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FEMS Microbiology Letters 250 (2005) 229–236

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Characterization of the IgA1 protease from the Brazilian purpuric fever strain F3031 of *Haemophilus influenzae* biogroup aegyptius

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Received 6 January 2005; received in revised form 21 June 2005; accepted 11 July 2005

First published online 28 July 2005

Edited by P.W. Andrew

Abstract

Brazilian purpuric fever is a severe vascular disease caused by an invasive clone of *Haemophilus influenzae* biogroup aegyptius, which normally causes self-limiting eye infections. A previous genome subtraction procedure resulted in the isolation of a DNA fragment, which encodes a putative IgA1 protease, specific to the F3031 Brazilian purpuric fever type strain. Cloning and sequencing of the entire F3031 *iga1* gene showed that the subtracted DNA fragment encompasses the *iga1* region encoding the active site and the cleavage specificity determinant of the protein, which are different from the cognate regions of the proteases produced by other *H. influenzae* strains. Western and IgA cleavage assays together with clustering analysis showed that the F3031 IgA1 protease is most similar to the type 2 proteases produced by *H. influenzae* type c and e strains. Analysis of the promoter region of the F3031 *iga1* gene revealed the presence of Fur binding sites. However, real-time PCR analysis and transcriptional fusion assays showed that the expression of *iga1* is not regulated by iron or hemin under the conditions tested.

Keywords: *Haemophilus aegyptius*; Brazilian purpuric fever; IgA1 protease

1. Introduction

Haemophilus influenzae biogroup aegyptius (*H. aegyptius*) is the causative agent of Brazilian purpuric fever (BPF), a pediatric infection that progresses from conjunctivitis to a severe and lethal hemorrhagic illness [1]. Since the original Brazilian outbreak in 1984, several laboratories have been investigating mechanisms of how this pathogen, which normally causes uncomplicated conjunctivitis, was transformed into a pathogen with invasive and cytotoxic abilities [2]. Our laboratory used genome subtraction hybridization to selectively enrich and identify DNA sequences that are present in the BPF strain F3031 but absent in the non-invasive non-BPF-causing *H. aegyptius* strain F1947 [3]. This approach resulted in the isolation of the F10 subtracted fragment, the product of which is predicted to be related to IgA1 proteases produced by numerous bacteria including typeable and non-typeable isolates of *H. influenzae* [4,5]. Researchers have determined that most BPF and non-BPF isolates of *H. aegyptius* produce type 2 and type 1 proteases, respectively; although the full nucleotide sequence of *iga1* from either of these isolates was not determined [4].

In this study we investigated why an *iga1* gene fragment specific to an invasive BPF isolate was selectively

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0378-1097/$22.00
doi:10.1016/j.femsle.2005.07.010

enriched for when the distribution of this gene is so prevalent among *Haemophilus* strains [5,6]. The data indicate that the *H. aegyptius* F3031-specific subtracted fragment F10 does not represent a new gene coding for a novel IgaA1 protease. Rather, it reflects the nucleotide sequence differences associated with the genetic determinants coding for the different types of IgA1 proteases produced by the *H. aegyptius* F3031 and F1947 strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Table 1 lists all bacterial strains and plasmids used in this study. The *Haemophilus* strains were grown on chocolate agar or on brain heart infusion agar supplemented with 2 μg/ml nicotinamide–adenine dinucleotide and 2 μg/ml hemin (sBHI) at 37 °C with 5% CO_2. This hemin concentration is enough to satisfy the nutrient requirement of this strain but does not create an iron-rich condition [7]. *Escherichia coli* strains were grown under standard conditions in Luria–Bertani medium [8] supplemented with appropriate antibiotics.

2.2. Cloning and sequencing of iga1

Colony blot screening [8] of a genomic library, made by cloning *Eco*RI-partially digested F3031 total DNA in pBR325, with the F10 subtracted fragment [3] as a probe resulted in the isolation of pMU97 harboring the *iga1* 5′ end. The *iga1* 3′ end was cloned in pMU115, which was isolated after a similar screening of a genomic library made by cloning *Eco*RI-partially digested F3031 total DNA in pZEro2.1. The nucleotide sequence of pMU97 and pMU115 was used for the PCR amplification of *iga1* and approximately 300 bp

Table 1
Bacterial strains and plasmids used in this work

Strains/plasmids	Relevant characteristics[a]	Source/reference[b]
Strains		
H. aegyptius		
F3031	Brazilian invasive BPF isolate, type strain	[1]
F3029	Brazilian invasive BPF isolate	[1]
F3033	Brazilian invasive BPF isolate	[1]
F3037	Brazilian invasive BPF isolate	[1]
F4380	Australian invasive BPF isolate	[13]
F1947	Brazilian non-invasive non-BPF isolate	[1]
H. influenzae		
Eagan	Type b	S. Goodgal
DL42	Type b	E. J. Hansen
DL63	Type b	E. J. Hansen
Rd KW20	Genome sequencing strain	H. O. Smith
Rd225DK	Same as Rd KW20 with 1-kb deletion in the *iga1* gene	A. Plaut
AMC 36-A-3	Type a	ATCC
AMC 36-A-5	Type c	ATCC
AMC 36-A-6	Type d	ATCC
AMC 36-A-7	Type e	ATCC
AMC 36-A-8	Type f	ATCC
E. coli		
DH5α	Host cloning strain	Gibco-BRL
Top10	Host cloning strain	Invitrogen
BN402	Strain with a wild-type *fur* allele	[18]
BN4020	Same as BN402 with *fur*::Tn5 allele	[18]
Plasmids		
pACYC177	Cloning vector, Amp^R, Km^R	ATCC
pBR325	Cloning vector, Amp^R, Cm^R, Tet^R	Gibco-BRL
pCR-Blunt II Topo	PCR cloning vector, Km^R, Zeo^R	Invitrogen
pKK232-8	Promoter cloning vector, Amp^R	Amersham/Pharmacia
pZEro2.1	Cloning vector, Km^R	Invitrogen
pMU97	5′ end of F3031 *iga1* cloned in the *Eco*RI site of pBR325, Amp^R, Tet^R	This work
pMU115	3′ end of F3031 *iga1* cloned in the *Eco*RI site of pZEro2.1, Km^R	This work
pMU121	F3031 *iga1* promoter cloned in pKK232-8, Amp^R, Cm^R	This work
pMU128	Amplicon harboring the *iga1-cat* fusion cloned in pACYC177, Amp^R, Cm^R	This work
pMU150	Amplicon harboring F3031 *iga1* cloned in pCR-Blunt II Topo, Km^R, Zeo^R	This work

[a] R, resistant; S, sensitive; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline; Km, kanamycin; Zeo, zeocin.
[b] CDC, Centers for Disease Control and Prevention; ATCC, American Type Culture Collection.

upstream and downstream of the proposed transcriptional start and stop sites of this gene, respectively, using F3031 total DNA as a template. The amplicon, which was obtained with *Pfu* DNA polymerase (Stratagene) and the primers IgA1F and IgA1R (Table 2), was cloned into pCR-Blunt II Topo, yielding pMU150. All nucleotide sequence data were generated using standard automated DNA sequencing methods. Primers CW and CCW (Table 2), which flank the *Eco*RI site of pBR325, were used to sequence the pMU97 DNA insert, and M13 forward and reverse universal primers were used to sequence the pMU115 DNA insert. The complete sequence of both strands was obtained with primer walking. Nucleotide sequences were analyzed with BLASTx and ORF Finder (http://www.ncbi.nlm.nih.gov), and predicted protein sequences were compared with PILEUP and BESTFIT (GCG software package, Accelrys). The *iga1* predicted promoter region was identified with the prokaryotic promoter predictor developed by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html). The nucleotide sequence for the *iga1* gene described in this study was deposited in GenBank under Accession No. AF369907.

2.3. Southern hybridization

Total DNA from various strains was digested with restriction enzymes, and the fragments were size-fractionated by agarose gel electrophoresis and transferred to nitrocellulose using standard methods [8]. *Hin*dIII-digested λ DNA was used as a size marker. Blots were probed with PCR products and *Hin*dIII-digested λ DNA radiolabeled with [α-^{32}P]dCTP [3]. Hybridizations were done under high stringency, and the signals were detected as described before [3].

2.4. Detection and activity of the IgA1 protease

Proteins from culture supernatants were size-fractionated by SDS–PAGE on 10% polyacrylamide gels and transferred to nitrocellulose as described previously [7]. The blot was incubated with a polyclonal antiserum raised against the β and protease domains of the *H. influenzae* Rd KW20 IgA1 protease. The immunocomplexes were detected with protein A conjugated to horseradish peroxidase (HRP) and chemiluminescence (Pierce). For detection of IgA1 cleavage, 50 μl samples of culture supernatants were incubated overnight at 37 °C with 50 μl of 2 μg/μl IgA1 from human colostrum (Sigma) [9]. The proteins were size-fractionated by SDS–PAGE, transferred to nitrocellulose, and incubated with anti-human IgA1 antibodies (Sigma). Chemiluminescent detection was done with HRP-conjugated protein A.

2.5. Expression analysis of the iga1 gene

The predicted *iga1* promoter region was PCR amplified with the Iga1pF primer containing a *Bam*HI restriction site and the Iga1R primer containing a *Hin*dIII restriction site (Table 2). The amplicon was ligated to *Bam*HI- and *Hin*dIII-digested pKK232-8, an approach that resulted in a derivative (pMU121) coding for chloramphenicol resistance. This resistance is due to the fusion of the predicted *iga1* promoter to the promoterless chloramphenicol acetyl transferase (*cat*) gene harbored by pKK232-8. pMU121 DNA was used as a template to PCR amplify the *iga1* promoter, the *cat* gene, and the first transcriptional terminator with the Iga1pF primer and a new minus strand primer containing an *Xma*I site (Table 2). The amplicon was ligated to *Bam*HI- and *Xma*I-digested pACYC177, giving pMU128. The plasmids pMU121 and pMU128 were sequenced to confirm the nature and orientation of the cloned fragment. pMU128 was transformed into *E. coli* BN402 and BN4020 strains using standard methods [8]. Strains BN402 and BN4020 containing pMU128 were cultured in M9 minimal medium [8] under iron iron-replete (100 μM $FeCl_3$) and iron-chelated [100 μM ethylenediamine-di-(*o*-hydroxyphenyl) acetic acid (EDDHA)] conditions, and total lysates were prepared as described before [7]. These samples were used to

Table 2
Oligonucleotide primers used in this work

Oligonucleotide	Sequence
IgA1F	5′-ACCGCCATTACGCAAAGAC-3′
IgA1R	5′-CACCGCACTTAATCACTG-3′
CW	5′-AGCACAAGTTTTATCCGGCC-3′
CCW	5′-ACAAGGGTGAACACTATCCC-3′
Iga1pF	5′-CGGGATCCCGACCGCCATTACGCAAAGAC-3′
Iga1R	5′-CCCAAGCTTGGGTAGGCGACAGTAAGCGC-3′
Minus strand primer	5′-CCCCCCGGGGGGAAATTCTGTTTTATCAG-3′
Iga-for	5′-AGCACTGAAAACCCTGTAGTGGA-3′
Iga-rev	5′-TAGGCTGACTAACAGTTCCTGTTGTC-3′
RecF-for	5′-GGCCCTCAAAAAGCGGATT-3′
RecF-rev	5′-TGTCCACGAGAAAGCACATCTT-3′

evaluate the differential expression of *cat* by immunoblotting using anti-Cat serum (Promega) and protein A conjugated-HRP as described above.

The differential expression of *iga1* was also assessed by real-time RT-PCR using Taqman assays. The primers and probes (MegaBases) were designed with Primer Express software (Applied Biosystems). The probes were labeled at the 5′ and 3′ end with the reporter dye 6-carboxyfluorescein (6FAM) and the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA), respectively. The Iga-for and Iga-rev primers and the RecF-for and RecF-rev primers (Table 2) were used for *iga1* and *recF* amplification, respectively. Nucleotide sequences for *iga1* and *recF* probes were 6FAM-TCCAGAGAATACCACTCAACCTGCGGTTAA-TAMRA and 6FAM-TCGTTTCAAAGCACAAGGATTGCCTGT-TAMRA, respectively. RNA was isolated from F3031 cells grown in sBHI in iron-replete (50 μg/ml hemin) or iron-chelated (100 μM EDDHA) conditions as described before [10]. RNA was isolated from three independent cultures at exponential phase, and each sample was tested in triplicate. Two of the RNA samples were isolated from cultures grown to an OD_{600} of 0.7, while the third sample was isolated from a culture grown to an OD_{600} of 0.85. Standard curves were made with genomic DNA from *H. aegyptius* strain F3031. *recF* was used to normalize TaqMan data because triplicate assays using three independent RNAs showed that transcription of *recF* was not significantly different ($P > 0.05$) in cells grown under iron-replete and iron-chelated conditions. The quantitative measurement of *iga1* expression is reported as its *recF*-normalized fold-difference in expression in iron-depleted relative to iron-chelated growth conditions.

3. Results

3.1. Cloning of the F3031 iga1 gene

Although pMU97 contained only 3955 bp of the *iga1* locus, it provided evidence about the genetic organization upstream of this gene (Fig. 1(a)). The 3′ region of *iga1* and a small portion of the downstream sequence were cloned separately (pMU115). Using the nucleotide sequence obtained with these two plasmids, the entire 5295-bp *iga1* gene was PCR amplified from the F3031 chromosome and cloned as a single fragment in pMU150 (Fig. 1(a)). The F10 subtracted fragment was located within the F3031 *iga1* gene (Fig. 1(a)), which, interestingly, contains the protease active site and a portion of the cleavage specificity determinant of the predicted protease [4]. A serine residue present in the active site is thought to be critical for IgA1 cleavage, and the cleavage specificity determinant is a region in the protease that is important in determining the type

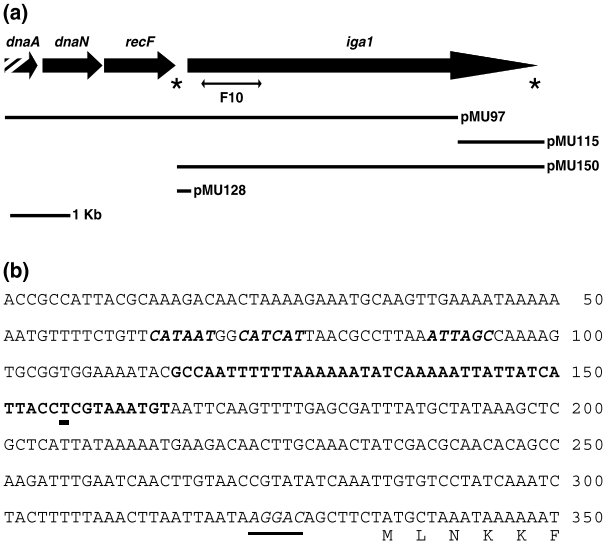

Fig. 1. Analysis of the DNA sequence of the *iga1* locus. (a) Graphic representation of the *iga1* locus. Thick arrows represent predicted ORFs with the broken arrow being an incomplete ORF. The double-headed arrow shows the boundaries of the F10 fragment generated from a genome subtraction procedure [3]. Straight lines represent F3031 cloned DNA used in this work. The position of DNA uptake signal sequences is indicated by the asterisks. (b) Nucleotide sequence of the DNA region containing the *iga1* promoter. Bold and italicized letters are separate proposed tripartite Fur binding sites which consist of two overlapping heptamer repeats (consensus tGATAATGA-TAATCATTATCa) [14]. Bold nucleotides identify the predicted *iga1* promoter, with the +1 nucleotide of the transcript underlined. A potential ribosome binding site is shown in underlined italics. The first six predicted amino acids of IgA1 are shown under the nucleotide sequence.

(1 or 2) of protease produced [11]. Two DNA uptake signal sequences (5′-AAGTGCGGT-3′) flank the *iga1* locus (Fig. 1(a)). Of note, our sequence is identical to part of the 5′ region of an *iga1* gene containing the cleavage specificity determinant from the strain F3034 [4], which was isolated from a BPF patient during an outbreak in Brazil. BESTFIT comparison between the F3031 IgA protease and the *H. influenzae* Rd KW20 HI0990 homolog [12] shows that the predicted amino acid sequences share 81.3% similarity.

3.2. The F3031 iga1-encoded protein product cleaves IgA1

The F3031 *iga1* locus cloned in pMU150 encodes a protein with IgA1 protease activity (Fig. 2(a)), which results in a major cleavage product that migrates slightly faster than the band produced by the *H. influenzae* Rd KW20 type 1 protease (compare lanes 2 and 8). Neither of these major degradation products was detected when human IgA was incubated with the culture supernatant of the *H. influenzae* Rd225DK *iga1* deletion derivative (lane 7) or *E. coli* DH5α harboring the cloning vector pCR2.1 (lane 3). This figure also shows that the non-invasive strain F1947 and *H. influenzae* Rd KW20

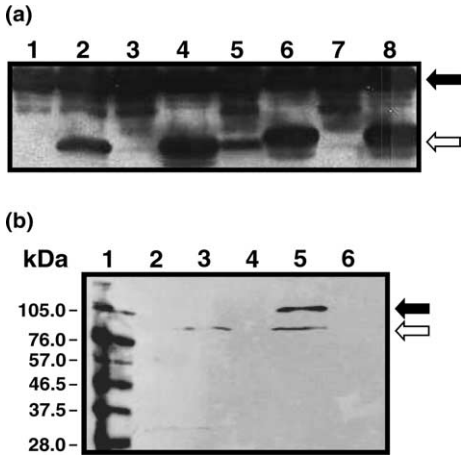

Fig. 2. Detection of the IgA1 protease and IgA1 cleavage activity. (a) In vitro IgA1 cleavage assay using culture supernatants from *E. coli* DH5α harboring pMU150 (lane 2), the negative control pCR2.1 transformed into DH5α (lane 3), *H. aegyptius* F3031 (lane 4), *E. coli* DH5α harboring pMU97 (lane 5), *H. aegyptius* F1947 (lane 6), *H. influenzae* Rd225DK *iga* minus mutant (lane 7), and *H. influenzae* Rd KW20 (lane 8). Culture supernatants were incubated with human IgA, and proteins were separated by SDS–PAGE and transferred to nitrocellulose. The blot was incubated with antiserum to human IgA1. Lane 1 contains uncleaved human IgA1. The black and open arrows denote the uncleaved and major IgA1 cleavage products, respectively. (b) Western blot analysis of supernatants from cultures of *H. aegyptius* F3031 (lane 3), *E. coli* DH5α harboring pMU97 (lane 4), *H. influenzae* Rd KW20 (lane 5), and *H. influenzae* Rd225DK *iga* deletion mutant (lane 6). Lane 1 is the biotin-labeled protein size standard, and lane 2 is sterile sBHI. The blot was incubated with an antiserum against the β and protease domains of the *H. influenzae* strain Rd KW20 IgA1 protease. The black and open arrows denote the IgA1 protease and β domains, respectively.

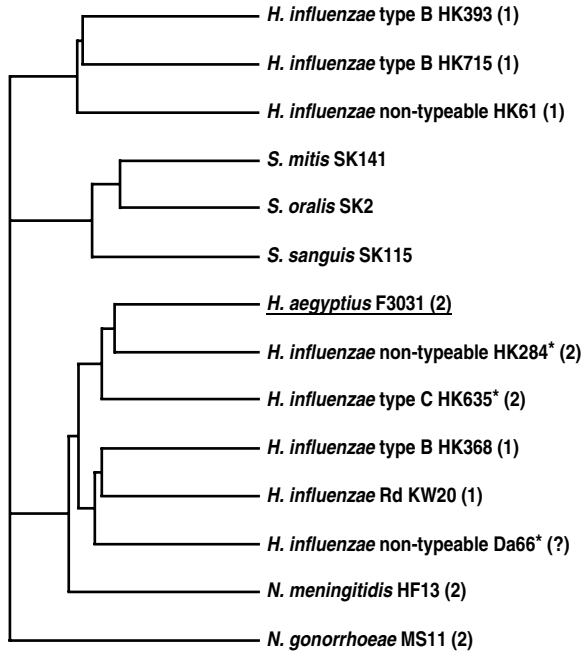

Fig. 3. Dendogram of a peptide alignment obtained using PILEUP. Numbers in parenthesis show the IgA1 cleavage type. *H. influenzae* Da66 has a protease with an undefined cleavage type. All known streptococcal IgA1 proteases share the same specificity. Entries with asterisks represent partial sequences (~600 amino acids of the N-terminus). GenBank Accession Numbers for the proteases from chosen strains are as follows: HK393, M87490; HK715, M87489; HK61, M87491; HK284, X82487; HK635, X82488; HK368, M87492; RdKW20, AAC22651; Da66, X82467; HF13, X82474; MS11, S75490; SK141, Y10285; SK2, Y10286; and SK115, Y13456.

produce proteases with similar substrate specificity (compare lanes 6 and 8). This assay also demonstrated that pMU97 encodes protease activity despite missing approximately 1300 bp in the 3′ region of *iga1* (lane 5). Taken together, these results indicate that the F3031 IgA1 protease is a type 2 protease, and, therefore, it cleaves IgA1 at a distinct site in the hinge region [4] when compared with type 1 enzymes.

Based on immunoblot analysis with antibodies to the ~80-kDa β domain of the IgA1 protease (Fig. 2(b)), which is responsible for autosecretion across the outer membrane, the IgA1 protease produced by *H. aegyptius* F3031 and *H. influenzae* Rd KW20 are similar. In contrast, the ~108-kDa fragment, which contains the protease active site, of the proteases produced by these strains is different enough to produce a signal with the product of *H. influenzae* Rd KW20 (lane 5) but not with the product of *H. aegyptius* F3031 (lane 3). The protease produced from the pMU97 construct did not react with this antiserum (lane 4), indicating that the portion of the β-domain important for antibody recognition but not protease activity is absent in the product of pMU97.

Fig. 3 shows the results of comparing the predicted protein sequences of numerous IgA1 proteases using PILEUP. This analysis is not intended to show relationships with a broad number of strains as has been done previously, but instead shows that the F3031 protease clusters closer to type 2 than to type 1 *H. influenzae* IgA1 proteases.

3.3. Distribution of F3031 iga1 homologous sequences in other H. influenzae isolates

All BPF isolates except the Australian isolate F4380 contained genomic regions related to the F10 probe indicating the conservation of the F3031 *iga1* gene among the Brazilian isolates obtained from BPF patients (Fig. 4(a)). The Australian strain F4380 is different from the Brazilian isolates and contains some but not all of the diagnostic BPF markers, although it produces clinical symptoms similar to the BPF disease caused by the Brazilian strains [13]. The F10 probe also detected the presence of related sequences in *H. influenzae* type c and e strains but not in the other typeable isolates tested in this work (Fig. 4(b) and (c)). These results are in accordance with the observation that type c and e strains and most BPF isolates of *H. influenzae* produce type 2 IgA1 proteases [4,9].

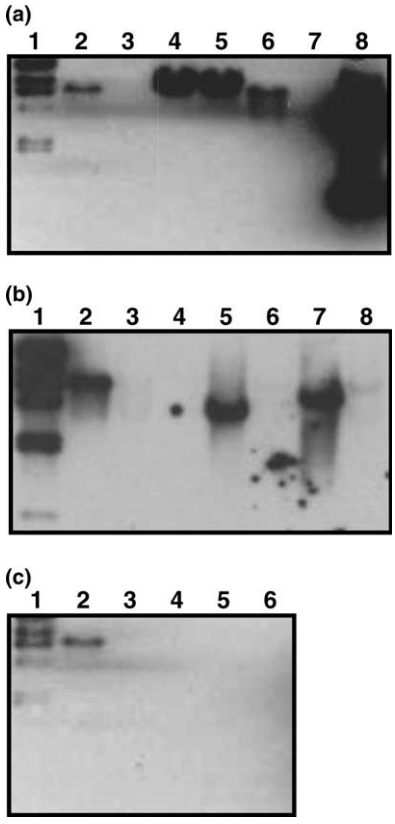

Fig. 4. Prevalence of *H. aegyptius* F3031 *iga1* related sequences in various *H. influenzae* isolates. (a) Total DNA from F3031 (lane 2), F1947 (lane 3), F3029 (lane 4), F3033 (lane 5), F3037 (lane 6), and F4380 (lane 7) digested with *Eco*RI. Lane 8 is a PCR amplicon of the F10 subtracted fragment used to make the radiolabeled probe. (b) Total DNA from F3031 (lane 2), type A (lane 3), type B Eagan (lane 4), type C (lane 5), type D (lane 6), type E (lane 7), and type F (lane 8) digested with *Eco*RI. (c) Total DNA from F3031 (lane 2), F1947 (lane 3), type B Eagan (lane 4), type B DL42 (lane 5), and type B DL63 (lane 6) digested with *Eco*RI. Lane 1 in (a), (b), and (c) is *Hin*dIII-digested λ DNA size standard. All three blots were probed with λ DNA and F10 PCR amplicons labeled with [α-^{32}P]dCTP.

3.4. Fur and iron regulation of iga1 expression

The *iga1* promoter region contains putative Fur binding sites [14] (Fig. 1(b)), implicating the Fur iron repressor protein and the availability of iron in regulating *iga1* transcription. In numerous microorganisms, including the F3031 BPF clone [7,15], Fur represses gene expression when iron is not limiting. Furthermore, the production of IgA1 protease in *N. gonorrhoeae* increases when bacterial cells are cultured under iron limitation [16]. Therefore, we tested the impact of Fur and iron in the regulation of *iga1* expression using Cat as a reporter. Immunoblotting showed that the intensity of the Cat protein signal was very similar in total lysates prepared from BN402 (*fur*$^+$) and BN4020 (*fur*$^-$) *E. coli* cells harboring the pMU128 reporter construct when cultured either under iron-rich or iron-chelated conditions (data not shown).

The failure to see iron and/or Fur regulation in *E. coli* could be due to the use of a heterologous host to express *Haemophilus* genes. Thus, the differential expression of *iga1* was assessed with real-time RT-PCR using RNA isolated from *H. aegyptius* F3031 cells grown under iron-replete and iron chelated-conditions. We have observed previously that the addition of 2 μg/ml hemin was enough to allow the growth of *H. influenzae* without creating an iron-rich environment, a condition that could be achieved by adding between 20 and 50 μg/ml hemin. These experimental conditions were appropriate to induce the differential expression of transferrin binding activity and the production of iron-regulated proteins [7], an observation that is similar to that made by other investigators working on similar problems with this bacterium. In addition, we consider that the addition of hemin rather than protoporphyrin IX, which can be used as an iron source by *H. aegyptius* F3031 [7], is more representative of the conditions found in the host where hemin could be present and used as an iron source for bacterial growth. Therefore, cells were cultured in sBHI containing either 100 μM EDDHA (iron chelated-condition) or 50 μg/ml of hemin (iron replete condition) and used to isolate total RNA. The *iga1* expression data were normalized to *recF* since its expression was not significantly different ($P < 0.05$) in the two growth conditions (data not shown). For the two cultures grown to an OD_{600} of 0.7, the mean fold difference of *iga1* gene expression in iron-chelated relative to iron-replete conditions was −1.6 and −1.4. The ratio was +1.4 when RNA isolated from cells grown to an OD_{600} of 0.85 was used as a template.

4. Discussion

The F3031 *iga1* gene was cloned and sequenced to assess the location and relevance of F10, a subtracted DNA fragment that represents sequences unique to this BPF invasive strain of *H. aegyptius* [3]. The *iga1* locus is preceded by ORFs having strong similarity to *dnaA*, *dnaN*, and *recF* both in *H. aegyptius* F3031 and *H. influenzae* Rd KW20 [12]. Thus, this fragment is not part of a genomic island specific to the F3031 strain. Rather, this fragment, which encodes the protease domain of the full-length IgA1 protease, reflects the biological differences between the type 1 and type 2 IgA1 proteases produced by these two *H. influenzae* strains. The data presented in this work also support the conclusion that the protease produced by *H. aegyptius* non-BPF strain F1947 is a type 1 protease with a protease domain different from that present in the protease made by the BPF type strain F3031. These results also help explain the observation that antibodies to IgA1 proteases from different *Haemophilus* strains could not inhibit IgA1 cleavage by BPF-specific IgA1 proteases [4].

The fact that the F3031 IgA1 protease clusters with the IgA1 proteases produced by non-typeable and type c *H. influenzae* strains is in agreement with previous research which showed that this non-typeable strain of *H. aegyptius* is most closely related to type c *H. influenzae* [17]. In addition, hybridization analysis of the F10 fragment showed that it was most similar to *iga1* from *H. influenzae* type c and type e strains, which typically produce type 1 IgA1 proteases. Interestingly, all BPF-causing *H. aegyptius* strains tested also hybridized with the F10 subtracted fragment except the Australian BPF-causing strain F4380, a finding that would support its difference from classical BPF strains [3]. Currently, it is not known if strain F4380 has an *iga1* similar to those found in type a, b, d, and f strains or if it is entirely missing the *iga1* gene.

The *iga1-cat* transcriptional fusion showed that the expression of this F3031 gene is not affected by the Fur iron repressor protein. Similarly, real-time RT-PCR analysis showed that the transcription of *iga1* was comparable under iron-rich and iron-chelated conditions when *H. aegyptius* F3031 cells were taken from cultures that attained an OD_{600} of 0.7, a condition that represents active cell growth and minimizes potential variations in RNA yield due to growth rate differences imposed by the conditions used to culture the bacterial cells. On the other hand, *iga1* expression in iron-limited conditions exceeded iron-replete conditions when the cultures reached an OD_{600} of 0.85. The difference of expression of *iga1* in cells that reached these two cell densities is less than two-fold, the minimum value being used widely by other investigators to select differentially transcribed genes for further studies. We also consider at the moment that the change in ratio from $-1.6/-1.4$ to $+1.4$ for the expression of this gene in cells that were cultured under the same conditions but reached different densities might not be significantly different under the experimental conditions used in this work. Thus, our data suggest that iron and Fur do not control the expression of *igA1*, although cell density may play a role. However, we consider that the potential role of iron, hemin, Fur and/or cell density in controlling the expression of *igA1* expression cannot be ruled out until the expression of this gene is tested in an experimental model that reflects the conditions found by this bacterial pathogen in the human host more accurately.

Acknowledgments

This work was funded by Miami University research funds and Public Health Grants R15AI37781-01 and R15AI44776-01A1 from the National Institutes for Health. We thank D.J. Brenner (CDC) for providing the *H. aegyptius* isolates; E.J. Hansen (University of Texas) for providing *H. influenzae* type B strains DL42 and DL63; S.H. Goodgal (University of Pennsylvania) for providing *H. influenzae* strain Eagan; H.O. Smith (Johns Hopkins) for providing *H. influenzae* Rd KW20; and A. Plaut (Tufts University) for providing the *H. influenzae* Rd KW20 *iga1* and the antiserum against IgA1 protease. Additional thanks go to J.M. Musser (NIH) for assistance in the expression analysis of *iga1* with real-time RT-PCR. We are grateful to C. Wood, coordinator of the Miami University Center of Bioinformatics and Functional Genomics, for his support and assistance with automated DNA sequencing and nucleotide sequence analysis.

References

[1] Brenner, D.J., Mayer, L.W., Carlone, G.M., Harrison, L.H., Bibb, W.F., de Cunto Brandileone, M.C., Sottnek, F.O., Irino, K., Reeves, M.W., Swenson, J.M., Birkness, K.A., Weyant, R.S., Berkley, S.F., Woods, T.C., Steigerwalt, A.G., Grimont, P.A.D., Cooksey, R.C., Arko, R.J. and Broome, C.V.The Brazilian Purpuric Fever Study Group (1988) Biochemical, genetic, and epidemiological characterization of *Haemophilus influenzae* biogroup aegyptius. J. Clin. Microbiol. 26, 1524–1534.

[2] Weyant, R.S., Quinn, F.D., Utt, E.A., Woreley, M., George, V.G., Candal, F.C. and Ades, E.W. (1994) Human microvascular cell toxicity caused by Brazilian purpuric fever-associated strains of *Haemophilus influenzae* biogroup aegyptius. J. Infect. Dis. 169, 430–433.

[3] Smoot, L.M., Franke, D.D., McGillivary, G. and Actis, L.A. (2002) Genomic analysis of the F3031 Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius by PCR-based subtractive hybridization. Infect. Immun. 70, 2694–2699.

[4] Lomholt, H. and Kilian, M. (1995) Distinct antigenic and genetic properties of the immunoglobulin A1 protease produced by *Haemophilus influenzae* biogroup aegyptius associated with Brazilian purpuric fever in Brazil. Infect. Immun. 63, 4389–4394.

[5] Lomholt, H., Poulsen, K. and Kilian, M. (1995) Comparative characterization of the *iga* gene encoding IgA1 protease in *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Haemophilus influenzae*. Mol. Microbiol. 15, 495–506.

[6] Reinholdt, J. and Kilian, M. (1997) Comparative analysis of immunoglobulin A1 protease activity among bacteria representing different genera, species, and strains. Infect. Immun. 65, 4452–4459.

[7] Smoot, L.M., Bell, E.C., Crosa, J.H. and Actis, L.A. (1999) Fur and iron transport proteins in the Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius. J. Med. Microbiol. 48, 629–636.

[8] Sambrook, J. and Russell, D.W. (2001) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[9] Mulks, M.H., Kornfeld, S.J., Frangione, B. and Plaut, A.G. (1982) Relationship between the specificity of IgA proteases and serotypes in *Haemophilus influenzae*. J. Infect. Dis. 146, 266–274.

[10] Graber, K., Smoot, L.M. and Actis, L.A. (1998) Expression of iron binding proteins and hemin binding activity in the dental pathogen *Actinobacillus actinomycetemcomitans*. FEMS Microbiol. Lett. 163, 135–142.

[11] Grundy, F.J., Plaut, A.G. and Wright, A. (1990) Localization of the cleavage site specificity determinant of *Haemophilus influenzae* immunoglobulin A1 protease genes. Infect. Immun. 58, 320–331.

[12] Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.-F., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J.D., Scott, J., Shirley, R., Liu, L.-I., Glodek, A., Kelley, J.M., Weidman, J.F., Phillips, C.A., Spriggs, T., Hedblom, E., Cotton, M.D., Utterback, T.R., Hanna, M.C., Nguyen, T., Saudek, D.M., Brandon, R.C., Fine, L.D., Fritchman, J.L., Fuhrmann, J.L., Geoghagen, N.S.M., Gnehm, C.L., McDonald, L.A., Small, K.V., Fraser, C.M., Smith, H.O. and Venter, J.C. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269, 496–512.

[13] Wilde, B.E., Pearman, J.W., Campbell, P.B., Swan, P.B. and Gurry, D.L. (1989) Brazilian purpuric fever in Western Australia. Med. J. Aust. 150, 344–346.

[14] Baichoo, N. and Helmann, J.D. (2002) Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J. Bacteriol. 184, 5826–5832.

[15] Smoot, L.M., Bell, E.C., Paz, R.L., Corbin, K.A., Hall, D.D., Steenbergen, J.N., Harner, A.C. and Actis, L.A. (1998) Molecular and genetic analysis of iron uptake proteins in the Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius. Front. Biosci. 3, d989–d996.

[16] Shoberg, R.J. and Mulks, M.H. (1991) IgA1 proteases of *Neisseria gonorrhoeae*: analysis of isogenic mutants In: Frontiers in Mucosal Immunology (Tsuchiya, H., Nagura, H., Hibi, T. and Moro, I., Eds.), Vol. 1, pp. 563–566. Elsevier Sciences Publishers B.V., Amsterdam, The Netherlands.

[17] Musser, J.M. and Selander, R.K. (1990) Brazilian purpuric fever: evolutionary genetic relationships of the case clone of *Haemophilus influenzae* biogroup aegyptius to encapsulated strains of *Haemophilus influenzae*. J. Infect. Dis. 161, 130–133.

[18] Bagg, A. and Neilands, J. (1985) Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J. Bacteriol. 161, 450–453.