A Serratia marcescens OxyR Homolog Mediates Surface Attachment and Biofilm Formation

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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 \( P_{fa} \)-mediated expression of famABCD 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 box 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 hydrophobic abiotic surfaces include the luxI homolog swrI, the two-component regulator genes rscA 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 MO1 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
S. MARCESCENS BIOFILMS REQUIRE OxyR

TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serratia marcescens strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-D</td>
<td><em>S. marcescens</em> D (pigmented)</td>
<td>Presque Isle Cultures</td>
</tr>
<tr>
<td>RSS1</td>
<td>WT-D <em>rpsL</em> mutation, Str′</td>
<td>R. M. Q. Shanks, unpublished</td>
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<tr>
<td>50A3</td>
<td>WT-D with <em>oxyR</em>-Tn-mariner, <em>oxyR</em>-I</td>
<td>This study</td>
</tr>
<tr>
<td>RSS3</td>
<td>WT-D with <em>oxyR</em>-2, <em>oxyR</em>-pRMQS133</td>
<td>This study</td>
</tr>
<tr>
<td>RSS4</td>
<td><em>oxyR</em> with wild-type <em>oxyR</em></td>
<td>This study</td>
</tr>
<tr>
<td>E50B5</td>
<td>WT-D with <em>oxyR</em>-Tn-mariner, <em>oxyR</em>-3</td>
<td>This study</td>
</tr>
<tr>
<td>50E2</td>
<td>WT-D with <em>fimC</em>-Tn-mariner</td>
<td>This study</td>
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<tr>
<td>59F12</td>
<td>WT-D with <em>fimB</em>-Tn-mariner</td>
<td>This study</td>
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<tr>
<td><strong>Escherichia coli strains</strong></td>
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<tr>
<td>SM10 <em>λ</em>-pir</td>
<td><em>thi-1</em> <em>thr</em> <em>tonA</em> lacY supE recA::RP4-2::Tc::Mu <em>pir</em></td>
<td>36</td>
</tr>
<tr>
<td>S17-1 <em>λ</em>-pir</td>
<td><em>thi pro hisD</em> <em>hisD-M</em> Δ<em>recA</em> RP4-2::Tc*:Mu<em>Km</em>::Tn7 <em>pir</em></td>
<td>36</td>
</tr>
<tr>
<td><strong>Saccharomyces cerevisiae InVSc1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MATa/MATa leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 <em>his3-D1/his3-D1</em></td>
<td>Invitrogen</td>
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</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
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<td>pBT20</td>
<td>Mariner delivery vector</td>
<td>29</td>
</tr>
<tr>
<td>pMQ118</td>
<td>oriR6K, suicide/allelic replacement vector</td>
<td>R. M. Q. Shanks and G. A. O'Toole, unpublished</td>
</tr>
<tr>
<td>pMQ125</td>
<td>p15a-yeast shuttle vector with <em>P</em>&lt;sub&gt;BAD&lt;/sub&gt;</td>
<td>R. M. Q. Shanks and G. A. O'Toole, unpublished</td>
</tr>
<tr>
<td>pMQ131</td>
<td>Broad-host-range yeast-pBBR1 vector</td>
<td>R. M. Q. Shanks and G. A. O'Toole, unpublished</td>
</tr>
<tr>
<td>pRMQS133</td>
<td>pMQ118 with internal fragment of <em>oxyR</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRMQS140</td>
<td>pMQ131 with <em>oxyR-prgL</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRMQS143</td>
<td>pMQ125 with <em>oxyR</em></td>
<td>This study</td>
</tr>
<tr>
<td>pRMQS145</td>
<td>Like pRMQS143 but C205S</td>
<td>This study</td>
</tr>
<tr>
<td>pRMQS147</td>
<td>Like pRMQS143 but H204R</td>
<td>This study</td>
</tr>
<tr>
<td>pRMQS169</td>
<td>Like pMQ118 with <em>P&lt;sub&gt;lac&lt;/sub&gt;-fimA&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>This study</td>
</tr>
</tbody>
</table>

**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 *λ*-pir 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 multwell 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<sub>600</sub>* 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<sub>600</sub>* was adjusted to 1.0 (~5 × 10<sup>7</sup> 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 (A660) 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 × 10^7 bacteria on an LB agar plate and placing a sterile 6-mm paper disk (catalog no. BB3103; BBL) on the plate, to which 10 μl of 30% H₂O₂ (Fisher Scientific catalog no. H3252) 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 Bsc-Light Live/Dead staining kit (Molecular Probes, Eugene, OR), and incubated at room temperature for 15 min. Red fluorescence and the A660 were determined using a plate reader (Synergy 2; Bio-tek, Winooski, VT). Numbers of relative fluorescence units (RFU) were normalized by dividing the red fluorescence units by the A660.

**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 normal yeast (5 × 10⁷) in a glass microfuge tube (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 colony segregants used to mask cultures that were agglutinated. Each 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 (A660, 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 A660 was determined; then the tubes were vortexed for 30 s at high speed, three 100-μl samples were removed from the upper aqueous phase of each tube, and the A660 was determined. The percentage of agglutination was determined as follows: 100 × (1 − A660 before vortexing/A660 after vortexing) for each genotype. 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 (Genta 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). The plasmid pRMQS145 is similar to pRMQS143 but contains versions of oxyR with point mutations that result in C205S and H204R mutations, respectively. These plasmids were used in yeast in vivo cloning techniques by recombining two PCR amplicons with the pMQR125 vector. The amplicons were made using primers that were engineered with the desired point mutation. These constructs were verified by sequencing.

**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 R5 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 hr. The inoculum was 10⁶ bacteria from a stationary-phase culture. The growth medium was removed at 24 h, and coverslips were resuspended in PBS and stained with a Bsc-Light kit, as described above. Coverslips were dipped three times in PBS and were mounted on hanging drop coverslips in PBS before imaging 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 objective and a z spacing of 1.47 μm between stacked images. Three-dimensional reconstructions were generated in Fluoview at 256 × 256 resolution.

**RNA isolation.** RNA 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 A600 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.

**RNAS isolation.** Strains were grown to stationary phase overnight and then subcultured 1:100 and grown until the A600 was 1. Each 5-ml liquid culture was transferred to 15 ml of Terrific Broth LS (Molecular Research Center, Inc.) and frozen at −80°C. RNA was cultured 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 × g for 60 min at 4°C. The pellets were washed with 90% 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 90% 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,
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 and virulence; therefore, priority was given to further characterization of the *S. marcescens* oxyR homolog.

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, 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-

![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*-I 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.](image-url)

TABLE 2. *oxyR* is required for biofilm formation at 20 h

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>A</em>&lt;sub&gt;bio&lt;/sub&gt; with the following substrate materials:</th>
<th>PVC</th>
<th>Polystyrene</th>
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<tr>
<td></td>
<td></td>
<td>30°C</td>
<td>37°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Wild type</td>
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<td>0.57±0.05</td>
</tr>
<tr>
<td><em>oxyR</em>-1</td>
<td></td>
<td>0.08±0.02</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td><em>oxyR</em>-2</td>
<td></td>
<td>0.19±0.03</td>
<td>0.22±0.04</td>
</tr>
<tr>
<td><em>oxyR</em>-3</td>
<td></td>
<td>0.31±0.04</td>
<td>0.09±0.03</td>
</tr>
<tr>
<td>Wild type + paxyR&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.43±0.05</td>
<td>0.50±0.09</td>
</tr>
<tr>
<td><em>oxyR</em>-1 + paxyR&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.55±0.04</td>
<td>0.46±0.05</td>
</tr>
<tr>
<td><em>oxyR</em>-2 revertant</td>
<td></td>
<td>0.45±0.07</td>
<td>0.51±0.06</td>
</tr>
<tr>
<td>fimC-50E2</td>
<td></td>
<td>0.20±0.04</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>fimC-50E2 + paxyR&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.20±0.08</td>
<td>0.14±0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>*A*<sub>bio</sub> of crystal violet-stained biofilms. The values are the averages ± standard deviations for 12 replicates from three independent cultures.

<sup>b</sup>paxyR is pRMQS140 and provides wild-type *oxyR* in trans.
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.

FIG. 2. OxyR mutants are defective in biofilm formation and primary attachment. (A) Photograph of crystal violet-stained biofilms on inverted PVC microtiter wells. pxyR 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.

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
Biofilm formation exhibited by flagellum mutants of mutant strains is reminiscent of the strong inhibition of early motility after 8 h of incubation at 30°C, the size of the zone for inhibited larger zones of swimming. In one representative experiment after 8 h of incubation at 30°C, the size of the zone 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 $1,390 \pm 150$ RFU (170 ± 19 RFU without $H_2O_2$) for the wild type and $2,391 \pm 255$ 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 the wild-type strain was $7.7 \pm 1.5$ mm, compared to $12.4 \pm 0.9$ mm for the oxyR-1 strain and $11 \pm 2$ 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 $13.5 \pm 2.0$ 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 fimbia-associated phenotypes.** In the biofilm screen, several mutations leading to biofilm-defective phenotypes mapped to predicted fimbral 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, 30).
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 ≥ 6) (Fig. 4A). The oxyR-1 mutant with pRMQS140 (poxR), 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 ≥ 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 ≥ 6).

A second method was developed to quantify the ability of wild-type and oxyR-1 cells to agglutinate eukaryotic cells. Normalized 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% agglutination, 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% ± 4.3% agglutination, whereas the oxyR-2 mutant exhibited 12.8% ± 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 PBAD 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% ± 6.4% yeast agglutination, while the strain grown in the presence of L-arabinose agglutinated 77.5% ± 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% ± 4.95% autoagglutination, and the oxyR-1 mutant exhibited 6.56% ± 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* fimбриae (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
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% ± 0.9%, compared to >80% for the wild type) (Fig. 4B). Addition of the pRMQS140 (poxYR) plasmid to the fimC strain did not rescue its deficiency in yeast agglutination (9.2% ± 4.1%), as it did the oxyR-I 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-I 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-I 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.

FIG. 5. Absence of surface structures on oxyR-I and fimC mutants grown under shaking conditions: transmission electron micrographs of negatively stained planktonic cells. (A) Wild-type cell. (B and C) oxyR-I mutants from different cultures on different days. (D) oxyR-I strain with pRMQS140 covered with fimbriae. (E) fimC mutant strain S0E2. (F) fimC strain with pRMQS140. The long arrow indicates flagella, and the short arrows indicate fimbriae. Bars = 100 nm. WT, wild type.

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-II 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-I mutant (Fig. 7A). The band from the wild type was excised and identified by mass spectroscopy to be the FimA protein. Addition of poxyR to the oxyR-I 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. 6. TEM of bacteria grown under static conditions. Surface fimbriae are evident on the wild type and complemented oxyR-I strain (pRMQS140) (A, C, and D) but not on the oxyR-I 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.
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 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 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 fimbia 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 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.

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REFERENCES


