

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

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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

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* as a single polycistronic mRNA.

(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 (Maresso & Schneewind, 2008).

SrtC1 is required for both the assembly of type 1 fimbriae in *A. oris* T14V and its adherence to saliva coated hydroxylapatite (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

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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 (Dramsı *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 1. Bacterial strains and plasmids

Plasmid or strain	Description*	Source
Plasmid		
p6Srt	Tm ^f , plasmid containing <i>srtC1</i> and both upstream downstream flanking regions, template for site directed mutagenesis	Chen <i>et al.</i> (2007)
p6Srt H184A	Tm ^f , analog of p6Srt, for creating H184A mutant	This study
p6Srt H204A	Tm ^f , analog of p6Srt, for creating H204A mutant	This study
p6Srt F213A	Tm ^f , analog of p6Srt, for creating F213A mutant	This study
p6Srt Y236A	Tm ^f , analog of p6Srt, for creating Y236A mutant	This study
p6Srt L263A	Tm ^f , analog of p6Srt, for creating L263A mutant	This study
p6Srt T265A	Tm ^f , analog of p6Srt, for creating T265A mutant	This study
p6Srt C266A	Tm ^f , analog of p6Srt, for creating C266A mutant	This study
p6Srt R275A	Tm ^f , analog of p6Srt, for creating R275A mutant	This study
p6Srt R282A	Tm ^f , analog of p6Srt, for creating R282A mutant	This study
<i>A. oris</i>		
T14V	Wild type strain, Km ^s Sm ^r	Cisar <i>et al.</i> (1988)
ΔSrtC1	SrtC1 deficient strain, Km ^r	Chen <i>et al.</i> (2007)
SrtC1 DHFR	Transformant of ΔSrtC1 obtained with p6Srt, Tm ^f	Chen <i>et al.</i> (2007)
H184A	Transformant of ΔSrtC1 obtained with p6Srt H184A, Tm ^f	This study
H204A	Transformant of ΔSrtC1 obtained with p6Srt H204A, Tm ^f	This study
F213A	Transformant of ΔSrtC1 obtained with p6Srt F213A, Tm ^f	This study
Y236A	Transformant of ΔSrtC1 obtained with p6Srt Y236A, Tm ^f	This study
L263A	Transformant of ΔSrtC1 obtained with p6Srt L263A, Tm ^f	This study
T265A	Transformant of ΔSrtC1 obtained with p6Srt T265A, Tm ^f	This study
C266A	Transformant of ΔSrtC1 obtained with p6Srt C266A, Tm ^f	This study
R275A	Transformant of ΔSrtC1 obtained with p6Srt R275A, Tm ^f	This study
R282A	Transformant of ΔSrtC1 obtained with p6Srt R282A, Tm ^f	This study

*Antibiotics: Km, kanamycin; Sm, streptomycin; Tm, trimethoprim.

Table 2. Primers used in RACE PCR and RT PCR

Primer	Sequence (5' 3')	Gene
1	CTTGTCAGTGAAGTCTCGTCATG	<i>fimQ</i>
2	CATCGGAGTAGATCGGACTGTTGACGT	<i>fimQ</i>
3	GTGAGAACCACCTTGCCGTAATTG	<i>fimP</i>
4	CACCGGCCCTTGACCACGTCACCCTTGA	<i>fimP</i>
5	ACGTAGAAGACGTCGCCCTTCTTG	<i>srtC1</i>
6	TCCGGGCCATGACCGGGTCGATGTCCA	<i>srtC1</i>
7	GCCATCAACCGCGAGTACTGGCTG	<i>fimQ</i>
8	GTCGATGGTGAGCCGTTCCGGGTC	<i>fimP</i>
9	GCGCCAACGACTACGTCAACGGTG	<i>fimP</i>
10	AGGAAGCGCTCACGCAGGACCTTG	<i>srtC1</i>
11	CACGGTCATCCTGCTGCGTCAG	<i>mt1</i>
12	GACACCATGGTCACAGGACGA	<i>fimQ</i>
13	ATGCAGACCTGGATGAAGGCCATC	<i>srtC1</i>
14	CAACCAATAGGAAGTCAGCAGCGA	<i>orfC</i>

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 DL threonine until the culture reached the early exponential phase with an OD_{600 nm} 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 Quik Change 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

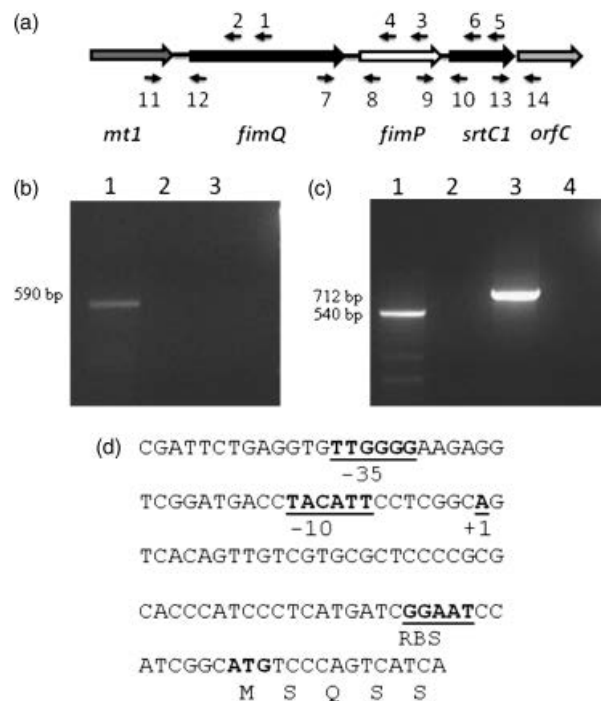


Fig. 1. (a) Schematic presentation of the type 1 fimbriae gene cluster in *Actinomyces oris* T14V (Chen *et al.*, 2007). Primers 1, 3 and 5 were used to initiate the first strand of cDNA synthesis from the regions of *fimQ*, *fimP* and *srtC1*, respectively. Primers 2, 4 and 6 were used for RACE PCR. Primers 7–14 were used for amplifying the junction regions of *fimQ* *fimP*, *fimP* *srtC1*, *mt1* *fimQ* 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 the 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 Δ SrtC1 (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

B. cereus-BC2509	AYADALNLGD	VMGYIEISKI	NIKLPIYQGT	SEEVLSRGIG	HLDFFSLPVG
C. diphtheriae_Srt1	AYEKLLEGEYP	AMAQISIPAI	SVNLPYHGT	SDATLLKGVG	HLYGTALPVG
C. diphtheriae_Srt2	EYLDQLDAND	AMARLIFPKI	KADLPYHGT	DNDTLQKGLG	HLFGSLPVG
GBS_SAG0647_sortase	EYARMLLEVKE	QIGHVPIPRI	NQDIPYAGS	AEENLQKGVG	HLEGTSLPVG
GBS_SAG0648_sortase	EYARMLLEVHE	QIGHVAIPSI	GVDIPYAGT	SETVLQKGS	HLEGTSLPVG
S. agalactiae-GBS1475	EYAHMLEITE	MIGYIDIPSI	KQKLPYAGT	TSSVLEKAG	HLEGTSLPIG
S. pneumoniae-SP0468	.YQVSDDPDA	VYGYLSIPSL	EIMEPVYLG	DYHHLGMGLA	HVDGTPPLD
S. pyogenes-SPY0135	ESLLQIENND	IMGYVEVPSI	KVTLPIYHYT	TDEVLTAKG	HLFGSALPVG
T14V_SrtC2	ANILKANNEG	LMARLKIPSI	SLDLVYHGT	ADDTLLKGLG	HLEGTSLPVG
T14V_SrtC1	AYLHEMDIDP	VMARIVIPSI	HVSLPIYHGT	DSRTLTEGVG	HLFGTSLPVG
					H184
B. cereus-BC2509	GENTHTILTG	HRGLPSAKLF	TDLDKLSKGD	LFYLSLSDKV	LAYKVDQIKV
C. diphtheriae_Srt1	GLGTRSVLTA	HSGIQKSTFF	DNLEKVKKGD	AIYVRNIGET	LKYQVRDIEI
C. diphtheriae_Srt2	GKGMHSVITG	HTGLANSTMF	DHLNKAEGD	TFYVQVSgek	LKYVVDQIKV
GBS_SAG0647_sortase	GESTHAVLTA	HRGLPTAKLF	TNLDKVTVGD	RFYIEHIGGK	IAYQVDQIKV
GBS_SAG0648_sortase	GLSTHSLVTA	HRGLPTARLF	TDLNKVKKQ	IFYVTNIKET	LAYKVVSIKV
S. agalactiae-GBS1475	GKSSHTVITA	HRGLPKAKLF	TDLDKLLKKGK	IFYIHNKEV	LAYKVDQISV
S. pneumoniae-SP0468	GTGIRSVIAG	HRAEPSHVFF	RHLDQLKVG	ALYYDNGQEI	VEYQMMDEI
S. pyogenes-SPY0135	GDGHTVISA	HRGLPSAEMF	TNLNLVKKGD	TFYFRVLNKV	LAYKVDQILT
T14V_SrtC2	GEGTRSVITG	HRGLAEATMF	TNLDKVKTG	SLIVEVFGEV	LYRVVTSTKV
T14V_SrtC1	GPSTHSLVLTG	HTGLSTATMF	DNLNQLKKG	VFYVSSLGQT	LKYEVNDITV
		H204	F213		Y236
B. cereus-BC2509	VLPHETDDLQ	IVQNKDYTTL	ITCTPYGINT	NRLLVGRERV	ELNEKEKQK.
C. diphtheriae_Srt1	IRPAEIDRIQ	PIPDRDLITL	VTCTPYGINT	HRLLVTAERV	PMEPEGADRA
C. diphtheriae_Srt2	VLPTETEDLR	PEQKDYITL	ITCTPYGINT	HRLMVRGHQV	PLAPEDHEVF
GBS_SAG0647_sortase	IAPDQLEDLY	VIQGEDHVTL	LTCTPYMINS	HRLLVGRKRI	PYVEKTVQK.
GBS_SAG0648_sortase	VDPTALSEVK	IVNGKDYITL	LTCTPYMINS	HRLLVKGERI	PYDSTEAEK.
S. agalactiae-GBS1475	VKPDNFSKLL	VVKGKDYATL	LTCTPYSINS	HRLLVRGHRI	KYVPPVKEKN
S. pneumoniae-SP0468	ILPSEWEKLE	SVSSKNIMTL	ITCDPIPTFN	KRLLVNFERV	AVYQKSDPQT
S. pyogenes-SPY0135	VEPDQVTSLS	GVMGKDYATL	VTCTPYGVNT	KRLLVRGHRI	AYHYKYYQQA
T14V_SrtC2	VEPEETEALR	VEEGKDLLTL	VTCTPLGINT	HRILLTGERI	YPTPAKDLAA
T14V_SrtC1	VKPEETDSLRL	KVPGRDLVTL	ITCTPYGVNS	HRLLVGTGERV	PMDPTAAAAE
		L263	T265	C266	R275

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).

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 TLXTC 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 That *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.

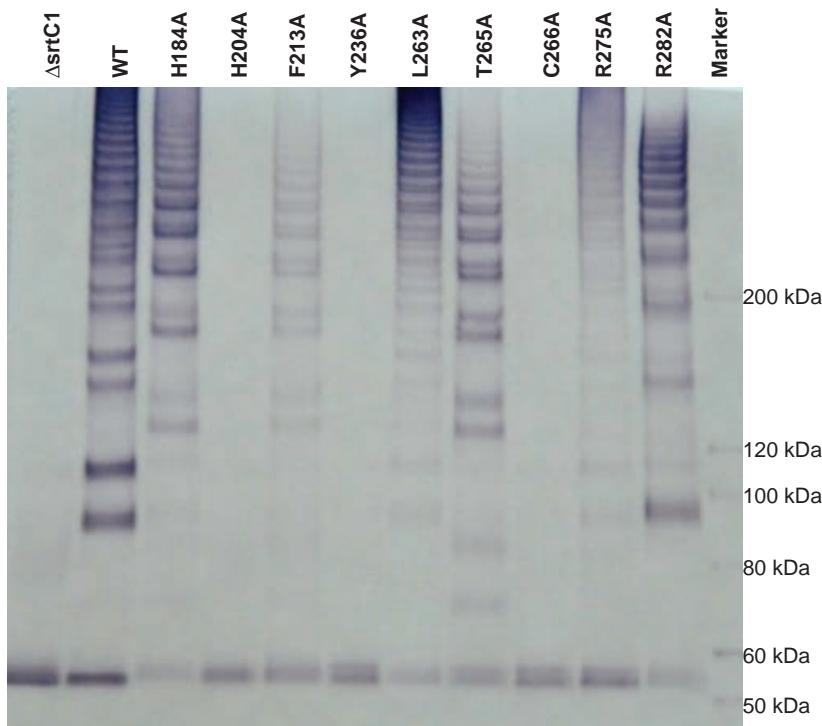


Fig. 3. Western blot analyses of type 1 fimbriae extracted from *Actinomyces oris* wild type and mutant strains. Cell surface proteins were extracted by mutanolysin digestion of bacteria, separated on a NuPAGE gel, transferred to a nitrocellulose membrane, probed with mAb 8A against an epitope of FimP and developed with Chromogenic Western Blot Immunodetection Kit (Invitrogen). Marker represents 10 μ L of Magic Mark XP Western Protein Standard (Invitrogen). WT, wild type *A. oris* T14; Δ SrtC1, *srtC* deletion mutant. Other lanes represent mutants that have one of the conserved amino acids replaced with alanine.

Table 3. Putative catalytic triad composition of different sortases

Sortase origin	Catalytic triad composition	Reference
SrtA of <i>S. aureus</i>	His120 Cys184 Arg197	Zong <i>et al.</i> (2004)
SrtC1 of <i>S. pneumoniae</i>	His131 Cys193 Arg202	Manzano <i>et al.</i> (2009)
SrtC1 of Group B <i>Streptococcus</i>	His157 Cys219 Arg228	Cozzi <i>et al.</i> (2011)
SrtC1 of <i>A. oris</i>	His204 Cys266 Arg275	Persson (2011)

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 His157 in SrtC1 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 SrtC1 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

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 *A. oris* consists of two components, FimP and FimQ, and both components are likely polymerized by SrtC1 (Chen *et al.*, 2007), it is possible that that *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 *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 *A. oris* T14V. These findings fill the knowledge gap with regard to the transcription and structure function of SrtC1 of *A. oris* T14V. The identification of these essential amino acid residues that are critical for the catalytic function of this enzyme in *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

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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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