1995

Cloning and characterization of the flanking region 3' to the flagellin gene (flaI) of Bartonella bacilliformis

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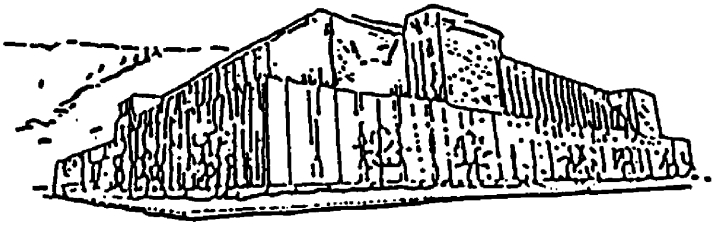

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Cloning and characterization of the flanking region 3' to the flagellin gene (*fla*I) of *Bartonella bacilliformis*

By Brian K. Clifton
B.S., The University of Montana--Missoula, 1992

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Cloning and characterization of the flanking region 3' to flagellin gene (*fla*I) of *Bartonella bacilliformis*

Director: Michael F. Minnick MFM

The flagellin gene (*fla*I) of *Bartonella bacilliformis* was obtained from a λ zap expression library (Stratagene) using rabbit anti–flagellin antiserum. The identified 3858-bp recombinant, pAULI, was used to further subclone the 3' flanking region of *fla*I. The resulting plasmid, pBKCI, contains a 1695-bp *Hind*III insert. Sequence analysis of pBKCI and pAULI identified three putative open reading frames (ORFs) termed *orf1*, *orf2*, and *xuoB*, in addition to *fla*I. The ORFs range in sizes of 62-bp, 152-bp, and 717-bp, respectively. *In vitro* transcription/translation of pBKCI and pAULI produced insert-encoded polypeptides of 3 kDa, 7 kDa, and 32 kDa, respectively. DNA hybridizations of total DNA isolated from *B. bacilliformis* strains KC583 and KC584, using the 1695-bp insert of pBKCI as a probe, indicate that the 3' region containing these genes is unique to *B. bacilliformis* and is not present in *Escherichia coli*. The largest of the 3 putative ORFs, *xuoB*, is a 717-bp ORF with a 39.2 G+C mol % content, preceded by a promoter region located 44 nucleotides upstream of the GTG start codon. The predicted (-35) region (TTGCTT) occurs at nucleotide -44, the (-10) region (TGTCAT) occurs at nucleotide -28, and a putative ribosomal binding site (RBS) consisting of GGGA occurs at nucleotide -13 to the start codon. The ORF ends with a single TAA stop codon. An apparant ρ–dependent transcriptional terminator occurs 17 bases 3' to the stop codon. Homology searches revealed a 47.3 % identity to an electron transfer flavoprotein-ubiquinone oxidoreductase from *Homo sapiens*, thus the gene was termed *xuoB* as ubiquinone oxidase B with an unknown target "x". No significant matches were made to any bacterial proteins of similar function. Homology searches with *orf1* and *orf2* revealed no significant matches to any proteins. The encoded XuoB protein has alternating hydrophilic and hydrophobic regions with a predicted pI of 5.74 and lacks a secretory signal sequence.

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CHAPTER I

Introduction

1.1 Chronicles and Characteristics

Bartonella bacilliformis is a gram negative, obligately aerobic bacterium which causes Oroya fever (also known as Carrion's disease) in humans (36). The disease is endemic to the Andes regions of Peru, Ecuador, and Colombia. Two distinct phases occur during infection. The primary or hematic phase produces severe hemolytic anemia due to a near depletion of circulating erythrocytes. The secondary or tissue phase produces hemangioma-like nodules believed to be caused by the induction of cellular proliferation of the capillary endothelial cells invaded by this organism. These nodules, known as verruga peruana, occur on the face and extremities (13,20).

The two distinct phases were shown to be caused by the same organism in 1885. A Peruvian medical student named Daniel Carrion innoculated himself with verruga peruana scrapings and then recorded the progression of disease. Carrion eventually died from a fatal case of Oroya fever (18). Koch's postulates were not fulfilled until 1926, when *B. bacilliformis* was re-isolated in pure culture from rhesus macaque monkeys that were inoculated with organisms obtained from patients afflicted with either the primary or secondary phase of bartonellosis (29)

Although *B. bacilliformis* is believed to have been reported as early as 100 A.D. to 1400 A.D., and is believed to have caused the death of the last ruler of the Incan Empire, i.e. Huayana Capac, (41), the first confirmed and documented case of the disease was in 1871. Hundreds of cases were reported by infected laborers of the Central

Railroad of Peru (12,18). Oroya fever was fatal in approximately 40% of these cases (18), but the fatality rate has since declined due to the discovery of antibiotics. With antibiotic therapy (penicillin, tetracycline, etc.) the disease is rarely fatal. About 10% of the population have been shown to be persistent carriers (9,10,20), and greater than 60% of the indigenous population have tested seropositive for the bacterium (19). The high fatality rate for untreated cases was witnessed as recently as 1987, when the disease claimed the lives of 14 Peruvian villagers who went undiagnosed until the disease had progressed beyond control (16). These surprising deaths reflect the serious need for early diagnosis and control of this disease.

1.2 Infection and invasion.

B. bacilliformis is an intracellular parasite of human erythrocytes and is transmitted by the bite of the female nocturnal sand flies within the genus *Phlebotomus*. Humans have been identified as the only reservoir for the bacterium (12,20,41). The vector appears to be restricted to an area 2^0 north of the equator to 13^0 south latitude and an area between 800 and 2600 meters in elevation. (18).

Previous studies provided evidence leading to several potential virulence factors that the bacterium may be using alone or in combination to invade human cells. One of these virulence factors is the bacterium's ability to produce an extracellular proteinaceous factor called deformin which independently deforms the erythrocyte membrane, producing deep-invaginations (25). Another virulence factor is the bacterium's ability to adhere to the surface glycolipids of erythrocytes (44). Finally, motility of the bacterium by its flagella

has been shown to be an important component for invasiveness of erythrocytes by this bacterium (3,40). All of these virulence factors and mechanisms of pathogenicity are still not fully understood and research has focused on the ability of *B. bacilliformis* to invade erythrocytes. A recent discovery by our laboratory has revealed two closely linked genes (*invA* and *invB*) which are able to convey the ability to invade human erythrocytes upon minimally invasive strains of *E. coli*. One of the genes showed a high degree of homology to another invasion-associated protein (Ail) from *Y. enterocolitica* (28). Invasion by *B. bacilliformis* probably relies on a combination of some of these factors possibly along with determinants yet to be discovered.

1.3 Phylogeny, patriarchal and present

The *Rickettsiales* order contains three families; *Rickettsiaceae, Bartonellaceae,* and *Anaplasmataceae* (48,49). *B. bacilliformis* has historically been placed in the *Rickettsiales* order based on its 16S rRNA sequence homology, cell size, and vector-mediated route of transmission (5,6). However, this phylogeny has been in question due to the bacterium's unique motility with its 1 to 10 polar flagella, and the fact that it can be grown axenically on blood-containing media. Recently the taxonomy of rickettsiae, utilizing the 16S rRNA sequencing studies by Weisburg and others (45,46,47), was re-examined. Utilizing the data from these earlier studies along with new data generated using DNA hybridization techniques, it has been proposed that the genera *Bartonella* and *Rochalimaea* do not belong in the order *Rickettsiales*. The evidence supports the transfer of the genus *Rochalimaea* from the family *Rickettsiaceae* to the family *Bartonellaceae*

and that the bacteria within genus *Rochalimaea* should now be considered as *Bartonella* species. The family *Bartonellaceae* would then be removed from the order *Rickettsiales* (5,6,7,30,32,33,34,35,50).

1.4 Objectives and goals

Given the importance of *Bartonella's* flagella in host cell invasion (3,40), we wanted to characterize the flanking regions of the flagellin gene (*fla*I). In this study we utilized a previously constructed 3858-bp clone (pAULI) containing the *flaI* gene of *B. bacilliformis*, to produce a 1695-bp subclone (pBKCI) to analyze the *flaI* 3' flanking region. The pBKCI plasmid was then double-strand sequenced and the resulting nucleotide sequence was fused with GenBank accession #L20677 containing the *fla*I gene. The fusion of these two sequences resulted in a 3858-bp sequence containing the *fla*I gene within the first 1484-bp and the genes characterized in this study within the remaining 2374-bp. Resulting putative ORF's were then analyzed and mapped. We then performed *In vitro* transcription/translation and analyzed the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on both pAULI and pBKCI plus their respective vectors pBK-CMV and pUC-19. Southern blot analysis of chromosomal DNA was then performed in order to confirm that the insert of pBKCI was unique to *B. bacilliformis* and was not present in *E. coli*. The largest ORF, *xuoB*, and its encoded protein, XuoB, were further analyzed with respect to homology, hydropathy, codon usage, signal sequences, and secondary structure prediction analysis.

Chapter II

Materials and Methods

BACTERIAL STRAINS AND GROWTH CONDITIONS

B. bacilliformis strain KC583 and KC584 were purchased from the American Type Culture Collection, Rockville, MD. *Bartonellae* were grown under high humidity conditions at 28° C on heart infusion agar (Difco Laboratories, Detroit, Michigan) enriched with 5% defribrinated sheep red blood cells and 5% filter-sterile sheep serum by volume (Colorado Serum Co., Denver, Colorado). *B. bacilliformis* cells were harvested 4 to 6 days post-inoculation. *Escherichia coli* strain DH5α was utilized in cloning and *E. coli* strain HB101 was utilized in DNA hybridization experiments. All *E. coli* strains were grown overnight in LB broth at 37° C with constant shaking. LB medium was supplemented with 0.1 mg/ml ampicillin or 0.05 mg/ml kanamycin when needed.

DNA ISOLATION AND PURIFICATION

B. bacilliformis chromosomal DNA was isolated by the methods of Ausubel et al. (2). Plasmid pAULI, a 3858-bp *Bam*HI insert in the vector pBK-CMV contains the flagellin gene *flaI*, and was obtained from a lambda zap expression library of *B. bacilliformis* by screening with rabbit anti-flagellin antiserum generated as previously described (40). Subcloning of pBKCI was done by fractionating the desired insert DNA on an ethidium-bromide stained 1% agarose gel followed by purification utilizing a GeneClean kit (Bio 101 LaJolla, California). The purified DNA fragment was then ligated

into the vector pUC19 (51) (Figure 2) by standard protocol (38). Transformation of *E. coli* DH5α with pUC19 recombinants was done by the methods of Chung et al. (8). Plasmids were extracted and purified using standard alkaline extraction (4). Large scale isolations of the purified plasmids were performed using a QIAGEN Midi-prep kit per the manufacturer's instructions (QIAGEN, Chatsworth, California).

DNA HYBRIDIZATION

Chromosomal DNA was isolated from *E. coli* strain HB101 and *B. bacilliformis* strains KC583 and KC584 (2) and digested to completion with *Bam*HI. Plasmid DNA from pBKCI was isolated and digested to completion with *Hin*dIII. Separation of DNA was then performed on an ethidium bromide-stained 1% agarose (w/v) gel. Blotting of the gel to 0.45υm pore size nitrocellulose (Schleicher & Schuell, Keene, N.H.) was done by the methods of Southern et al. (42). The nitrocellulose was then baked at 80^0C for 1 hour. The 1695-bp *Hin*dIII fragment of pBKCI was obtained from an ethidium-bromide stained 1% agarose (w/v) gel and purified with a GeneClean (Bio101) kit. The pBKCI insert DNA was then labeled by random primer extension (11) using [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.) and the Klenow fragment of *E. coli* polymerase I (Gibco-BRL, Gaithersburg, MD) and subsequently used as the probe. Probing of the nitrocellulose blot with the ^{32}P-labeled pBKCI insert was done overnight at 50^0C and was washed at high stringency (65^0C) as previously described (27). The blot was then exposed on Kodak (Sigma) XAR-5 X-OMAT film.

Figure 1. **Vector pBK-CMV (Stratagene Inc.) used to generate pAULI.**

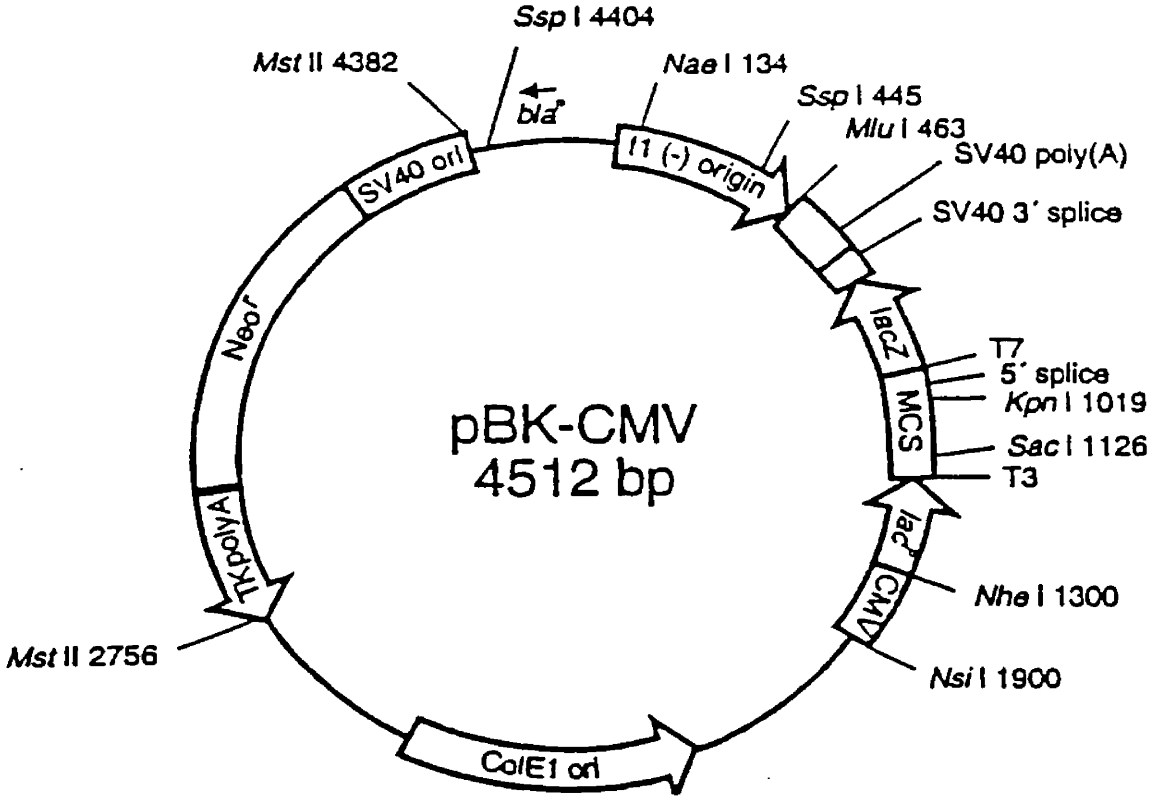
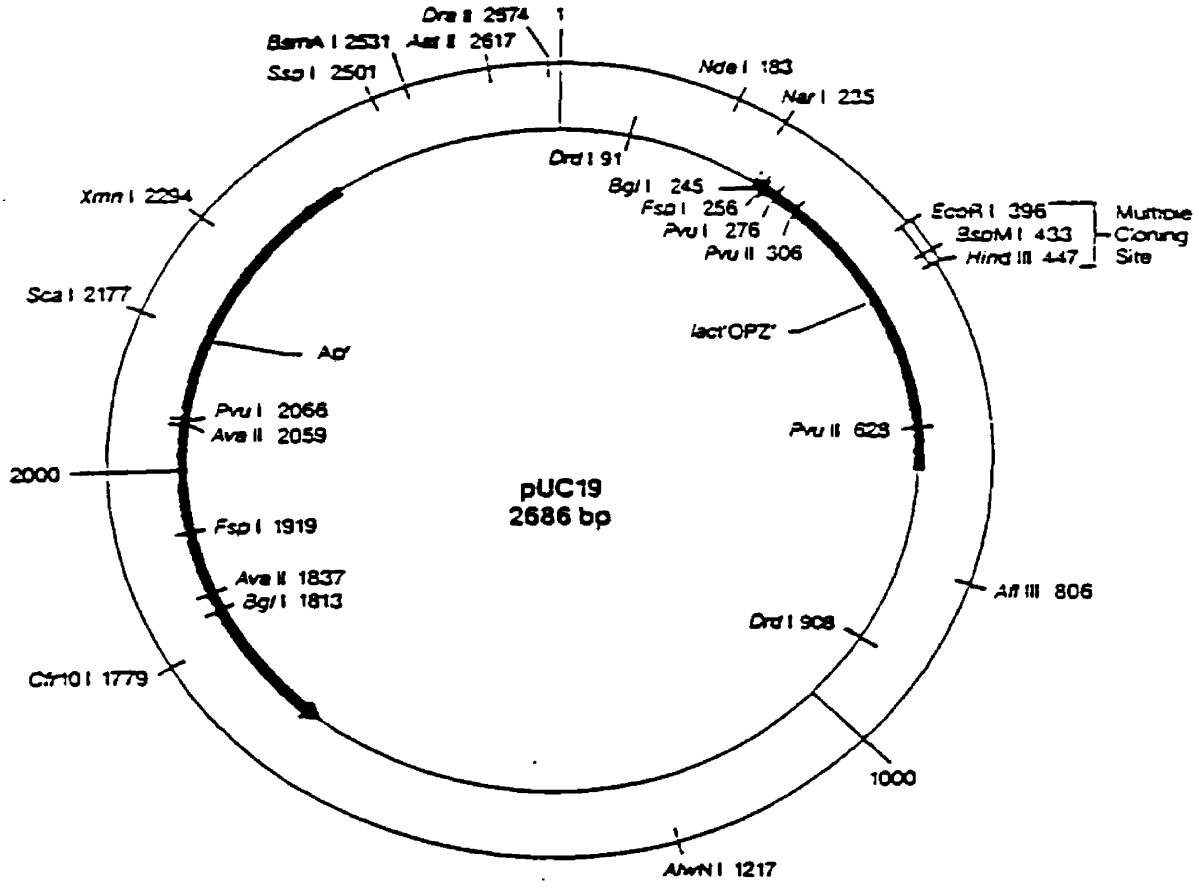

Ssp I 4404
Mst II 4382
Nae I 134
Ssp I 445
Mlu I 463
SV40 poly(A)
SV40 3' splice
bla
f1 (-) origin
SV40 ori
lacZ
T7
5' splice
Kpn I 1019
MCS
Sac I 1126
T3
Neor
pBK-CMV
4512 bp
lacI
CMV
Nhe I 1300
Nsi I 1900
TKpolyA
Mst II 2756
ColE1 ori

Figure 2. Vector pUC19 (51) used to generate pBKCI


Ora I 2574
1
BamH I 2531
Sso I 2501
Aat II 2617
Nde I 183
Nar I 235
Ora I 91
Bgl I 245
Fsp I 256
Pvu I 276
Pvu II 306
EcoR I 396
BspM I 433
Hind III 447
Multiple
Cloning
Site
lacI'OPZ'
Pvu II 623
Aat III 806
Ora I 908
1000
AhaN I 1217
Cla I 1779
Fsp I 1919
Ava II 1837
Bgl I 1813
2000
Pvu I 2066
Ava II 2059
Amp'
Sca I 2177
Xmn I 2294
pUC19
2686 bp

DNA SEQUENCING

Double stranded sequencing of the *Hind* III insert of pBKCI was performed by the methods of Sanger et al. (39) using an ABI model 373A automated nucleic acid sequencer (Applied Biosystems, Foster City, Calif.). Sequencing primers included M13 universal forward and reverse, plus primers designed from the template synthesized by an ABI model 394 DNA synthesizer (Applied biosystems). Translational start and stop codons, hydropathy plots, codon usage tables, signal sequence analysis, predicted secondary structure, and homology analyses were performed using PCGENE 6.8 software (Intelligenetics, Mountain View, Calif.) and the National Center for Biotechnology Information's sequence-homology search program, BLAST (1).

GENE EXPRESSION

DNA of pUC19, pBKCI, pAULI, and pBK-CMV was purified using a QIAGEN Midi-prep kit per the manufacturer's instructions (Qiagen, Chatsworth, California). *In vitro* transcription and translation of these plasmids was then performed utilizing a prokaryotic DNA transcription/translation kit (Amersham Co., Arlington Heights, Illinois). Proteins were prepared for analysis by labeling with an [^{35} S] methionine/cysteine mix (Express;NEN, Boston, Mass.), and boiling for 10 minutes in Laemmli sample buffer (LSB) (22). Denatured samples were fractionated on a 0.1% SDS-PAGE gel (12.5% acrylamide; w/v) and the gene products of pBKCI and pAULI DNA were visualized by exposing the dried gel overnight to KODAK (Sigma) XAR-5X-OMAT film.

CHAPTER III

RESULTS

3.1 DNA SEQUENCING

Mapping of the *Hin*dIII insert of pBKCI allowed for a computer-generated fusion of the sequence with GenBank accession #L20677 (sequence containing *fla*I) producing the entire nucleotide sequence of pAULI. A partial restriction map of the *Bam*HI insert of pAULI is shown in Figure 3. Nucleotide sequencing of pBKCI using M13 universal forward and reverse primers along with synthetic oligodeoxynucletide primers indicated 3 ORF's of 62-bp, 152-bp, and 717-bp lengths are present. These ORF's have been designated *orf1*, *orf2*, and *xuoB*, respectively. All three of these ORF's are in opposite orientation to the *lacZ'* promoter on the pUC19 cloning vector, and all three have potential endogenous promoter sites.

The *xuoB* gene, a 717-bp ORF, begins at nucleotide number 1423 and utilizes a GTG translational-start codon and extends through to nucleotide number 2140, a TAA stop codon (Figure 4). A putative promoter region was located 44 bases 5' to the start codon containing a -35 region (TTGCTT) and a -10 region (TGTCAT). Ten bases separates these hexameric sequences which have similarities to the *E. coli* consensus promoter sequence (23). A putative ribosomal binding site (RBS) was located 13 bases 5' to the start codon. This polypurine-rich sequence (GGGA) is similar to other RBS sequences previously discovered in *E. coli* (15,43). Analysis of the region 3' to the stop codon revealed a 5-nucleotide inverted repeat located 17 bases downstream from the stop codon which may serve as a ρ–dependent transcription terminator (37).

Figure 3. **Partial restriction endonuclease map of the pAULI and pBKCI inserts**. Bold arrows indicate positions of open reading frames, *xuoB, orf1,* and *orf2*. Arrowheads indicate direction of *lacZ'* promoter in the pUC19 and pBK-CMV cloning vectors of pBKCI and pAULI, respectively.

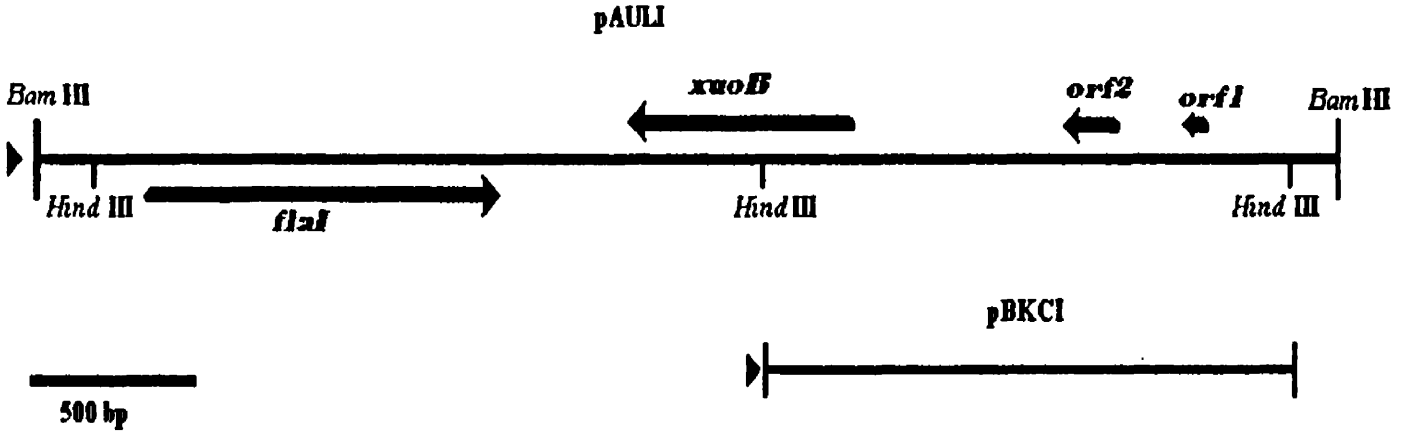

pAULI
Bam III
xuoB
orf2
orf1
Bam III
Hind III
flaI
Hind III
Hind III
pBKCI
500 bp

Figure 4. **Nucleotide sequence analysis of the pBKCI insert.** ORFs are shown in bold capital letters and from top to bottom include *orf1*, *orf2*, and *xuoB*. Putative -35 and -10 promoter regions are indicated along with the predicted ribosomal binding sites (RBS). Stop codons are indicated with a bold asterisk. Predicted ρ-dependent termination signals are indicated with bold opposing arrows.

```
                    ccatgaagacacaaaaacaccgatgatttttcaggcatttgcgtttt
                    gcttagccttgatcttattcccgcttttctcagcacgaggcatagtcat
                    ctctgcatgggtcaatacactcttactgtgcacagccctgatcgaaac
181-  attcctaaaaatcggggaaatgaatagactgagtagcaagaatgtttgccaaatagttgct
                          -35       -10              RBS   M  S  G  V
241-  gtctttaatggtaaaaTAGACCAtccTATAATtgtagcgattaGCCAcATGAGTGGTGTA
       R  E  R  R  R  L  S  C  I  F  Q  R  V  M  R  L  M  *
301-  AGAGAACGGCGCAGATTATCCTGCATTTTCCAGCGTGTTATGAGGTTAATGTGAcggttc

361-  tcaaagagatagggcaataattgccaatcaccgcgtgtccaacgatgatggcgagcaaca
421-  tcaacattataagctgtaggataatcttcaattacttctacatcactaactagcgcggca
                                                          -35
481-  cgtgcatagccaccttctaaaagatctgatcaccttcttttgctgccttTAATGtgcctg
                          -10 RBS  M  A  K  P  T  N  K  A  H  T  I
541-  taacaacagcttcctgtccAATATAAAGATGGCAAAACCCACCAATAAGGCCCATACCAT
       Q  L  A  C  F  F  L  E  T  A  N  Q  K  H  F  T  I  S  I  D
601-  ACAATTGGCCTGCTTTTTCCTCGAAACGGCGAATCAAAAGCATTTCACGATAAGCATTGA
       F  L  F  F  R  K  M  G  Y  L  R  C  P  T  Y  R  L  S  S  I
661-  TTTCCTCTTCTTTCGTAAAATGGGCTATTTGCGCTGTCCTACATACCGTCTTAGCAGTAT
       *
721-  TTAAtaatgcagcattctcaaccgatgcagaatttttttgatccgttctgccatattca
781-  actcccttcgatgacgacccgtaaataacccctacgaacaaaagttacaagcattataaa
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1141-  taaatatgatagctgaaatagcttacaacccaaaccgtcataacgggcaatataaagtgc
1201-  gttttgattgatcatacaagtggtggtggatttctctatcaccaagaaaacaacttagtt
1261-  tctgtcggctttgttgttcacttggattataaaaatccttatctttctccttttgaagaa
1321-  tttcagcgctttaaaacacaccctaacttgtatgaaatctttaaagatgccaaacgccTT
      -35              -10              RBS              V  P  K  L  T  F
1381-  GCTTatggcgcacgTGTCATcagtgaaggGGGAtggcaatctGTGCCAAAACTCACTTTT
       P  G  G  A  L  I  G  C  A  A  G  F  I  N  V  P  R  I  K  G
1441-  CCTGGTGGTGCACTGATTGGCTGTGCTGCTGGCTTTATCAATGTTCCTCGGATTAAAGGC
       S  H  N  A  I  L  S  G  I  L  A  A  D  K  I  A  D  A  L  A
1501-  TCACATAATGCCATATTATCTGGCATATTGGCAGCCGATAAAATTGCTGACGCTCTTGCA
       K  N  R  S  H  D  E  V  K  E  I  E  D  Q  W  R  K  G  P  I
1561-  AAAAATCGCTCTCATGATGAAGTTAAAGAAATTGAAGATCAATGGCGCAAGGGCCCTATT
       G  K  D  L  Y  K  V  R  N  T  K  P  L  W  A  K  Y  G  T  K
1621-  GGAAAAGACCTTTACAAAGTGCGTAATACCAAACCCCTTTGGGCAAAATACGGCACAAAA
       Y  G  I  K  L  A  G  F  D  L  W  W  Q  Q  L  F  S  F  S  L
1681-  TATGGAATAAAGCTTGCTGGATTTGATCTATGGTGGCAACAGCTCTTCAGCTTTTCCTTA
       F  R  T  L  S  H  G  K  P  D  H  E  Y  L  E  P  A  K  K  F
1741-  TTTAGAACTCTTTCTCATGGAAAACCAGATCATGAATATCTTGAACCCGCAAAAAAATTT
       Q  P  I  P  Y  P  K  P  D  G  I  V  T  F  D  R  L  S  S  V
1801-  CAACCTATTCCTTATCCAAAACCAGATGGTATTGTGACTTTCGATCGCCTTTCAAGTGTT
       A  L  S  N  T  H  H  E  D  N  Q  P  C  H  L  K  I  T  S  L
1861-  GCGCTTTCCAATACCCACCATGAAGACAACCAACCTTGTCATTTGAAAATAACTTCACTG
       E  K  Q  K  N  S  E  Y  A  I  Y  G  G  P  S  T  R  Y  C  P
1921-  GAAAAACAAAAAAATTCTGAATATGCAATCTACGGAGGACCTTCTACACGTTATTGCCCT
       A  G  V  Y  E  W  L  T  P  N  D  H  K  T  Y  I  I  N  A  S
1981-  GCTGGTGTCTATGAATGGCTGACCCCTAATGATCATAAAACGTATATCATCAATGCTTCA
       N  C  I  H  C  K  T  C  D  I  K  D  P  N  Q  N  I  N  W  T
2041-  AACTGCATACATTGTAAAACATGTGACATTAAAGACCCAAACCAAAATATTAACTGGACT
       C  P  Q  G  N  E  G  P  V  Y  P  N  M  *                 ---
2101-  TGTCCTCAAGGTAATGAAGGACCTGTTTATCCAAATATGTAAtcagcacctcatcacCTC
       -->   <----
2161-  TTGctaCAAGAtccgttctttctaaaaatgtatcatttat
```

A codon usage table generated illustrates the AT richness of this bacterium. *B. bacilliformis* has an obvious codon bias in the 3rd position of codons. Of all possible codon combinations for the amino acids shown in this table, *B. bacilliformis* utilizes an A or T in the 3rd position of the codons whenever possible. For example, Lysine has an A in the third codon position 20 times in comparison to a G used in the third codon position only 2 times (Table 1).

Table 1. **Codon usage table analysis of *xuoB*.**
Percentages of each codon used by *xuoB* are given.

TTT	Phe	6	2.4%	TCT	Ser	6	2.4%	TAT	Tyr	8	3.3%	TGT	Cys	5	2%
TTC	Phe	2	.8%	TCC	Ser	2	.8%	TAC	Tyr	3	1.2%	TGC	Cys	2	.8%
TTA	Leu	2	.8%	TCA	Ser	5	2%	TAA	---	1	.4%	TGA	---	0	0%
TTG	Leu	2	.8%	TCG	Ser	0	0%	TAG	---	0	0%	TGG	Trp	6	2.4%
CTT	Leu	8	3.3%	CCT	Pro	11	4.5%	CAT	His	8	3.3%	CGT	Arg	2	.8%
CTC	Leu	2	.8%	CCC	Pro	2	.8%	CAC	His	1	.4%	CGC	Arg	3	1.2%
CTA	Leu	1	.4%	CCA	Pro	6	2.4%	CAA	Gln	7	2.8%	CGA	Arg	0	0%
CTG	Leu	3	1.2%	CCG	Pro	0	0%	CAG	Gln	1	.4%	CGG	Arg	1	.4%
ATT	Ile	9	3.7%	ACT	Thr	5	2%	AAT	Asn	11	4.5%	AGT	Ser	1	.4%
ATC	Ile	4	1.6%	ACC	Thr	3	1.2%	AAC	Asn	4	1.6%	AGC	Ser	1	.4%
ATA	Ile	5	2%	ACA	Thr	3	1.2%	AAA	Lys	20	8.2%	AGA	Arg	1	.4%
ATG	MET	1	.4%	ACG	Thr	1	.4%	AAG	Lys	2	.8%	AGG	Arg	0	0%
GTT	Val	4	1.6%	GCT	Ala	7	2.8%	GAT	Asp	8	3.3%	GGT	Gly	5	2%
GTC	Val	1	.4%	GCC	Ala	2	.8%	GAC	Asp	5	2%	GGC	Gly	6	2.4%
GTA	Val	0	0%	GCA	Ala	6	2.4%	GAA	Glu	10	4.1%	GGA	Gly	7	2.8%
GTG	Val	3	1.2%	GCG	Ala	1	.4%	GAG	Glu	0	0%	GGG	Gly	0	0%

3.2 DNA HYBRIDIZATION

High stringency DNA hybridizations (allowing for approximately 7% DNA mismatch) were performed in order to confirm that the pBKCI insert had originated from *B. bacilliformis*. Probing of Southern blots with the ^{32}P-labeled pBKCI insert showed hybridization signals of 7200 bp in both *Bam*HI-digested chromosome strains KC583 and KC584 of *B. bacilliformis*, and the pBKCI *Hin*dIII insert (positive control). *Bam*HI - digested chromosomal DNA from *E. coli* HB101 showed no hybridization signal. (Figure 5).

3.3 COMPUTER ANALYSIS OF XuoB

Using the search algorithm BLAST (1), a significant amino acid sequence identity (47.3%) between XuoB and a portion of a human electron transfer flavoprotein-ubiquinone oxidoreductase protein was discovered (Figure 6). Statistical analysis of this homology indicates a p value of 1x10 e -67. The predicted XuoB protein contains 237 amino acid residues and a predicted molecular mass of approximately 28,000 daltons. The protein contains alternating hydrophilic and hydrophobic regions (Figure 7), an isoelectric point of 5.74, and evidence that it may be a peripheral protein of the cytosol. Further computer analysis indicates that no secretory sequence is present in XuoB supporting the prediction that XuoB is a peripheral protein of the cytosol and not surface exposed (Figure 8). Secondary structure prediction of the protein reveals the particular helical, turn, coil, and extended conformations (Figure 9) predicted to be present in this protein.

Figure 5. **Detection of sequences with homology to the pBKCI insert in chromosomal DNA from *B. bacilliformis*..**
(A) Ethidium bromide-stained agarose gel (1% agarose; w/v) containing lanes: 1, λ*Hin*dIII DNA size standard; 2, *Bam*HI-digested chromosomal DNA *B.bacilliformis* strain KC583; 3, *Bam*HI-digested chromosomal DNA *B.bacilliformis* strain KC584; 4, *Bam*HI-digested chromosomal DNA of *E.coli* strain HB101; 5, empty; 6, *Hin*dIII fragment used for hybridization probe. (B) The corresponding autoradiograph after DNA hybridization with the ^{32}P-labeled pBKCI insert DNA. Open arrow indicates the hybridization signal from *B. bacilliformis* strains KC583 and KC584.. Bold arrows indicate the location of the *Hin*dIII insert of pBKCI producing the hybridization signals of approximately 7200 bp.

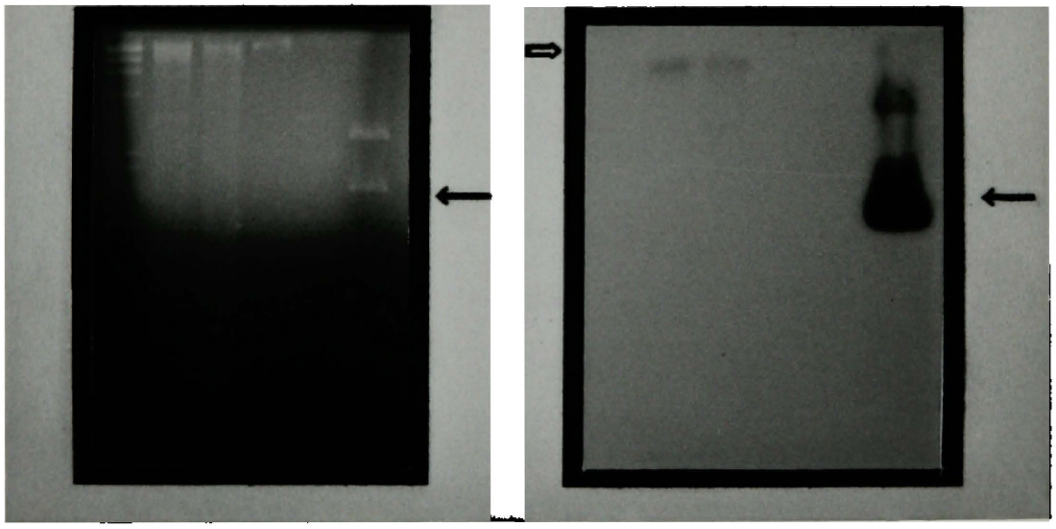

A
1 2 3 4 5 6
B
1 2 3 4 5 6

Figure 6. **Alignment of the *B. bacilliformis* XuoB protein with *Homo sapiens* electron transfer protein-ubiquinone oxidoreductase** (GenPept Accession # S69232). Amino acid residue numbers are to the left of each sequence. Solid lines represent exact identity between amino acid residues.


B. bacilliformis 1- VPKLTFPGGALIGCAAGFINVPRIKGSHNAILSGILAADKIADAL
H. sapiens 374- IPKLTFPGGLLIGCSPGFMNVPKIKGTHTAMKSGILAAESIFNQL
B. bacilliformis 46- AKNRSHDEV----------KEIEDQWRKGPIGKDLYKVRNTKPL-
H. sapiens 419- TS-----ENLQSKTIGLHVTEYEDNLKNSWVWKELYSVRNIRPSC
B. bacilliformis 91- ----------WAKYGTKYGIKLAGFDLWWQ
H. sapiens 464- HGVLGVYGGMIYTGIFYWILRGMEPW----------
B. bacilliformis 136- QLFSFSLFRTLSHGKPDHEYLEPAKKFQPIPYPKPDGIVTFDRLS
H. sapiens 509- ---------TLKHKGSDFERLKPAKDCTPIEYPKPDGQISFDLLS
B. bacilliformis 181- SVALSNTHHEDNQPCHLKITSLEKQKNSEYAIYGGPSTRYCPAGV
H. sapiens 554- SVALSGTNHEHDQPAHLTLRDDSIPVNRNLSIYDGPEQRFCPAGV
B. bacilliformis 226- YEWLTPNDHKTYII----------NASNCIHCKTCDIKDPNQNI
H. sapiens 599- YE---------FVPVEQGDGFRLQINAQNCVHCKTCDIKDPSQNI
B. bacilliformis 271- NWTCPQGNEGPVYP
H. sapiens 644- NWVVPEGGGGPAYN

Figure 7. **Hydropathy plot of the predicted XuoB protein of *B. bacilliformis* utilizing the method of Kyte and Doolittle (20).** Values above the dotted line indicate hydrophobic regions. Values below the dotted line indicate hydrophilic regions. Numbers on the x axis indicate amino acid residue number. Numbers on the y axis indicate the relative hydrophobicity.

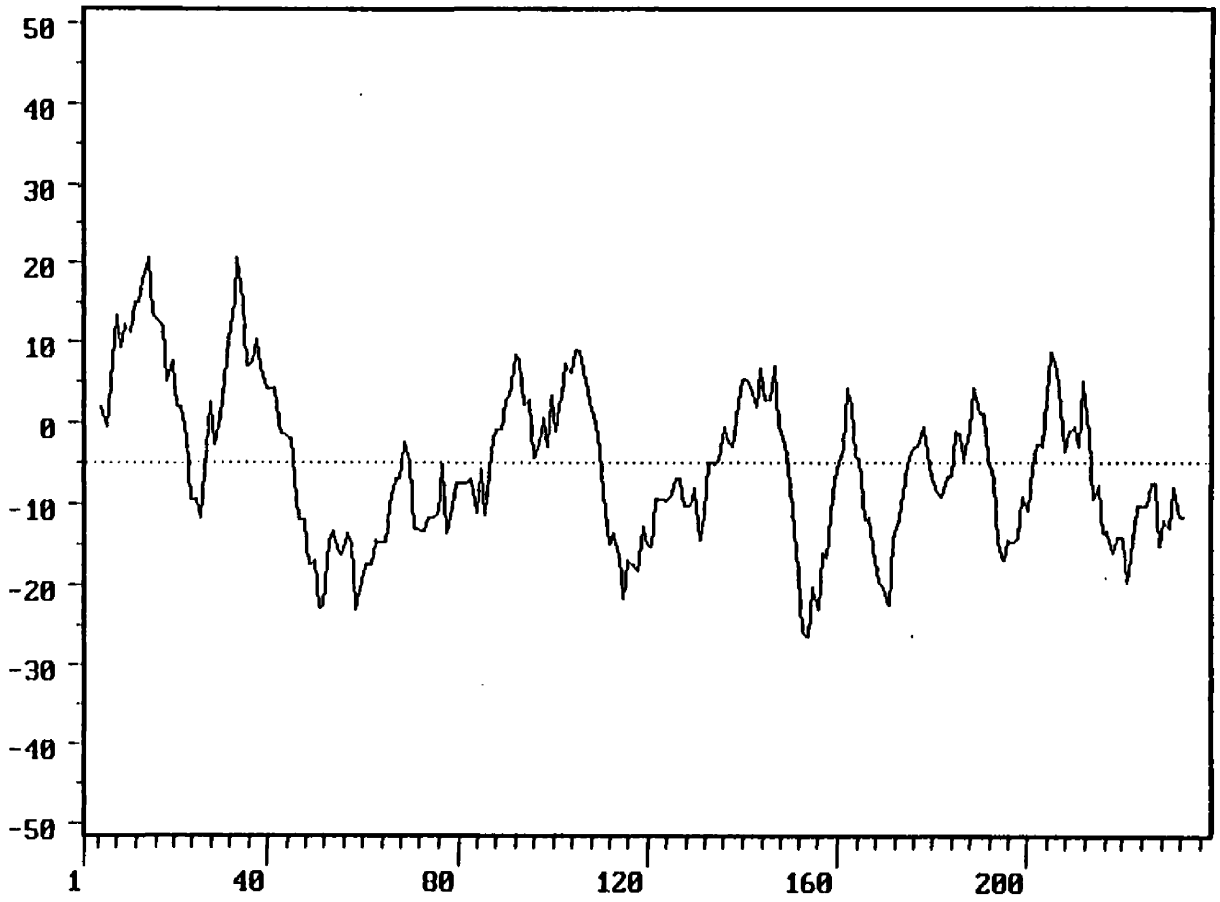
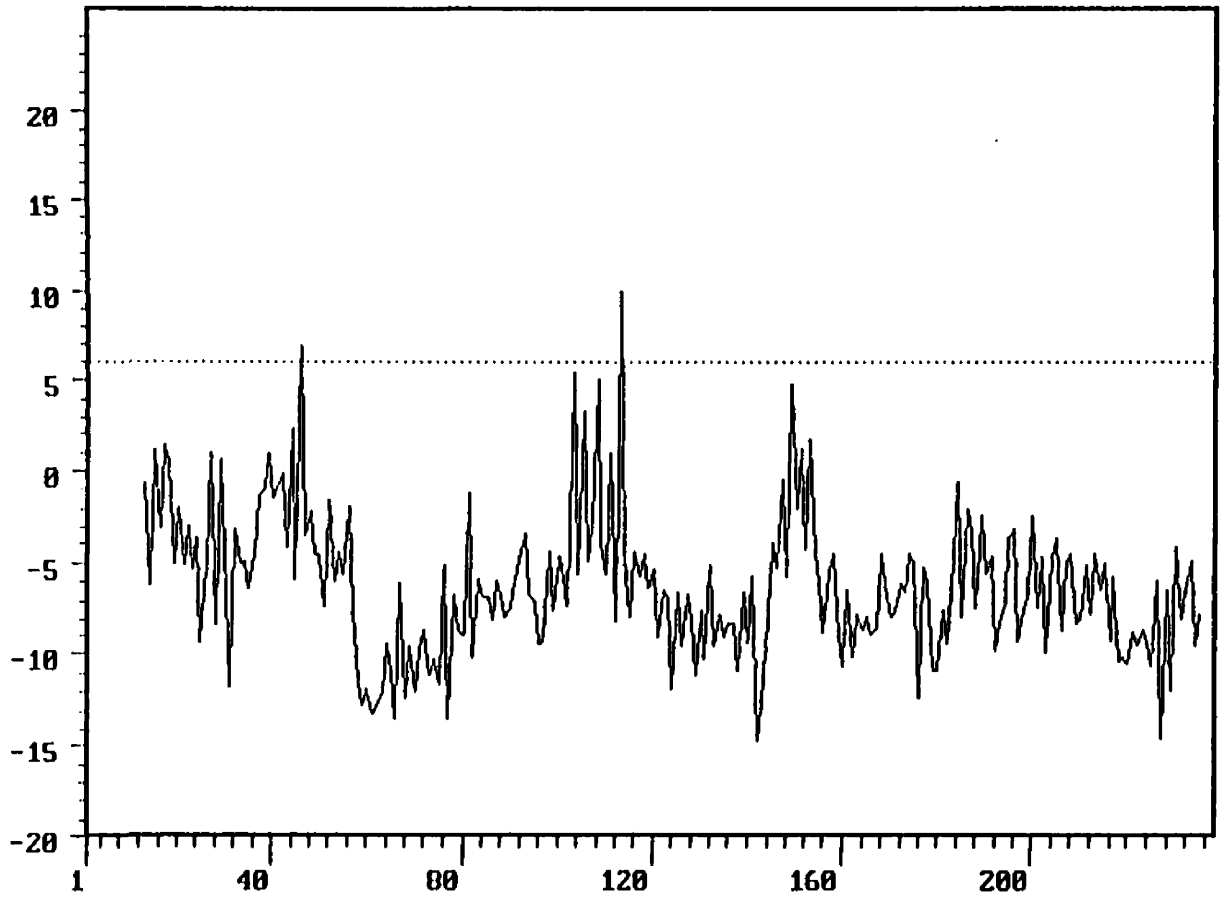

50
40
30
20
10
0
-10
-20
-30
-40
-50
1
40
80
120
160
200

Figure 8. **Secretory signal sequence analysis of the XuoB protein of *B. bacilliformis* as predicted by PCGENE ver. 6.8**
The x axis illustrates the amino acid residue number and the y axis illustrates the probability of a signal sequence present.


20
15
10
5
0
-5
-10
-15
-20
1
40
80
120
160
200

3.4 GENE EXPRESSION

Utilizing a prokaryote-directed DNA expression kit (Amersham), characterization of the proteins encoded by pAULI and pBKCI recombinants was performed. SDS-PAGE analysis of the *in vitro* products of pBKCI showed three insert-specific bands of 3 kDa, 7 kDa, and 10 kDa with no corresponding bands for these gene products in the cloning vector (pUC19)(Figure 10). The 10 kDa product is believed to be a result of partial XuoB gene being contained within pBKCI (Figures 2 and 10). SDS-PAGE analysis of the *in-vitro* products of pAULI indicate the predicted gene product of *xuoB* at 32 kDa with no corresponding band for this gene product seen from expression of the cloning vector pBK-CMV (Figure 11).

Figure 9. **Secondary structure prediction of the XuoB protein of *B. bacilliformis* by the method of Garnier (14).**
Amino acid residues and their numbers are given above the secondary structure prediction. Helical conformations are indicated by an (X), extended conformation by a (-), turn conformation by a (>), and coil conformations by an (*).

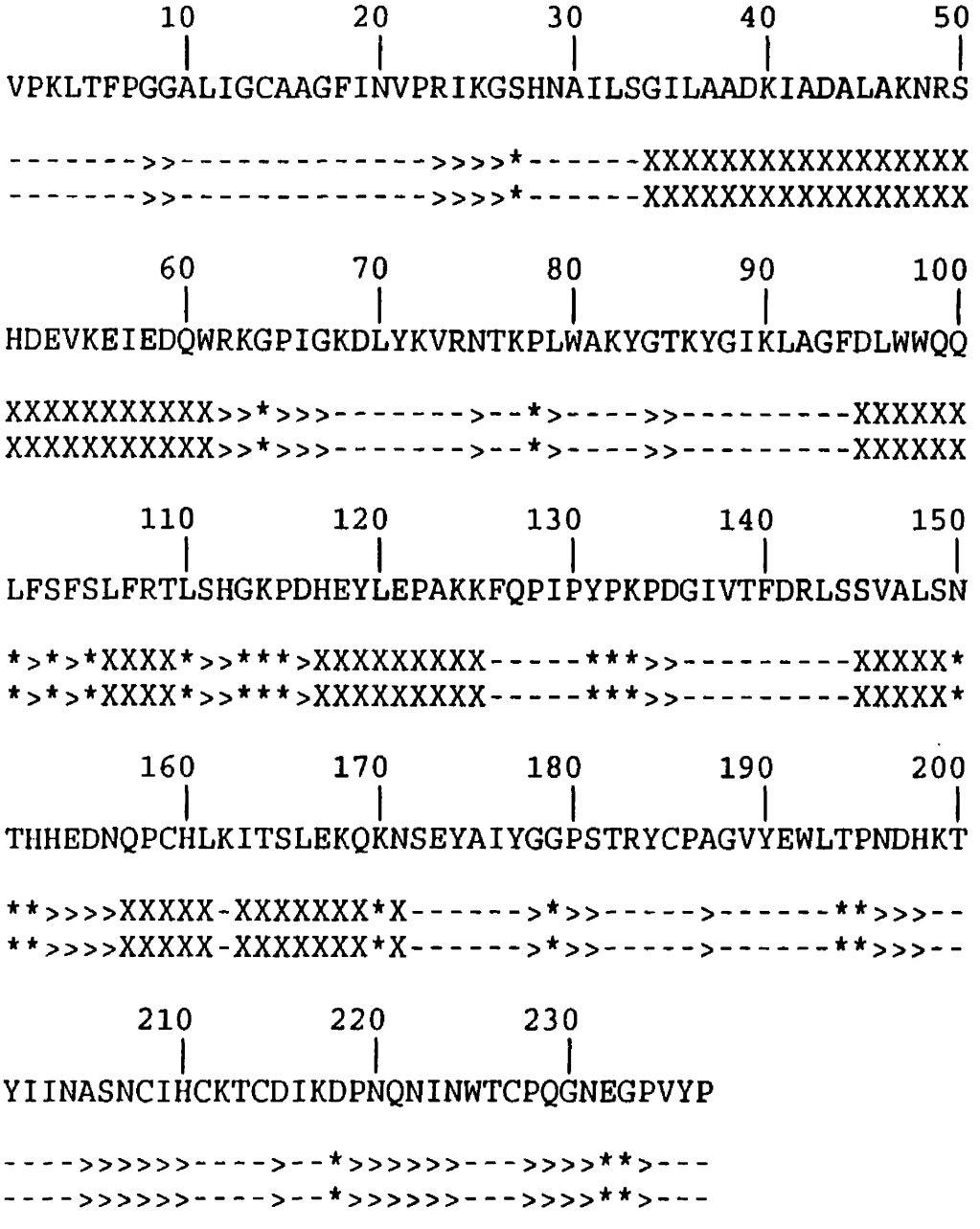

10 20 30 40 50
VPKLTFPGGALIGCAAGFINVPRIKGSHNAILSGILAADKIADALAKNRS
-------->>--------------->>>>*------XXXXXXXXXXXXXXXXX
-------->>--------------->>>>*------XXXXXXXXXXXXXXXXX
60 70 80 90 100
HDEVKEIEDQWRKGPIGKDLYKVRNTKPLWAKYGTKYGIKLAGFDLWWQQ
XXXXXXXXXXX>>*>>>-------->--*>---->>---------XXXXXX
XXXXXXXXXXX>>*>>>-------->--*>---->>---------XXXXXX
110 120 130 140 150
LFSFSLFRTLSHGKPDHEYLEPAKKFQPIPYPKPDGIVTFDRLSSVALSN
>>*XXXX*>>***>XXXXXXXXX-----***>>---------XXXXX*
>>*XXXX*>>***>XXXXXXXXX-----***>>---------XXXXX*
160 170 180 190 200
THHEDNQPCHLKITSLEKQKNSEYAIYGGPSTRYCPAGVYEWLTPNDHKT
>>>>XXXXX-XXXXXXX*X------>*>>----->------>>>--
>>>>XXXXX-XXXXXXX*X------>*>>----->------>>>--
210 220 230
YIINASNCIHCKTCDIKDPNQNINWTCPQGNEGPVYP
---->>>>>>----->--*>>>>>>--->>>>**>---
---->>>>>>----->--*>>>>>>--->>>>**>---

Figure 10. ***In-vitro*** **transcription and translation and SDS-PAGE analysis of pBKCI and its vector pUC19.**
Lanes: 1, ^{14}C-labeled protein standards with corresponding M_r values to the left in kDa; 2, pUC19 protein products; 3, pBKCI protein products. Arrows from bottom up indicate position of ORF1 (3 kDa), ORF2 (7kDa), and truncated XuoB (10 kDa) protein product respectively.

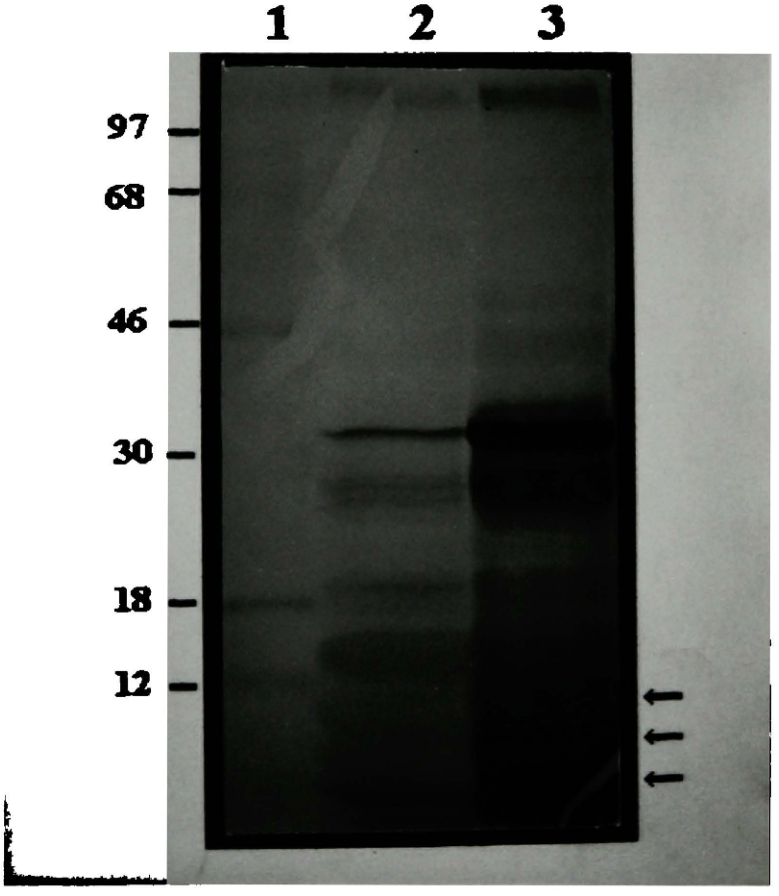
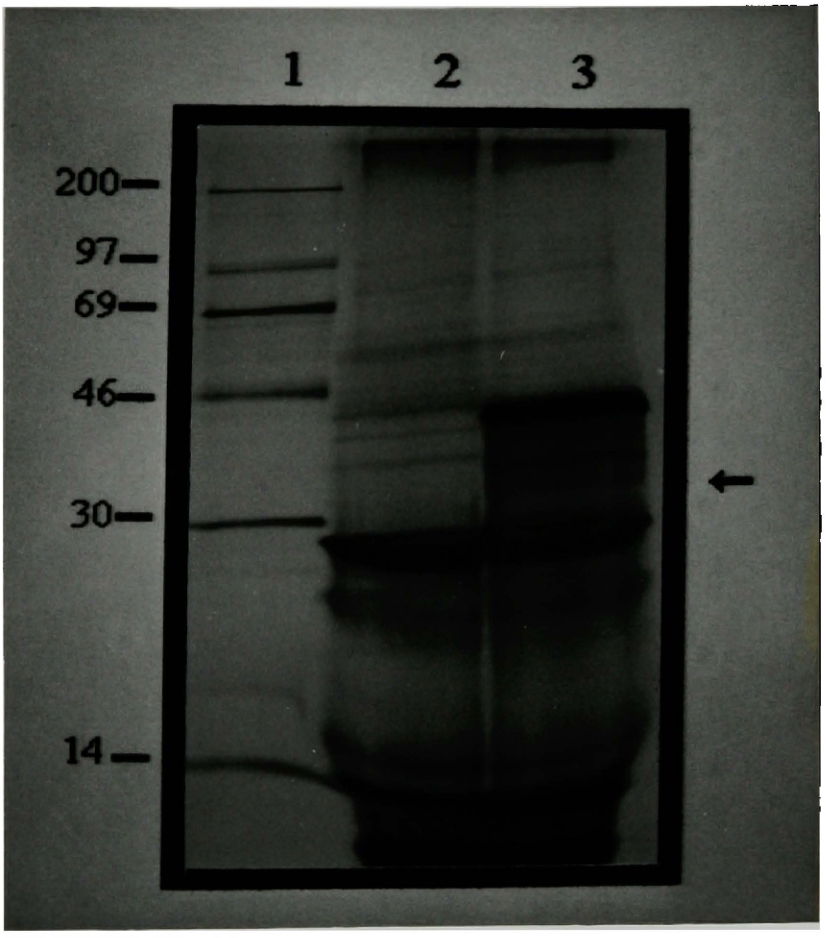

1
2
3
97
68
46
30
18
12

Figure 11. ***In-vitro*** **transcription and translation and SDS-PAGE analysis of pAULI and its vector pBK-CMV.**
Lanes: 1, ^{14}C-labeled protein standards with corresponding M_r values to the left in kDa; 2, pBK-CMV protein products; 3, pAULI protein products. Arrow indicates the full length XuoB (measured M_r of 32 kDa) protein product.


1
2
3
200
97
69
46
30
14

CHAPTER IV

DISCUSSION

Previous studies on *B. bacilliformis* have provided significant evidence supporting the importance of flagella for invasiveness. Irreversible deformation of the erythrocyte membrane by *B. bacilliformis* has been observed by Benson et al. (3) and in their studies they attributed this ability to the functional motility of the bacterium. They theorized that the motile bacteria were capable of creating enough force to "drill" their way into the erythrocyte thus causing, possibly along with other factors, the alteration seen in the erythrocytic membrane. This postulation was further supported when the authors found that nonmotile mutants were unable to produce this same deformation. Thus they theorized that the loss of motility greatly reduced the drilling effect or that nonmotile bacteria perhaps lacked a surface receptor required for attachment.

Mernaugh and Ihler (25) identified a proteinaceous factor which is released by *B. bacilliformis* and has the ability to deform the erythrocyte membrane in the absence of the pathogen. This deformation factor, termed deformin, is believed to be responsible for the invaginations seen in the erythrocytic membrane which Benson et al (3). believed was caused by a drilling effect from the flagella.

Hill et al. (17) performed invasion studies of human dermal fibroblasts, laryngeal epithelial cells, and umbilical vein endothelial cells by *B. bacilliformis*. They found that when bacteria were treated with a whole-cell antiserum, invasiveness was reduced by approximately 50%. These results correlate well with the 41% to 99.8% reduction in

invasiveness of erythrocytic cells by *B. bacilliformis* treated with an antiflagellin antiserum reported by Scherer et al. (40).

Walker and Winkler (44) reported that a significant number of bacteria were attached to erythrocytes by fiber-like structures emmenating from one pole of *B. bacilliformis.* These electron microscopy studies are very interesting since *B. bacilliformis* contains multiple polar flagella. Finally, the most recent study published on the invasiveness of *B. bacilliformis* reveals two genes which may also play a significant role in this bacterium's ability to invade. In this study, Mitchell and Minnick (28) discovered two invasion-associated genes in *B. bacilliformis* which, when transformed into a non-invasive strain of *E. coli,* were able to confer the ability to invade human erythrocytes.

All of these previous studies, although implying that many different virulence factors may be involved in conjunction with each other, still suggest that functional flagella are required for the bacterium to invade effectively. Determining the role that flagella play in virulence is important because the mechanism of the invasion process has not been elucidated and that one of the most severe hemolytic anemias occurring in humans results from this bacterium's ability to invade circulating erythrocytes. In the primary phase of this disease, *B. bacilliformis* infects nearly all the circulating erythrocytes in humans in as little as 3 days. Considering the damage this bacterium is capable of producing, and after examing all of the previous studies conducted on invasion by this bacteria, we set out to determine if other invasion-associated or motility-associated genes were located downstream of the *B. bacilliformis* flagella gene, *flaI.*

In this study we characterized 3 putative ORFs located 3' to the *flaI* gene of *B.*

bacilliformis. Nucleic acid sequencing of pAULI , utilizing a pBK-CMV vector, revealed the locations of these putative ORF's (Figure 3 and 4). By creating a 1644-bp *Hind*III subclone termed pBKCI, we were able to positively identify all 3 *in vitro* protein products of 3 kDa, 7 kDa, and 32 kDa, from these 3 putative ORF's (Figures 10 and 11). DNA hybridization analysis confirmed that the insert DNA originated from *B. bacilliformis* strains KC583 and KC584, and not from *E. coli* HB101 (Figure 5). We further characterized the largest ORF, *xuoB*, a 717-bp gene encoding a 32 KDa *in vitro* expressed protein termed XuoB.

Using a computer search algorithm (BLAST) (1) we found a 47.26% sequence identity to a human electron transfer flavoprotein-ubiquinone oxidoreductase (Figure 6). This human protein protein is encoded by a 617-bp ORF and computer prediction estimates a molecular mass of about 24 kDa compared to XuoB in this study of 32 kDa in size. Proteins of similar nature are believed to be responsible for the transfer of electrons to a mobile electron carrier coenzyme Q for the metabolism of lipids (22). Interestingly, homology searches produced no significant degree of homology to any known bacterial electron transfer proteins. The homology of *B. bacilliformis* XuoB to that of *Homo sapiens* is possibly due to some process requiring electron transfer. This protein may play a role in lipid metabolism or perhaps in the generation of ATP. A potential involvement in ATP generation could affect rotation of the flagella of *B. bacilliformis.* It is known that phosphorylation in bacteria comes from peripheral proteins located on the inner, phospholipid-containing cytoplasmic membrane, where a proton motive force is created by lowering the concentration of hydrogen ions in the cytosol. This allows for a difference

in electrical charge across the inner membrane to occur. The energy created from this proton motive force allows for several processes to occur. First, active transport of food molecules and inorganic ions are able to cross into the cytosol and second, the generation of ATP from ADP utilizing cytoplasmic membrane-bound ATP synthetases occurs. It is possible, that the XuoB protein in *B. bacilliformis* is involved in the generation of energy to drive the flagella.

Upon further analyzing the 32 kDa XuoB protein of *B. bacilliformis*, we were able to predict a secondary structure (Figure 9), and utilizing a hydropathy plot, we hypothesize that XuoB is peripherally located within the cytosol (Figure 7). Supporting evidence for it not being a surface protein is the absence of any predicted signal sequence (Figure 8).

A number of theories have been studied as to how this bacterium is able to invade t erythrocytes. Mechanical and chemical deformation factors (3, 40, 44), adherence and invasive factors (3,25), two newly discovered invasion genes capable of making a non-invasive *E. coli* strain invasive for erythrocytes(28), and finally flagellum-based motility (3, 40, 44), are all strategies believed to be used by the pathogen.

The central mechanism for *B. bacilliformis* invasiveness is thought to be the flagellar-associated motility. Drilling through erythrocytes by the direct power of the proton-motive force driven flagella has been demonstrated using light, scanning-electron, and transmission-electron microscopy (44). Adherence to human erythrocytes has also been theorized as dependent upon flagella in an analogous fashion to *Vibrio cholerae* (44). Finally, a recent publication by our laboratory revealed that monospecific anti-flagellin

antibody incubated with *B. bacilliformis* significantly reduced the bacterium's ability to invade human erythrocytes *in vitro*.

It was with this information in mind that we went searching for gene products which may facilitate this bacterium's ability to invade and/or might be involved in motility. What we discovered was a gene encoding a protein which may serve to transfer electrons in *B. baciliformis*. The actual role of XuoB in *B. bacilliformis* metabolism or pathogenesis remains to be determined.

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