Analysis of Functional Domains of the *Enterococcus faecalis* Pheromone-Induced Surface Protein Aggregation Substance

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Pheromone-inducible aggregation substance (AS) proteins of *Enterococcus faecalis* are essential for high-efficiency conjugation of the sex pheromone plasmids and also serve as virulence factors during host infection. A number of different functions have been attributed to AS in addition to bacterial cell aggregation, including adhesion to host cells, adhesion to fibrin, increased cell surface hydrophobicity, resistance to killing by polymorphonuclear leukocytes and macrophages, and increased vegetation size in an experimental endocarditis model. Relatively little information is available regarding the structure-activity relationship of AS. To identify functional domains, a library of 23 nonpolar 31-amino-acid insertions was constructed in Asc10, the AS encoded by the plasmid pCF10, using the transposons TnlacZ/in and TnphoA/in. Analysis of these insertions revealed a domain necessary for donor-recipient aggregation that extends further into the amino terminus of the protein than previously reported. In addition, insertions in the C terminus of the protein also reduced aggregation. As expected, the ability to aggregate correlates with efficient plasmid transfer. The results also indicated that an increase in cell surface hydrophobicity resulting from AS expression is not sufficient to mediate bacterial aggregation.

*Enterococcus faecalis* has become a growing health concern as a mediator of the spread of antibiotic resistance and a leading agent of nosocomial infections (for review, see reference 10). The surface protein aggregation substance (AS) appears to play a role in both antibiotic resistance spread and in the pathogenesis of enterococcal infections. Expression of AS, which is encoded on the sex pheromone plasmids of *E. faecalis*, is induced by small 7- to 8-amino-acid peptide pheromones (26). AS on the surface of the donor cell then binds its receptor, enterococcal binding substance, on the recipient cell, mediating close cell contact that leads to conjugative transfer of the plasmid. It is thought that AS has no role in forming the DNA channel machinery, as efficient conjugation can occur if AS is expressed on either the donor or recipient cells (26).

Over 20 different pheromone plasmids have been identified. Often, these pheromone plasmids express antibiotic resistance genes and other virulence factors, and many clinical isolates have multiple pheromone plasmids (36). The AS genes from the three most-studied plasmids, Asa1 from pAD1, Asp1 from pPD1, and Asc10 from pCF10 (encoded by the *prgB* gene), have been sequenced and show high identity (see below). The gene encoding Asa373, the AS protein of the pheromone plasmid pAM373, has also been sequenced but shows little homology with the other known AS proteins and appears to aggregate through a different mechanism (21). Expression of AS, which is normally tightly controlled in laboratory cultures, is induced in serum (13).

A number of functions of AS that may contribute to virulence have been identified. A major function of AS is host cell adhesion. Kreft et al. found that Asa1 increased adherence to cultured pig renal tubular cells (13). Increased uptake mediated by Asc10 into epithelial cells originating from the colon and duodenum but not from the ileum has also been observed (25, 30). Along these lines, Asa1 increases invasion in an ex vivo model of the colonic mucosa but does not increase translocation (11). Asc10 has been found to increase adherence to and uptake by polymorphonuclear leukocytes, possibly by binding the integrin CR3 (35). In other studies, Asc10-expressing enterococci had higher intracellular survival rates in polymorphonuclear leukocytes (27). Likewise, adherence to and survival inside macrophages were increased with the expression of Asa1 (33). In vivo examination of the role of AS has centered on the rabbit experimental endocarditis model. Infection of a rabbit with a damaged heart valve leads to development of a mass of bacteria, platelets, and fibrin known as a vegetation (17). Two studies have found more severe vegetation formation induced by AS-expressing enterococci (4, 31). Asc10 has also been shown to increase adherence to fibrin and cell surface hydrophobicity (8).

Although much study has focused on the functions of AS, it is unclear how the structure of the protein mediates these functions. Like most gram-positive surface proteins, Asc10 has an N-terminal signal sequence and C-terminal LPXTG cell wall anchor motif (see Fig. 1A). Analysis of the three sequenced genes encoding closely related proteins reveals striking conservation of >90% identity in the majority of the protein, excluding a variable region of 30 to 50% identity located between amino acids 266 and 559 in the N terminus of AS (36). All three proteins also have conserved Arg-Gly-Asp (RGD) motifs that have been implicated in binding to integrins (13, 29, 33, 35). Secondary structural analysis yields little information with the exception of a predicted alpha-helix domain from amino acids 200 to 280 (36). Isolation of AS yields both a full-length version of the protein (137 kDa) and a specific, 78-kDa, N-terminal cleavage product (9). Scanning electron...
microscopy of Asa1 on the cell surface suggests that the N terminus of the protein is more exposed than the C terminus (9). Finally, the only structural analysis of AS done to date found that an aggregation domain of Asa1 from amino acids 525 to 617 (of the mature protein with the signal sequence removed) exists and that the C terminus plays no essential role in aggregation (20).

One dilemma with the use of conventional biochemical approaches to AS structure-function analysis is the high instability of purified protein. For this reason, we have taken a genetic approach to probe the protein for functional domains using the transposons TnαlacZ/in and TnphoA/in (15, 16). In-frame insertions can be identified by functional fusions to the 5′ LacZ or PhoA reporter protein. Digestion of the insertion with BamHI removes most of the transposon but leaves an in-frame 31-amino-acid insertion. These transposons have been successfully used to analyze the structure-function relationship of a number of membrane and cytosolic proteins (14, 15, 19, 22, 23), but this is the first attempt to use them in the analysis of a gram-positive surface protein.

A library of 23 insertion mutants distributed throughout the length of the prgB gene has been constructed. The stability of the AS protein expressed by these mutants was examined, and most proteins were found to be stable on the surface of E. faecalis. Phenotypic analysis of the insertion mutants in aggregation and conjugation revealed that both the N and C termini of the protein play significant roles in these processes. The ability of wild-type and mutant Asa10 proteins to increase cell surface hydrophobicity was also analyzed, and it was shown that increased hydrophobicity is not sufficient for aggregation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. E. faecalis was grown at 37° or 30°C as indicated with gentle shaking in Todd-Hewitt broth (Difco). For DNA isolation and manipulation, Escherichia coli was grown at 37°C with shaking in Luria-Bertani (LB) medium or brain heart infusion broth (Difco) for erythromycin selection. Agar plates contained 1.5% agar. The antibiotic concentrations were erythromycin at 50 μg/ml (in LB) and 10 μg/ml, and rifampin at 200 μg/ml (in LB) and 2.5 g of NaCl per liter supplemented with 0.2% maltose and 10 mM MgSO4). PCR was performed with a Promega, Gibco BRL, and New England BioLabs. PCR was performed with a"
Plasmids

- **pWM402**: *E. coli, E. faecalis* shuttle vector
- **pGEX-4T**: Expression-glutathione S-transferase fusion vector from the *tac* promoter
- **pTRKH2**: Shuttle vector
- **pINY1801**: pCF10 positive control region from *prgX* through *prgC*
- **pMSP3601**: KpnI-EcoRI fragment of pTRKH2 blunt ended and inserted into BamHI site of pINY1801
- **pMSP3602**: BamHI sites of pMSP3601 filled, first mutagenesis target
- **pMSP3603.1**: XhoI-BstXI PCR cloned into pGEXT-Easy
- **pCF10**: Nisin-inducible *prgB*
- **pCW175**: Tn917 insertion into *prgB* of pCF10

### *prgB* insertions in pMSP7517

- **pCW196**: TnphoA/in insertion at base 96
- **pCW1258**: TnphoA/in insertion at base 258
- **pCW1324**: TnlacZ/in insertion at base 324
- **pCW1438**: TnlacZ/in insertion at base 438
- **pCW1468**: TnlacZ/in insertion at base 468
- **pCW11074**: TnlacZ/in insertion at base 1074
- **pCW11077**: TnlacZ/in insertion at base 1077
- **pCW11299**: TnlacZ/in insertion at base 1299
- **pCW11317**: TnlacZ/in insertion at base 1317
- **pCW11419**: TnlacZ/in insertion at base 1419
- **pCW11551**: TnlacZ/in insertion at base 1551
- **pCW11638**: TnlacZ/in insertion at base 1638
- **pCW12049**: TnlacZ/in insertion at base 2049
- **pCW12064**: TnlacZ/in insertion at base 2064
- **pCW12085**: TnlacZ/in insertion at base 2085
- **pCW12421**: TnlacZ/in insertion at base 2421
- **pCW12601**: TnlacZ/in insertion at base 2601
- **pCW12760**: Reverse in-frame TnlacZ/in insertion at base 2760
- **pCW12979**: TnlacZ/in insertion at base 2979
- **pCW13102**: TnlacZ/in insertion at base 3102
- **pCW133183**: Reverse in-frame TnlacZ/in insertion at base 3183
- **pCW13414**: TnlacZ/in insertion at base 3414
- **pCW13599**: Out-of-frame TnlacZ/in insertion at base 3599 resulting in a stop codon and no cell wall anchor

### Notes

- All have the BamHI fragment of the transposon removed.

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**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain, phage, or plasmid</th>
<th>Strain or description</th>
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<td>pCF175</td>
<td>Tn917 insertion into <em>prgB</em> of pCF10</td>
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**Plasmid transfer.** The insertional mutants were induced with nisin as previously described with the exception that no antibiotic was added to the medium. The donor strain, OG1SSppCF175, an Asc10+ pCF10 derivative, was induced in the same manner except that 25 ng of cCF10/ml was added instead of nisin. The recipient strains expressed either wild-type Asc10 or one of the insertion mutants, as AS can increase plasmid transfer when expressed on either the donor or recipient cell. The induced donor and recipient cultures were mixed at a ratio of 1:10 respectively and were incubated at 37°C for 30 min. Transconjugants were enumerated by serial dilution on Todd-Hewitt broth with rifampin and tetracycline.

**Quantification of aggregation using flow cytometry and spectrophotometry.** Aggregation was quantified by two methods. Nisin-induced cultures were directly analyzed on a Beckman DU-70 Spectrophotometer.

- The donor strain, OG1SS(pCF175), an Asc10+ pCF10 derivative, was induced in the same manner except that 25 ng of cCF10/ml was added instead of nisin.
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**Aggregation was quantified by two methods. Nisin-induced cultures were directly analyzed on a Beckman DU-70 Spectrophotometer.**
The positions of insertion mutations in prgB are shown on a linear map of the gene. Each mutation consists of an in-frame 31-amino-acid insertion. The insertions at r2760 and r3183 are in frame but in the reverse orientation, while the insertion at s3599 is out of frame and produces a stop codon. Structural map: SS, signal sequence; helix, predicted N-terminal helix domain; variable, unconserved AS region; essential, aggregation domain identified by Muscholl-Silberhorn; and cleavage, site of cleavage that produces the characteristic N-terminal 78-kDa fragment. (B) The mutant Asc10 proteins were expressed using the nisin-inducible Asc10 expression vector pMSP7517.

**RESULTS**

**Construction of PrgB insertion mutants.** To scan Asc10 for potential functional domains, the transposons TlaczZin and TnphoA in were used to generate in-frame, nonpolar 31-amino-acid insertion mutations throughout prgB (15, 16). Previous work with the well-defined LacI repressor protein using these transposons identified the important functional motifs, validating their use in structure-function analysis of less-well-defined proteins (22). Briefly, prgB, carried on a shuttle plasmid, was targeted by TlaczZin or TnphoA in E. coli strain CC160. Relevant insertions were isolated and sequenced. The bulk of the transposon was then removed from prgB by BamHI restriction digestion and religation, leaving a 31-amino-acid in-frame insertion. The 31-amino-acid insertion consists of 84 bp provided by the transposon and 9 bp derived from the duplicated target sequence. The prgB insertions were electroporated into E. faecalis and were analyzed for surface expression, loss of function of aggregation and of conjugation, and increase in cell surface hydrophobicity. The designation of the insertion mutations generated in this study indicates the nucleotide residue of prgB that immediately precedes the insertion junction (Table 1). A number of different plasmids were used to construct the insertion mutants (see Materials and Methods for rationale), but all of the functional analysis was performed with the insertions in the prgB gene of the nisin-inducible construct pMSP7517, allowing for controlled expression of the mutations.

**Analysis of surface localization of Asc10 mutants.** The stability of the mutant proteins was addressed by analyzing their surface expression in E. faecalis. Surface expression of the Asc10 insertion mutations in E. faecalis was analyzed by generating cell wall extracts of each mutant (7). An equivalent amount of total protein (measured by a bicinchoninic acid protein assay [Pierce Chemical] and silver staining) was electrophoresed on a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and was Western blotted with a polyclonal antibody generated against the first 333 amino acids of Asc10 (18) (Fig. 2). When isolated from the cell wall, wild-type AS becomes very unstable and usually forms a laddering pattern on a Western blot. Most of the surface protein extracts from the insertion mutants reacted well in a Western blot, indicating normal cell wall localization. Only three mutants, ω3524, ω2049, and ω2064, had reduced levels of reactive protein. One mutant, ω2085, had no reactive protein on the cell surface and was removed from the functional analysis. Surprisingly, ωs3599, which has no cell wall anchor, also had reactive protein on the cell surface. Likely, anchorless Asc10 released from the cell immediately binds the AS receptor, enterococcal binding substance, on the cell surface. A Western blot of ωs3599 expressed in the enterococcal strain INY3000 (negative for enterococcal binding substance) (34) had no reactive protein on the surface of the cell (data not shown). The mutant proteins also have different laddering patterns on the Western blot, suggesting differences in protein stability on the surface of the cell or during the extraction procedure. When different preparations of the same mutant protein were examined on different blots, they exhibited variable laddering patterns, making it difficult to draw conclusions about the mutant stability from the laddering patterns.

**Identification of two domains that mediate aggregation.** Aggregation of the insertion mutants was quantified by two methods. Figure 3A shows representative data from five mutants. First, the forward scatter and side scatter profiles of induced cultures were determined on a flow cytometer. Larger particles have larger forward scatter and side scatter profiles. Popula-
tions were separated into four quadrants (Fig. 3A, bottom). The vector control (3535) had very few events located in the upper right quadrant (0.03%), while a much larger percentage of the wild-type Asc10 nisin-induced (7517) population was located in the upper right quadrant (4.65%) (Fig. 3A and B). The profile of each mutant was determined two to three times (Fig. 3B). Many mutations throughout the gene resulted in complete inhibition of aggregation, while others maintained wild-type aggregation levels. Some mutants, Ω1077, Ωr2760, and Ωr3183, had intermediate levels of aggregation. Ω1317 and Ω1419 had very low but statistically significant levels of aggregation. Interestingly, Ω1299, Ω2064, and Ω3414 had statistically significant, increased levels of aggregation relative to wild-type Asc10. As expected, Ωs3599 was unable to aggregate.

<table>
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<th>r2760</th>
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<td>6.98</td>
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</table>

FIG. 3. Aggregation of the mutants was measured by spectrophotometry and flow cytometry. Representative data for Asc10<sup>1</sup> (7517), the vector control (3535), a functional mutant (1299), intermediate mutant (r2760), and a nonaggregating mutant (3102) are shown. UR, upper right. (A). The aggregation of the mutants was measured by flow cytometry as a percentage of the induced populations in the (UR) quadrant (B). The OD<sub>600</sub> of induced cultures after 1 h of settling was also determined (C). A decrease in OD<sub>600</sub> indicates aggregation. #, P < 0.05 from 7517; @, P < 0.1 from 7517; *, P < 0.05 from 3535; $, P < 0.1 from 3535.
Aggregation was also quantified by determination of the OD_{600} of induced cultures after 1 h of settling (Fig. 3A, top, and C). For these data, increased aggregation is indicated by a decreased OD. Data obtained in this manner generally agreed with the results generated on the flow cytometer. In this method, no significant differences could be distinguished between cells expressing wild-type Asc10 and cells expressing mutant proteins that were functional aggregators. Likewise, no statistical difference was found between vector control 3535 and the nonaggregators V1317 and V1419. However, mutants displaying an intermediate level of aggregation when measured on the flow cytometer (V1074, V1077, Vr2760, and Vr3183) were also intermediate when measured by the spectrophotometer. Comparison of the two methods suggests that flow cytometry is more sensitive in quantifying small differences in aggregation levels.

The data measured by these two methods indicated a distinct aggregation domain expressed in the gene from nucleotide residues 1317 to 1638 (corresponding to amino acids 439 to 663 of the Asc10 protein). Also, many insertions in the C terminus (V2421, Vr2760, V3102, and Vr3183) had reduced or abolished aggregation, indicating regions in the C terminus that are involved in aggregation. However, two C-terminal functional aggregators (V2601 and V2979) were interspersed among the nonaggregators, suggesting that the entire region does not participate in aggregation.

Temperature-sensitive stability of Asc10 from V2049 and V2421. Growth of V2049 and V2421 at 30°C resulted in increased aggregation to near-wild-type levels (Fig. 4A). Analysis of surface extracts of these strains by Western blotting revealed that much higher levels of Asc10 were isolated from cells grown at 30°C. Note that increased exposure times would show reactive protein in the 37°C extract of 2421, confirming the presence of reactive protein seen in Fig. 2. Interestingly, more wild-type Asc10 can be seen from cultures grown at 30°C as well (Fig. 4B). These data suggest that the failure of Ω2049 and Ω2421 to aggregate at 37°C was likely due to instability of the mutant proteins rather than to disruption of an aggregation functional domain. The mechanism of this increased stability at 30°C is unknown. Growth at 30°C did not affect the aggregation phenotype of any of the other insertion mutants.

Plasmid transfer of insertion mutants correlates with aggregation. To determine the plasmid transfer capability of the insertion mutants, nisin-induced mutant cultures were used as recipients and were mixed with an E. faecalis OG1SSp (pCF175) donor strain. pCF175 is a pCF10 derivative that has aTn917 insertion in the prgB gene, rendering it incapable of aggregation or efficient conjugation. Thus, aggregation could only be mediated by the recipient Asc10 mutants, as expression of AS can lead to increased conjugation when expressed on either the donor or recipient cell of the mating pair (26). Plasmid transfer is expressed as the number of transconjugant cells/donor. As expected, induced 7517 gave high transfer levels at 3.5 \times 10^2, while the vector control transferred at 6.5 \times 10^2. Various transfer levels were observed for the mutants (Fig. 5A), and as expected, transfer levels correlated with aggregation ability (Fig. 5B). Interestingly, the three mutants that had significantly higher aggregation levels as measured by flow cytometry, Ω1299, Ω2064, and Ω3414, did not have statistically significantly higher transfer levels.

Hydrophobicity. Cell surface hydrophobicity of the insertion mutants was measured by determining the percentage of cells
that could be extracted from aqueous solution into hexadecane (Fig. 6A). A number of mutants maintained high levels of cell surface hydrophobicity, with some mutants having an even higher percentage of cell surface hydrophobicity than wild-type Asc10. Likewise, many mutants showed low levels of surface hydrophobicity comparable to those of non-Asc10-expressing strains. The amino acid sequences generated by both the forward and reverse insertions are relatively hydrophilic, with a percentage of polar amino acids at 63 and 50%, respectively. However, the actual amino acids of the insertion seemed to have little effect on the overall hydrophobicity of the mutants. The hydrophilicity, surface probability, and antigenicity indices were calculated for the amino acid sequence of Asc10 using the program PEPTIDESTRUCTURE (Wisconsin Package Version 10.1; Genetic Computer Group [GCG], Madison, Wis.). The values for a window of 10 amino acids adjacent to the insertion mutations were not predictive of the effect on cell surface hydrophobicity (data not shown).

The effect of cell surface hydrophobicity on aggregation was examined by plotting aggregation ability on the x axis versus the percent hydrophobicity on the y axis (Fig. 6B). No strong correlation between cell surface hydrophobicity and aggregation levels was observed.

**DISCUSSION**

The functional analysis of the 23 prgB insertion mutants generated in this study led to four important conclusions: (i) the domain that mediates bacterial aggregation extends into the variable region of Asc10, farther into the amino terminus than previously reported (13), (ii) the C terminus of the protein does contribute to aggregation, (iii) efficient conjugation directly correlates with functional aggregation, and (iv) increased cell surface hydrophobicity caused by Asc10 is not sufficient to mediate aggregation.

The transposons Tn5lacZin and TnphoA/in have been successfully used to analyze the structure-function relationship of a number of gram-negative membrane and cytosolic proteins (14, 15, 22, 23) and even a mouse mammary tumor virus superantigen (19), but to our knowledge, this study is the first attempt to use these transposons for mutagenesis of a protein from a gram-positive organism. Although we had problems with *E. coli* transcription machinery recognizing artifactual promoters and inefficient export of the fusion proteins, 23 nonpolar, in-frame 31-amino-acid insertions were generated that were spaced throughout the prgB gene. The insertion mutants have good coverage of the protein, with the largest gap found from nucleotide residues 468 to 1074. This region was resistant to transposition, as multiple mutagenesis attempts yielded no insertions. Interestingly, previous Tn5 mutagenesis studies that targeted pCF10 DNA also had a large gap in the prgB gene that corresponds to the same transposition-resistant region identified in this study (26).

Western blotting of the surface extracts of induced mutant cultures revealed that most proteins were expressed on the surface of the cell. Full-length protein was difficult to observe in some mutants, but this result is not unexpected, as purified protein is very unstable. Three mutants, Ω324, Ω2049, and Ω2064, had reduced expression of Asc10 at 37°C, while Ω2085 showed no reactive protein. Consequently, Ω2085 was removed from the functional analysis. Interestingly, the insertion mutants Ω2049 and Ω2421 were found to aggregate to near-wild-type levels when grown at 30°C, suggesting that their lack of aggregation at 37°C is due to protein instability. The increased stability of these proteins at 30°C may be due to alterations in folding conformations or differences in activity of an enterococcal cell surface protease.

Insertions in two major regions of the protein inhibited or abolished aggregation. Insertions in the N terminus inhibited aggregation in agreement with Muscholl-Silberhorn’s previous identification of an aggregation functional domain for Asa1 (20). The insertions generated in this study identify an aggregation functional domain in the gene from nucleotide residues 1317 to 1638, but it is not clear how far the functional domain extends beyond residue 1638. However, as Ω2049 aggregates at 30°C, the aggregation domain does not extend this far into the gene. The aggregation domain identified in Asa1 extends from base numbers 1704 to 1980. Combining the two identified regions would indicate an aggregation domain from 1317 to 1980 (Fig. 7). Also, a comparison of the two results indicates that the aggregation functional domain for Asc10 extends farther into the amino terminus of the protein than previously reported for Asa1. Interestingly, the domain identified in this study extends into the variable region, suggesting that it may play a role in aggregation. This result is supported by the observation that an Asa1-Asp1 variable region chimera fails to aggregate (20). Most of the insertions in the extreme N terminus of the protein, residues 258 to 1299, had no effect on aggregation levels with the exception of Ω96 and Ω324. Mutation Ω96 was in the signal sequence of the protein and likely.
between the members of the donor-recipient pair. Interestingly, the mutants that had statistically significant higher levels of aggregation as measured by flow cytometry did not have higher levels of plasmid transfer, suggesting that AS has evolved aggregation levels that maximize plasmid transfer.

The increased cell surface hydrophobicity elicited by Asc10 expression was also measured for each mutant. Various levels of hydrophobicity could be seen for the insertion mutants, but no one distinct domain necessary for increasing hydrophobicity was detected. Comparison of the hydrophobicity and aggregation levels reveals that increased cell surface hydrophobicity is not sufficient for aggregation. Specifically, Ω1551 is unable to aggregate but induces very high levels of cell surface hydrophobicity. Likewise, mutants that are intermediate aggregators have hydrophobicity levels equivalent to those of strong aggregators. Thus, AS mediates aggregation through a specific interaction, as opposed to a generalized alteration of cell surface hydrophobicity. It should be noted that no mutants that were completely deficient in increased cell surface hydrophobicity were able to aggregate, possibly indicating that hydrophobicity may be necessary but not sufficient for aggregation.

This study reports the construction of a library of 23 31-amino-acid insertions in Asc10, the AS of pCF10. By using Western blotting of surface extracts, it was shown that most of the insertion mutants were expressed on the surface of E. faecalis. Analysis of aggregation identified both N- and C-terminal domains important in aggregation and showed that the variable region may play a role in aggregation. Moreover, an increase in cell surface hydrophobicity is not sufficient for aggregation. This insertion library will be further analyzed for functional domains of Asc10 involved in the pathogenesis of E. faecalis infections.

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