Role of OmpA1 and OmpA2 in Aggregatibacter actinomycetemcomitans and Aggregatibacter aphrophilus serum resistance

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\textbf{ABSTRACT}
Aggregatibacter actinomycetemcomitans and Aggregatibacter aphrophilus belong to the HACEK group of fastidious Gram-negative organisms, a recognized cause of infective endocarditis. \textit{A. actinomycetemcomitans} is also implicated in aggressive forms of periodontitis. We demonstrated that \textit{A. aphrophilus} strains, as \textit{A. actinomycetemcomitans} are ubiquitously serum resistant. Both species encode two Outer membrane protein A paralogues, here denoted OmpA1 and OmpA2. As their respective pangenomes contain several OmpA1 and OmpA2 alleles, they represent potential genotypic markers. A naturally competent strain of \textit{A. actinomycetemcomitans} and \textit{A. aphrophilus}, respectively were used to elucidate if OmpA1 and OmpA2 contribute to serum resistance. Whereas OmpA1 was critical for survival of \textit{A. actinomycetemcomitans} D7S in 50\% normal human serum (NHS), serum resistant ompA1 mutants were fortuitously obtained, expressing enhanced levels of OmpA2. Similarly, OmpA1 rather than OmpA2 was a major contributor to serum resistance of \textit{A. aphrophilus} HK83. Far-Western blot revealed that OmpA1\textsuperscript{AA}, OmpA2\textsuperscript{AA}, and OmpA1\textsuperscript{AP} can bind to C4-binding protein, an inhibitor of classical and mannose-binding lectin (MBL) complement activation. Indeed, ompA1 mutants were susceptible to these pathways, but also to alternative complement activation. This may at least partly reflect a compromised outer membrane integrity but is also consistent with alternative mechanisms involved in OmpA-mediated serum resistance.

\textbf{Introduction}

The HACEK group of fastidious Gram-negative organisms is a recognized cause of infective endocarditis, responsible for 1.4 to 3\% of cases [1], with the genus Aggregatibacter now being the dominant etiology of HACEK endocarditis [2]. Colonization by the human oral bacterium, \textit{Aggregatibacter actinomycetemcomitans} is strongly associated with aggressive forms of periodontitis in adolescents and young adults [3,4]. \textit{Aggregatibacter aphrophilus} is a closely related organism (14.7–23.7\% difference in gene content relative to \textit{A. actinomycetemcomitans} [5]), which belongs to the indigenous human oral microflora. Infective endocarditis and cerebral abscesses are the most frequent invasive \textit{A. aphrophilus} infections, whereas this organism is widely considered to have low virulence in periodontitis [2].

\textit{A. actinomycetemcomitans} exhibits large genetic diversity, and serotypes form genetically divergent lineages [4]. Highly leukotoxic \textit{A. actinomycetemcomitans} genotypes, JP2 and \textit{cagE}, respectively (serotype b) are strongly linked to periodontal attachment loss progression in North and West African adolescents [3,6]. \textit{A. actinomycetemcomitans} produces outer membrane vesicles (OMVs), which have been demonstrated to internalize into host cells and act as a trigger of innate immunity [7]. The systemic role of \textit{A. actinomycetemcomitans}, in addition to its involvement in endocarditis, includes its association with cases of soft tissue abscesses, and osteomyelitis [8], and the species can be detected in atheromatous plaque [9]. It is not known if there are specific genotypes of \textit{A. actinomycetemcomitans} (or \textit{A. aphrophilus}) that are more prone to translocate to the peripheral circulation.

Serum resistance is an important pathogenicity determinant of Gram-negative bacteria that enter the bloodstream and cause infection, allowing their evasion of the innate defenses present in serum, including complement and antimicrobial peptides. Resistance to complement-mediated killing by human serum appears to be important for \textit{A. actinomycetemcomitans} virulence, and it is suggested to be a common trait among strains of this species [10,11]. It is not known whether serum resistance is also frequent among \textit{A. aphrophilus} strains. Mechanisms of bacterial resistance against complement-mediated killing include the production of protective extracellular polysaccharide capsules, and expression of factors that inhibit or interfere with the...
complement cascade [12]. Outer membrane integrity is important for serum resistance of Gram-negative bacteria, and in a number of species, outer membrane proteins (OMPs) have been shown to be associated with serum resistance, e.g., Ail [13], OmpW [14], PagC [15], and OmpA [16,17]. OmpA protein family members represent key components in the structural integrity of the outer membrane of bacteria and have several described pathogenic roles [18-20].

Hence, OmpA inhibition offers a strategy to combat virulence of Gram-negative organisms [21]. C4b-binding protein (C4bp) is a major inhibitor of the classical and mannose-binding lectin (MBL) pathways of the complement system [22]. Evidence has been presented that upon interacting with C4bp, Escherichia coli OmpA inhibits the classical complement activation cascade [23,24]. On the other hand, in Acinetobacter baumannii, OmpA was shown to enhance serum resistance via trapping of the alternative complement inhibitor Factor H [16].

Among the OMPs identified in A. actinomycetemcomitans, Omp100 (also known as ApiA) was earlier demonstrated to be important for serum resistance in some serotype b, and d strains, and to bind to the alternative pathway regulator Factor H in vitro [10,25]. Evidently, A. actinomycetemcomitans OMPs are immunoreactive in the human host [26]. As presence of antibodies towards OMPs is a known trigger of classical complement activation [27], serum resistance of Aggregatibacter spp. would be expected to include mechanisms blocking this activation. A = 35-kDa, 346-amino acid heat-modifiable OmpA-like protein (also known as Omp29, and Omp34) is the most abundant A. actinomycetemcomitans surface protein, and a major component of outer membrane vesicles [26,28]. The A. actinomycetemcomitans OmpA-like protein is associated with the bacterial entry into gingival epithelial cells [29], however, its role in serum resistance has not been elucidated. Whether A. aphrophilus OMPs, hitherto only subjected to a preliminary characterization [30], may possibly contribute to serum resistance is not known. The aim of the present work was to investigate if OmpA proteins play a role in serum resistance in A. actinomycetemcomitans and A. aphrophilus.

Materials and methods

Ethics considerations

All procedures were conducted in accordance with the guidelines of the local ethics committee at the Medical Faculty of Umeå University, which are in compliance with the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, October 2013). For the assays using normal human serum, blood was sampled from healthy volunteers after informed consent.

Bacterial strains and growth conditions

A. actinomycetemcomitans strain D7SS is a naturally genetic competent, smooth-colony derivative of D7S (serotype a), which was originally isolated from a patient with aggressive periodontal disease [31]. Mutant A. actinomycetemcomitans derivatives, i.e., D7SS ompA1::spe [SpeE], D7S ompA1::spe [SpeE], D7SS ompA2::kan [KmR], D7SS ompA1::spe, ompA2::kan [SpeE, KmR], D7SS omp100::kan [KmR], and D7SS omp100::spe [KmR, SpeE] were generated in the present work. CCUG 3715 and NI8700 are type strains of A. aphrophilus [32,33]. The naturally genetic competent A. aphrophilus strains HK83 (CCUG 49494), and CCUG 11575 were originally sampled from saliva, and a brain abscess, respectively [33]. DNA from NI8700 was used to transform HK83 and CCUG11575 into a V factor-independent growth phenotype, following a described procedure [33]. Mutant derivatives of HK83, i.e., HK83 ompA1::spe [SpeE], HK83 ompA2::kan [KmR], and HK83 ompA1::spe, ompA2::kan [SpeE, KmR] were generated in the present work. Strains AH1-351, IH-90256, and IH-90274 are part of the collection of clinical isolates of A. aphrophilus in our laboratory, established by Dr. Sirkka Asikainen. The A. aphrophilus strains 4 Aap-K, 12 Aap-K, 13 Aap-K, 21 Aap-K, 29 Aap-K, 30 Aap-K, 32 Aap-K, and 53 Aap-K belong to our bacterial strain collection at the clinical laboratory, Oral Microbiology. The A. actinomycetemcomitans and A. aphrophilus strains were routinely cultivated in air supplemented with 5% CO2, at 37°C, on blood agar plates (5% defibrinated horse blood, 5 mg hemin/l, 10 mg Vitamin K3/l, Columbia agar base). Alternatively, for transformation assays, the strains were grown on Trypticase soy broth supplemented with 0.1% yeast extract, 5% heat-inactivated horse serum, and 1.5% agar (sTSB agar), and when needed, supplemented with 100 µg/ml (final concentration) spectinomycin, or kanamycin. E. coli K-12 laboratory strain DH5α was used for maintenance of plasmids and was cultured aerobically at 37°C in Luria-Bertani (LB) broth, or on LB broth solidified with 1.5% (w/v) agar.

Construction of outer membrane protein gene replacement mutants in A. actinomycetemcomitans and A. aphrophilus

A PCR-based approach following standard cloning procedures was used to construct gene replacement mutants in naturally competent strains of A. actinomycetemcomitans (D7SS), and A. aphrophilus (HK83). For A. actinomycetemcomitans, the strain D7S-1 complete genome (GenBank accession CP003496) was used as reference in oligonucleotide synthesis. For A. aphrophilus, strain NI8700 was used as reference genome (GenBank accession CP001607). Whole genome sequence data of the A. aphrophilus...
 modelo strain HK83 was kindly communicated by Niels Nørskov-Lauritsen and has been deposited at DDBJ/ENA/GenBank under the accession QMG50000000. Sequences of the oligonucleotide primers used for mutant construction and outer membrane protein gene database entries are listed in Table 1. In brief, PCR fragments flanking the target genes were amplified. The PCR primers contained BamH1 or Sall restriction sites, allowing ligation of the PCR fragments to flank either the spectinomycin resistance gene from plasmid pK-Spe [34], or the kanamycin resistance gene from pUC4K [35]. Ligation products were then used to transform D7SS and HK83 on agar plates using procedures described earlier [31]. Confirmation of allelic replacements and the orientation of the inserted resistance cassette were done by DNA sequencing and PCR. For this we used a target gene-specific F1, and R2 oligonucleotide primer (Table 1), respectively in combination with a primer specific for the appropriate antibiotic determinant, i.e., spectinomycin (Spe1: 5'-CCACTCTCAACTCCTGATCC-3') or kanamycin (H7R: 5'-GGACGGCGGCTTTGTTG AATAAAATCG-3').

SDS-PAGE and Western blot analysis

The procedures used for SDS-PAGE and Western blot analysis have been described previously [36]. Gels were stained using Coomassie Brilliant Blue R-250 (Bio-Rad) or Pierce’s Silver Stain Kit (Thermo Fisher Scientific). Selected protein bands after Coomassie- or Silver-staining were excised from gels and subject to liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Proteomics Core Facility at Chemical Biological Centre, Umeå University and Swedish University of Agricultural Sciences. For Western blot, we used a rabbit polyclonal antisera specific for E. coli OmpA [37] (final dilution 1:10,000), and a patient serum from a periodontitis subject, which exhibits strong reactivity to A. actinomycetemcomitans antigens [38] (1:2,000). As secondary antibody, anti-rabbit or anti-human horseradish peroxidase (HRP)-conjugate was used (Jackson ImmunoResearch, Newmarket, UK) (1:10,000). Immunoreactive bands were visualized using ClarityTM Western ECL Substrate (Bio-Rad) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), and the ChemiDocTM XRS + System (Bio-Rad).

Far-Western blot analysis

To detect binding of the complement regulator, C4b-binding protein to OmpA, the immunoblotted membrane was blocked in tris-buffered saline, 0.1% tween 20 (TBS-T) with milk (5% w/v) overnight at 4°C, and then incubated with 1 μg/ml (final dilution) human recombinant C4bp (Abcam) in TBS-T with milk (0.5% w/v) for 2 h at room temperature. After washing four times with TBS-T, the membrane was incubated with a rabbit polyclonal antisera specific

### Table 1. Target ompA1 and ompA2 genes in the A. actinomycetemcomitans and A. aphrophilus reference strains D7S-1 and NJ8700, respectively, and oligonucleotide primers used for the generation of allelic replacement mutants.

| Target gene | GenBank accession number, and definition in database | Oligonucleotide* (F: forward; R: reverse) | PCR product length (bp) |
|-------------|-----------------------------------------------------|------------------------------------------|----------------------|
| Primers for A. actinomycetemcomitans | | | |
| ompA1 | AF86243 | membrane protein | F1: 5’- CTGGCTTCCAATAAAAGCGCAGAGGAG-3’ | 1,295 |
| | R1: 5’- GATGCGGATGTCGCCATTTTGGATGATCC-3’ | 1,287 |
| | R2: 5’-AAATGGCGGTCGAGTTCAATAATGAA-3’ | 1,061 |
| ompA2 | AF86283 | membrane protein | F1: 5’-GACTCCGCTGCAGATCGATC-3’ | 1,269 |
| | R1: 5’-GTCGTTAATTGGTTTCTTCTG-3’ | 1,081 |
| | R2: 5’-GTCGAGCAGATTGCTCAGGA-3’ | 1,386 |
| omp100 | AF86288 | membrane protein | F1: 5’-GATGCGGATGTCGCCATTTTGGATGATCC-3’ | 1,208 |
| | R1: 5’-GTCGAGCAGATTGCTCAGGA-3’ | 1,386 |

| Primers for A. aphrophilus | | | |
| ompA1 | ACS596989 | ‘outer membrane protein A’ | F1: 5’-GTCGGATTGGACGCCACTCTGTGC-3’ | 1,081 |
| | R1: 5’-GATGCTCTCTCTAGAACCTGCTC-3’ | 2,180 |
| | R2: 5’-GATGCTCTCTCTAGAACCTGCTC-3’ | 1,280 |
| ompA2 | ACS57183 | ‘outer membrane protein A’ | F1: 5’-GGGCGGCGAATTGAGCGTTACG-3’ | 1,081 |
| | R1: 5’-AAATGGCGGTCGAGTTCAATAATGAA-3’ | 1,328 |
| | R2: 5’-GGGCGGCGAATTGAGCGTTACG-3’ | 1,280 |

*Primers introducing BamH1 (GGATCC) and Sall (GTCGAC) restriction sites (sequences in underlined bold) as indicated.
*Predicted based on genome sequences of D7S-1 and NJ8700, respectively.
*Also referred to as Omp29, Omp34, and OmpA-like protein.
*Encoded protein exhibits = 76% amino acid identity with OmpA1AA.
*Also referred to as ApiA.
*Encoded protein exhibits = 83% amino acid identity with OmpA1AA.
*Encoded protein exhibits = 71% amino acid identity with OmpA1AA.
for human C4bp (Abcam) (final dilution 1:1,500). After four washes with TBS-T, anti-rabbit HRP-conjugate (Jackson ImmunoResearch, Newmarket, UK) was used at 1:10,000, and immune detection was then performed as described above.

**Electron microscopy**

Analyses were carried out at the Umeå Core Facility for Electron Microscopy (UCEM). For transmission electron microscopy of OMV preparations, 3.5 µl of samples was applied to glow discharged formvar and carbon coated Cu-grids. The grids were washed and negatively stained in 1.5% Uranyl acetate for 2 × 15 sec. Samples were examined with a Talos 120C transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV. Micrographs were acquired with a Ceta 16M CCD camera (FEI, Eindhoven, The Netherlands) using TEM Image & Analysis software version 4.14 (FEI, Eindhoven, The Netherlands). For scanning electron microscopy, small pieces of agar containing bacterial colonies were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C overnight, then dehydrated in graded series of ethanol, critical point dried, and metal-coated (4 nm). The morphology of the samples was analyzed with a field-emission scanning electron microscope (Zeiss Merlin FESEM).

**Bacterial serum killing assay**

To monitor the sensitivity of bacterial strains to normal human serum (NHS), NHS was taken from healthy volunteers. We largely followed procedures described previously [10,27], using *A. actinomycetemcomitans*, and *A. aphrophilus* strains grown on agar. In brief, prior to being used in the assays, bacteria were harvested and suspensions were adjusted to 1.0 × 10^9 cells/ml in PBS. Reaction mixtures contained 105 µl NHS, 95 µl PBS, and 10 µl bacterial suspension and were incubated at 37°C for 2 h. Cell survival was assessed by plating serial dilutions on agar. Heat-inactivated (56°C, 30 min) NHS (HI-NHS) was used as control. Survival rates were calculated as the ratio (%) of colony forming units (CFU) NHS/HI-NHS. When needed to distinguish between the classical and MBL, and the alternative pathway of complement activation, serum killing assays were performed in the presence of 5 mM MgCl₂, and 10 mM EGTA (Mg²⁺/EGTA), which selectively permits the alternative pathway only [39]. To obtain NHS depleted from the MBL pathway, serum was filtered through D-mannose-agarose beads (Sigma-Aldrich, St. Louis, USA), as described previously [27].

**Statistical analysis and image processing**

The statistical significance of the data was calculated using two-tailed Student’s t-test. The level of
statistical significance was set to $P < 0.05$, based on at least three independent experiments unless otherwise stated. Data are expressed as means ± standard errors of the means (SEM). Images for figures were assembled using Adobe Photoshop CS6 or Microsoft PowerPoint.

**Results**

**Both A. actinomycetemcomitans and A. aphrophilus encode two OmpA proteins**

To assess the prevalence of *ompA* genes in these species, we screened all whole genome sequences of *A. actinomycetemcomitans* strains (n = 38) and *A. aphrophilus* (n = 7) available in the National Center for Biotechnology (NCBI) database in September 2017. The result of this *in silico* analysis is summarized in Supplementary Table 1. Of the *A. actinomycetemcomitans* strains, 37 were found to encode the major OmpA protein (in the present work referred to as OmpA1AA). Strain SA3733 (serotype d), encoded a partial OmpA1 sequence. In total, six OmpA1AA alleles were identified exhibiting 99–100% amino acid identity to the strain D7S protein. A 356-amino acid protein sharing approximately 76% amino acid identity with OmpA1AA is in this work referred to as OmpA2AA. The database searches revealed OmpA2AA to be encoded by 37 out of the 38 *A. actinomycetemcomitans* genomes (not identified only in SA3733). In total, six OmpA2AA alleles were identified exhibiting 97–100% amino acid identity to the strain D7S protein. As there were allele combinations specific for some serotypes, e.g., OmpA1AA-4 and OmpA2AA-4 in serotype b, these gene and protein sequences may represent potential genotyping markers. According to the database searches, *A. aphrophilus*, similar to *A. actinomycetemcomitans* encodes two OmpA paralogues. These proteins (346 and 363–366 amino acids, respectively) exhibit ~74% amino acid identity, and we have in this study referred to them as OmpA1AP and OmpA2AP. OmpA1AP was encoded in all *A. aphrophilus* genomes, and in total three alleles were identified exhibiting 96–100% amino acid identity to the strain NJ8700 protein. A gene encoding OmpA2AP was identified in all *A. aphrophilus* genomes, except ATCC7901 and in total five alleles were identified exhibiting 96–100% amino acid identity to the strain NJ8700 protein. Thus, a large majority of *A. actinomycetemcomitans* and *A. aphrophilus* strains encode two OmpA proteins.

**OmpA1 is important for serum resistance in *A. actinomycetemcomitans* strains D7SS and D7S**

To investigate if OmpA1AA contributes to serum resistance, the *ompA1AA* gene was subject to allelic replacement in strains D7S and D7SS. The abolished expression of OmpA1 in the mutants was confirmed by SDS-PAGE and Western blot, analyzing OMP (Figure 1(a,c)), and OMV preparations (Figure 1(b, d, and e)), respectively. These analyses revealed distinct protein bands at approximately 35 and 25 kDa, respectively, which were also confirmed to represent OmpA1AA using LC-MS/MS, and which were absent in the *ompA1* mutant derivatives. According to serum killing assays using 50% NHS (Figure 2), strain D7SS was completely serum resistant (survival rate determined to 161.3%) whereas its *ompA1* mutant derivative exhibited high susceptibility (survival rate 8.0%). Essentially similar survival rates were obtained when assessing the rough-colony D7SS parental strain, D7S (161.4% ± 21.9% [SEM]), and its *ompA1* mutant derivative (1.1% ± 0.9% [SEM]). In contrast to the observations with *ompA1*, inactivation of *omp100* in strain D7SS did not result in a low survival rate (106.6% ± 19.8% [SEM]). Lack of Omp100 production in the mutant was confirmed by Western blot (Figure 1(e)). Consistent with a major role of OmpA1 in serum resistance, inactivation of the *ompA1* gene in the D7SS *omp100* mutant led to a severely reduced survival rate, 3.1% (Figure 2). Based on these results together, we concluded that OmpA1 was a major contributor to the serum resistance of *A. actinomycetemcomitans* D7SS, and its parental strain D7S.

*A. actinomycetemcomitans* strain D7SS *ompA1* mutant derivatives regained serum resistance upon enhanced production of OmpA2

We made attempts to clone *ompA1AA* behind the leukotoxin promoter using the pVT1503 *A. actinomycetemcomitans* shuttle vector, and the same approach as for the complementation of *bilR1* mutants [40]. Unfortunately, this resulted solely in clones with a truncated C-terminus (data not shown), suggesting that overexpression of OmpA1 was toxic to the *E. coli* laboratory strain used. Nonetheless, colonies of the D7SS *ompA1AA* mutant were fortuitously obtained after the incubation in 50% NHS. After isolation of such clones from independent experiments, they were found to exhibit a serum survival rate, comparable to D7SS. Interestingly, according to SDS-PAGE analysis of OMV preparations from one selected serum resistant *ompA1* mutant clone, denoted D7SS *ompA1* R1 (survival rate 186.5% ± 62.1% [SEM]), there were two enhanced protein bands at approximate sizes very similar to OmpA1, i.e. ~35 and ~25 kDa (Figure 1(b,d)). These proteins, which appeared to be produced at a low level in the serum-sensitive D7SS *ompA1* mutant derivative, were subject to LC-MS/MS analysis. This revealed that both protein bands corresponded to...
Thus, we concluded that the protein identified in the lower molecular weight band likely represented a degradation product of full length OmpA2. To investigate if serum resistant D7SS ompA1 derivatives may represent a heterogeneous population of mutants, OMV preparations from five additional clones were analyzed by SDS-PAGE (Supplementary Figure 1). This revealed upregulated OmpA2 production in four of the tested clones, suggesting that this is a common characteristic among D7SS ompA1 mutants surviving in 50% NHS. The role of OmpA2 was evaluated in D7SS and D7SS ompA1-ompA2 mutants, by allelic replacement of ompA2AA. As judged by the very low to non-existing serum survival rates of the double mutants, D7SS ompA1 ompA2 (0%), and D7SS ompA1-R1 ompA2 (1.8% ± 0.6% [SEM]), OmpA2 indeed contributed to serum resistance. In contrast, allelic replacement of ompA2AA in an ompA1AA+ gene background did not result in an apparent decrease in serum resistance (Figure 2). The survival rates of the wild-type strain and its mutant derivatives were similar in heat-inactivated 50% NHS (data not shown), whereas there was a somewhat reduced growth rate of the D7SS ompA1 single and ompA1 ompA2 double mutants in liquid cultures (Supplementary Figure 2). Electron microscopy revealed that OMV preparations from both D7SS and D7SS ompA1 ompA2 contained vesicles of various sizes (diameter approximately 30–200 nm) (Figure 3(a-b)). However, larger membrane vesicle-like structures were occasionally observed projecting from the cell surface of the double mutant, which was not observed assessing the wild-type (Figure 3(c-d)). This suggests that the serum sensitivity was accompanied by at least partially impaired membrane stability. Based on these results together, we concluded that in the absence of OmpA1, serum resistance of A. actinomycetemcomitans strain D7SS could be regained via increased production of OmpA2. As judged by DNA sequencing, background mechanisms why OmpA2 appeared to be produced at enhanced

Figure 1. SDS-PAGE analysis of outer membrane protein, and outer membrane vesicle preparations obtained from A. actinomycetemcomitans strains. OMP preparations were analyzed using Coomassie-staining (a), and Western blot using a polyclonal antiserum specific for E. coli OmpA (c). OMV preparations were analyzed using Silver-staining (b), and Western blot using a polyclonal antiserum specific for E. coli OmpA (d), or an A. actinomycetemcomitans-responsive serum from a periodontitis subject (e). Samples from the following strains are shown in the panels: D7SS (lane 1), D7SS omp100 ompA1 (lane 2 in panel a–d), D7SS ompA1 (lane 3), D7SS ompA1 R1 (lane 4), D7SS ompA1 ompA2 (lane 5), and D7SS omp100 (lane 2 in panel e). Selected protein bands are indicated with arrows. Sizes (kDa) of the proteins in the pre-stained molecular weight marker (M) are indicated along the left side.
levels in the serum resistant D7SS ompA1 mutant clones did not include alterations in the ompA2 promoter and upstream region, as amplified using the primers ompA2\textsuperscript{AA} F1 and ompA2\textsuperscript{AA} R1 (data not shown).

**Serum resistance in A. aphrophilus strains**

Prompted by our findings with *A. actinomycetemcomitans*, we wanted to investigate if OmpA proteins may contribute to serum resistance in an additional
species of this genus, i.e., the close relative *A. aphrophilus*. In serum killing assays using 50% NHS, we screened our available \((n = 15)\) *A. aphrophilus* strains in our collections, observing that they displayed moderate to high levels of serum resistance (Supplementary Table 2). Albeit this initial screening suggested that the degrees of serum resistance may vary among strains, it supported the notion that serum resistance may be ubiquitous also among *A. aphrophilus* strains. Strain HK83 was selected for genetic analysis as it is a naturally competent strain, facilitating the genetic analysis.

**OmpA protein expression in *A. aphrophilus* strain HK83**

Analysis of whole genome sequencing data of *A. aphrophilus* strain HK83 confirmed that it encodes OmpA\(^{1\text{AP}}\) and OmpA\(^{2\text{AP}}\), both sharing approximately 96% amino acid identity with the corresponding proteins of the *A. aphrophilus* reference strain NJ8700. We noted that the OmpA\(^{1\text{AP}}\) protein sequence of HK83 is identical to that of *A. aphrophilus* strains ATCC 7901 and W10433 (accession numbers OBY53818 and AKU63521, respectively), and that the HK83 OmpA\(^{2\text{AP}}\) protein is identical to that of strain W10433 (accession number AKU63719). Strain HK83 was used to generate ompA\(^{1\text{AP}}\) and ompA\(^{2\text{AP}}\) single, and ompA\(^{1\text{AP}}\) ompA\(^{2\text{AP}}\) double mutants, respectively. To estimate the relative levels of produced OmpA\(^{1\text{AP}}\) and OmpA\(^{2\text{AP}}\) in HK83, SDS-PAGE and Western blot were used to analyze OMP preparations. In addition, OMV preparations were assessed as *A. aphrophilus* strains were found to produce outer membrane vesicles, at a level similar to *A. actinomycetemcomitans* (Figure 5). These analyses revealed distinct protein bands at approximately 35 and 25 kDa, respectively, which were confirmed to represent OmpA\(^{1\text{AP}}\) using LC-MS/MS, and which were absent in the ompA\(^{1\text{AP}}\) mutant derivatives (Figure 4). This observation is consistent with the notion that OmpA\(^{1\text{AP}}\) was the major OmpA protein produced under the growth conditions used. Moreover, SDS-PAGE revealed two protein bands with somewhat increased intensity, at approximately 37 kDa in OMP and OMV preparations from the HK83 ompA\(^{1\text{AP}}\) ompA\(^{2\text{AP}}\) double mutant (Figure 4(a,b)). According to LC-MS/MS analysis of these protein bands, the slightly larger-sized protein was identified as OmpC (AKU63902), a homologue to *A. actinomycetemcomitans* Omp39 (≈73% amino acid identity). This may represent compensatory expression of OmpC due to lack of OmpA\(^{1\text{AP}}\) and OmpA\(^{2\text{AP}}\). The second protein band was identified as a methyl-galactoside ABC transporter substrate-binding protein (OBY51871).

**OmpA1 is important for the serum resistance of *A. aphrophilus* strain HK83**

To elucidate if OmpA\(^{1\text{AP}}\) and/or OmpA\(^{2\text{AP}}\) might be involved in *A. aphrophilus* serum resistance, strain HK83 and its ompA\(^{1\text{AP}}\) and ompA\(^{2\text{AP}}\) single, and ompA\(^{1\text{AP}}\) ompA\(^{2\text{AP}}\) double mutants were assessed in serum killing

![Figure 4](image-url)
experiments using 50% NHS. The ompA1 mutant exhibited a survival rate of 12.1%, i.e. almost four times lower than the parental strain, HK83 (Figure 6), clearly supporting that OmpA1 \( {}^{ap} \) was contributing to serum resistance. Similar to the findings with \( A. \) actinomyces strain D7SS, inactivation of \( \text{ompA}_2 \) \( {}^{ap} \) in HK83 did not significantly affect the serum survival. However, serum resistance was almost abolished in the \( \text{ompA}_1 \text{ompA}_2 \) double mutant (Figure 6), indicating that OmpA2 \( {}^{ap} \) contributed to the serum resistance in this background. The survival rates of the wild-type strain and its mutant derivatives were similar in heat-

![Figure 5](image1.png)

**Figure 5.** Release of OMVs from \( A. \) aphrophilus strains. Atomic force micrographs of bacterial strains cultivated on agar. \( A. \) aphrophilus strains HK83 (a and c), CCUG 3715 (b), and HK83 \( \text{ompA}_1 \text{ompA}_2 \) (d), which produces large numbers of OMVs relative to HK83. Arrows indicate examples of the released \( A. \) aphrophilus vesicles in a and b. Bars = 300 nm (a, b), and 500 nm (c, d).

![Figure 6](image2.png)

**Figure 6.** Analysis of the serum survival of \( A. \) aphrophilus strain HK83 and its mutant derivatives. \( 1.0 \times 10^9 \) bacterial cells were incubated in 50% normal human serum (NHS), or 50% heat-inactivated (HI)-NHS at 37°C for 2 h. The assay was performed in the absence (blue bars) or presence (red bars) of \( \text{Mg}^{2+} \)/EGTA. Bacterial serum survival was determined by viable count and expressed as ratio (%) of CFU in NHS/HI-NHS. Shown are means ± SEM from at least three independent experiments. *\( P < 0.04 \), HK83 vs HK83 \( \text{ompA}_1 \) for both conditions.
Inactivated NHS (data not shown), whereas like the A. actinomycetemcomitans strains, there was a somewhat reduced growth rate of the HK83 ompA1 single and ompA1 ompA2 double mutants in liquid cultures (Supplementary Figure 2). As judged by the hypervesculation phenotype of HK83 ompA1 ompA2 revealed by AFM (Figure 5(d)), its serum sensitivity was most likely accompanied by a disruption of the outer membrane integrity. Analogously to our findings with the A. actinomycetemcomitans strain, clones of the HK83 ompA1 mutant were fortuitously obtained after the incubation in 50% NHS, which after isolation were found to exhibit serum resistance at a level comparable to the parental strain. As judged by SDS-PAGE analysis of OMV preparations from selected ompA1 mutant clones with enhanced serum resistance, there was no noticeable difference in the levels of outer membrane proteins relative to the parental strain (data not shown). One clone with enhanced serum resistance, HK83 ompA1R1 (survival rate 56.1% ± 12.6% [SEM]), was subject to inactivation of ompA2AP. As this resulted in essentially abolished serum survival (< 1%), we concluded that OmpA2 contributed to the serum resistance in this background.

Complement activation pathways effective in elimination of serum-sensitive OmpA mutant derivatives

To initially investigate mechanisms for OmpA-mediated serum resistance, we assessed which pathways of complement activation were effective in elimination of serum-sensitive ompA1 single and ompA1 ompA2 double mutant strains of the two species. To this end, serum killing assays were performed in the presence of Mg2+/EGTA, which allow alternative complement activation but inhibits the classical and MBL pathways (Figure 2). Whereas blocking the classical and MBL pathways had no effect on survival of the serum resistant A. actinomycetemcomitans strain D7SS, its ompA1 mutant displayed a largely increased survival rate (66.0%). Comparably, in the presence of Mg2+/EGTA there was much enhanced survival rates of the D7SS ompA1 ompA2 (25.3%), and ompA1 omp100 (31.7%), double mutants, respectively, and in serum-killing assays using MBL-depleted NHS, the survival rate of the D7SS ompA1 mutant was relatively high, i.e. 77.9% ± 27.6% [SEM]). This together supports that the serum susceptibility of the ompA1AP mutant derivatives to a relatively large extent was mediated via classical/MBL complement activation. However, as in the presence of Mg2+/EGTA, none of the serum-sensitive D7SS mutant derivatives exhibited a survival rate corresponding to that of their respective parental strains (Figure 2), we concluded that they were also susceptible to alternative complement activation.

In the presence of Mg2+/EGTA the serum survival rate of the A. aphrophilus wild-type strain, HK83, and its ompA2 derivative was 71.5% and 45.3%, respectively, consistent with a partial susceptibility to both classical/MBL and alternative complement activation (Figure 6). Likewise, although there was enhanced survival of the HK83 ompA1 mutant in the presence of Mg2+/EGTA, its survival rate was still as low as 19.3%, whereas the HK83 ompA1 ompA2 double mutant was essentially serum-sensitive. Moreover, the survival rate of the HK83 ompA1 mutant was enhanced when MBL-depleted serum was used (39.3% ± 13.9% [SEM]). Hence, taken together, these results support the notion that the classical, MBL, and alternative complement activation pathways were all involved in the elimination of the serum-sensitive ompA mutant derivatives tested. This suggests that alternate mechanisms may be involved in OmpA-mediated serum resistance.

OmpA-dependent trapping of C4b binding protein (C4bp)

To investigate possible mechanisms how the Aggregatibacter OmpA proteins may protect the bacterial cells from complement-mediated killing, we investigated whether OmpA1AP and OmpA1AP, similar to E. coli OmpA [24], might be able to bind to C4bp. For this, OMVs obtained from A. actinomycetemcomitans D7SS and A. aphrophilus HK83, and their respective ompA1 mutants, were assayed by Far-Western analysis using recombinant human C4bp, and polyclonal anti-C4bp, and anti-OmpA antibodies, respectively. The detection using the anti-C4bp antibody indicated that there was binding of C4bp as represented by a protein band on the membrane at a position corresponding to the size of OmpA, which was observed in the case of both wild-type strains (Figure 7(a)). This observation was confirmed using the anti-OmpA antibody (Figure 7(a)). In contrast, there was no such C4bp binding detected assessing the OMV samples from the ompA1AP and ompA1AP mutant, respectively (Figure 7(a)). Moreover, also OmpA2AP was found to bind to C4bp, assessing OMVs from the serum resistant strain D7SS ompA1-R1 (Figure 7(b)). These results together are consistent with the notion that both A. actinomycetemcomitans and A. aphrophilus can exhibit OmpA-dependent binding of C4bp.

Discussion

In the present work we have used naturally competent model strains of A. actinomycetemcomitans (D7SS; serotype a), and its close relative A. aphrophilus (HK83) to demonstrate the role of OmpA proteins in serum resistance of these organisms.
human serum is consistent with their association with extra-oral infections such as infective endocarditis and cerebral abscesses [2]. A role of OmpA proteins in serum resistance of *A. actinomycetemcomitans* and *A. aphrophilus* is in agreement with findings with a number of other bacterial species, including *A. baumannii* and *E. coli* [16,17]. In both *Aggregatibacter* species tested in the present work, the major OmpA protein, OmpA1, was important for the serum survival, albeit not essential, as a low frequency of the *ompA1*AA and *ompA1*AP mutant derivatives survived in the presence of 50% NHS. The interesting finding that *ompA1*AA mutants fortuitously regained their serum resistance upon increased OmpA2AA production, supported that OmpA2AA here acted as a functional homologue to OmpA1AA, which is consistent with the high degree of amino acid identity of these proteins, and with previous observations of compensatory OMP expression upon loss of major outer membrane porins [47]. Similarly, our present work suggests that there was compensatory production of OmpC and/or ABC transporter substrate protein in the *ompA1*AP *ompA2*AA double mutant, which would be in accordance with the role of homologues to these proteins in bacterial serum resistance [48,49].

The finding that no clones could be obtained expressing the full length OmpA1AA protein for mutant complementation, but rather a C-terminally truncated form, implies that overexpression of OmpA1 may be deleterious to the *E. coli* laboratory strain used. The phenomenon that OmpA of some bacterial species can be toxic in *E. coli* strains has earlier been described e.g., with OmpA of *Haemophilus influenzae, Mycobacterium tuberculosis,* and *Rhodopseudomonas blastica* [50–52]. In line with the essentially abolished serum survival of the *ompA1* ompA2 double mutants of both species, EM and AFM, respectively revealed that they exhibited larger membrane-vesicle like structures projecting from the cell surface, and/or a hypervesiculation phenotype, consistent with earlier observations with *ompA* mutants exhibiting impaired membrane stability [19,53–56], and reduced growth [57].

In this work, we have also attempted to investigate putative mechanism(s) for OmpA-mediated protection of the bacterial cells from complement-mediated killing. Similar to earlier findings with *Klebsiella pneumo-

niae* [58], we observed that several complement activation pathways appeared to be effective in the elimination of serum-sensitive *ompA1* single, and *ompA1* *ompA2* double mutant derivatives of *A. actinomycetemcomitans* and *A. aphrophilus*. This may, at least partly reflect a compromised outer membrane integrity in these mutants, which would be consistent with their somewhat reduced growth rates, observed in liquid cultures. However, evidently, OmpA proteins have been demonstrated to interact with serum components

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**Figure 7.** Far-Western analysis to detect binding of OmpA1AA and OmpA1AP to C4b-binding protein (C4bp). (a) OMVs obtained from *A. actinomycetemcomitans* D7SS (lane 1), and D7SS ompA1 (lane 2), and from *A. aphrophilus* HK83 (lane 3), and HK83 ompA1 (lane 4) were subjected to SDS-PAGE. For Far-Western analysis, the membrane was first incubated with C4bp (1 μg/ml final dilution) prior to immunodetection using an anti-C4bp polyclonal antibody. OmpA was detected using a polyclonal antiserum specific for *E. coli* OmpA. (b) Far-Western analysis of OMVs obtained from *A. actinomycetemcomitans* D7SS ompA1-R1 (lane 1), and D7SS ompA1 (lane 2), respectively. Sizes (kDa) of the proteins in the pre-stained molecular weight marker (M) are indicated along the right side.
that regulate both the classical/MBL, and alternative pathways of complement activation, respectively [16,23,24]. Our finding that A. actinomycetemcomitans OmpA1 and OmpA2, and A. aphrophilus OmpA1 could bind to the classical/MBL pathway negative regulator, C4bp is consistent with observations with E. coli OmpA. It remains, however, to be elucidated to which extent this interaction per se plays an active role in conferring serum resistance, similar to what was demonstrated with E. coli OmpA [23,24]. In contrast to earlier findings with a couple of A. actinomycetemcomitans serotype b and d strains [10,25], inactivation of the omp100 gene locus in the serotype a strain D7SS did not render the cells serum-susceptible. The reason for this discrepancy is not known but may reflect the relative levels of produced OMPs in the different strains under the conditions used. Such a scenario is plausible as judged by observations with Salmonella Enteritidis that the patterns of OMP expression vary among strains exhibiting different degrees of serum sensitivity [59]. The notion that A. actinomycetemcomitans and A. aphrophilus may exploit alternate mechanisms to evade complement-mediated killing is supported by recent findings that strains display different patterns of complement activation pathway component consumption in vitro [60]. Moreover, it has been demonstrated that human serum induces global stress responses to varying degrees in A. actinomycetemcomitans strains [46]. Thus, it is likely that serum resistance in both Aggregatibacter species, similar to A. baumannii [48], is highly complex, relying on a large number of gene products.

In summary, the present work has revealed the importance of primarily OmpA1, but also OmpA2 in serum resistance of A. actinomycetemcomitans and A. aphrophilus. The recognition of bacterial products mediating serum resistance represents an approach to vaccine and drug development. The existence of different alleles of OmpA1 and OmpA2 among strains in these species can possibly be utilized in the development of strain-specific vaccines and agents that reduce the serum resistance.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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