Identification of the srtC1 transcription start site and catalytically essential residues required for Actinomyces oris T14V SrtC1 activity

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Abstract
In Actinomyces oris T14V, sortase SrtC1 mediates the assembly of type 1 fimbriae. We analyzed the effects of the conserved residues (H184, H204, F213, Y236, L263, T265, C266 and R275) on the SrtC1 activity by site directed mutagenesis. We identified three essential conserved residues (H204, Y236 and C266) that are critical for the assembly of type 1 fimbriae in this organism. Rapid amplification of cDNA ends analyses and reverse transcriptase-PCR results indicate that srtC1 was transcribed together with the putative adhesin gene fimQ and major structural subunit gene fimP respectively as a single polycistronic mRNA.

Introduction
Actinomyces oris T14V (Henssge et al., 2009), formerly known as Actinomyces naeslundii T14V, a member of A. naeslundii genospecies 2 family, is considered as one of the primary colonizers for the formation of dental plaque on tooth surfaces (Li et al., 2004). Actinomyces oris T14V possesses two immunologically distinct types of fimbriae, which mediate the attachment of this species to both hard and soft tissue surfaces (Cisar et al., 1988). These fimbriae were among one of the first observed in gram positive bacteria (Girard & Jacius, 1974). Type 1 fimbriae promote the binding of this organism to tooth surfaces mediated by the adsorbed salivary acidic proline rich proteins and statherin. These salivary proteins serve as receptors for type 1 fimbriae (Clark et al., 1984; Gibbons et al., 1988). Type 2 fimbriae mediate the adherence of A. oris to oral mucosal epithelial cells and lactose sensitive coaggregations with certain oral streptococci. Such interactions with other bacteria further promote the formation of dental plaque initiated by type 1 fimbriae of the organism (Palmer et al., 2003).

Previously, we demonstrated that the biogenesis of functional type 1 fimbriae in A. oris T14V required three genes (Yeung et al., 1987; Chen et al., 2007): the putative adhesin gene fimQ, the major structural subunit gene fimP and the type 1 fimbria specific sortase gene srtC1. Sequence alignment indicates that A. oris SrtC1 contains all three conserved domains (D1, D2 and D3) that are present in all sortases and an extra C terminal hydrophobic domain. According to the sortase classification (Dramsi et al., 2005), SrtC1 belongs to class C sortase family.

Sortases are a group of bacterial thiol transpeptidases responsible for the covalent attachment of specific surface proteins to the cell wall envelope of gram positive bacteria (Marraffini et al., 2006). These enzymes are involved in the expression of several virulence factors and the assembly of fimbriae, and have been considered as a target of anti infective therapy (Maresco & Schneewind, 2008).

SrtC1 is required for both the assembly of type 1 fimbriae in A. oris T14V and its adherence to saliva coated hydroxyapatite (Chen et al., 2007). Accordingly, preventing the formation of type 1 fimbriae in A. oris by inhibiting the function of this sortase may reduce the colonization of this organism and consequently the dental plaque formation. In order to provide the foundation for developing inhibitor(s) for this sortase at the genetic level and/or protein level, we were interested in how this sortase gene was transcribed and...
Identification of the srtC1 transcription start site and catalytically essential residues required for Actinomyces oris T14V SrtC1 activity.

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which amino acid residue(s) was critical for the enzyme activity. This enzyme possesses a number of conserved residues, which include H204, F213, Y236, L263, T265, C266 and R275 that are commonly present among different classes of sortases from various bacteria. These conserved residues are located primarily in domains D2 and D3 (Dramsi et al., 2005). For example, H204 and F213 are located in domain D2, Y236 is positioned between domains D2 and D3, and L263, T265, C266 and R275 are found in Domain D3. Thus, the roles of these conserved residues may provide valuable information for developing potent and selective inhibitors for both this particular sortase and other sortases. Herein, we report the identification of the transcription starting site of the srtC1 determined by rapid amplification of cDNA ends (RACE) method and several conserved residues essential for its catalytic function revealed by site directed mutagenesis.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The Escherichia coli strains used for subcloning and plasmid isolation were grown in Luria Bertani medium (Difco Laboratories, Detroit, MI) at 37 °C in the presence of the appropriate selective substances. Actinomyces oris T14V and its mutants were grown in Todd Hewitt broth (THB) (Difco Laboratories), or as otherwise indicated, at 37 °C without agitation. When needed, kanamycin and trimethoprim were included in growth media at concentrations of 50 and 100 μg mL⁻¹, respectively.

RNA isolation and transcription start site

Total RNA from exponentially growing wild type A. oris cells was extracted using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Residual DNA in the total RNA samples was removed by DNase I treatment. Total RNA was concentrated by ethanol precipitation, resuspended in a small volume of RNase free water and stored at −80 °C.

To determine the transcription start site(s) of A. oris srtC1, 5’RACE PCR experiments were carried out using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA) with 3 μg of total RNA. The sequences of oligo primers used are shown in Table 2. Briefly, the first strand of cDNA synthesis was carried out at 42 °C for 1.5 h using a gene specific primer: primer 1 for fimQ, primer 3 for fimP and primer 5 for srtC1. RACE PCR was performed using the above cDNA as the template and using SMART Table 2. Bacterial strains and plasmids

<table>
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<tr>
<th>Plasmid or strain</th>
<th>Description*</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>p6Srt</td>
<td>Tm', plasmid containing srtC1 and both upstream downstream flanking regions, template for site directed mutagenesis</td>
<td>Chen et al. (2007)</td>
</tr>
<tr>
<td>p6Srt H184A</td>
<td>Tm', analog of p6Srt, for creating H184A mutant</td>
<td>This study</td>
</tr>
<tr>
<td>p6Srt H204A</td>
<td>Tm', analog of p6Srt, for creating H204A mutant</td>
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<td>Tm', analog of p6Srt, for creating F213A mutant</td>
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<tr>
<td>p6Srt Y236A</td>
<td>Tm', analog of p6Srt, for creating Y236A mutant</td>
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<tr>
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<td>p6Srt T265A</td>
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<tr>
<td>p6Srt R282A</td>
<td>Tm', analog of p6Srt, for creating R282A mutant</td>
<td>This study</td>
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<td>A. oris T14V</td>
<td>Wild type strain, Km° Sm'</td>
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</tr>
<tr>
<td>A.srtC1</td>
<td>SrtC1 deficient strain, Km'</td>
<td>Chen et al. (2007)</td>
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<td>Transformant of A.srtC1 obtained with p6Srt, Tm'</td>
<td>Chen et al. (2007)</td>
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<td>R275A</td>
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<td>R282A</td>
<td>Transformant of A.srtC1 obtained with p6Srt R282A, Tm'</td>
<td>This study</td>
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*Antibiotics: Km, kanamycin; Sm, streptomycin; Tm, trimethoprim.
PCR primer UPM and gene specific primers: primer 2 for fimQ, primer 4 for fimP and primer 6 for srtC1.

The amplified PCR products were further cloned into Zero Blunt TOPO vector (Invitrogen, Carlsbad, CA) and transformed into E. coli competent cells. Plasmid DNAs were isolated with QIAprep Spin Miniprep Kit (Qiagen). Cloned fragments were sequenced in both directions (ACGT Inc., Wheeling, IL) using an ABI automated sequencer and Dye Terminator Cycle Sequencing Kit, and the transcription start site was determined.

Transformation

*Actinomyces oris* transformation was performed with a modification to a previous method (Yeung & Kozelsky, 1994). Briefly, bacteria were grown in 150 mL of THB in the presence of 0.05% Tween 80 and 20 mM L-threonine until the culture reached the early exponential phase with an OD600nm of 0.2. The culture was chilled on ice for 30 min, and the bacteria were harvested by centrifugation and washed extensively with ice cold sterile distilled water and 10% glycerol in distilled H2O. Cells from the 150 mL culture were suspended in 0.6 mL of 10% glycerol. One hundred microliters of suspended cells were used for each electroporation, which was conducted in a chilled 2 mm Gap cuvette using a Pulser model of ECM630 (BTX, San Diego, CA) with the following settings: 2.5 kV, 25 μF capacitor and 400 Ω resistor. One milliliter of THB with 0.05% Tween 80 was added to the pulsed cells. After 2 h incubation at 37°C, the samples were plated on TH agar plates with appropriate selective substance(s).

Construction of *A. oris* mutants

Nine plasmid p6Srt derivatives were created with a QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA): H184A, H204A, F213A, Y236A, L263A, T265A, C266A, R275A and R282A using the primer sets listed in Supporting Information, Table S1. The presence of the desired mutation in each plasmid was confirmed by sequencing the mutagenized plasmids. *Actinomyces oris* mutants were constructed by transforming SrtC1 deficient strain *A. oris* ΔSrtC1 with corresponding p6Srt derivative plasmids based on the allelic exchange mechanism.

Extraction of surface proteins from *A. oris*

Surface proteins were solubilized from *A. oris* T14V and its mutants using a procedure modified from a mutanolysin digestion method as described previously (Demuth et al., 1996). Briefly, cells from a 10 mL overnight culture were harvested by centrifugation and washed twice with sterile water. The washed cells were suspended in the extraction buffer at a ratio of 4 μL of buffer per milligram of wet cells. The extraction buffer consisted of 26% melezitose, 10 mM MgCl2, 10 mM phosphate buffer (pH 7.0) and 1000 U mL−1 mutanolysin. After a 5 h incubation at 37°C, the suspension was centrifuged (10 000 g, 10 min, 4°C). The supernatant was dialyzed against distilled water using a 10 kDa molecular weight cut off mini Dialysis Units (Pierce, Rockford, IL) and stored at −20°C for analyses. All chemicals used in the extraction were obtained from Sigma Aldrich Corp. (St. Louis, MO).

Western blot analysis

Extracted surface proteins were separated on 3% 8% Tris Acetate NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes. These membranes were incubated with 1 μg mL−1 monoclonal antibody C8A4 directed against the structural subunit (FimP) of T14V type 1 fimbriae (Cisar et al., 1991). Membranes were washed, incubated with a secondary antibody and developed according to the instructions of WesternBreeze Chromogenic Immunodetection System kit (Invitrogen).

Results and discussion

Previously, we identified three essential genes (fimQ, fimP and srtC1) for the biosynthesis of type 1 fimbriae in *A. oris* T14V (Chen et al., 2007). These three genes were bordered by methyltransferase gene (mt1) at the upstream and putative orfC gene at downstream (Fig. 1a). A BLAST search indicates that OrfC contains a PAP2 domain (type 2 phosphatidic acid phosphatase) and may be a PAP2 like superfamily member. In order to localize the promoter(s) for these three genes, RACE analyses were performed to determine the transcription start site(s). As shown in Fig. 1b, we were able to obtain a RACE fragment only from RACE PCR reactions initiated within fimQ (lane 1), not from the reactions initiated within either fimP (lane 2) or fimQ (lane 3).
we amplified the junctional regions of
Actinomyces oris fimP
that the transcription of either
srtC1
was not initiated immediately upstream of these two respective
genes. It is likely that both fimP and srtC1 were transcribed together with fimQ as a single mRNA unit. To confirm this,
we amplified the junctional regions of fimQ fimP and fimP
srtC1. As shown in Fig. 1c, lanes 1 and 3, when the same
cDNA generated by the use of primers 3 or 5 were used as the templates, both junctional regions were amplified. The

fimP
srtC1

Fig. 1. (a) Schematic presentation of the type 1 fimbriae gene cluster in Actinomyces oris T14V (Chen et al., 2007). Primers 1, 3 and 5 were used to initiate the first strand of cDNA synthesis from the regions of fimQ, fimP and srtC1, respectively. Primers 2, 4 and 6 were used for RACE PCR. Primers 7–14 were used for amplifying the junction regions of fimQ fimP, fimP srtC1, mt1 fimP and srtC1 orfC. (b) RACE fragments amplified from RACE PCR reactions initiated within fimQ (lane 1), fimP (lane 2), srtC1 (lane 3). The size of the fragment is shown on the left. (c) Reverse transcriptase PCR showing that fimQ, fimP and srtC1 were transcribed as a contiguous transcript. fimQ fimP and fimP srtC1 junction regions were amplified with the cDNA templates synthesized in the RACE reactions initiated with primer 3 (lane 1) and primer 5 (lane 3), respectively, not with total RNA sample as the template (lanes 2 and 4). The sizes of two amplified fragments are shown on the left. (d) The nucleotide sequence of the operon promoter region is shown. The transcription start site, as determined by 5′RACE analysis, is indicated with +1 and underlined. The deduced 35 and 10 promoter sequences and the putative ribosome binding site (RBS) are also underlined.

srtC1

(lane 3). The size of the RACE fragment is consistent with the sequence derived size of 590 bp. The results suggest that the transcription of either fimP or srtC1 was not initiated immediately upstream of these two respective genes. It is likely that both fimP and srtC1 were transcribed together with fimQ as a single mRNA unit. To confirm this, we amplified the junctional regions of fimQ fimP and fimP srtC1. As shown in Fig. 1c, lanes 1 and 3, when the same cDNA generated by the use of primers 3 or 5 were used as the templates, both junctional regions were amplified. The two PCR products have the expected sizes of 540 and 712 bp. These results indicated that fimQ fimP and fimP srtC1 are together at the mRNA level. Therefore, these data confirmed that fimQ, fimP and srtC1 were transcribed as a single polycistronic mRNA. In addition, no amplification was observed when total RNA was used as the template (Fig. 1c, lanes 2 and 4). The results suggest that there is no DNA contamination in the RNA preparation and the amplicons produced were derived from the cDNA generated in the RACE reactions. When similar reverse transcriptase PCR reactions were performed on the junctional regions of mt1 fimQ and srtC1 orfC, no amplicon was obtained (data not shown). These results reveal that fimQ is the first gene and srtC1 is the last gene in a tricistronic operon. This assignment is consistent with our previous findings that orfC is not required for the type 1 fimbriae biosynthesis (Chen et al., 2007).

To locate the transcription start site, the amplified RACE PCR product (Fig. 1b, lane 1) was cloned into Zero Blunt TOPO vector and sequenced. Based on the DNA sequence obtained, the fimQ (and the fimP and srtC1) transcription start site was located at the adenine residue that is 58 bp upstream of the proposed ATG start codon (Fig. 1d). The identified transcription start point was subsequently used to deduce the putative promoter sequence of the type 1 fimbriae gene cluster based on the consensus sequences observed in promoters from prokaryotic organisms. The deduced −35 (TTGGGG) and −10 (TACATT) boxes for the promoter of the gene cluster are separated by a spacer of 16 nucleotides (Fig. 1d). The −10 box sequence is consistent with the promoters’ −10 consensus sequence (Hawley & McClure, 1983), TATAaT, whereas the −35 hexamer shares only three out of the six bases with the consensus sequence TTGACa.

Sortases catalyze the assembly of surface proteins and fimbriae in the cell wall envelope of gram positive bacteria. SrtC1 is required for the biosynthesis of type 1 fimbriae in A. oris T14V (Chen et al., 2007). In order to better understand the structure function of this sortase, we analyzed the role of eight conserved amino acid residues. The amino acids to be mutated were chosen based on the sequence alignment of several class C family sortases (Fig. 2). Each mutation was first introduced in vitro into plasmid p6Srt carrying the srtC1 gene (Chen et al., 2007) by site directed mutagenesis to replace each conserved amino acid with an alanine residue. The desired mutations were confirmed by sequencing and the integrity of all plasmid constructs was verified by enzyme digestions and sequencing. The mutated srtC1 copies were introduced into the srtC1 deletion host strain ΔsrtC1 (Chen et al., 2007) by transformation.

The resultant transformants were confirmed for the presence of mutated srtC1 introduced by allelic exchange. Cell surface proteins from these mutants were extracted, separated on gel and probed with monoclonal antibody against the type 1 structural subunit FimP. The ability to assemble type 1 fimbriae, as indicated by the polymerization...
of FimP, was used to evaluate the activity of mutated sortases. As shown by the results of the Western blot (Fig. 3), five mutants (H184A, L263A, T265A, F213A and R275A) produced patterns of surface proteins similar to those of the wild type, displaying the polymeric form of the structural subunits in the high molecular weight region as revealed by the anti FimP antibody. However, only the monomeric form of FimP was observed in the other three mutants, H204A, Y236A and C266A. The results indicate that each of these three mutations either abolished the SrtC1 activity, or reduced the activity to an undetectable level as revealed by the blot method, or that these three mutated sortases might not be expressed and/or stable compared with the wild type SrtC1. Dot blot results indicate that there are less FimP components on the surfaces of these three mutants than on those of the wild type strain and other mutants (Fig. S1).

There is a conserved TLTXC motif in all identified sortases. The Cys residue in this motif is essential for any sortase activity. Based on the newly published crystal structure of SrtC1 (Persson, 2011), the nucleophile Cys 266 is located at the centre of the active site. The effect of C266A mutation is consistent with the hypothesis that this catalytic cysteine residue is used in the nucleophilic attack of the Thr-Gly peptidic bond in the target’s LPXTG motif. A similar mutation effect has also been reported for both nonpilus related and pilus related sortases from other organisms. For example, Cys 184 in SrtA from *Staphylococcus aureus* (Ton et al., 1999, 2002; Frankel et al., 2007), Cys 193 in SrtC1 from *Streptococcus pneumoniae* (Manzano et al., 2008) and Cys 219 in SrtC1 from Group B *Streptococcus* (Cozzi et al., 2011) are critical for each of their corresponding sortase activities. When two other residues (Leu263 and Thr265) in this motif were changed to Ala, the effect on the enzyme activity was minimal (Fig. 3). Regarding whether these two residues are important for sortase activity, there are no mutagenesis data available for comparison in other pilus related sortases.

Fig. 2. Partial sequence alignment of class C sortases from different bacteria. Identical residues are indicated with pink letters. The sequences were aligned using the Vector NTI program (Invitrogen).
However, in the nonpilus related SrtA, the corresponding L181A mutation has modest effect on catalytic efficiency, while T183A mutation resulted in a 1200 fold decrease in $k_{cat}$ relative to wild type SrtA (Frankel et al., 2007). Because our polymerization assay only indicates the presence or the absence of activity, not the rate of the enzymatic activity, quantitative methods need to be developed to address the effect of these mutations more precisely. To this end, our dot blot results did show that there are less FimP components on the surface of T265A mutant than on the surface of the wild type strain (Fig. S1).

It has been proposed that sortases use a catalytic triad composed of His Cys Arg during the catalytic process (Table 3). The His204 residue is most likely the His residue in the catalytic triad. The His 204 residue is located 6 Å from the Cys 266 SG atom (Persson, 2011). The H204A mutation effect is consistent with what has been reported about other histidine residues located in the catalytic triads. For instance, in pilus related sortases, its counterparts His 131 in SrtC 1 of S. pneumoniae and His 157 in SrtC 1 of Group B Streptococcus were essential for pilus fiber formation in both organisms (Manzano et al., 2009; Cozzi et al., 2011). In the nonpilus related SrtA from S. aureus, His120 residue at a similar location in relation to the essential Cys184 residue is also critical for the catalytic process (Ilangovan et al., 2001; Ton That et al., 2002). However, the catalytic function of this critical histidine residue is still a subject of debate. It is speculated that the His residue, because it is being positively charged, might contribute to the electrostatic environment essential for the catalytic activity (Zong et al., 2004).

Although the newly published crystal structure (Persson, 2011) showed that Arg275 is part of the His Cys Arg catalytic triad, our results indicate that this Arginine residue is not important for the SrtC1 activity in A. oris T14V. In contrast, its counterparts Arg202 in SrtC 1 of S. pneumoniae and Arg228 in SrtC 1 of Group B Streptococcus are essential for the activity of the corresponding sortases (Manzano et al., 2009; Cozzi et al., 2011). Even in the nonpilus related sortase SrtA, the Arginine 197 residue was identified to be important for the enzyme’s activity(Frankel et al., 2007). However, in SrtA, the Arg197 residue is 13 amino acids away from the essential Cys194 residue instead of the nine amino acid distance between the arginine and cysteine residues in the SrtC catalytic triads. To eliminate the possibility that the triad Arg residue is located a bit farther away from the triad Cys residue.

![Fig. 3. Western blot analyses of type 1 fimbriae extracted from Actinomyces oris wild type and mutant strains. Cell surface proteins were extracted by mutanolysin digestion of bacteria, separated on a NuPAGE gel, transferred to a nitrocellulose membrane, probed with mAb 8A against an epitope of FimP and developed with Chromogenic Western Blot Immunodetection Kit (Invitrogen). Marker represents 10 µL of Magic Mark XP Western Protein Standard (Invitrogen). WT, wild type A. oris T14; ΔSrtC1, srtC deletion mutant. Other lanes represent mutants that have one of the conserved amino acids replaced with alanine.](image)

Table 3. Putative catalytic triad composition of different sortases

<table>
<thead>
<tr>
<th>Sortase origin</th>
<th>Catalytic triad composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SrtA of S. aureus</td>
<td>His120 Cys184 Arg197</td>
<td>Zong et al. (2004)</td>
</tr>
<tr>
<td>SrtC1 of S. pneumoniae</td>
<td>His131 Cys193 Arg202</td>
<td>Manzano et al. (2009)</td>
</tr>
<tr>
<td>SrtC1 of Group B Streptococcus</td>
<td>His157 Cys219 Arg228</td>
<td>Cozzi et al. (2011)</td>
</tr>
<tr>
<td>SrtC1 of A. oris</td>
<td>His204 Cys266 Arg275</td>
<td>Persson (2011)</td>
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out, we also mutated the Arg282 residue. Western blot results indicate that Arg 282 is not critical for the activity either (Fig. 3). Considering that the type 1 fimbriae of \textit{A. oris} consists of two components, FimP and FimQ, and both components are likely polymerized by SrtC1 (Chen \textit{et al.}, 2007), it is possible that that \textit{A. oris} SrtC1 may be more flexible compared with other class C sortases, and only use Cys 266 His 204 catalytic dyad, instead of Arg 275 Cys 266 His 204 catalytic triad, for the catalytic process. The role that the critical residue Tyr 236 plays with regard to SrtC1 activity in \textit{A. oris} is presently unknown and will be the subject of our future study.

In summary, we have identified the promoter (transcription start site) for the type 1 fimbria gene cluster and the three essential amino acid residues critical for the SrtC1 activity in \textit{A. oris} T14V. These findings fill the knowledge gap with regard to the transcription and structure function of SrtC1 of \textit{A. oris} T14V. The identification of these essential amino acid residues that are critical for the catalytic function of this enzyme in \textit{A. oris} may reveal potential targets for therapeutic use to prevent or reduce dental plaque formation initiated by this oral colonizer.

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**Statement**

The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

**References**


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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Dot immunoblot analyses of type 1 fimbriae on the surfaces of *Actinomyces oris* wild type and mutant strains.

Table S1. Primers used for site directed mutagenesis.

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