G. A. O'Toole, unpublished
pMQ131	Broad-host-range yeast-pBBR1 vector	R. M. Q. Shanks and G. A. O'Toole, unpublished
pRMQS133	pMQ118 with internal fragment of *oxyR*	This study
pRMQS140	pMQ131 with *oxyR-prgL*	This study
pRMQS143	pMQ125 with *oxyR*	This study
pRMQS145	Like pRMQS143 but C205S	This study
pRMQS147	Like pRMQS143 but H204R	This study
pRMQS169	pMQ118 with P_{lac}-*fimA'*	This study

gonorrhoeae; however, the mechanism responsible for these phenotypes has not been determined (34, 56).

In addition to antigen 43, fimbriae and fimbria-like adhesins are important in biofilm formation in *E. coli* and other organisms (5, 6, 12, 14, 20, 33, 49, 55, 59). These large surface structures also play a key role in attachment to epithelial cells and pathogenesis (11, 31, 40). These appendages are also important in pellicle formation and impart growth advantages in some environmental niches (16, 42). A previous study showed that while OxyR is important for regulation of antigen 43, it apparently is not important for the expression of type I fimbriae in *E. coli* (21).

Here, we identified an *oxyR* homolog in a screen for genes that contribute to *S. marcescens* biofilm formation. Similar to what is observed with *N. gonorrhoeae*, contrary to what is reported for *E. coli* and *B. pseudomallei*, *S. marcescens* oxyR protein exhibit reduced biofilm formation and decreased autoagglutination (cell-cell interactions). The *S. marcescens* OxyR protein was found to contribute to both oxidative stress survival and the production of functional fimbriae.

MATERIALS AND METHODS

Bacterial strains, media, and growth. Microorganisms used in this study are listed in Table 1. All bacteria were grown in lysogeny broth (LB) (0.5% yeast extract, 1% tryptone, 0.5% NaCl). Autoagglutination assays were performed in PG medium (1% peptone, 1% glycerol). Helvick medium (0.5% peptone, 0.5% tryptone) was used for swimming and swarming assays (0.3% [wt/vol] agar for swimming and 0.6% agar for swarming). The antibiotics used in this study were gentamicin (10 μg/ml), tetracycline (10 μg/ml), kanamycin (100 μg/ml), and ampicillin (100 μg/ml).

Transposon mutagenesis and genetic screens. *E. coli* strain SM10-λpir bearing the mariner-based transposon delivery plasmid pBT20 (29) was mated with a red-pigmented wild-type *S. marcescens* strain. Briefly, 0.5-ml portions of overnight cultures of both strains were mixed in a Microfuge tube, spun down, resuspended in 200 μl Tris-EDTA (TE), incubated at 42°C for 10 min, spotted onto an LB plate, and incubated at 30°C for 3 to 12 h. A portion of the cells was taken up with a toothpick and suspended in 1 ml of TE. Aliquots were plated onto LB plates supplemented with gentamicin (10 μg/ml) to select for transposon recipients and with tetracycline (10 μg/ml) to select against *E. coli* growth. Plates were incubated for 2 to 3 days at 30°C.

Pigmented colonies were picked and placed into individual wells of a flat-bottom 96-well dish (Corning Costar catalog no. 3595) in 0.1 ml of LB and incubated at 37°C for 16 to 18 h. A 96-prong multiwell transfer device (Dan-Kar catalog no. MC96) was used to transfer aliquots of mutant libraries into individual wells of round-bottom 96-well polyvinylchloride (PVC) dishes (Falcon catalog no. 3911) containing 85 μl LB, which were incubated at 37°C for 6 or 24 h (11,000 mutants were incubated for 6 h, and 3,000 separate mutants were tested after 24 h). Biofilm formation was then assessed using previously described protocols (43). Transposon insertion sites were mapped using arbitrary PCR (44).

Biofilm assays. Static microplate assays were performed as described by O'Toole and Kolter, with modifications (43). Cells at an A_{600} of 0.1 were incubated in LB for 6 h (as described below) before biofilm formation was assessed (43). Relative biofilm formation was determined by solubilizing crystal violet with 30% glacial acetic acid and determining the absorbance at 590 nm. Biofilm assays were performed using at least four replicates from triplicate independent cultures for each genotype and were repeated at least two times on different days.

Primary attachment assay. Bacteria were attached to tissue culture-treated polystyrene (Costar catalog no. 3513) as follows. Bacterial cultures were grown to saturation with aeration at 30°C and washed with phosphate-buffered saline (PBS), and the A_{600} was adjusted to 1.0 (~5×10^9 cells/ml). One milliliter of LB was added to each well of a 24-well dish, and then 100 μl of the washed culture was added to each well and incubated at room temperature for 20 min. LB and nonattached cells were then removed, and wells were washed five times with 1 ml of PBS. One milliliter of PBS was added, and bacterium-surface interactions

were observed by phase-contrast microscopy at a magnification of ×400. Digital micrographs were taken, and the number of attached bacteria per field was determined using Metamorph software. At least 14 fields from three independent cultures per strain were used, and the experiment was repeated twice.

Growth curves. The growth rates of the wild type and an isogenic *oxyR* mutant strain were determined in 5 ml of LB incubated at 30°C with rotation at high speed on a TC-7 tissue culture roller (New Brunswick, Edison, NJ). Triplicate 100-μl aliquots were removed at various time points, and the culture turbidity (A_{600}) was determined using a plate reader (Synergy HT; Bio-Tek, Winooski, VT). This experiment was performed twice on different days.

Oxidative stress sensitivity assays. Disk diffusion assays were done by spreading ~5×10^8 bacteria on an LB agar plate and placing a sterile 6-mm paper disk (catalog no. BB31039; BBL) on the plate, to which 10 μl of 30% H_2O_2 (Fisher Scientific catalog no. H325) was added, and incubating the preparation overnight at 30 and 37°C. Four replicates from four independent cultures were used per experiment, and each experiment was repeated at least three times on different days.

Hexidium iodide is a quantitative fluorescent DNA dye that stains cells with compromised membranes and is used as a "dead stain." Cultures that were exposed to oxidative stress were spun down, resuspended in saline (0.85% NaCl) with hexidium iodide using a Bac-Light Live/Dead staining kit (Molecular Probes, Eugene, OR), and incubated at room temperature for 15 min. Red fluorescence and the A_{600} were determined using a plate reader (Synergy 2; Bio-tek, Winooski, VT). Numbers of relative fluorescence units (RFU) were determined by dividing the red fluorescence units by the A_{600}.

Fimbria activity assays. Timed agglutination of yeast cells was performed by placing 25 μl of bacteria that had been washed and resuspended in PBS and normalized (A_{600}, 2.0) onto a glass microscope slide (Corning, Corning, NY). Simultaneously, 25 μl of *Saccharomyces cerevisiae* (Sigma, product no. YSC2, 2% [wt/vol] in PBS) was added to the slide along with the bacteria, and a timer was started. Concurrently, a second researcher turned the orbital shaker on to mix the bacteria and yeast cells (Lab-line model 2309; setting 10). The researcher with the timer determined the moment at which visible aggregates appeared and was unaware of the genotype of the experimental strain. For each genotype, individual single colonies were used to make three cultures that were tested. The experiment was performed at least three times on separate days for each genotype.

Yeast agglutination was measured spectrophotometrically by mixing 1.5 ml PBS with 500 μl of yeast cells (as described above) and 400 μl of bacteria in PBS (A_{600}, 1.0). The cultures were vortexed at moderate speed for 5 s and allowed to stand at room temperature for 10 min. Three 100-μl samples were then removed from the upper aqueous phase of each tube, and the A_{600} was determined; then the tubes were vortexed for 30s at high speed, three 100-μl samples were removed from the upper aqueous phase of each tube, and the A_{600} was determined. The percentage of agglutination was determined as follows: $100 \times (1 - A_{600}$ before vortexing/A_{600} after vortexing). For each genotype, single colonies were used to make three cultures that were tested. The experiment was performed at least three times on separate days for each genotype.

Plasmid construction and genetic manipulations. Chromosomal DNA preparations were made using a kit (Gentra catalog no. D5500A). DNA was amplified using a high-fidelity polymerase (TripleMaster; Eppendorf), except where noted below, and was cloned using either T4 DNA ligase (Quick Ligase; New England Biolabs) or yeast in vivo cloning (57).

Targeted mutagenesis of *oxyR* was carried out using a single-crossover strategy. A 426-bp internal fragment of *oxyR* was amplified using *Taq* DNA polymerase (New England Biolabs, Beverly, MA), cloned into pCR2.1 (Invitrogen), and subcloned into pMQ118, a suicide vector in *S. marcescens* (R. M. Q. Shanks and G. A. O'Toole, unpublished results). The resulting plasmid, pRMQS133, was introduced into *S. marcescens* via conjugation as described above, except that kanamycin was used as the selectable marker. pRMQS133 chromosomal integrations were verified by PCR. The resulting *oxyR-2* mutation has an internal duplication and has pMQ118 inserted at bp 435 (the total length of *oxyR* is 936 bp).

A wild-type revertant of *oxyR-2* was created by growing the *oxyR-2* mutant to saturation four times in the absence of antibiotic selection to allow a recombination event to loop out the integrated plasmid. The bacterial culture was streaked to obtain single colonies on LB plates, and replicates were generated on LB plates and LB plates containing kanamycin with a replica block. The wild-type *oxyR* status of kanamycin-sensitive isolates was confirmed by PCR and phenotype. One isolate was designated RSS4 and used as a control in further experiments (Table 1).

pRMQS140 is a medium-copy-number shuttle plasmid bearing a wild-type copy of *oxyR* and the adjacent gene *prgL*. The *S. marcescens* DNA was amplified using a high-fidelity polymerase and was cloned into pMQ131 using yeast in vivo cloning (57).

pRMQS143, a p15a-based shuttle plasmid bearing a wild-type *oxyR* open reading frame under control of the P_{BAD} promoter, was made using yeast in vivo cloning (57). The *oxyR* gene was amplified from the wild-type *S. marcescens* WT-D strain and recombined into pMQ125 under control of the P_{BAD} promoter.

pRMQS145 and pRMQS147 are similar to pRMQS143 but contain versions of *oxyR* with point mutations that result in C205S and H204R mutations, respectively. These plasmids were made using yeast in vivo cloning techniques by recombining two PCR amplicons with the pMQ125 vector. The amplicons were made using primers that were engineered with the desired point mutation. These constructs were verified by sequencing.

pRMQS169 was designed to place the chromosomal *fimABCD* operon under control of P_{lac} through an integration event. A 335-bp region of *fimA* including 20 bp upstream of the start codon was cloned 30 bp downstream of the P_{lac} promoter in pMQ118 using techniques described above.

Microscopy. Phase-contrast microscopy and fluorescent microscopy were performed using a Nikon Eclipse TE2000-U microscope equipped with a CoolSnap HQ charge-coupled device camera (Photometrics, Tucson, AZ), and images were acquired using either RS Image or Metamorph software. Confocal laser scanning microscopy was performed with bacteria grown under flow conditions, using the Kadori System 2 method as previously described (35) except that we used LB diluted 10-fold in water, glass coverslips were inserted in the wells, and the flow rate was 16.5 ml per h. The inoculum was 10^8 bacteria from a stationary-phase culture. The growth medium was removed at 24 h, and coverslips were resuspended in PBS and stained with a Bac-Light kit, as described above. Coverslips were dipped three times in PBS and were mounted on hanging drop microscope slides filled with PBS to maintain hydration. Images were acquired with an Olympus FV1000 confocal system equipped with an Olympus IX81 microscope, 405/458/488/515/543/633 nm lasers, and Fluoview software. The initial acquisition was at 12 bits (512 × 512; dwell time, 10 ms/pixel) with an NA 0.85 × 20 oil objective and a z spacing of 1.47 μm between stacked images. Three-dimensional reconstructions were generated in Fluoview at 256 × 256 resolution.

Bacterial samples were prepared for transmission electron microscopy (TEM) in one of two ways. Test tubes (25 by 150 mm) containing 5 ml of LB were inoculated with bacteria and incubated at 30°C. The resulting cultures were classified as "agitated" or "static" cultures based on the growth conditions. Agitated culture tubes were incubated on a TC-7 tissue culture roller (New Brunswick Scientific, New Jersey) rotating at maximum speed, and samples were taken at 20 to 24 h, whereas static cultures were placed vertically in a rack and incubated for 48 to 72 h without agitation. Samples were washed and resuspended in PBS to a final A_{600} of 1.0. Cells were incubated with a Formvar-coated copper grid, negatively stained with 1% uranyl acetate, and observed with a Jem-1210 electron microscope.

RNA isolation. Strains were grown to stationary phase overnight and then subcultured 1:100 and grown until the A_{600} was 1. Each 5-ml liquid culture was then mixed with 15 ml of TriReagent LS (Molecular Research Center, Inc.) and frozen at −80°C until RNA was precipitated. RNA was isolated using the TriReagent LS protocol. After a 5-min incubation at room temperature, 4 ml of chloroform was added to each tube, which was then shaken vigorously and incubated at room temperature for 5 min. The mixtures were transferred into 15-ml gel phase lock tubes (Eppendorf) and centrifuged. Each aqueous layer was then mixed with isopropanol and glycogen prior to overnight precipitation at −80°C. The tubes were thawed and centrifuged at $1,900 \times g$ for 60 min at 4°C. The pellets were washed with 80% ethanol and dried prior to suspension in nuclease-free water (USB). The quantity of RNA was determined using a DU800 spectrophotometer (Becton Dickenson), and the quality was determined using an Agilent 2100 Bioanalyzer. The RNA was treated with DNase to remove any contaminating DNA using a Turbo DNA-*free* kit (Ambion). In short, 50 μg of nucleic acid was mixed with 0.1 volume of 10× Turbo DNase buffer, 1 μl of DNase, and nuclease-free water to obtain a final volume of 50 μl. The mixture was incubated at 37°C for 30 min, and then an additional 1 μl of DNase was added and the mixture was incubated for 30 min at 37°C. The reaction mixture was inactivated by using the manufacturer's protocol, with additional centrifugation to ensure complete removal of the inactivation reagent. The remaining nucleic acids were precipitated with isopropanol and ammonium acetate. The resulting pellet was washed with 80% ethanol and dried before it was suspended in nuclease-free water. The quantity and quality were assessed as described above.

PAGE analysis of surface proteins. Surface proteins from cells grown overnight on LB plates at 30°C were prepared as previously described (31). Trichloroacetic acid-precipitated proteins were washed twice with cold acetone, dried,
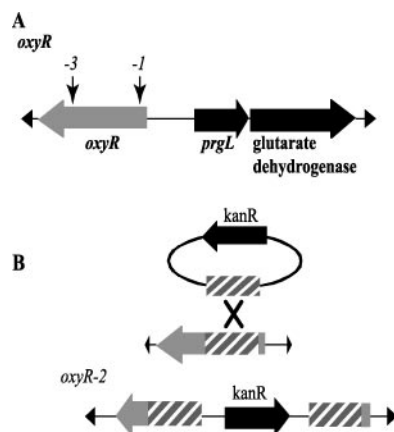
FIG. 1. Genomic context of the *S. marcescens oxyR* homolog. (A) Approximate positions of *oxyR* transposon mutations required for biofilm formation are indicated by the vertical arrows. The mariner transposon from *oxyR-1* inserted in the 13th codon and in codon 266 for *oxyR-3*. The predicted OxyR protein is 311 amino acids long. (B) Schematic representation of the single-crossover strategy used to disrupt *oxyR* with plasmid pRMQS133 to generate the *oxyR-2* allele.

resuspended in 10 μl of TE and 15 μl of polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled, and run on an 8 to 16% gradient gel. Identification of proteins by mass spectroscopy was performed by the University of Pittsburgh Genomics and Proteomics Core Laboratories.

Real-time PCR. DNase-treated RNA (1 μg) was used in the cDNA synthesis reaction. Duplicate RNA samples were incubated with random primers (catalog no. 48190-011; Invitrogen) and deoxynucleoside triphosphates for 5 to 10 min at 70°C and cooled on ice. To one of the duplicates, SuperScript III reverse transcriptase (RT) (Invitrogen), 0.1 M dithiothreitol, and 5× First Strand buffer were added according to the manufacturer's directions. For the other duplicate, water was substituted for the SuperScript III RT. The samples were incubated for 60 to 120 min at 50°C. The cDNA was then diluted with nuclease-free water (UBS) to obtain a final dilution of 1:5,000 for use in the real-time PCR. Diluted cDNA template (5 μl) was added to a 96-well PCR plate (Bio-Rad). Separate master mixtures were prepared for each primer set according to manufacturer's protocol (iQ SYBR green Supermix; Bio-Rad). A two-step program was used with a Bio-Rad iQ5 real-time PCR machine. The data are the average ± standard error fold changes relative to the wild type for three biological replicates. To calculate the fold change, the average cycle threshold (C_T) for the control gene (*rplU*) was subtracted from the average C_T for the experimental gene (*fimC* or *fimA*), which resulted in the ΔC_T. The wild-type expression ΔC_T was subtracted from the mutant strain ΔC_T to determine the $\Delta \Delta C_T$, which was transformed ($2^{-\Delta\Delta C_T}$), giving the average fold change relative to the wild type. The primer sequences are as follows: F-rplU, GCTTGGAAAAGCTGGACATC; R-rplU, TACGGTGGTGTTTACGACGA; F-fimA-RT, ACTACACCCTGCGTTTC GAC; R-fimA-RT, GCGTTAGAGTTTGCCTGACC; F-fimC-RT, AAGATCG CACCGTACAAACC; and R-fimC-RT, TTTGCACCGCATAGTTCAAG.

RESULTS

Identification of genes required for biofilm formation in *S. marcescens*. To gain insight into the molecular mechanism of *S. marcescens* biofilm formation, a library of random mutations was generated in *S. marcescens* using a mariner transposon. This library was screened to identify genes required for biofilm formation using a method similar to that of O'Toole and Kolter (43). Among 14,000 mutants, approximately 50 were found with reproducibly altered biofilms. Two biofilm-defective isolates had mutations that mapped to a putative regulatory factor gene, a homolog of *oxyR* that shares more than 90% amino acid identity with the OxyR gene of *E. coli* (Fig. 1A and Table 2). The mutant alleles were designated *oxyR-1* and

oxyR-3, and the mutants had biofilm phenotypes quite similar to those of isolates whose mutations mapped to fimbrial genes (Fig. 1A and Tables 1 and 2). The *oxyR* mutants exhibited 5- to 50-fold reductions in biofilm staining (Table 2). At least three of the isolates with mutations in putative chaperone-usher pathway fimbria genes had independent mutations in the same predicted type I fimbrial usher gene, *fimC*. The sequence of this gene was submitted to GenBank by another group of workers, and this gene has been shown to be important in adherence of *S. marcescens* strain MG1 to corneal epithelial cells (GenBank accession no. EEF193059) (31). These usher gene mutations result in a severe reduction in biofilm formation on both hydrophobic and hydrophilic substrates (Table 2 and data not shown).

OxyR is a LysR family transcription factor that is conserved in many gram-negative and -positive bacteria. OxyR has been implicated as a pathogenicity factor in several species and regulates the transcription of an *E. coli* adhesive factor (25, 26, 32, 52). OxyR is important for the response to a bacterium's redox state (60). Bacterial adhesive factors and the bacterial response to local environmental conditions both play important roles in biofilm formation and virulence; therefore, priority was given to further characterization of the *S. marcescens oxyR* homolog.

OxyR is required for *S. marcescens* biofilm formation and initial attachment. Complementation analysis of the *oxyR-1* mutant was performed using a plasmid bearing a wild-type copy of *oxyR* (Table 1). The biofilm defect of the *oxyR-1* mutant was fully complemented by wild-type *oxyR* added in *trans* (Fig. 2A and Table 2). This result is consistent with the hypothesis that the transposon mutation of *oxyR* is responsible for the biofilm defect of strain 50A3 (*oxyR-1*) (Table 2). Similar patterns of biofilm formation were obtained using tissue culture-treated polystyrene as a substrate rather than PVC for the wild-type, *oxyR-1*, and *oxyR-3* strains (Table 2). Tissue culture-treated polystyrene is a hydrophilic surface, whereas PVC is relatively hydrophobic. A persistent biofilm defect on both PVC and polystyrene suggests that an OxyR-dependent adhesive factor(s) mediates attachment to diverse substrates.

Directed mutation of the chromosomal copy of *oxyR* was performed as a second test to ensure that the biofilm-defective phenotype was *oxyR* dependent rather than the result of another mutation. A single-crossover knockout strategy was cho-

TABLE 2. OxyR is required for biofilm formation at 20 h

Strain	A_{590} with the following substrate materials[a]:			
	PVC		Polystyrene	
	30°C	37°C	30°C	37°C
Wild type	0.54 ± 0.05	0.57 ± 0.05	0.31 ± 0.05	0.55 ± 0.05
oxyR-1	0.08 ± 0.02	0.08 ± 0.02	0.01 ± 0.02	0.01 ± 0.01
oxyR-2	0.19 ± 0.03	0.22 ± 0.04	0.06 ± 0.03	0.05 ± 0.05
oxyR-3	0.11 ± 0.04	0.09 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
Wild type + p*oxyR*[b]	0.43 ± 0.05	0.50 ± 0.09	0.38 ± 0.03	0.48 ± 0.04
oxyR-1 + p*oxyR*	0.55 ± 0.04	0.46 ± 0.05	0.47 ± 0.04	0.44 ± 0.08
oxyR-2 revertant	0.45 ± 0.07	0.51 ± 0.06	0.38 ± 0.03	0.57 ± 0.03
fimC-50E2	0.20 ± 0.04	0.18 ± 0.02	0.06 ± 0.02	0.15 ± 0.02
fimC-50E2 + p*oxyR*	0.20 ± 0.08	0.14 ± 0.02	0.06 ± 0.02	0.13 ± 0.01

[a] A_{590} of crystal violet-stained biofilms. The values are the averages ± standard deviations for 12 replicates from three independent cultures.
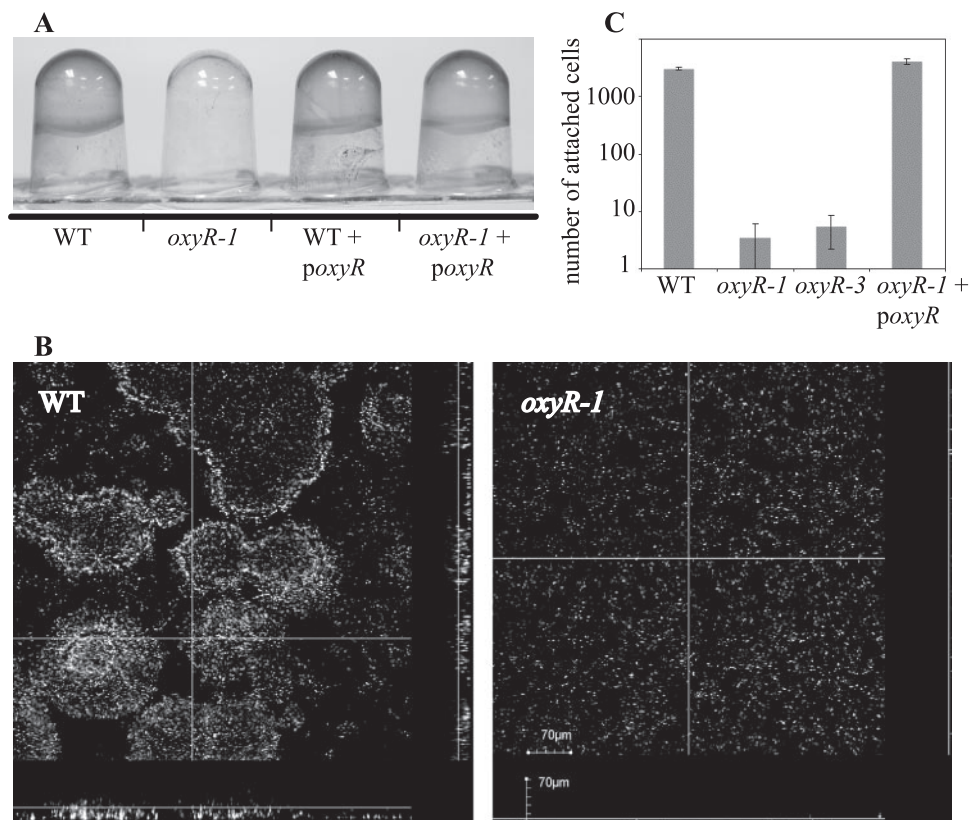[b] p*oxyR* is pRMQS140 and provides wild-type *oxyR* in *trans*.

FIG. 2. OxyR mutants are defective in biofilm formation and primary attachment. (A) Photograph of crystal violet-stained biofilms on inverted PVC microtiter wells. p*oxyR* is pRMQS140. (B) Confocal micrographs of Syto 9-stained biofilms that were formed under flow conditions for 24 h on glass. The micrographs were taken at the same magnification. (C) Quantification of bacterial attachment to polystyrene after 20 min of incubation in LB. The error bars indicate one standard deviation. WT, wild type.

sen to disrupt *oxyR*, which yielded a second mutant allele, *oxyR-2* (Fig. 1B). Like *oxyR-1* and *oxyR-3* mutants, *oxyR-2* mutants were defective in biofilm formation on both PVC and polystyrene. *oxyR-2* mutants exhibited a significant defect in biofilm formation compared to the wild type, but they produced severalfold more biofilm than the *oxyR-1* or *oxyR-3* mutants on polystyrene ($P < 0.001$) (Table 2). The method by which the *oxyR-2* allele was generated allowed us to isolate wild-type revertants from the *oxyR-2* strain. We took advantage of this to generate strain RSS4, which is an *oxyR-2* strain with a restored wild-type *oxyR* gene. RSS4 served as a complementation strain without a multicopy plasmid and exhibited biofilm phenotypes equivalent to those of the wild-type strain (Table 2).

Confocal microscopic analysis of the *oxyR-1* mutant and the wild type grown under flow conditions confirmed the dramatic reduction in biofilm formation by the *oxyR-1* mutant relative to the wild type seen with the microtiter plate assay. At 24 and 48 h wild-type cells produced very large microcolonies, whereas the *oxyR-1* mutant exhibited no biofilm architecture whatsoever, and only scattered cells were associated with the surface (Fig. 2B and data not shown). Similar results were obtained with epifluorescence microscopy of biofilms grown under static conditions on both PVC and glass coverslips (data not shown). The decrease in attached cells suggests that there is a defect in the initial attachment of the bacteria to the surface.

The initial attachment of the wild-type, *oxyR-1*, and *oxyR-3* strains and the *oxyR-1* strain bearing pRMQS140 (pMQ131 plus *oxyR*) to polystyrene was determined after 20 min of incubation with a surface. After nonattached cells were washed away, the number of bacteria attached per microscopic field was reduced approximately 1,000-fold for the *oxyR* mutants. By supplying wild-type *oxyR* in *trans*, full levels of attachment were restored, suggesting that OxyR contributes to biofilm formation through promoting the initial attachment of bacteria to surfaces (Fig. 2C). We noted that whereas cells of the wild-type and complemented strains were associated with the surface along their longitudinal axis, the few cells observed for the *oxyR* mutant strains were polarly attached and spinning as if they were attached by a flagellum. The lack of longitudinal attachment of *oxyR* mutants compared to the wild type is characteristic of a defect in the "reversible-to-irreversible" attachment step of biofilm formation, as exhibited by *lapA*, *sadB*, and certain *pho* regulon mutants of *Pseudomonas* species (9, 22, 39). Sauer and colleagues first characterized the "reversible-to-irreversible" attachment switch as a developmental step in early biofilm formation after primary attachment to a substrate, which may represent bacteria sensing the environment (53). This phenomenon was quantified by determining the ratio of the number of nonmotile, longitudinally attached cells per field to the total number of cells per field using microscopy. *oxyR-1* mutants exhibited a significant reduction in irreversibly

TABLE 3. *oxyR* sensitivity to hydrogen peroxide

Genotype	Inhibition zone (mm)[a]	
	30°C	37°C
Wild type	16.0 ± 1.3	13.4 ± 1.4
oxyR-1	21.5 ± 0.8[b]	17.8 ± 0.9[b]
oxyR-2	21.3 ± 1.9[b]	17.5 ± 2.9[b]
oxyR-3	ND	23.6 ± 1.7[b]
oxyR-2 revertant[c]	ND	13.2 ± 0.6

[a] The values are the sizes of zones of inhibition obtained using disk diffusion assays from three separate experiments performed on different days with ≥8 independent cultures per strain and are the averages ± standard deviations for ≥10 plates. ND, not determined.
[b] Significantly different from the wild-type value (*P* < 0.01).
[c] Strain RSS4, which is wild type for *oxyR*.

attached cells (23.3% ± 8.7% compared with 96.9% ± 0.5% for the wild type; *n* > 700 cells for each strain). A similar reluctance for longitudinal surface association with the surface was exhibited by the *oxyR-1* mutant under flow conditions at 24 h; less than 9% of surface-associated *oxyR-1* cells were longitudinally attached, whereas the wild-type strain coated the surface.

oxyR gene is required for hydrogen peroxide resistance. To determine whether the *S. marcescens* OxyR homolog has a conserved function, its role in oxidative stress was assessed using a disk diffusion assay. Hydrogen peroxide was employed as an oxidative stress response agent. The *oxyR-1* mutant exhibited elevated sensitivity to H_2O_2 compared to the isogenic wild-type strain (Table 3). The *oxyR-2* and *oxyR-3* mutants were also hypersensitive to H_2O_2. Similar trends were seen at 30 and 37°C, suggesting that OxyR is important at both environmental and body temperatures (Table 3).

A secondary assay was used to quantify the contribution of OxyR to oxidative stress resistance. Hexidium iodide is a fluorescent dye that can be used for quantitative analysis of dead or severely compromised cells. We used hexidium iodide to stain the DNA of bacteria with compromised membranes. The ratio of hexidium iodide fluorescence to culture optical density (RFU) was determined using a plate reader. Within 4 h of exposure to H_2O_2, significant sensitivity was observed in *oxyR-1* mutants (*P* = 0.012). The values obtained were 1,390 ± 150 RFU (170 ± 19 RFU without H_2O_2) for the wild type and 2,391 ± 255 RFU (190 ± 17 RFU without H_2O_2) for the *oxyR-1* mutant.

Mutation of oxyR does not result in reduced growth rates or a defect in swimming motility. The reduced level of biofilm formation exhibited by *oxyR-1* mutants could be a result of a lower growth rate. The growth rates of the *oxyR-1* mutant and the isogenic wild-type strain were assessed. No difference in the growth rates was observed (Fig. 3A).

The strong reduction in biofilm formation at 6 h by *oxyR* mutant strains is reminiscent of the strong inhibition of early biofilm formation exhibited by flagellum mutants of *Pseudomonas aeruginosa* (43, 53). The flagellum-based swimming motility through a semisolid agar matrix was determined for all three *oxyR* mutants, and no defect was observed (Fig. 3B and data not shown). In fact, the mutant strains reproducibly exhibited larger zones of swimming. In one representative experiment after 8 h of incubation at 30°C, the size of the zone for
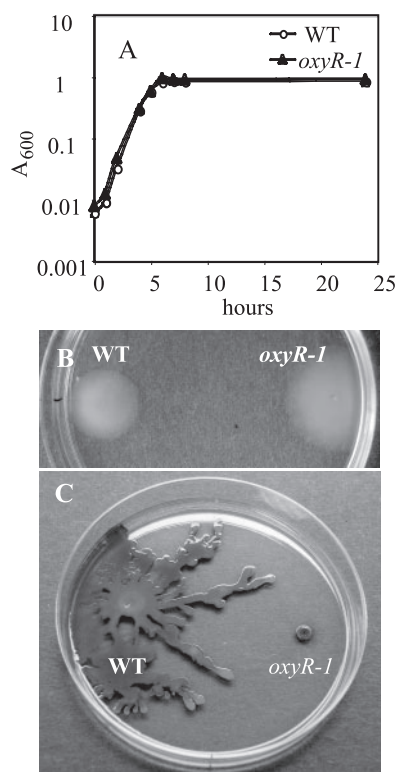

FIG. 3. *S. marcescens* OxyR is required for swarming but not for swimming or growth. (A) The wild type and *oxyR* mutant have nearly identical growth rates. (B) The *oxyR-1* mutant swims further through semisolid media than the wild type. (C) The *oxyR-1* mutant is defective in swarming motility. WT, wild type.

the wild-type strain was 7.7 ± 1.5 mm, compared to 12.4 ± 0.9 mm for the *oxyR-1* strain and 11 ± 2 mm for the *oxyR-2* strain (*n* = 5 plates per strain; *P* < 0.02 for both mutant strains compared to the wild type). This suggests not only that flagella are made and functional but that the chemotaxis function is intact, as chemotaxis mutants appear to be negative in swimming motility agar assays.

The *oxyR-1* and *oxyR-2* strains were assessed to determine their surface motility (swarming), a group activity akin to biofilm formation in which populations of bacteria move in groups to form finger-like projections. A radical difference was observed. Whereas the wild-type strain was able to cover much or all of a plate in 48 h, the isogenic *oxyR-1* strain did not form finger-like swarming projections under our experimental conditions (Fig. 3C and data not shown). In a sample experiment, after 24 h of incubation at room temperature on swarming plates, the wild type swarmed a maximum of 13.5 ± 2.0 mm from the point of inoculation, compared to the absence of any swarming for the *oxyR-1* mutant (*n* = 5 plates per strain).

OxyR is important for fimbria-associated phenotypes. In the biofilm screen, several mutations leading to biofilm-defective phenotypes mapped to predicted fimbrial genes. Most of these mutations resulted in severe defects in biofilm formation similar to those seen in the *oxyR-1* strain (Table 2 and data not shown). Fimbriae have previously been described as structures that play a role in biofilm formation on abiotic surfaces and primary attachment in other organisms, such as *E. coli* (7, 49,
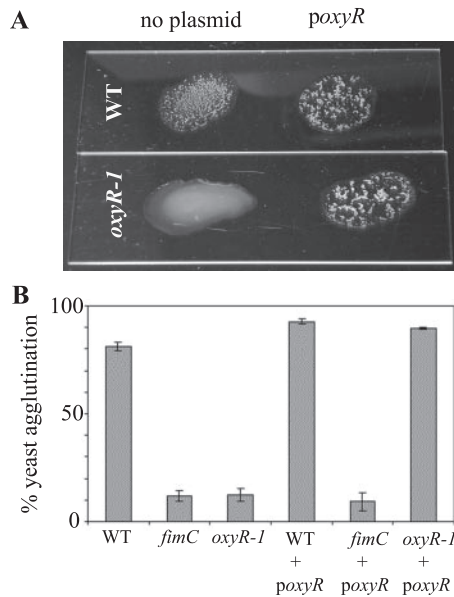
A

no plasmid   p*oxyR*

B

FIG. 4. OxyR is required for aggregation of eukaryotic cells. (A) The *oxyR-1* mutant is defective for agglutination of yeast on microscope slides, and this defect can be complemented by *oxyR* added in *trans* using pRMQS140. (B) Semiquantitative yeast agglutination assay. The *oxyR-1* mutant is defective in yeast agglutination, and the levels are restored to wild-type levels by complementation with *oxyR* added in *trans* using pRMQS140. The *fimC* defect in yeast agglutination is not complemented by pRMQS140. The error bars indicate one standard deviation. WT, wild type.

59). Recently, phenotypic variants arising from *S. marcescens* biofilms have been isolated that display elevated levels of piliation and increased abilities to form biofilms, correlating the presence of surface pili to biofilm formation in this species (27). While OxyR has not been described as a protein that plays a role in regulating fimbrial expression in *E. coli*, it regulates the surface protein antigen 43 (21). Based on these observations, we hypothesized that *S. marcescens* OxyR positively regulates fimbrial expression, resulting in reduced biofilm formation in the *oxyR* mutant strains.

Fimbria activity is commonly measured through agglutination of eukaryotic cells, such as *S. cerevisiae* cells or erythrocytes (24, 37, 38). The *oxyR-1* mutant was found to be severely deficient in the ability to agglutinate budding yeast cells (Fig. 4A). An assay that determined the time of appearance of agglutinated cells on a glass slide was performed. We noted that whereas visible aggregates appeared with the wild-type strain in 4.3 ± 0.6 s, no aggregates appeared with the mutant strain during the period of observation (60 s; $n \geq 6$) (Fig. 4A). The *oxyR-1* mutant with pRMQS140 (p*oxyR*), which provides wild-type *oxyR* in *trans*, was able to form aggregates rapidly (2.7 ± 0.4 s; $n = 6$), indicating that the agglutination defect is linked to *oxyR*. A similar deficiency was observed with type I fimbrial mutants (*fimB* and *fimC*) (no agglutination at 60 s; $n \geq 6$). The *oxyR-2* and *oxyR-3* mutants also exhibited a severe yeast agglutination phenotype with no agglutination at 60 s, and the *oxyR-2* mutant restored to the wild-type strain (RSS4) agglutinated yeast in 2.9 ± 0.7 s ($n \geq 6$).

A second method was developed to quantify the ability of wild-type and *oxyR-1* cells to agglutinate eukaryotic cells. Nor-

malized quantities of bacteria and yeast cells were mixed in PBS and allowed to interact, form aggregates, and settle for 5 min. The percentage of agglutination was determined spectrophotometrically by comparing the optical densities before and after aggregation. The wild-type strain exhibited >80% aggregation, whereas the *oxyR-1* strain agglutinated only 12.5% of the yeast cells ($P < 0.001$; $n = 6$) (Fig. 4B). The level of agglutination of the *oxyR-1* mutant was >80% when wild-type *oxyR* was provided in *trans* (Fig. 4B). Mutation of the type I fimbrial usher gene *fimC* resulted in similar reductions in yeast agglutination; however, this defect could not be complemented by addition of the wild-type *oxyR* gene on a plasmid (Fig. 4B). This suggests that the *oxyR* mutant defect is fimbria dependent. *oxyR-2* and *oxyR-1* mutants were similarly defective in yeast agglutination. In a representative experiment done in triplicate with three cultures grown from separate single colonies, the wild-type strain exhibited $86.9\% \pm 4.3\%$ agglutination, whereas the *oxyR-2* mutant exhibited $12.8\% \pm 4.0\%$ agglutination ($P < 0.001$). Expression of wild-type *oxyR* complemented the fimbria-deficient phenotype conferred by the *oxyR-2* mutation. The *oxyR* open reading frame was cloned under control of a P_{BAD} promoter (pRMQS143), and yeast agglutination was determined after overnight growth in the presence or absence of 10 mM L-arabinose. In the absence of arabinose the *oxyR-2* strain exhibited $4.7\% \pm 6.4\%$ yeast agglutination, while the strain grown in the presence of L-arabinose agglutinated $77.5\% \pm 2.1\%$ of the yeast cells ($P < 0.001$; $n = 6$).

Autoagglutination (bacterium-bacterium interactions) of bacterial cells can also be dependent upon type I fimbriae and other pili (10, 41). It was noted that wild-type cultures grown in PG medium became flocculent, whereas the *oxyR-1* mutant produced homogeneous, turbid cultures. The extent of autoagglutination in PG medium was determined spectrophotometrically after tubes were allowed to stand without agitation for 1 h. In a representative experiment done in triplicate using three different single colonies, the wild-type culture exhibited $48.14\% \pm 4.95\%$ autoagglutination and the *oxyR-1* mutant exhibited $6.56\% \pm 6.0\%$ autoagglutination ($P < 0.001$). Similar results were obtained with bacteria grown in brain heart infusion medium (data not shown).

Electron microscopy of fimbriae and *oxyR-1* mutants reveals a lack of surface pili. TEM was utilized to directly determine the effect of fimbriae and *oxyR-1* mutations on *S. marcescens* surface structures. Aliquots of agitated overnight cultures were observed using TEM. The wild-type strain was covered with pilus-like surface projections (60.3% of cells; $n = 189$) (Fig. 5A), whereas the *oxyR-1* mutant strain had no surface structures other than flagella (0.7% of cells; $n = 140$) (Fig. 5B and C), similar to the *fimC* (50E2) mutant (2.7% of cells; $n = 109$) (Fig. 5E). Moreover, *oxyR-1* mutants bearing a high-copy-number plasmid with *oxyR* (pRMQS140) exhibited a high frequency of piliation (95.7% of cells; $n = 254$) (Fig. 5D). These data suggest that overexpression of *oxyR* leads to hyperpiliation, while the absence of *oxyR* leads to the absence of piliation. The appearance of the fimbriae was similar to the appearance of previously reported *Serratia* fimbriae (2).

The *oxyR* plasmid was added to the *fimC* mutant strain (50E2) to determine whether the enhanced pilus phenotype exhibited by cells bearing pRMQS140 was directly dependent
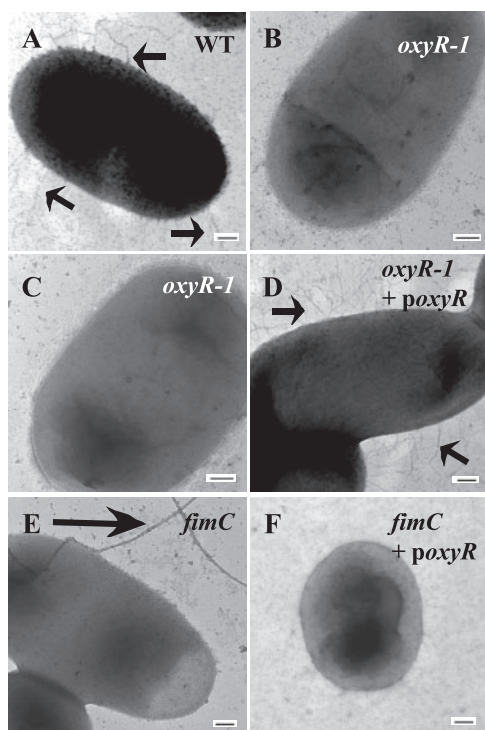
FIG. 5. Absence of surface structures on *oxyR-1* and *fimC* mutants grown under shaking conditions: transmission electron micrographs of negatively stained planktonic cells. (A) Wild-type cell. (B and C) *oxyR-1* mutants from different cultures on different days. (D) *oxyR-1* strain with pRMQS140 covered with fimbriae. (E) *fimC* mutant strain 50E2. (F) *fimC* strain with pRMQS140. The long arrow indicates flagella, and the short arrows indicate fimbriae. Bars = 100 nm. WT, wild type.
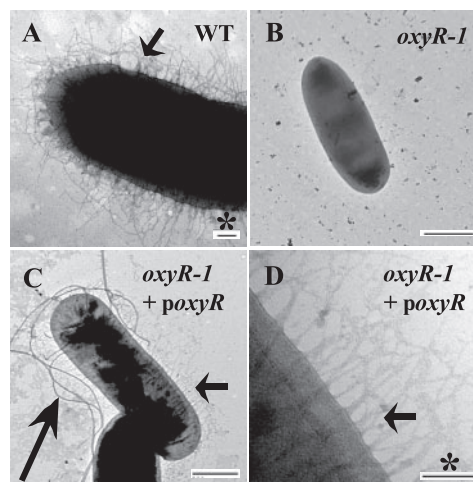

FIG. 6. TEM of bacteria grown under static conditions. Surface fimbriae are evident on the wild type and complemented *oxyR-1* strain (pRMQS140) (A, C, and D) but not on the *oxyR-1* strain, which is devoid of obvious surface structures (B). The short arrows indicate type I fimbriae, and the long arrow indicates flagella. (A and D) Bars = 100 nm. (B and C) Bars = 500 nm. WT, wild type.

upon fimbrial genes. No cells with convincing fimbriae were observed for the *fimC* mutant with the multicopy *oxyR* plasmid, and a conservative count indicated that 4.5% of the cells exhibited a single projection that could be a fimbria ($n = 89$) (Fig. 5F). Consistently, the *fimC* mutant was defective in agglutination of yeast cells ($11.8\% \pm 0.9\%$, compared to $>80\%$ for the wild type) (Fig. 4B). Addition of the pRMQS140 (p*oxyR*) plasmid to the *fimC* strain did not rescue its deficiency in yeast agglutination ($9.2\% \pm 4.1\%$), as it did the *oxyR-1* mutant (Fig. 4B). Similar TEM results were observed with a *fimB* (chaperone) mutant bearing pRMQS140; 2.7% of the cells exhibited one or more potential fimbriae ($n = 110$). These results show that the hyperpiliation phenotype caused by multiple copies of *oxyR* is dependent upon type I fimbrial genes.

To assess the importance of OxyR in fimbria production under the most stringent conditions, bacteria were grown in static conditions in which fimbria production is highly stimulated in *E. coli* (42). Almost all cells from wild-type static cultures were covered with multitudinous fimbriae (98%; $n = 101$) (Fig. 6A), whereas only 2.6% of the cells from an *oxyR-1* culture exhibited a surface projection that resembled a fimbria-like structure ($n = 40$) (Fig. 6B). The pRMQS140 plasmid was able to complement the fimbria-deficient phenotype conferred by the *oxyR-1* mutation, as described above (100% fimbria positive; $n = 31$) (Fig. 6C and D). Together, these TEM data suggest that *oxyR* acts as a positive regulator of fimbria expression.

Specific mutations in the *oxyR* gene of *E. coli* can lock it into the "oxidized" or "reduced" state. The mutations responsible for these phenotypes in *E. coli* create the amino acid changes H198R (oxidized) and C199S (reduced). There is nearly complete identity between *E. coli* OxyR and *S. marcescens* OxyR in this region, which is thought to respond to a cell redox/thiol-disulfide status and in turn to regulate surface adhesins (48, 49). The analogous mutations were created in *S. marcescens oxyR* to generate C205S and H204R mutations to test whether fimbria production was inhibited when the alleles were expressed in the *oxyR-2* mutant strain background. The mutant alleles were placed under the control of an arabinose-inducible promoter in the pMQ125 vector to create pRMQS145 and pRMQS147. Expression of the wild-type *oxyR* gene and the *oxyR-C205S* and *oxyR-H204R* alleles supported the production of fimbriae at similar levels when preparations were assessed by yeast agglutination and TEM analysis, while the vector alone did not support the production of fimbriae (data not shown). This result implies that fimbria expression in *S. marcescens* is independent of the disulfide status of OxyR.

OxyR mediates expression of type I fimbriae. The surface-associated proteins of *S. marcescens* were observed by PAGE analysis to further assess the effect of mutation of *oxyR* on fimbria production. The surface protein migration pattern exhibited by the wild-type strain used in this study is very similar to that recently reported for strain MG1 (31). A major band migrating at about 20 kDa, which corresponds to the predicted mass of the FimA subunit (18.1 kDa), was observed in the wild-type strain but was absent in the *oxyR-1* mutant (Fig. 7A). The band from the wild type was excised and identified by mass spectroscopy to be the FimA protein. Addition of p*oxyR* to the *oxyR-1* mutant reproducibly restored the presence of FimA (as verified by mass spectroscopy) (Fig. 7A). The predicted protein was absent in a *fimC* mutant, as expected for a fimbrial subunit, as the FimA protein would not be secreted in the absence of an usher protein (Fig. 7A). A band corresponding to flagellin (37
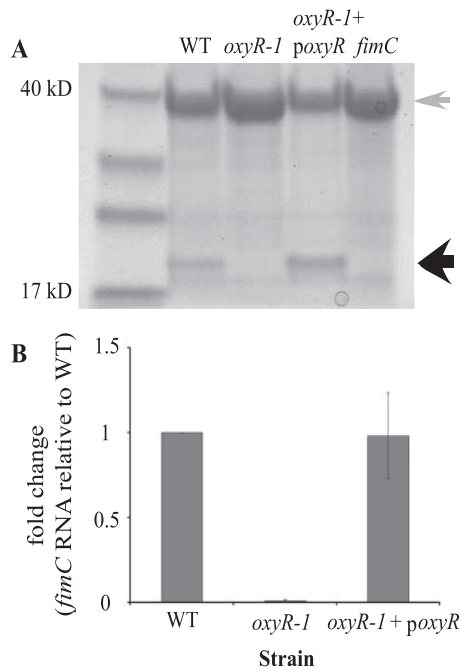
FIG. 7. PAGE analysis of sheared surface proteins and quantitative real-time PCR of *fimC* expression in wild-type and mutant *S. marcescens*. (A) Sheared surface proteins were separated on a 8 to 16% polyacrylamide gel. The major fimbrial subunit (FimA, identified by mass spectroscopy) has a predicted mass of 18.1 kDa and is indicated by the black arrow. The gray arrow indicates the flagellin protein (identified by mass spectroscopy). (B) Expression of *fimC* was measured by the $\Delta\Delta C_T$ method, in which *rpLU* was used as the internal reference gene and wild-type cells were used as the calibrator. Fold changes in *fimC* with respect to the wild type are shown for the *oxyR-1* mutant and the *oxyR-1* mutant containing pRMQS140. The data are the averages ± standard errors of three independent experiments. WT, wild type.

kDa) was observed in the tested strains, as expected, and was identified by mass spectroscopy (Fig. 7A).

To determine whether the OxyR transcription factor regulates fimbrial gene expression at a transcriptional level, we utilized real-time RT-PCR. The data show that *oxyR* mutants have undetectable levels of *fimC* usher gene expression relative to the wild-type strain (Fig. 7B). In contrast, the *oxyR* mutant with pRMQS140 (*oxyR* multicopy plasmid) exhibited restored levels of *fimC* RNA (Fig. 7B). While these data indicate that OxyR positively regulates *fimC* gene expression, it remains unclear whether the effect is direct or indirect. An identical pattern of expression was observed with primers specific for the *fimA* gene in the same operon (data not shown).

OxyR-independent *fimABCD* expression restores biofilm formation to *oxyR-1* mutants. In has not been determined whether expression of type I fimbriae is sufficient to suppress the attachment-deficient phenotypes of the *oxyR-1* mutant. The chromosomal *fimABCD* operon was placed under control of the P_{lac} promoter to make its expression independent of OxyR through integration of plasmid pRMQS169 (Fig. 8A). To confirm that this construct conferred a Fim⁺ phenotype to the *oxyR-1* strain, we utilized the yeast agglutination assay (Fig. 8B). As a control we integrated pRMQS169 into the chromosome of a *fimC* mutant. P_{lac}-mediated expression of *fimABCD*
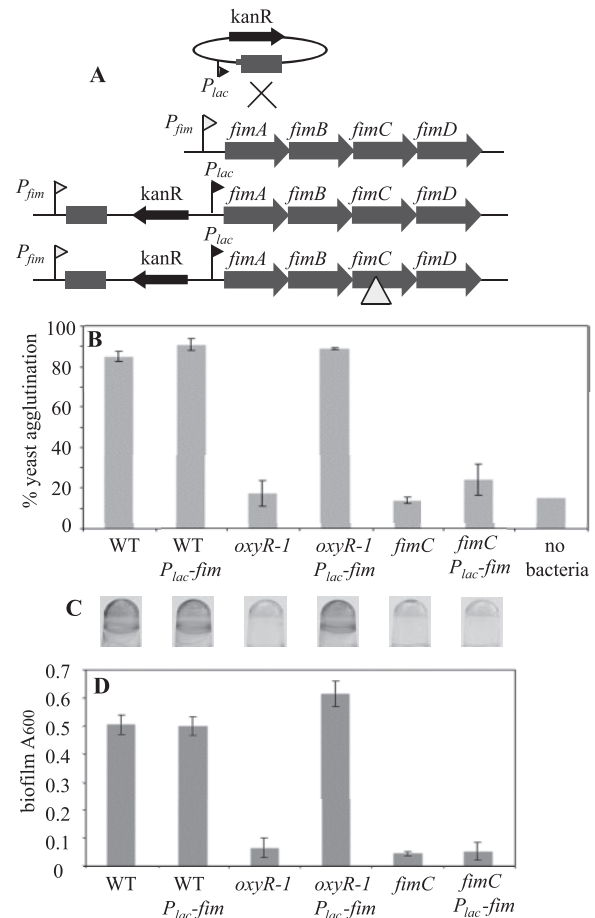

FIG. 8. P_{lac}-mediated expression of *fimABCD* is sufficient to suppress *oxyR-1* attachment defects. (A) The chromosomal *fimABCD* operon of wild-type, *oxyR-1*, and *fimC* strains was placed under control of the P_{lac} promoter through integration of a plasmid. (B) A chromosomal copy of P_{lac}-*fimABCD* significantly increased yeast agglutination with the *oxyR-1* mutant ($P = 0.002$) but not with the wild type or the *fimC* mutant. The experiment was repeated twice on separate days with triplicate independent samples, producing very similar results. (C and D) A chromosomal copy of P_{lac}-*fimABCD* significantly increased biofilm formation by the *oxyR-1* mutant ($P < 0.001$) but not by the wild type or the *fimC* mutant. The experiment was repeated three times on separate days with identical results. The error bars indicate one standard deviation. WT, wild type.

in this strain should not yield functional fimbriae due to the transposon mutation in *fimC*. The *oxyR-1* strain with P_{lac}-*fimABCD* was able to agglutinate yeast cells, but P_{lac}-*fimABCD* did not rescue the *fimC* mutant defects (Fig. 8B and data not shown). Consistent with a model indicating that *oxyR* mutants are defective in biofilm formation due to severely reduced fimbria production, P_{lac} expression of *fimABCD* restored wild-type levels of biofilm formation to the *oxyR-1* mutant (Fig. 8 C-D).

DISCUSSION

This report describes the isolation of biofilm-defective mutants of *S. marcescens* and the characterization of one of the mutations, which maps to an *oxyR* homolog. The *oxyR* mutant

was defective in attachment to surfaces, the oxidative stress response, swarming, yeast agglutination, and bacterial cell-cell interactions. Fimbria-like structures were absent from the surface of *oxyR* mutant cells observed by electron microscopy, whereas the presence of *oxyR* in a multicopy plasmid led to a hyperpiliated phenotype in a fimbria-dependent manner. Consistently, the transcriptional expression of a fimbria usher gene (*fimC*) and the major subunit (*fimA*) from the same operon is dependent upon OxyR. These data, in addition to the finding that mutations in fimbrial structural and processing genes are required for *S. marcescens* biofilm formation, led us to the model that OxyR contributes to *S. marcescens* biofilm formation as a positive regulator of fimbria expression. To our knowledge, this is the first example of an OxyR protein regulating expression of fimbriae.

The role of an oxidative stress response regulator in biofilm formation is consistent with the importance of environmental context to the choice between biofilm formation and planktonic lifestyles. The environmental signals could originate from the status of plant and animal defense systems, tissue type, competing microorganisms, and a range of other conditions, including sensing of the air-liquid interface (13, 17, 50, 55). Expression of stress response genes has been shown to be upregulated in biofilms, which makes sense as biofilms are highly heterogeneous and some cells are likely to find themselves in inhospitable niches (7). In the case of OxyR, stress signals likely occur before attachment, leading to altered expression of surface adhesins, attachment to a surface, and an increased chance of survival. Consistent with the hypothesis that stress responses are important in biofilm formation, we have also genetically identified three different predicted oxidoreductases and a *pspA* (phage shock protein) homolog as mediators of *S. marcescens* biofilm formation (data not shown). The addition of hydrogen peroxide to biofilms did not induce an increase in biofilm formation under our experimental conditions (data not shown). The expression of fimbriae in response to various forms of oxidative stress is currently being studied.

Many of the genes required for biofilm formation in other organisms were not identified in this screen. Flagella are important in the initial steps of biofilm formation in many gram-negative organisms, and the many genes involved in making functional flagella make this a large target when random mutagenesis is performed. Although only 14,000 different candidates were screened, it is likely that a gene important for flagellum function would have been disrupted, as numerous genes are required for building functional flagella. This suggests either that the flagellum machinery was not mutated in our screen or that under our experimental conditions *S. marcescens* does not require the flagella for biofilm formation.

Another gene expected to be found in our biofilm screen is a homolog of the gene encoding antigen 43. In *E. coli*, antigen 43 is a major biofilm factor and is negatively regulated by OxyR. A search of the *S. marcescens* strain Db11 genome at the Sanger Center did not reveal a gene similar to the gene that codes for antigen 43 or antigen 43-related domains. An obvious antigen 43 homolog gene was also absent in the *Serratia proteamaculans* 568 genome. Recently, it has been shown that *oxyR* mutants of *E. coli* have antigen 43-dependent defects in flagellum motility, whereas *S. marcescens oxyR* mutants are

proficient in flagellum-based movement (58). Additionally, *oxyR* mutants of *E. coli* exhibit enhanced biofilm formation due to overexpression of antigen 43, whereas *S. marcescens oxyR* mutants are defective for biofilm formation (13). Together, these data are consistent with the absence of an antigen 43-like factor in *S. marcescens*. In *E. coli*, antigen 43 provides a mechanism for cell-cell interactions. Here we provide evidence that fimbriae are important in cell-cell interactions as the *oxyR* mutant is defective in both cell-cell interactions and fimbria production and the presence of *oxyR* on a multicopy plasmid stimulates both cell-cell interactions and fimbria production.

S. marcescens biofilm formation likely increases the opportunities of this organism to infect humans through attachment to abiotic surfaces (catheters, contact lens cases, etc.). OxyR is a good candidate for further study because it is important for both the attachment of bacteria to abiotic and biotic surfaces and the ability of the bacteria to withstand hostile environments, giving it a double role in successful pathogenesis.

REFERENCES

1. **Acar, J. F.** 1986. *Serratia marcescens* infections. Infect. Control **7:**273–278.
2. **Adegbola, R. A., and D. C. Old.** 1982. New fimbrial hemagglutinin in *Serratia* species. Infect. Immun. **38:**306–315.
3. **Alexandrakis, G., E. C. Alfonso, and D. Miller.** 2000. Shifting trends in bacterial keratitis in south Florida and emerging resistance to fluoroquinolones. Ophthalmology **107:**1497–1502.
4. **Almeida, C. C., C. V. Romao, P. F. Lindley, M. Teixeira, and L. M. Saraiva.** 2006. The role of the hybrid cluster protein in oxidative stress defense. J. Biol. Chem. **281:**32445–32450.
5. **Anriany, Y., S. N. Sahu, K. R. Wessels, L. M. McCann, and S. W. Joseph.** 2006. Alteration of the rugose phenotype in *waaG* and *ddhC* mutants of *Salmonella enterica* serovar *Typhimurium* DT104 is associated with inverse production of curli and cellulose. Appl. Environ Microbiol. **72:**5002–5012.
6. **Austin, J. W., G. Saunders, W. W. Kay, and S. K. Collinson.** 1998. Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. FEMS Microbiol. Ecol. **162:**295–301.
7. **Beloin, C., J. Valle, P. Latour-Lambert, P. Faure, M. Kzreminski, D. Balestrino, J. A. J. Haagensen, S. Molin, G. Prensier, B. Arbelle, and J.-M. Ghigo.** 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol. Microbiol. **51:**659–674.
8. **Bourcier, T., F. Thomas, V. Borderie, C. Chaumeil, and L. Laroche.** 2003. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. Br. J. Ophthalmol. **87:**834–838.
9. **Caiazza, N. C., and G. A. O'Toole.** 2004. SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **186:**4476–4485.
10. **Chiang, S. L., R. K. Taylor, M. Koomey, and J. J. Mekalanos.** 1995. Single amino acid substitutions in the N-terminus of *Vibrio cholerae* TcpA affect colonization, autoagglutination, and serum resistance. Mol. Microbiol. **17:**1133–1142.
11. **Connell, H., W. Agace, P. Klemm, M. A. Schembri, S. Marild, and C. Svanborg.** 1996. Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. Proc. Natl. Acad. Sci. USA **93:**9827–9832.
12. **Cookson, A. L., W. A. Cooley, and M. J. Woodward.** 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing *Escherichia coli* in adherence to abiotic surfaces. Int. J. Med. Microbiol. **292:**195–205.
13. **Danese, P. N., L. A. Pratt, S. L. Dove, and R. Kolter.** 2000. The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. Mol. Microbiol. **37:**424–432.
14. **de Oliveira-Garcia, D., M. Dall'Agnol, M. Rosales, A. Azzuz, N. Alcantara, M. Martinez, and J. Giron.** 2003. Fimbriae and adherence of *Stenotrophomonas maltophilia* to epithelial cells and to abiotic surfaces. Cell. Microbiol. **5:**625–636.

15. **Diaz, P. I., N. Slakeski, E. C. Reynolds, R. Morona, A. H. Rogers, and P. E. Kolenbrander.** 2006. Role of *oxyR* in the oral anaerobe *Porphyromonas gingivalis.* J. Bacteriol. **188:**2454–2462.

16. **Friedman, L., and R. Kolter.** 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol. Microbiol. **51:**675–690.

17. **Fujiwara, S., Y. Miyake, T. Usui, and H. Suginaka.** 1998. Effect of adherence on antimicrobial susceptibility of *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Proteus mirabilis.* Hiroshima J. Med. Sci. **47:**1–5.

18. **Fux, C. A., J. W. Costerton, P. S. Stewart, and P. Stoodley.** 2005. Survival strategies of infectious biofilms. Trends Microbiol. **13:**34–40.

19. **Gabig, M., A. Herman-Antosiewicz, M. Kwiatkowska, M. Los, M. S. Thomas, and G. Wegrzyn.** 2002. The cell surface protein Ag43 facilitates phage infection of *Escherichia coli* in the presence of bile salts and carbohydrates. Microbiology **148:**1533–1542.

20. **Genevaux, P., P. Bauda, M. S. DuBow, and B. Oudega.** 1999. Identification of Tn10 insertions in the *rfaG*, *rfaP*, and *galU* genes involved in lipopolysaccharide core biosynthesis that affect *Escherichia coli* adhesion. Arch. Microbiol. **172:**1–8.

21. **Hasman, H., T. Chakraborty, and P. Klemm.** 1999. Antigen-43-mediated autoaggregation of *Escherichia coli* is blocked by fimbriation. J. Bacteriol. **181:**4834–4841.

22. **Hinsa, S. M., M. Espinosa-Urgel, J. L. Ramos, and G. A. O'Toole.** 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. Mol. Microbiol. **49:**905–918.

23. **Hume, E. B., and M. D. Willcox.** 2004. Emergence of *Serratia marcescens* as an ocular surface pathogen. Arch. Soc. Esp. Oftalmol. **79:**475–477.

24. **Jingushi, S., M. Mitsuyama, T. Moriya, and K. Amako.** 1987. Antigenic analysis of *Serratia marcescens* fimbriae with monoclonal antibodies. Infect. Immun. **55:**1600–1606.

25. **Johnson, J. R., C. Clabots, and H. Rosen.** 2006. Effect of inactivation of the global oxidative stress regulator *oxyR* on the colonization ability of *Escherichia coli* O1:K1:H7 in a mouse model of ascending urinary tract infection. Infect. Immun. **74:**461–468.

26. **Kehres, D. G., M. L. Zaharik, B. B. Finlay, and M. E. Maguire.** 2000. The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. **36:**1085–1100.

27. **Koh, K. S., K. W. Lam, M. Alhede, S. Y. Queck, M. Labbate, S. Kjelleberg, and S. A. Rice.** 2007. Phenotypic diversification and adaptation of *Serratia marcescens* MG1 biofilm-derived morphotypes. J. Bacteriol. **189:**119–130.

28. **Kolter, R., and E. P. Greenberg.** 2006. The superficial life of microbes. Nature **441:**300–302.

29. **Kulasekara, H. D., I. Ventre, B. R. Kulasekara, A. Lazdunski, A. Filloux, and S. Lory.** 2005. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial cup genes. Mol. Microbiol. **55:**368–380.

30. **Labbate, M., S. Y. Queck, K. S. Koh, S. A. Rice, M. Givskov, and S. Kjelleberg.** 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. J. Bacteriol. **186:**692–698.

31. **Labbate, M., H. Zhu, L. Thung, R. Bandara, M. R. Larsen, M. D. P. Willcox, M. Givskov, S. A. Rice, and S. Kjelleberg.** 2007. Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. J. Bacteriol. **189:**2702–2711.

32. **Lau, G. W., B. E. Britigan, and D. J. Hassett.** 2005. *Pseudomonas aeruginosa* OxyR is required for full virulence in rodent and insect models of infection and for resistance to human neutrophils. Infect. Immun. **73:**2550–2553.

33. **Lavender, H. F., J. R. Jagnow, and S. Clegg.** 2004. Biofilm formation in vitro and virulence in vivo of mutants of *Klebsiella pneumoniae.* Infect. Immun. **72:**4888–4890.

34. **Loprasert, S., R. Sallabhan, W. Whangsuk, and S. Mongkolsuk.** 2002. The *Burkholderia pseudomallei oxyR* gene: expression analysis and mutant characterization. Gene **296:**161–169.

35. **Merritt, J. H., D. E. Kadouri, and G. A. O'Toole.** 2005. Growing and analyzing static biofilms, p.1B.1.1–1B.1.17. *In* Current protocols in microbiology. John Wiley & Sons, Inc., New York, NY.

36. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR.* J Bacteriol. **170:**2575.

37. **Mirelman, D., G. Altmann, and Y. Eshdat.** 1980. Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeasts. J. Clin. Microbiol. **11:**328–331.

38. **Mizunoe, Y., Y. Nakabeppu, M. Sekiguchi, S. Kawabata, T. Moriya, and K.**

Amako. 1988. Cloning and sequence of the gene encoding the major structural component of mannose-resistant fimbriae of *Serratia marcescens.* J. Bacteriol. **170:**3567–3574.

39. **Monds, R. D., P. D. Newell, R. H. Gross, and G. A. O'Toole.** 2007. Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0-1 biofilm formation by controlling secretion of the adhesin LapA. Mol. Microbiol. **63:**656–679.

40. **Mulvey, M. A., Y. S. Lopez-Boado, C. L. Wilson, R. Roth, W. C. Parks, J. Hueuser, and S. J. Hultgren.** 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli.* Science **282:**1494–1497.

41. **Nataro, J. P., D. Yikang, J. A. Giron, S. J. Savarino, M. H. Kothary, and R. Hall.** 1993. Aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli* requires two unlinked plasmid regions. Infect. Immun. **61:**1126–1131.

42. **Old, D. C., and J. P. Duguid.** 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. J. Bacteriol. **103:**447–456.

43. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.

44. **O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter.** 1999. Genetic approaches to the study of biofilms. Methods Enzymol. **310:**91–109.

45. **Pagan-Ramos, E., S. S. Master, C. L. Pritchett, R. Reimschuessel, M. Trucksis, G. S. Timmins, and V. Deretic.** 2006. Molecular and physiological effects of mycobacterial *oxyR* inactivation. J. Bacteriol. **188:**2674–2680.

46. **Paget, M. S., and M. J. Buttner.** 2003. Thiol-based regulatory switches. Annu. Rev. Genet. **37:**91–121.

47. **Pair, S. D., B. D. Bruton, F. Mitchell, J. Fletcher, A. Wayadande, and U. Melcher.** 2004. Overwintering squash bugs harbor and transmit the causal agent of cucurbit yellow vine disease. J. Econ. Entomol. **97:**74–78.

48. **Patterson, K. L., J. W. Porter, K. B. Ritchie, S. W. Polson, E. Mueller, E. C. Peters, D. L. Santavy, and G. W. Smith.** 2002. The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, Acropora palmata. Proc. Natl. Acad. Sci. USA **99:**8725–8730.

49. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285–293.

50. **Queck, S. Y., M. Weitere, A. M. Moreno, S. A. Rice, and S. Kjelleberg.** 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environ Microbiol. **8:**1017–1025.

51. **Rice, S. A., K. S. Koh, S. Y. Queck, M. Labbate, K. W. Lam, and S. Kjelleberg.** 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. J. Bacteriol. **187:**3477–3485.

52. **Rocha, E. R., C. D. Herren, D. J. Smalley, and C. J. Smith.** 2003. The complex oxidative stress response of *Bacteroides fragilis:* the role of OxyR in control of gene expression. Anaerobe **9:**165–173.

53. **Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. **184:**1140–1154.

54. **Schaefer, F., O. Bruttin, L. Zografos, and Y. Guex-Crosier.** 2001. Bacterial keratitis: a prospective clinical and microbiological study. Br. J. Ophthalmol. **85:**842–847.

55. **Schembri, M. A., and P. Klemm.** 2001. Coordinate gene regulation by fimbriae-induced signal transduction. EMBO J. **20:**3074–3081.

56. **Seib, K. L., H. J. Wu, Y. N. Srikhanta, J. L. Edwards, M. L. Falsetta, A. J. Hamilton, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings.** 2007. Characterization of the OxyR regulon of *Neisseria gonorrhoeae.* Mol. Microbiol. **63:**54–68.

57. **Shanks, R. M. Q., N. C. Caiazza, S. M. Hinsa, C. M. Toutain, and G. A. O'Toole.** 2006. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. Appl. Environ Microbiol. **72:**5027–5036.

58. **Ulett, G. C., R. L. Webb, and M. A. Schembri.** 2006. Antigen-43-mediated autoaggregation impairs motility in *Escherichia coli.* Microbiology **152:**2101–2110.

59. **Vallet, I., J. W. Olson, S. Lory, A. Lazdunski, and A. Filloux.** 2001. The chaperone/usher pathways of *Pseudomonas aeruginosa:* identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. Proc. Natl. Acad. Sci. USA **98:**6911–6916.

60. **Zheng, M., and G. Storz.** 2000. Redox sensing by prokaryotic transcription factors. Biochem. Pharmacol. **59:**1–6.