

The gene encoding pyolysin, the pore-forming toxin of *Arcanobacterium pyogenes*, resides within a genomic islet flanked by essential genes

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Abstract

The *plo* gene, encoding the *Arcanobacterium pyogenes* cholesterol-dependent cytolysin, pyolysin (PLO), was localized to a 2.7-kb genomic islet of reduced %G+C content and alternate codon usage frequency. This islet, conserved among isolates from diverse hosts and geographical locations, separated the housekeeping genes *smc* and *ftsY*, which are found adjacent in many prokaryotes. The *ftsY* and *ffh* genes, located downstream of the *plo* islet, encode components of the signal recognition particle. Mutational analysis suggested that these genes were essential for viability in *A. pyogenes*. The *A. pyogenes ffh* gene was unable to complement a conditional *ffh* mutant of *Escherichia coli* and its overexpression was toxic in *E. coli*. Mutagenesis of the islet-encoded *orf121* did not affect *plo* expression, indicating that it may not be involved directly in the regulation of *plo* expression. Regardless, the presence of the *plo* gene as part of a genomic islet inserted between genes essential for normal growth may provide selective pressure for the retention of this important virulence factor. © 2003 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Pyolysin; Cholesterol-dependent cytolysin; Pathogenicity island; Signal recognition particle; Pathogenesis; Virulence

1. Introduction

Arcanobacterium pyogenes is a Gram-positive, facultative anaerobe that can exist in the host both as a commensal of the mucous membranes and as an opportunistic pathogen, invading after physical or microbial induced trauma [1]. As an opportunistic pathogen, *A. pyogenes* has been identified in a number of animal species, including cattle, where it has been isolated from liver abscesses [2] and mastitis [3], which have a significant economic impact on the beef and dairy industries.

A. pyogenes secretes a cholesterol-dependent cytolysin (CDC), pyolysin (PLO) [4], which is both a host-protective

antigen and virulence factor [5]. An *A. pyogenes plo* mutant was attenuated for virulence in a mouse model, and immunization with recombinant PLO protected mice from infection with wild-type *A. pyogenes* [5]. PLO is cytolytic for host cells including erythrocytes, murine peritoneal macrophages, and bovine and ovine polymorphonuclear leukocytes [5], through the formation of large pores in the host cell membrane, characteristic of the CDC family of toxins [6].

The genetic regulation of CDC expression is not well understood, although some are regulated by genes closely associated with the CDC structural gene. The *Listeria monocytogenes* CDC, listeriolysin O (LLO) is encoded, along with its positive regulator, PrfA, on the pathogenicity island LIPI-1 [7]. Similarly, *Clostridium perfringens* perfringolysin O (PFO) is encoded immediately downstream of its putative regulator, PfoR [8]. In an effort to identify factors involved in the expression of PLO, the nucleotide sequence of regions flanking the *A. pyogenes plo* gene was determined. Here we report the localization of *plo* and an upstream open reading frame (ORF), *orf121*, on a genomic islet flanked by conserved housekeeping genes.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

A. pyogenes strains were grown on brain heart infusion (BHI) agar supplemented with 5% bovine blood at 37°C with 5% CO₂ for 48–72 h, or in BHI supplemented with 10% bovine calf serum with shaking at 37°C. *Escherichia coli* was grown on Luria–Bertani (LB) agar at 37°C, or in LB broth with shaking at 37°C. The *E. coli* *ffh* conditional mutant WAM113 [9], in which *ffh* was under the control of the *araB* promoter, was grown on LB agar with 0.2% arabinose, for maintenance of wild-type levels of *ffh* expression. Media were supplemented with antibiotics at the following concentrations: for *A. pyogenes*: erythromycin (Erm) 15 µg ml⁻¹, kanamycin (Kan) 30 µg ml⁻¹, and streptomycin (Str) 200 µg ml⁻¹; and for *E. coli*: ampicillin (Amp) 100 µg ml⁻¹, chloramphenicol (Cam) 30 µg ml⁻¹, Erm 200 µg ml⁻¹, Kan 50 µg ml⁻¹, Str 50 µg ml⁻¹, and tetracycline 10 µg ml⁻¹.

2.2. Recombinant DNA techniques

A. pyogenes genomic DNA was isolated by the method of Pospiech and Neumann [10]; plasmid DNA isolation and electroporation protocols for *A. pyogenes* were performed as previously described [11]. *E. coli* plasmid DNA preparations and transformations, agarose gel electrophoresis, restriction endonuclease digestions, and ligation reactions were performed as previously described [12]. Polymerase chain reactions (PCRs) were performed on template derived from isolated colonies of *A. pyogenes* using *Taq* DNA polymerase (Fisher Scientific) and its supplied buffer in a standard protocol with an initial hot start for 5 min at 94°C, followed by 35 cycles consisting of 1 min at 94°C (DNA denaturation), 30 s at 55°C (primer annealing) and 1 min kb⁻¹ at 72°C (DNA synthesis).

2.3. Nucleotide sequencing and computer analysis

The *plo* gene was originally identified on the cosmid ApH1 and sequenced by Billington et al. [4]. Sequencing of the *plo* flanking regions was performed on subclones of ApH1 using either vector or insert specific primers (Sigma-Genosys). Sequencing was performed on both strands on a 377A DNA sequencer (Applied Biosystems Inc.) at the University of Arizona's Genomic Analysis Technology Core Facility. Sequences were assembled using Sequencher[™] 3.1 (GeneCodes), and database searches were performed using the BlastX and BlastP programs [13]. Further analyses, including the construction of %G+C and codon usage tables, analysis of patterns and possible structural motifs, and multiple sequence alignments were conducted using the GCG suite of programs (Accelrys).

The nucleotide sequence data reported in the paper have

been deposited in the GenBank nucleotide sequence database under the accession number U84782.2.

2.4. Allelic exchange mutagenesis

To construct an *A. pyogenes* strain carrying a mutation in *orf121*, a 2.4-kb PCR product containing *orf121*, amplified using primers SmcF1 (5'-GACGAGGTCGAGG-CACAC-3') and FtsYR3 (5'-GCAAGTTCCTCGTGT-CCG-3'), was cloned into the vector pHSS19 [14] to generate pJGS436. An Erm resistance cassette derived from pNG2 [15] was cloned into the unique *KpnI* site within *orf121*, which was previously blunt-ended with T4 DNA polymerase (New England Biolabs), to construct pJGS438, which was then introduced into BBR1 by electroporation. In strain JGS534, the wild-type copy of *orf121* was replaced by homologous recombination with the insertionally inactivated copy, as confirmed by PCR. To assess the effect of overexpression of *orf121*, a 1.3-kb *orf121*-containing fragment was cloned directly downstream of P_{lac} in pJGS181, a Str-resistant derivative of the multicopy shuttle vector pEP2, to generate pJGS440.

The construction of *A. pyogenes* *ftsY* and *ffh* mutants was carried out using both allelic exchange and vector integration strategies. A 1.4-kb PCR product containing *ftsY* was amplified using primers FtsYF1 (5'-GGTG-CGCTCTGATCGCGAAGGC-3') and FtsYR1 (5'-AAGCTGATTGACGGCGTTGGGG-3'), cloned into pHSS19 and disrupted at the unique *NruI* site with a 2.0-kb Str resistance cassette derived from pKRP13 [16], to construct pJGS222. Similarly, a 1.7-kb PCR product containing *ffh* was amplified using primers FfhF1 (5'-CTACCTGAGGGCATAATGTTTA-3') and FfhR1 (5'-CGTCAGTGCCCGCAAAGACCC-3'), cloned into pHSS19 and disrupted at the unique *EcoRV* site with the pKRP13 2.0-kb Str resistance cassette, to construct pJGS225. Each of these constructs was introduced independently into *A. pyogenes* BBR1 by electroporation, selecting for Str resistance, indicating allelic exchange. Alternatively, internal fragments of *ftsY* or *ffh* amplified with primers FtsYF4 (5'-GGACGTCTGGCCGCTTCTGG-TG-3') and FtsYR5 (5'-CCCACGTGGAGAGCTGATCGGC-3'), or FfhF2 (5'-AACGCCGTCAATCAGCTT-CAGG-3') and FfhR2 (5'-CTCCTCGCTCCAGGTCTT-TTCG-3') were cloned into pHSS19 to construct pJGS313 and pJGS315, respectively. These plasmids were introduced into BBR1, selecting for the vector-encoded Kan resistance and, therefore, disruption of either the wild-type *ftsY* or *ffh*, resulting in two incomplete copies of the gene.

2.5. Hemolytic assays

Hemolytic assays were performed on *A. pyogenes* culture supernatant essentially as described [4]. Hemoglobin release was measured at A₄₁₀, with one hemolytic unit

(HU) representing the amount of hemolysin required to release 50% of the hemoglobin from 200 μ l of 0.25% ovine erythrocytes in 1 h.

2.6. Complementation of an *E. coli* *ffh* conditional mutant

A 1.6-kb *NotI* fragment carrying the *A. pyogenes* *ffh* gene was blunt-ended with DNA polymerase I Klenow fragment (Promega) and cloned into *SmaI*-digested pDK7 [17] to construct pJGS369. In this vector, *ffh* is under the control of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible P_{tac} . pJGS369 and pDK7 were introduced into WAM100, and its conditional *ffh* mutant WAM113 by electroporation. The resulting strains were grown on LB agar plates supplemented with 1 μ M–10 mM IPTG to induce expression from the *ffh* gene on pJGS369, in the presence of decreasing arabinose (0.2–0.002%) to limit transcription from the endogenous *ffh* gene in WAM113. In a separate experiment, *E. coli* DH5 α derivatives carrying pDK7 or pJGS369 were inoculated into LB broth with or without 100 μ M IPTG to determine the effect of the *A. pyogenes* *ffh* gene on the growth of *E. coli*.

3. Results and discussion

3.1. The *A. pyogenes* *plo* gene and *orf121* are located on a genomic islet

In an attempt to identify genes physically linked to *plo* involved in the expression of PLO activity, the nucleotide sequence 2160 bp upstream and 4121 bp downstream of *plo* was determined. The organization of ORFs in the *plo* region is shown in Fig. 1. Identified in the sequence upstream of *plo* was one complete ORF, *orf121*, which encoded a 13.4-kDa protein with no homologs in the GenBank database, and the 3'-end of a partial ORF, which encoded a protein with similarity to Smc proteins from a

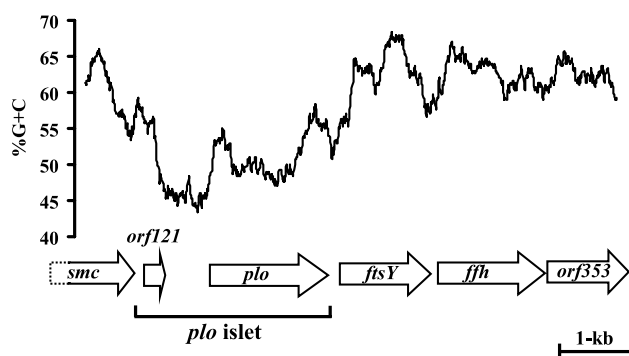


Fig. 1. The *plo* islet has a reduced %G+C content. The genetic map of the *plo* region of *A. pyogenes* strain BBR1 is shown below a plot of the %G+C content. The extent of the *plo* islet is indicated below the gene map. The %G+C plot was generated by the Window program (GCG). The scale is indicated by the 1-kb bar.

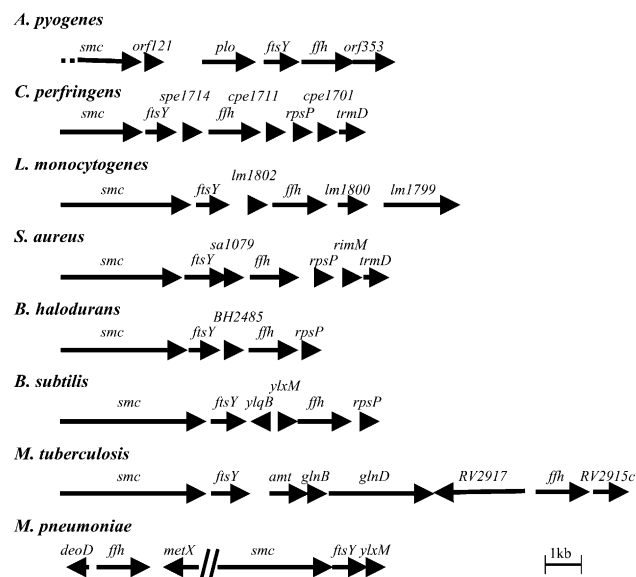


Fig. 2. Comparison of the *A. pyogenes* *smc-orf353* chromosomal arrangement to similar regions in other prokaryotic species. Arrangements and genetic distances were compiled from genomic sequences available in the GenBank database. The figure is shown to scale except that the *Mycoplasma pneumoniae* *smc* gene is separated from *metX* by 378 kb. The scale is indicated by the 1-kb bar. GenBank accession numbers for the completed genomes are as follows: *C. perfringens*: NC_003366, *L. monocytogenes*: NC_003210, *S. aureus*: NC_002745, *B. halodurans*: NC_002570, *B. subtilis*: NC_000964, *M. tuberculosis* H37Rv: NC_000962, and *M. pneumoniae*: NC_000912.

number of bacterial species, and most similarity to *Streptomyces coelicolor* Smc (67.9% identity, 78.5% similarity across the amino acids examined) (GenBank accession number T35661). Smc proteins are responsible for the structural maintenance of chromosomes and are essential for chromosome partitioning in *Bacillus subtilis* [18] and *E. coli* [19]. Downstream of the *plo* transcriptional terminator [4] were two ORFs which encoded proteins with similarity to the protein components of the signal recognition particle (SRP), FtsY and Ffh, from a number of bacterial species. Interestingly, the *A. pyogenes* FtsY and Ffh proteins shared the highest similarity with the *S. coelicolor* FtsY (58.2% identity, 66.8% similarity) (GenBank accession number CAA22423) and Ffh (74.7% identity, 65.4% similarity) (GenBank accession number CAA19378) proteins, respectively. Immediately downstream of *ffh* was *orf353*, whose translated product shared similarity to a *S. coelicolor* conserved hypothetical protein (37.2% identity, 48.7% similarity) [20].

The conserved *smc* and *fisY* genes are genetically linked in many prokaryotes, particularly Gram-positive bacteria (Fig. 2). In *A. pyogenes*, they are separated by *orf121* and *plo*, suggesting the insertion of a small genomic islet. Further analysis of this region identified a lower %G+C content for *orf121* (51.5%) and *plo* (54.5%) than the average %G+C content of *A. pyogenes* housekeeping genes (62.5%) (S.J. Billington, S.T. Rudnick and B.H. Jost, unpublished data) and genes flanking the proposed islet such as *smc*

(62.3%), *ftsY* (63.5%), *ffh* (62.3%) and *orf353* (62.1%) (Fig. 1). Furthermore, the codon usage of *plo* and *orf121* was different compared to that of *smc*, *ftsY*, *ffh*, and *orf353*, with a significant AT bias in the wobble position of the codons for Ala, Gln, Asn, Thr, and His within the *plo* islet. For example, 62% of His-encoding codons in non-islet genes are CAC, while in islet genes, 73% of these codons are represented by the alternate codon CAT.

The identification of *plo* on a genomic islet of reduced %G+C content and alternate codon usage is suggestive of horizontal transfer of this virulence gene. The acquisition of *plo* may have converted the commensal *A. pyogenes* into a potential pathogen, similar to the acquisition of the LIPI-1 pathogenicity island of *L. monocytogenes* [21]. The mechanism by which the *plo* islet was incorporated into the *A. pyogenes* chromosome is unknown. Insertion via transposition, or bacteriophage integration, seems unlikely, as neither integrase, transposon, insertion sequence, nor repeat sequences, often found associated with pathogenicity islands [22], were identified within or flanking the *plo* islet. However, it is possible that this islet may have inserted into the intergenic region between *smc* and *ftsY* via homologous recombination, as was hypothesized for *sly* on the *Streptococcus suis* chromosome [23]. The integration of the *plo* islet between *smc* and *ftsY* may provide a selective advantage for retention of the islet since both *smc* and *ftsY* are required for normal bacterial growth and deletion of the islet may affect expression of either of these genes.

3.2. The *plo* islet is conserved across geographically diverse isolates

To examine the distribution of the *plo* islet among different isolates, several bovine and porcine strains from geographically diverse locations were analyzed by PCR for conservation of this region. Primers to the 3'-end of *smc*, SmcF1 (5'-GACGAGGTCGAGGCAGCAC-3'), and the 5'-end of *ftsY*, FtsYR3 (5'-GCAAGTTCCTCGTGTCG-3'), were used to amplify a 3.2-kb product containing the entire *plo* islet. All isolates tested were positive for this amplification product (Fig. 3). Furthermore, PCRs using primers internal to *plo*, 2125 (5'-GGCCCGAATGTCACCGC-3') and 2127 (5'-AACTCCGCCTCTAGCGC-3'), in conjunction with SmcF1 or

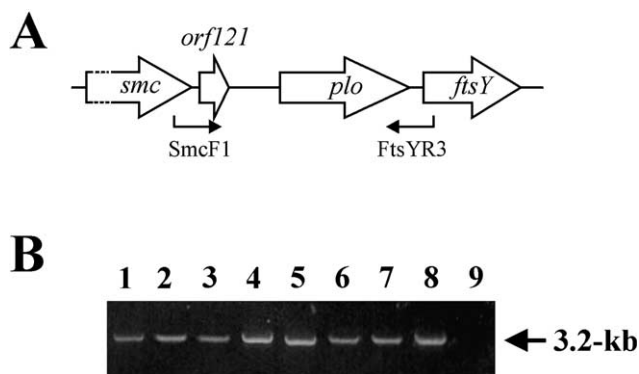


Fig. 3. The *plo* pathogenicity islet is conserved among *A. pyogenes* isolates. A: The positions of primers SmcF1 and FtsYR3, outside the *plo* islet are shown on the gene map. B: PCR products were amplified with primers SmcF1 and FtsYR3 from *A. pyogenes* strains (1) BBR1 (bovine isolate, Marana, AZ, USA), (2) ATCC19411 (porcine isolate), (3) ATCC9732 (bovine isolate), (4) E1DE (bovine isolate, Colorado State University, Fort Collins, CO, USA), (5) #67 (porcine isolate, Victoria Institute of Animal Science, Victoria, Australia), (6) 98-4277-1 (bovine isolate, Arizona Veterinary Diagnostic Laboratory, Tucson, AZ, USA), (7) 52785-99 (porcine isolate, Rollins Animal Disease Diagnostic Laboratory (RADDL), Raleigh, NC, USA), and (8) 14373-00-1 (porcine isolate, RADDL, Raleigh, NC, USA). A no template negative control is shown in lane 9. The 3.2-kb PCR product is indicated by the arrow on the right.

FtsYR3, confirmed the location of *plo* between *smc* and *ftsY* in each of the isolates (data not shown). These results indicate that the location of the *plo* islet is invariant, and that if it was horizontally acquired, its introduction occurred prior to isolate diversification and may represent an ancestrally derived virulence islet.

3.3. *orf121* does not regulate *plo* expression

Two homologs of PLO, LLO and PFO, are both regulated by proteins encoded by linked ORFs [7,8]. As *orf121* was identified immediately upstream of *plo*, and within the *plo* islet (Fig. 1), this ORF was considered a candidate for a regulator of PLO expression. To analyze the role of *orf121* on *plo* expression, an allelic exchange mutant of *orf121*, JGS534, was constructed. Hemolytic activities of culture supernatants collected at points during a growth curve were compared for JGS534 and BBR1. Growth rates and hemolytic activities in log and early stationary phase were similar between the two strains (Table 1), in-

Table 1
Role of *orf121* in PLO expression

Strain	Growth rate ^a (h)	Hemolytic activity ^b (HU ml ⁻¹)	
		Mid-log phase	Early stationary phase
BBR1	1.4	248	2087
JGS534	1.2	235	1982
BBR1(pJGS181)	1.6	123	743
BBR1 (pJGS430)	1.6	135	611

^aDoubling time in log phase.

^bEach value represents the average of duplicate assays from one representative experiment, and duplicate experiments demonstrated the same trends.

dicating that inactivation of *orf121* has no observable effect on *plo* expression under these conditions. However, the effects of *orf121* may be subtle, as has been observed with *pfoR* and *sloR*, putative regulatory genes of CDCs from *C. perfringens* and *Streptococcus pyogenes*, respectively [8,24]. To determine the effect of *orf121* overexpression on *plo* production, *orf121* was cloned downstream of P_{lac} , on pJGS440. BBR1 derivatives carrying either pJGS181 or pJGS440 were grown in broth culture with Str selection to retain the plasmid. Both strains demonstrated similar growth rates in log phase and similar hemolytic activities in both log and stationary phase (Table 1). While it is possible that *orf121*, or some *trans*-acting factor necessary for its effect on *plo* expression, is not expressed in either of these systems *in vitro*, the combined results of these experiments, including the fact that computer analyses did not identify Orf121 DNA binding motifs, suggest that *orf121* does not have a direct role in the expression of *plo* *in vitro*.

3.4. The *A. pyogenes* SRP protein component genes are physically linked and essential for growth

The *A. pyogenes* *ftsY*, *ffh* and *orf353* genes were oriented in an operon-like arrangement and may be co-transcribed (Fig. 1), as *ftsY* and *ffh* were separated by 85 bp and *ffh* and *orf353* by only 10 bp, and no obvious transcriptional terminators were identified in the intergenic sequences. This is an unusual arrangement for *ftsY* and *ffh* when compared to other bacteria. Analysis of the annotated prokaryotic genomes currently present in the GenBank database indicated that in only *C. perfringens*, *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus halodurans*, *B. subtilis*, and *Mycobacterium tuberculosis* were *ftsY* and *ffh* found in close proximity, but in each case separated by at least one ORF (Fig. 2), while in most prokaryotic species, *ftsY* and *ffh* were separated by over 100 kb. The association of these genes in *A. pyogenes* could be indicative of an ancestral organization, which has been dispersed through evolution.

In *E. coli*, SRP is primarily a targeting mechanism for inner membrane proteins (IMPs) [25], although in Gram-positive organisms, SRP may also function in the targeting of secreted products [26]. The identification of *ftsY* and *ffh* in close association with *plo* lead to the hypothesis that SRP may be involved in the targeting of PLO to the membrane for secretion. To address this hypothesis, strategies were devised to disrupt the *A. pyogenes* *ftsY* and *ffh* genes. Plasmid constructs carrying either *ftsY* or *ffh* disrupted by a Str resistance cassette, or constructs containing internal fragments of *ftsY* or *ffh* were introduced into BBR1. The former strategy attempted to disrupt the wild-type genes by allelic exchange, and the latter through a single crossover recombination event resulting in two defective copies of the target gene. Neither of these strategies resulted in viable recombinants with inactivated *ftsY* or *ffh* genes,

despite the fact that *plo* mutants of BBR1 could be isolated at a frequency of 2.5×10^2 mutants μg^{-1} plasmid DNA using a previously constructed *plo* mutagenic plasmid, pJGS79 [5]. While these experiments do not directly confirm that *ftsY* and *ffh* are essential in *A. pyogenes*, the number of failed attempts to construct stable mutants strongly suggests that they are essential under the conditions tested. Furthermore, these results are consistent with the essential nature of SRP genes in other bacteria [27].

3.5. The *A. pyogenes* *ffh* gene can not complement an *E. coli* conditional *ffh* mutant and has a dominant negative effect on normal *E. coli* growth

Since allelic exchange mutations in *ftsY* or *ffh* were not obtained in *A. pyogenes*, the ability of the *A. pyogenes* *ffh* gene to complement an *E. coli* *ffh* conditional mutant was investigated. *E. coli* WAM113, a conditional *ffh* mutant of WAM100, contains *ffh* under tight control of the *araB* promoter, such that depletion of arabinose results in severe growth defects [9]. The *A. pyogenes* *ffh* gene was placed under the control of P_{lac} , on pJGS369. To determine if the *A. pyogenes* *ffh* could circumvent the growth defects observed in the absence of arabinose in WAM113, a range of IPTG concentrations (1 μM –10 mM) was used to induce expression of *ffh* on pJGS369 in the presence of decreasing arabinose concentrations (0.2–0.002%) on LB agar. Analysis of WAM113(pJGS369) showed that while decreasing arabinose alone, as expected, negatively affected the growth of this strain, addition of IPTG did not rescue growth (data not shown). Furthermore, induction of the *A. pyogenes* *ffh* from pJGS369, with IPTG concentrations ≥ 100 μM , inhibited growth of WAM100(pJGS369). These results not only suggest that the *A. pyogenes* *ffh* does not complement the *E. coli* *ffh* deficiency, but that its expression is toxic to *E. coli*. The lack of complementation of WAM113 by the *A. pyogenes*

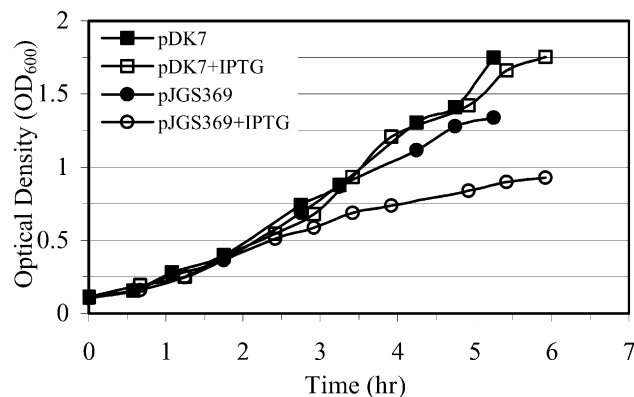


Fig. 4. Effect of heterologous expression of *A. pyogenes* *ffh* in *E. coli*. Growth curves of *E. coli* DH5 α strains carrying pDK7 or pJGS369. Strains were grown in LB broth with 30 $\mu\text{g ml}^{-1}$ Cam, with or without 100 μM IPTG. Data are shown from a single experiment representative of three independent experiments.

ffh gene was surprising as the *E. coli* and *A. pyogenes ffh* genes share 50.2% identity and 60.7% similarity. The *Streptococcus mutans ffh* gene has previously been demonstrated to complement the growth defect in WAM113 [28]. However, expression of the mammalian SRP 54-kDa homolog of Ffh in WAM113 was unable to complement pleiotropic defects characterized by cell elongation, and increased accumulation of precursor proteins [29].

To confirm the toxic nature of *A. pyogenes ffh* in *E. coli*, growth curves of DH5 α (pDK7) and DH5 α (pJGS369) were performed in the presence and absence of 100 μ M IPTG, levels that were found to be detrimental, but not completely inhibitory to WAM100(pJGS369). While the growth of DH5 α (pDK7) was not affected by the addition of IPTG, DH5 α (pJGS369) exhibited a significant decrease in growth in the presence of IPTG (Fig. 4).

These results suggest that *A. pyogenes ffh* encodes an active protein that affects normal growth of *E. coli*, but does not complement the loss of *E. coli* Ffh production. Therefore, expression of *A. pyogenes ffh* had a dominant negative effect on the normal functioning of the *E. coli* SRP system, probably resulting from the inability of *A. pyogenes* Ffh to interact with some components of the *E. coli* SRP, e.g. the *E. coli* FtsY, as is the case with the mammalian SRP 54 kDa [29]. In toto, these results suggest that the *E. coli* system cannot currently be used as a heterologous system to study the role of the *A. pyogenes* SRP on targeting of *A. pyogenes* proteins. Future analyses may require the construction of conditional expression strains of *A. pyogenes*.

4. Conclusions

The *A. pyogenes plo* gene was localized to a genomic islet conserved across geographically diverse isolates. The identification of this and other CDCs on genomic islets suggests that the introduction of these genomic regions may have increased the virulence of previously non-pathogenic bacteria. Furthermore, the presence of *plo* on a genomic insertion between two essential genes may provide selective pressure for the maintenance of this important virulence gene during periods when *A. pyogenes* is a commensal on the mucosal membrane of its host. While we report here that *orf121*, located on the *plo* pathogenicity islet, does not play a direct role in the expression of *plo* in vitro, its role in vivo has yet to be determined.

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