

Mutagenesis and Chemical Cross-Linking Suggest that Wzz Dimer Stability and Oligomerization Affect Lipopolysaccharide O-Antigen Modal Chain Length Control[∇]

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In *Shigella flexneri*, the polysaccharide copolymerase (PCP) protein Wzz_{SF} confers a modal length of 10 to 17 repeat units (RUs) to the O-antigen (Oag) component of lipopolysaccharide (LPS). PCPs form oligomeric structures believed to be related to their function. To identify functionally important regions within Wzz_{SF}, random in-frame linker mutagenesis was used to create mutants with 5-amino-acid insertions (termed Wzz_i proteins), and DNA sequencing was used to locate the insertions. Analysis of the resulting LPS conferred by Wzz_i proteins identified five mutant classes. The class I mutants were inactive, resulting in nonregulated LPS Oag chains, while classes II and III conferred shorter LPS Oag chains of 2 to 10 and 8 to 14 RUs, respectively. Class IV mutants retained near-wild-type function, and class V mutants increased the LPS Oag chain length to 16 to 25 RUs. *In vivo* formaldehyde cross-linking indicated class V mutants readily formed high-molecular-mass oligomers; however, class II and III Wzz_i mutants were not effectively cross-linked. Wzz dimer stability was also investigated by heating cross-linked oligomers at 100°C in the presence of SDS. Unlike the Wzz_{SF} wild type and class IV and V Wzz_i mutants, the class II and III mutant dimers were not detectable. The location of each insertion was mapped onto available PCP three-dimensional (3D) structures, revealing that class V mutations were most likely located within the inner cavity of the PCP oligomer. These data suggest that the ability to produce stable dimers may be important in determining Oag modal chain length.

Lipopolysaccharide (LPS) of *Shigella flexneri* is an important virulence factor, providing protection against host defenses and affecting interaction with host cells. LPS is composed of three regions: the hydrophobic lipid A membrane anchor, the core sugar region, and the O-antigen (Oag) polysaccharide chain (18). The basic Oag repeat unit of *S. flexneri* is a tetrasaccharide consisting of three rhamnose sugars and one *N*-acetylglucosamine sugar (19). The contribution of *Shigella* Oag to establishing virulence has been extensively investigated, and results indicate that regulated Oag modal length is required for virulence (8, 23, 25). Loss of Oag modal chain length regulation affects virulence due to the masking of the outer membrane (OM) protein IcsA (8, 23), and the type III secretion system is also affected by Oag chain length (24).

The current model for Oag biogenesis in *S. flexneri* involves the initiation of Oag repeat unit synthesis on the cytoplasmic face of the inner membrane (IM) and continues with a series of successive sugar transferase reactions. The repeat units are assembled on the lipid carrier undecaprenol phosphate (Und-P), and transported across the IM by the Wzx flippase to the periplasmic face of the IM. Polymerization of Oag repeat units is catalyzed by the Wzy polymerase, linking the individual oligosaccharide repeat units into a chain; the nascent chain is transferred from its lipid carrier to the nonreducing end of the

newly flipped oligosaccharide repeat unit. The resulting chain is then ligated to the lipid A core by WaaL ligase (18, 26) to form LPS.

The regulation of the chain length of the Oag polysaccharide is controlled by the Wzz protein, a member of the polysaccharide copolymerase 1a (PCP1a) family (13, 21). *S. flexneri* Wzz (Wzz_{SF}) confers an average chain modal length of 10 to 17 Oag repeat units. In addition to determining the Oag chain modal length, PCP proteins are involved in enterobacterial common antigen (ECA) modal chain length regulation and biosynthesis and in capsule polysaccharide (CPS) and exopolysaccharide (EPS) biosynthesis (13). The PCP1a proteins are located in the IM and have two transmembrane (TM) regions, TM1 and TM2 (14). TM1 is located close to the N-terminal end, and TM2 is located near the C-terminal end, while the hydrophilic region between TM regions is located in the periplasm (14). PCPs exhibit a conserved motif, proximal to and partly overlapping the TM2 region, rich in proline and glycine residues (2, 3, 13). Site-directed mutagenesis studies targeting a number of these conserved residues, singularly or in combination, indicate that changes to this region have a significant effect on the resulting Oag modal chain length (4). Many mutagenesis studies on residues throughout Wzz indicate that function may be an overall property of the protein and may not be limited to one particular region (4, 6, 21). Despite studies conducted to probe the Wzz structure function relationship, little is known about the mode of action in determining Oag modal chain length. Recently, the periplasmic domain structures of a collection of PCP proteins including *Salmonella enterica* serovar Typhimurium WzzB (Wzz_{ST}) and *Escherichia coli* O157 FepE and WzzE have been solved, and it has been deduced that

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these structures show marked similarities at the protomer and oligomer levels (21). These protomers are elongated and consist of two structural components: a trapezoidal α/β base domain close to the membrane and an extended α -helical hairpin containing an ~ 100 -Å-long helix forming anti-parallel coiled-coil interactions with two helices that fold back toward the membrane (21). The protomers self-assemble into bell-shaped oligomers displaying comparable structural features, with Wzz_{ST} forming pentameric oligomers, WzzE assembling into octameric oligomers, and FepE assembling into nonameric structures (21). In contrast, a recent study from Larue et al. reports that Wzz_{ST}, FepE, and Wzz_{K40} favor hexameric structures (9). A previous study on the oligomeric status of *S. flexneri* WzzB (Wzz_{SF}) via *in vivo* cross-linking with formaldehyde indicated that Wzz_{SF} has the ability to form hexamers and high-order oligomers, suggesting that oligomerization is important in function (4). Related to this, Marolda et al. have shown that the ECA-associated Wzx can fully complement an LPS Oag-associated Wzx-deficient mutant if the remaining ECA gene cluster is deleted, providing genetic evidence that proteins involved in Oag/ECA biosynthesis and processing may function as a complex (11).

Several models of the likely mechanisms of Oag chain regulation have been proposed. Bastin et al. initially suggested that Wzz acts as a molecular timer, allowing polymerization to occur to a particular point, hence increasing the number of repeat units added to the chain (1). An alternative model proposed by Morona et al. suggested that Wzz acts as a molecular chaperone, facilitating the interaction between Wzy and WaaL, and modal length is the result of the ratio of Wzy and WaaL (14). Published data indicated that the ratio of Wzy and Wzz was important in determining Oag modal chain length, which is supportive of the latter model (5). With recent developments in solving the PCP three-dimensional (3D) structure and oligomeric arrangement, a new model has been proposed by Tocilj et al. in which the Wzz oligomers act as molecular scaffolds for multiple Wzy polymerase molecules and the growing Oag chain is transferred from one Wzy to another Wzy molecule (21).

In a previous study, site-directed mutagenesis analysis was conducted on Wzz_{SF} (4). Although mutational alterations targeting the TM regions caused dramatic changes in the resulting LPS Oag chain length, mutations targeting the periplasmic domain generally did not have an obvious effect on the resulting LPS Oag chain length. This was also shown for mutations in FepE (17). Hence, we decided that a more severe approach to Wzz_{SF} mutagenesis was needed to investigate the relationship between Wzz structure and function by increasing the likelihood of acquiring Wzz mutants displaying phenotypic changes. In this study, we have investigated the structure and function of Wzz_{SF} by constructing a library of in-frame linker mutants with 5-amino-acid (aa) insertions throughout the Wzz_{SF} protein. We have identified regions in Wzz_{SF} which alter the modal length in different ways and present biochemical evidence acquired by *in vivo* chemical cross-linking that indicates oligomeric differences exist between Wzz mutants and the wild type (WT). We also present evidence that suggests the dimeric form of Wzz_{SF} is important in establishing modal length.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are *S. flexneri* RMA2741 serotype Y SFL1 wzz::Km^r, cured of virulence plasmid and pHS-2, carrying F' (*lacI^q Tet^r*), from our laboratory collection, and *Escherichia coli* strain Top10 F'[(*lacI^q Tet^r*) *mcrA* Δ (*mrr-hsd RMS-mcrBC*) ϕ 80 *lacZ* Δ M15 *ΔlacX74 deoR recA1 araD139* Δ (*ara-leu*) 7697 *galU galK rpsL endA1 nupG*] from Stratagene. Strains were grown in Luria-Bertani broth (10 g/liter Tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) with aeration for 16 to 18 h. Eighteen-hour cultures were diluted 1/50 into fresh broth and grown to log phase (optical density at 600 nm [OD₆₀₀] of 0.7). During induction conditions, cultures had 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) added and were grown for a further 1 to 1.5 h.

Mutagenesis. An *in vitro*-based, random mutagenesis kit (mutagenesis generation system; Finnzymes) was employed to produce a library of in-frame wzz_{SF} mutant constructs as per the manufacturer's instructions. Plasmid pRMCD30, a pQE-30 (Qiagen)-based construct with the wzz_{SF} open reading frame (ORF) expressed as a His₆-tagged protein His₆-Wzz_{SF} (4) and containing SacI and SmaI sites flanking the coding region, was incubated with the kanamycin resistance conferring the Mu *entranceposon* DNA sequence element (harboring NotI sites very close to its ends) to allow its random formation of transposition complexes. These were electroporated into Top10 F', and transformants which were resistant to kanamycin were screened via PCR using primers #2197 (5'-AGGGTA GAGTCAGAGTAGAAAAT-3') and #2198 (5'-GTTACCCGGGGAGCAG GTGTGA-3'), corresponding to the 5' and 3' region of wzz_{SF}, respectively. Selected plasmids were restricted with NotI to remove the *entranceposon*, leaving a 15-bp insertion which contained a NotI restriction site. The NotI mini-primer (5'-TGCGGCCGCA-3') was used to identify the approximate site of insertion via PCR. The precise position of the 15-bp insertion within the coding region was determined by cycle sequencing (AGRF, Queensland, Australia). The mutational alterations were mapped on 3D images created using Pymol software (DeLano Scientific LLC 2008).

LPS PAGE and silver staining. LPS was prepared as described previously (15, 22). Briefly, 1×10^9 cells were harvested by centrifugation, resuspended in lysing buffer (10% [wt/vol] glycerol, 2% [wt/vol] SDS, 4% [wt/vol] β -mercaptoethanol, 0.1% [wt/vol] bromophenol blue, 1 M Tris-HCl, pH 7.6) and incubated with 2 μ g/ml of proteinase K for approximately 16 h. The isolated LPS samples were electrophoresed on a 15% polyacrylamide gel for 16 to 18 h at 12 mA. The gel was stained with silver nitrate and developed with formaldehyde (15).

SDS-PAGE and Western immunoblotting. Log-phase bacteria were induced as described above, harvested by centrifugation, and resuspended in $1 \times$ sample buffer (10). SDS-PAGE and Western immunoblotting were performed as previously described (4, 17), and samples were run alongside a molecular mass marker (Invitrogen). The nitrocellulose was blocked with 5% skim milk and incubated overnight with affinity-purified anti-Wzz polyclonal, prepared by Daniels et al. (4), at a concentration of 1:1,000 in 2.5% (wt/vol) skim milk. The membrane was incubated with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (Biomediq DPC). The CPS3 chemiluminescence substrate (Sigma) was applied to the membrane for 5 min, exposed to film, and developed.

Chemical cross-linking analyses. Bacteria grown as described above were induced, and 5×10^8 cells were harvested and washed with chilled buffer (10 mM KPO₄, 10 mM Tris, pH 6.8) and then incubated with 0.5% (vol/vol) formaldehyde (Sigma) in buffer at 25°C for 1 h (4, 16). Samples were also incubated at 25°C without formaldehyde as a control. Both cross-linked and control samples were washed once more with chilled buffer and heated to 60°C for 5 min and electrophoresed on an SDS-12% polyacrylamide gel and either subjected Western immunoblotting as described above or stored at -20°C until needed. Cross-linked samples were also heated to 100°C for 5 min, electrophoresed, and subjected to Western immunoblotting as described above.

RESULTS

Location of 5-aa insertions within Wzz_{SF}. As random mutagenesis of Wzz_{SF} has not previously been published, a random in-frame linker mutagenesis method was employed to create a library of Wzz_{SF} proteins with 5-aa insertions in different positions. An *in vitro* system (see Materials and Methods) was employed that resulted in random insertions of the Mu *entranceposon* sequence within the wzz_{SF} ORF in pRMCD30. Following excision of the *entranceposon* with NotI, transformants were screened by PCR and restriction enzyme

TABLE 1. Different classes of Wzz mutants based on the length of Oag they confer when complementing *S. flexneri* strain RMA2741^a

Wzz _i mutant	Insertion site with 5-aa insertion	Class ^b	Protein detection ^c
; ₃₂	M32 TAAAM T33	I	+
; ₆₆	D66 CGRTD V67	I	+
; ₈₀	I80 CGRII Y81	IV	+
; ₈₁	Y81 CGRIY G82	IV	+
; ₉₂	Q92 VRPQQ E93	III	+
; ₁₂₈	Q128 LRPHQ Q129	V	+
; ₁₃₁	P131 LRPQP L132	V	+
; ₁₃₈	G138 CGRIG Q139	III	+/-
; ₁₆₁	Q161 DAAAQ E162	I	+/-
; ₁₆₃	L163 VRPQL E164	I	-
; ₁₉₁	E191 LRPOE Q192	II	-
; ₁₉₉	Q199 IAAAA I200	I	+
; ₂₁₉	Q219 MRPQQ T220	II	+
; ₂₃₁	L231 VRPHL G232	II	+/-
; ₂₄₇	P247 CGRSP L248	II	+/-
; ₂₅₅	Y255 CGRNY Q256	II	-
; ₂₇₉	Y279 CGRSY V280	I	-
; ₂₉₀	D290 CGRSD S291	I	+

^a The 5-amino-acid insertion sequences of each Wzz_i mutant are listed, along with the classes and confirmation of Wzz_i protein detection.

^b Class I, random Oag modal chain length; class II, very short (2 to 10 repeats); class III, shorter (8 to 14 repeats); class IV, wild type (11 to 19 repeats); class V, longer (16 to 25 repeats).

^c +, wild type; +/-, less than wild type; -, not detected.

analysis to identify approximate sites of insertion, as each insertion possessed a NotI restriction site. DNA sequencing identified 18 unique mutant constructs encoding proteins termed Wzz_i, which were then assessed for protein production and impact on LPS O-antigen modal chain length distribution. Due to the nature of this in-frame mutagenesis, each Wzz_i mutant has a unique 5-aa sequence at the site of insertion. These insertion sequences are listed in Table 1.

LPS Oag modal chain length conferred by Wzz_i mutants.

The Wzz_i-encoding plasmids were transformed into RMA2741, an *S. flexneri* Y strain with a wzz_{SF}::Km^r mutation and lacking both pHS-2 (encoding Wzz_{pHS-2}) and the large virulence plasmid. Following analysis of the resulting LPS Oag modal chain length distribution by SDS-PAGE and silver staining, the Wzz_i mutants were grouped into five different phenotypic classes (Table 1 and Fig. 1). Seven of the 18 mutants, categorized into class I, had lost the ability to regulate Oag modal chain length under the conditions used in these experiments and hence displayed LPS with random-length Oag chains (represented by ;₃₂; Fig. 1A, lane 3). Five mutants conferred significantly reduced average Oag modal chain lengths from wild type to between 2 to 6 and 2 to 10 Oag repeat units (represented by ;₂₁₉, class II; lane 4). Two mutants con-

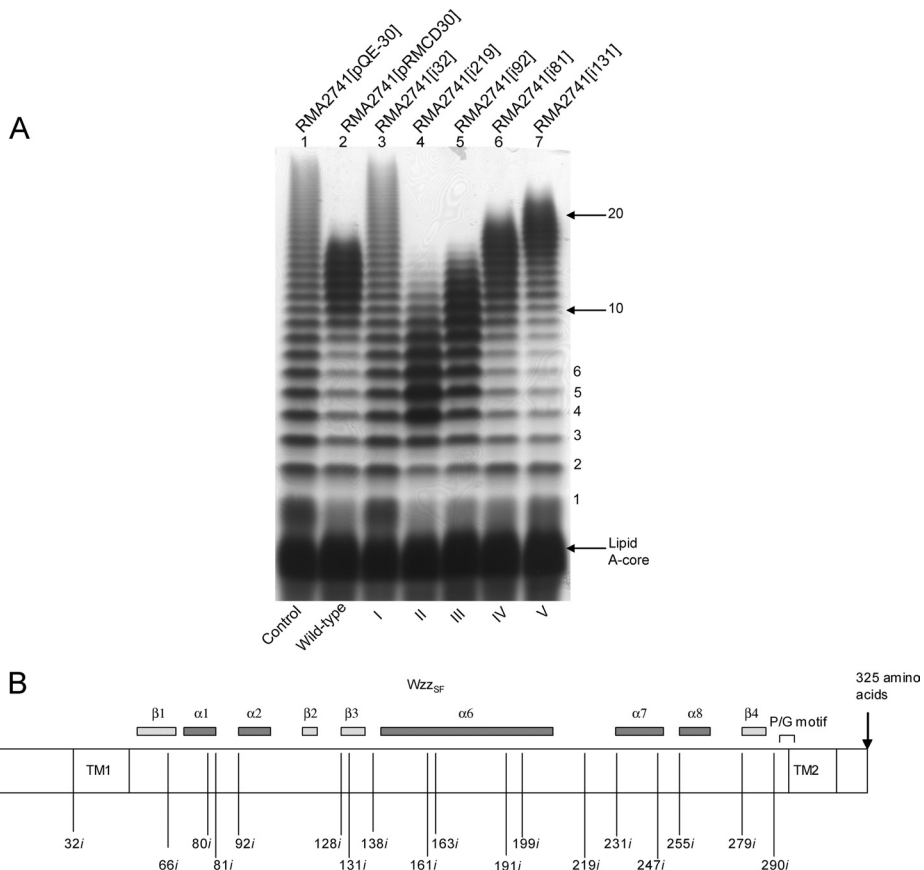


FIG. 1. Oag modal chain length conferred by the different classes of Wzz_i mutants expressed in *S. flexneri* RMA2741. (A) LPS samples were prepared, electrophoresed on a 15% SDS-polyacrylamide gel, and silver stained as described in Materials and Methods. The strains in each lane are as follows: 1, RMA2741(pQE-30); 2, RMA2741(pRMCD30); 3, RMA2741 (;₃₂, class I); 4, RMA2741 (;₂₁₉, class II); 5, RMA2741 (;₉₂, class III); 6, RMA2741 (;₈₁, class IV); and 7, RMA2741 (;₁₃₁, class V). (B) Schematic representation of the locations of each insertion within Wzz_{SF}, illustrating the insertions within Wzz_{SF} that were designated titles according to the last uninterrupted amino acid preceding the 5-aa insertion. The proline rich motif is indicated (P/G motif). Secondary structural features are based on the 3D structure of Wzz_{SF}.

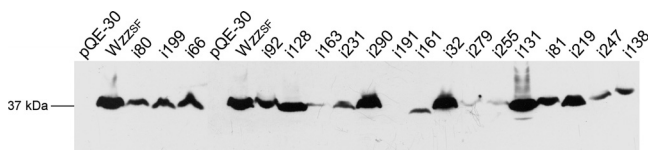


FIG. 2. Western immunoblotting performed on whole-cell lysates from *S. flexneri* strain RMA2741 carrying *Wzz_i* plasmids. Strains were grown and induced, and whole-cell lysates were detected with affinity-purified *Wzz* polyclonal antibodies. Each lane contained approximately 1×10^8 bacterial cells.

ferred a reduced Oag modal chain length of 8 to 14 repeat units (represented by i_{92} , class III; lane 5), and two mutants conferred a near-wild-type Oag modal chain length between 11 and 19 repeat units (represented by i_{81} , class IV; lane 6). Interestingly, the two mutants categorized into class V conferred an increased Oag modal chain length from the wild-type modal length to 16 to 25 repeat units (represented by i_{131} , class V; lane 7).

Whole-cell lysates from *S. flexneri* strains carrying plasmids encoding the *Wzz_i* mutants were subjected to Western immunoblotting using anti-*Wzz* antibodies. The majority of plasmids encoding *Wzz_i* proteins resulted in detectable *Wzz* protein under induced conditions; however, a number of *Wzz_i* proteins could not be detected (class I i_{163} , class II i_{191} , class II i_{255} , and class I i_{279} mutants; Table 1 and Fig. 2). The class I i_{161} , class II i_{231} , class II i_{247} , and class III i_{138} mutant proteins were detected but at a lower intensity than wild type (summarized in Table 1 and Fig. 2).

In vivo chemical cross-linking of *Wzz_i* mutants. Wild-type *Wzz_{SF}* and representative *Wzz_i* mutants which were expressed at a level comparable to wild-type *Wzz_{SF}* (class I i_{290} , class II i_{219} , class III i_{92} , class V i_{128} , and class V i_{131} mutants) were analyzed for the ability to form higher-order oligomers by cross-linking with 0.5% formaldehyde (4). Wild-type *Wzz_{SF}* monomeric (36 kDa) and dimeric (72 kDa) forms were readily detected in the non-cross-linked sample and also what appears to be a tetrameric form (144 kDa) (Fig. 3, lane 3). The cross-linked sample revealed bands at ~ 30 kDa and ~ 36 kDa, a doublet band at ~ 72 kDa, and a smeared high-molecular-mass band of >180 kDa, which indicated the presence of higher-order oligomerization (Fig. 3, lane 4). The class I mutant i_{290} also exhibited the presence of both monomeric (~ 36 kDa) and dimeric (~ 72 kDa) bands in both non-cross-linked and cross-linked samples and higher-molecular-mass bands around the >180 -kDa region (Fig. 3, lanes 9 and 10). The presence of a 30-kDa protein was detected in the non-cross-linked sample of i_{290} (Fig. 3, lane 9), although this band was only identified in the presence of formaldehyde for *Wzz_{SF}* and other *Wzz_i* proteins (Fig. 3, lanes 4, 6, 8, 10, 12, and 14). There is also an additional i_{290} band of approximately 89 kDa which is not detected in other *Wzz_i* cross-linked profiles (Fig. 3, lane 9).

The class II mutant i_{219} (Fig. 3, lanes 13 and 14) had both monomeric (36 kDa) and dimeric (72 kDa) protein forms in cross-linked and non-cross-linked samples, and the 30-kDa protein was also detected in the cross-linked sample (Fig. 3, lane 14). Higher-molecular-mass bands were decreased in the cross-linked sample (Fig. 3, lane 14) compared to the wild type. The class III mutant i_{92} displayed a cross-linking profile com-

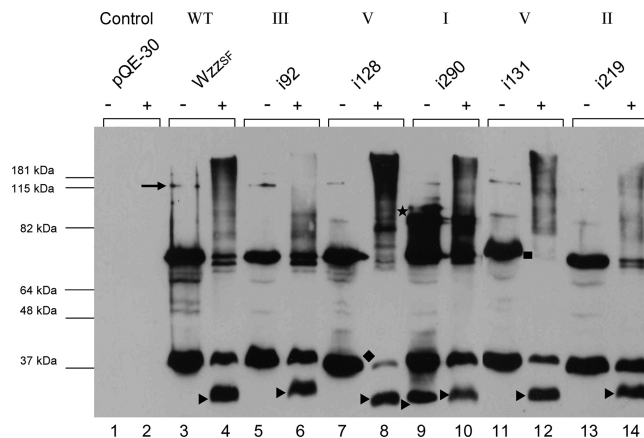


FIG. 3. Analysis of the *Wzz_i* mutant by cross-linking with formaldehyde. *S. flexneri* RMA2742 strains carrying plasmid-encoded *Wzz_i* proteins were harvested, washed in 10 mM KPO_4 , and exposed to 0.5% formaldehyde at 25°C (+); controls were incubated without formaldehyde (-) as described in Materials and Methods. Samples were heated at 60°C and electrophoresed on a 12% polyacrylamide gel. Western immunoblotting was performed with affinity-purified *Wzz_{SF}* polyclonal antisera. \blacktriangleright , ~ 30 -kDa *Wzz_{SF}* conformation; \star , extra band in i_{290} ; \blacklozenge , depleted monomeric form of i_{128} ; \blacksquare , lack of the dimeric form of i_{131} . Each lane contained approximately 4×10^8 bacterial cells.

parable to *Wzz_{SF}*, having bands of ~ 36 kDa, ~ 72 kDa, and ~ 144 kDa in the non-cross-linked sample (Fig. 3, lane 5) and ~ 30 kDa, ~ 36 kDa, and ~ 72 kDa in the cross-linked sample (Fig. 3, lane 11). However, the high-molecular-mass band around the 180-kDa region was not detected, similar to i_{219} .

The two class V mutants, i_{128} and i_{131} , exhibited bands of sizes 36 kDa and 72 kDa and weak bands at ~ 144 kDa in the non-cross-linked sample (Fig. 3, lanes 7 and 11). However, the cross-linked profile was very different from those of the other *Wzz_i* proteins: monomeric protein (36 kDa) and dimeric protein (72-kDa) forms were significantly reduced in the cross-linked sample (Fig. 3, lanes 8 and 12), very little monomeric protein and dimeric protein was detected in the i_{128} cross-linked sample, and no dimeric form of i_{131} was detected under cross-linking conditions. Larger bands greater than 180 kDa were detected in i_{131} cross-linked samples (Fig. 3, lane 12), and in the case of class V mutant i_{128} , *Wzz*-related bands greater than 180 kDa were very readily detected and were comparable to the higher-molecular-mass oligomers detected in the wild-type cross-linked sample (Fig. 3, lane 4). In summary, the data indicate that following cross-linking, class V mutants i_{128} and i_{131} , which confer a longer modal length, exhibit high-molecular-mass oligomers and reduced monomeric and dimeric forms, whereas high-molecular-mass oligomers are not detected in the class II and III mutants (i_{92} and i_{219}), which confer modal lengths shorter than wild type.

Stability of *Wzz* dimers. Previous studies have shown that *Wzz_{SF}* dimers can be detected even after SDS-PAGE and also that formaldehyde cross-linked *Wzz_{SF}* dimers are still able to be detected after being heated to 100°C (4). Representative *Wzz_i* mutants were chemically cross-linked, heated to 100°C, and along with non-cross-linked samples subjected to Western immunoblotting to ascertain whether the mutants exhibited different dimeric properties compared to wild-type *Wzz_{SF}*.

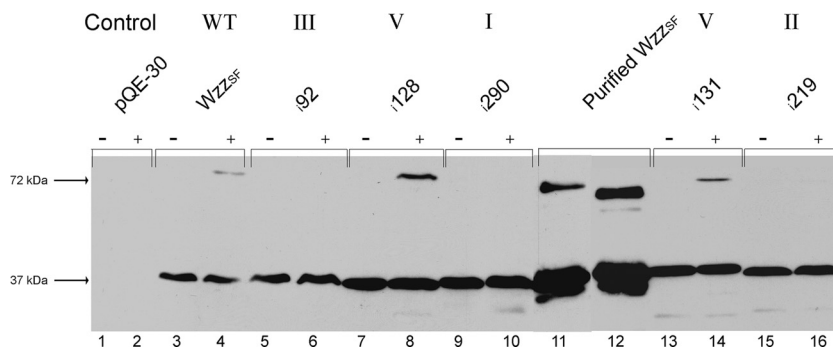


FIG. 4. Comparison of stabilities of Wzz_{SF} and Wzz_i dimers. *S. flexneri* RMA2742 strains carrying plasmid-encoded Wzz_i proteins were harvested, washed in 10 mM KPO_4 , and exposed to 0.5% formaldehyde at 25°C (+); controls were incubated without formaldehyde (-) as described in Materials and Methods. Both cross-linked and non-cross-linked samples of Wzz_{SF} and Wzz_i mutants were heated to 100°C for 5 min, electrophoresed on a 12% SDS-polyacrylamide gel, and subjected to Western immunoblotting. The strains in each lane are as follows: 1 and 2, RMA2741(pQE-30); 3 and 4, RMA2741(pRMCD30); 5 and 6, RMA2741 (i_{92} , class III); 7 and 8, RMA2741 (i_{128} , class V); 9 and 10, RMA2741 (i_{290} , class I); 11 and 12, purified Wzz_{SF} ; 13 and 14, RMA2741 (i_{131} , class V); and 15 and 16, RMA2741 (i_{219} , class II). Each lane contained approximately 1×10^8 bacterial cells.

Wild-type Wzz_{SF} dimers were detected in the cross-linked samples (Fig. 4, lane 4), as were dimers of class V mutants i_{128} (Fig. 4, lane 8) and i_{131} (Fig. 4, lane 14). Mutants i_{290} , i_{219} , and i_{92} from classes I, II, and III, respectively, did not form stable dimers under these conditions (Fig. 4, lanes 6, 10, and 16).

Location of insertions mapped to PCP 3D structures. The location of the Wzz_i 5-aa insertions could be mapped onto 3D structures of several PCP proteins (21). The last uninterrupted amino acid of each Wzz_i mutant was mapped onto the monomeric and oligomeric structures of Wzz_{ST} , $WzzE$, and $FepE$ (Fig. 5). The three structures exhibit comparable secondary structure characteristics (21). Analysis was primarily conducted on Wzz_{ST} , as it exhibits the greatest sequence identity to Wzz_{SF} (13). The six class I (no activity) mutant insertions that were mapped onto the structures were i_{66} , i_{161} , i_{163} , i_{199} , i_{279} , and i_{290} . Mutation i_{66} is predicted to be located on the very first turn of the first α helix, $\alpha 1$ (Fig. 5A, B, and C). Mutations i_{161} and i_{166} were predicted to be located about one-third of the way into $\alpha 6$, the long α helix extending from the α/β base domain, while i_{199} was predicted to be located toward the uppermost region of $\alpha 6$ (Fig. 5A, B, and C). Mutations i_{279} and i_{290} were predicted to be located within a β sheet of the α/β base domain ($\beta 4$) and the loop closest to TM2 of the determined 3D structure, respectively (Fig. 5A, B, and C).

In the oligomeric structures, i_{66} is predicted to be on the periphery of the monomer, with close proximity to $\alpha 2$ and the long extended $\alpha 6$ helix of neighboring monomers (Fig. 5D to G). Mutants i_{161} and i_{163} , with insertions predicted to be located on the bottom half of $\alpha 6$, appear to be embedded within the monomer, close to $\alpha 2$. Mutant i_{199} , was predicted to have the insertion sequence located at the very summit of $\alpha 6$, isolated from other structural features (Fig. 5F). Mutant i_{279} was predicted to contain the insertion in the central region of the $\beta 4$ sheet, on the fringe of the monomeric structure, very close to the neighboring $\alpha 2$ (Fig. 5F). Mutation i_{290} is predicted to be located on the lowest points in the oligomer and would most likely have close proximity to the transmembrane regions. The insertions from this class were mapped to both

internal and external locations on the oligomeric structures (Fig. 5D to G).

Only three of the five class II (very short Oag chain modal length) mutants (i_{191} , i_{247} , and i_{255}) were able to be mapped onto the 3D structure, as i_{219} and i_{231} are located in regions where structural data are unavailable for all three PCP proteins. Mutation i_{191} was predicted to be located on the uppermost quarter of $\alpha 6$. Mutation i_{247} was predicted to be located on the loop between $\alpha 7$ and $\alpha 8$, and the insertion of mutant i_{255} was mapped onto the second turn on $\alpha 8$ (Fig. 5A, B, and C).

Class II mutations were also mapped onto the oligomeric structures, which indicated that i_{191} , i_{247} , and i_{255} are all on the upper half of the oligomer (Fig. 5B to F). Mutation i_{191} , predicted to be mapped to the uppermost region of $\alpha 6$, appeared to be quite remote from other helices or structural components of Wzz , and i_{247} was predicted to be positioned on the cusp of $\alpha 7$ and $\alpha 8$, close to $\alpha 6$ of neighboring monomers (Fig. 5A to C and F). Mutation i_{255} , located on the second turn of $\alpha 8$, appeared to be situated closer to the inner surface of the cavity (Fig. 5F). These insertions are all mapped to be located on external regions of the oligomeric structures (Fig. 5D to G).

The two class III mutants i_{92} and i_{138} (conferring an Oag modal length slightly shorter than wild type) contain insertions predicted to be located in the second α helix ($\alpha 2$; Fig. 5A and C) and at the base of the monomer, respectively. The insertion of mutant i_{92} was predicted to be mapped onto the uppermost region of $\alpha 2$: on the second turn for Wzz_{ST} and on the first turn in $FepE$ (Fig. 5A, B, and C). This region of the structure is absent in $WzzE$. The insertion of mutant i_{138} was predicted to be mapped either on the very top of $\beta 3$ (Wzz_{ST} and $WzzE$) or on the loop between $\beta 3$ and $\alpha 6$ ($FepE$), at the α/β base domain (Fig. 5A, B, and C). When these insertions were mapped onto the oligomeric structures, i_{92} , positioned within $\alpha 2$, appears to be situated close to the lining of the inner cavity (Fig. 5F). Mutation i_{138} was predicted to be situated at the base of the oligomer, embedded between $\alpha 6$ and $\beta 3$, close to the membrane surface. These insertions appear to be primarily located to internal regions of the oligomeric structures (Fig. 5D to G).

The class IV mutants i_{80} and i_{81} , conferring wild-type LPS

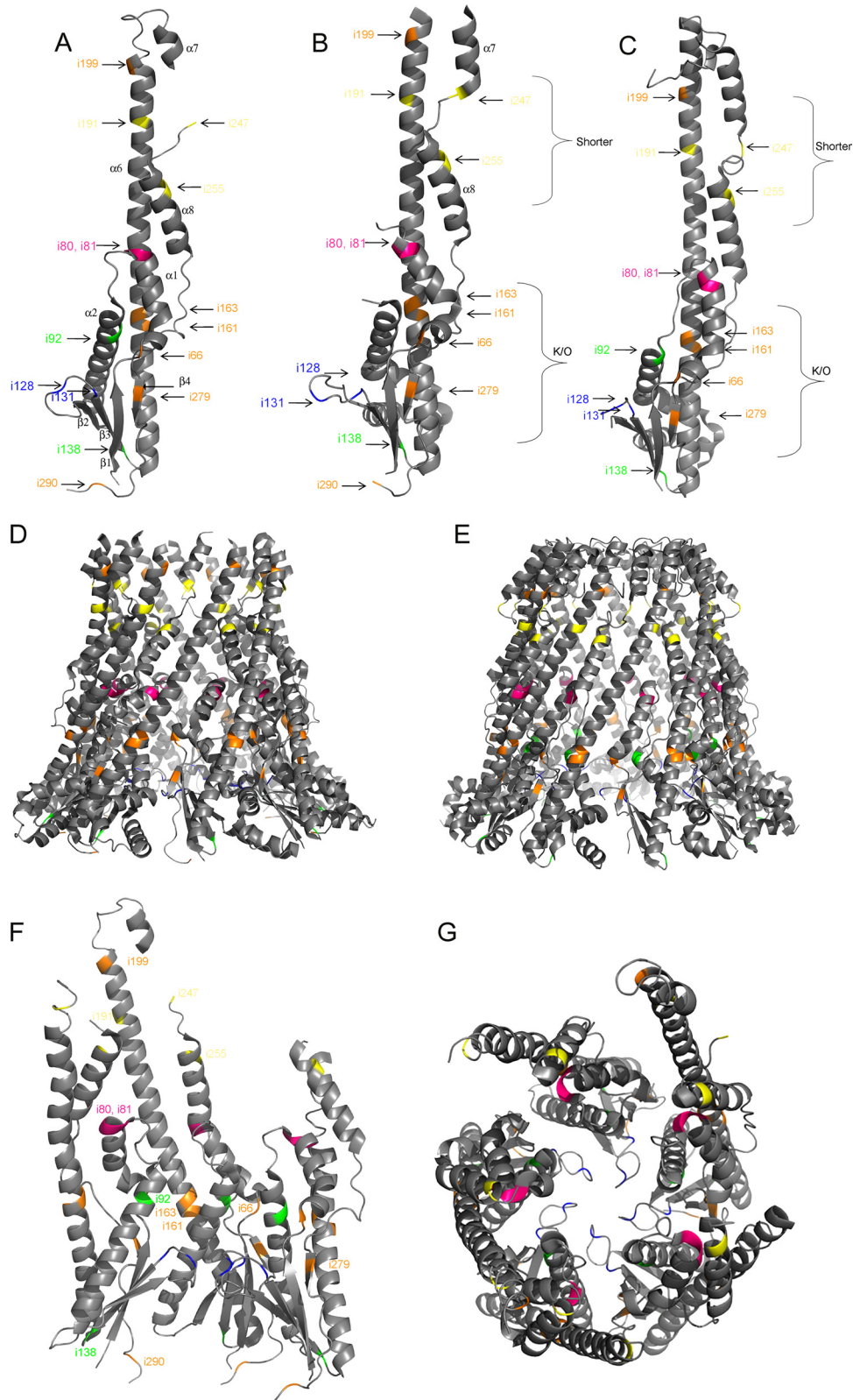


FIG. 5. Location of the Wzz_i insertions mapped on the 3D structures of Wzz_{ST} (Protein Data Bank [PDB] no. 3b8p), $WzzE$ (PDB no. 3b8o), and $FepE$ (PDB no. 3b8n). The last uninterrupted amino acid of Wzz_{SF} was mapped on each monomeric and oligomeric structure. (A) Wzz_{ST} monomer; (B) $WzzE$ monomer; (C) $FepE$ monomer; (D) $WzzE$ octomer; (E) $FepE$ nonamer; (F) three Wzz_{ST} monomers; (G) Wzz_{ST} pentamer (top view). The mutant classes are illustrated in different colors: class I mutants are orange, class II mutants are yellow, class III mutants are green, class IV mutants are magenta, and class V mutants are blue.

Oag modal length, have insertions which are predicted to be located within the last turn of $\alpha 1$ (Fig. 5A, B, and C). In the oligomeric prediction, this region is securely embedded internally between $\alpha 2$ and $\alpha 8$ (Fig. 5F and G).

Class V mutations ;128 and ;131 , conferring longer Oag modal lengths, were mapped on the loop between two β strands, $\beta 2$ and $\beta 3$, toward the base of the structure (Fig. 5A, B, and C). This loop is located within the inner cavity in the oligomeric structures (Fig. 5G).

DISCUSSION

In this study, we constructed and characterized a collection of mutant Wzz_i proteins. Previous mutagenesis studies (4, 6) suggested that there is not one particular region within Wzz which controls function: rather, Wzz Oag chain length regulation is a function of the entire protein structure. In our study, it is interesting to note that only 7 of the 18 characterized mutants appeared to be phenotypic knockout mutants (class I) and did not have Oag chain length regulation capability (Fig. 1). Considering that Wzz has such a key regulatory function and functionality is perceived to be a result of the 3D protein structure as a whole, it might be predicted that the insertion of 5 aa would abolish regulation of Oag chain length in most mutants. However, not only did approximately half of the mutants retain function, a range of Oag chain length phenotypes were observed. Class II mutants conferred Oag modal length of 2 to 10 repeat units, class III mutants resulted in shorter-than-wild-type Oag length (8–14), and the two class IV mutants conferred near-wild-type Oag chain length, while class V mutants increased the resulting Oag modal length to 16 to 25 repeat units.

Although approximately half of the Wzz_i mutants exhibited control over Oag chain length regulation, in many cases, protein band intensities are not comparable to wild-type levels of detection. Strains with mutants ;163 , ;191 , ;255 , and ;279 produced protein below the limit of detection, and mutants ;138 , ;161 , ;231 , and ;247 were detected at a level apparently lower than that of wild-type Wzz_{SF} (Fig. 2). All other mutants (;32 , ;66 , ;80 , ;81 , ;92 , ;128 , ;131 , ;199 , ;219 , and ;290) were detected at a level comparable to wild type (Fig. 2). However, less-than-wild-type protein detection does not appear to correlate with a particular resulting Oag modal length, as these mutants are from a range of different phenotypic classes. It is possible that many of these mutant proteins exhibit instability and are targets for proteases such as DegP, as this protease acts on Wzz_{PHS-2} mutant proteins (17). We cannot rule out that these 5-aa insertions have disrupted, concealed, or altered particular epitopes that the antibody binds to, hence altering protein detection in some mutants. It is also possible that several class I mutants lack control of Oag modal chain length due to low protein production and/or protein misfolding, although it is interesting that a collection of the seven class I mutants (;32 , ;66 , ;199 , and ;290) have detectable protein and are phenotypically inactive, whereas other mutants from which protein could not be detected (e.g., ;191 of class II) still exhibit control over Oag modal chain length. It would appear that a very small amount of Wzz protein is required to establish a regulated Oag modal chain length, consistent with other studies (21).

The insertions in each Wzz_i mutant were mapped onto the

3D PCP oligomeric structures to determine if the interrupted regions appeared to be structurally critical or important in intra- or intermonomer interactions. Despite the fact that each mutant exhibits a unique 5-aa insertion and the different amino acids may be affecting the structure and resulting phenotype, the classes generally consist of insertions that are predicted to be located in a similar region, with the exception of class I. The expected insertion positions relative to the Wzz 3D structure for class I mutants span many regions of the structure (Fig. 5A). Protein function appears to be sensitive to 5-aa insertions at these positions. The α/β base domain, comprised of β sheets $\beta 1$ to $\beta 4$, α helices $\alpha 1$ and $\alpha 2$, and the lowest region of $\alpha 6$ (in Wzz_{ST}), and $\alpha 1$ to $\alpha 4$ and the lowest region of $\alpha 6$ (in FepE and WzzE), appears to play an important role in intermonomeric interactions and oligomerization (21). The mutations that are predicted to be located within this region are ;66 , ;80 , ;81 , ;92 , ;128 , ;131 , and ;290 (Fig. 5). There are obvious differences in the LPS phenotypes of these mutations: ;66 and ;290 do not impart any Oag modal chain length control, whereas the others do. Mutation ;66 is also predicted to exist on the periphery of the monomer and hence may be in a position to influence hydrophobic interactions between monomers, thereby affecting stability and oligomerization (Fig. 5A). The β strands comprising the central feature of the α/β base domain are key structural elements in bringing the N and C termini closer together, which may also bring the TM segments closer (21). Mutation ;279 , predicted to be located on the final β strand ($\beta 4$), may be interfering with this process, perhaps by steric hindrance. In the case of the class IV ;80 and ;81 mutations, it is unclear as to why this region allows a 5-aa insertion with little alteration in resulting Oag chain length; however, the insertions appear to be located in a region embedded within $\alpha 2$ and $\alpha 8$ and may not be destabilizing intramonomeric interactions (Fig. 5A to C and F). Mutation ;92 (class III) is predicted to be located close to the top of an α helix; however, ;92 differs from both ;80 and ;81 in the way the $\alpha 2$ helix is more exposed to the inner cavity than $\alpha 1$, hence possibly being slightly less tolerant to a 5-aa insertion (Fig. 5F). The other class III ;138 insertion is predicted to be located close to the base of the oligomeric structure, toward the end of $\beta 3$ (Fig. 5A to C). Considering its predicted proximity to the α/β base domain (Fig. 5A to C and F), it is surprising that the resulting phenotype is merely a slight decrease in the LPS Oag modal chain length. This mutant insertion, however, is predicted to be located toward the end of $\beta 3$ (Wzz_{ST}) or on the loop between $\beta 3$ and $\alpha 6$ (FepE), and this position may be able to sustain extra amino acids without drastically altering nearby structural features, as it appears to be on the cusp of the oligomer (Fig. 5D and F). In the case of ;290 , as it is close to TM2, the insertion may be influencing a key region needed for function (Fig. 1B). In previous Wzz_{SF} mutagenesis studies, P292, one of the highly conserved prolines present in the proline-glycine-rich motif, was found to knock out Wzz function when converted to an alanine (4). This proline is theorized to be in *cis* conformation, hence providing rigidity and stability and influencing the orientation of the base domain and TM2 (12, 21). Mutation ;290 , being located so closely to this conserved residue, may undermine this critical arrangement. It is also possible that this mutation destabilizes any anchoring of the oligomer at the

membrane surface, as residues close to the inner membrane are considered to interact with lipid head groups (21).

There are a number of mutations predicted to be located on the long extended hairpin $\alpha 6$, such as $\text{i}161$, $\text{i}191$, and $\text{i}199$ (Fig. 5A). Mutant $\text{i}191$ is in class II, and $\text{i}161$ and $\text{i}199$ are class I mutants (Fig. 1 and Table 1). From the 3D crystal structure analyses, it appears as though the $\alpha 6$ helix is involved in maintaining intramonomeric stability, by interacting with $\alpha 2$ via conserved hydrophobic residues (21). Indeed, all mutations predicted to exist within $\alpha 6$ are in classes I and II and have similar phenotypes. The upper region of the oligomer is speculated to be involved in interacting with outer membrane proteins such as those involved in LPS export (21). It is possible that perturbation in the central α helix either results in disruption of local protein conformation or affects interaction with outer membrane proteins. Various conserved residues, including I237 and L240 in FepE, are present which form a leucine zipper motif (21). If this leucine zipper is critical for monomeric or oligomeric stability and/or interactions in Wzz_{SF} , it is possible that the insertion in $\text{i}199$, predicted to be located within two turns of this region, may severely disrupt these interactions.

Mutations $\text{i}247$ and $\text{i}255$ are predicted to be mapped to the upper region of the oligomer, within or close to $\alpha 7$ and $\alpha 8$ (Fig. 5). These α helices play a role in intermonomeric stability, as they interact with the long extended $\alpha 6$ helix on neighboring monomers (21). The mutations $\text{i}247$ and $\text{i}255$, both class II, are located on the outermost region of the oligomer, facing outwards and high up on the structure (Fig. 5). It is interesting to note that mutations that are located on the exterior region of the oligomer can play such a role in Wzz function; however, previous mutagenesis conducted on Wzz_{SF} indicates that a K267N mutation, predicted to be located on the outer side of the oligomer on the lower region of $\alpha 8$, results in an increase in Oag modal chain length (21). Hence, it appears that residues on the exterior face of Wzz oligomers have the ability to influence Wzz function, as previously proposed (21).

The class V mutants $\text{i}128$ and $\text{i}131$, conferring longer Oag chain length modality, were predicted to be located in the loop between $\beta 2$ and $\beta 3$ in the α/β base domain, directly in the central cavity of the oligomer (Fig. 5G). The phenotype resulting from the 5-aa insertions at this location is not observed for any other Wzz_i mutant. Previous Wzz_{SF} mutagenesis studies have not yielded mutants which increase the Oag modal chain length to this degree. We can speculate that the cause for such a dramatic modal length change may be attributed to the change this 5-aa insertion exerts on the cavity width and that the increase in the number of amino acid residues within this cavity is widening it by increasing the size of the α/β base domain. In general, it appears as though class V, class IV, and class III mutants are mapped to internal regions on the oligomeric structure, whereas class II mutants have their insertions mapped exclusively to external regions, and class I mutant insertions are mapped to both internal and external regions (Fig. 5D to F).

Previous cross-linking studies show Wzz can oligomerize (4, 7, 9), and recent studies have suggested that *E. coli* O86:H2 Wzz can form tetrameric oligomers (20), while others show Wzz forms hexameric oligomers (9). In our study, higher-order oligomers were easily detected in wild-type Wzz_{SF} and a num-

ber of select mutants (Fig. 3). It is interesting that the mutants conferring longer Oag chain length have comparable cross-linking profiles to wild type, whereas mutants resulting in random or shorter chain lengths do not appear to oligomerize as well, as judged by cross-linking. The lack of detectable oligomers in the resulting cross-linking profiles of $\text{i}92$, $\text{i}219$, and $\text{i}290$ may also be attributed to weak stability of the mutant proteins. It is possible that various Wzz_i mutant proteins may be able to form oligomers (e.g., $\text{i}290$); however, they either cannot be stably maintained or are perhaps incapable of successful interactions with other Oag processing proteins (or putative OM binding partners) to confer wild-type Oag modal chain length. An unusual feature of mutant $\text{i}290$ is the presence of the extra 30-kDa band in the non-cross-linked sample (Fig. 3, lane 9). This band appears to be Wzz related as it is readily detected by the Wzz antibody and is present in all other mutants (including wild-type Wzz), although never detected in the absence of cross-linking. The 30-kDa variation might have an altered conformation and may be nonfunctional, and it is possible that the presence of this variant in the non-cross-linked sample of $\text{i}290$ is linked to the fact that $\text{i}290$ appears to be nonfunctional. In contrast, monomers and dimers of class V Wzz_i mutants were detected at a much lower intensity when subjected to cross-linking than other mutants, and higher-order oligomers were easily detected. Previous findings have shown that Wzz_{SF} dimers appear to be very stable, as being heated at 100°C in the presence of SDS does not cause complete disassociation (4). In this study, Wzz_{SF} and Wzz_i cross-linked and non-cross-linked samples were heated to 100°C for 5 min and subjected to Western immunoblotting to ascertain whether or not the Wzz_i mutant dimers exhibit the similar stable trait of the wild-type dimers by being able to withstand the presence of SDS at 100°C. Only the wild-type Wzz_{SF} and class V $\text{i}128$ and $\text{i}131$ mutant dimers were detected after this treatment (Fig. 4). From these data, it appears as though there is a positive correlation between dimeric stability and wild-type-or-longer Oag modal chain length determination. These experiments show that the mutants which exhibit higher-order oligomers, as judged by *in vivo* formaldehyde cross-linking (Fig. 3), possess stable dimers, and it may be possible that this feature is a key factor in the ability to form oligomers, perhaps by providing a scaffolding element. Our results support previous findings that Wzz function is not restricted to one particular region on the structure and that oligomerization is related to modal length chain determination.

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