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Type 3 Fimbriae Encoded on Plasmids Are Expressed from a Unique Promoter without Affecting Host Motility, Facilitating an Exceptional Phenotype That Enhances Conjugal Plasmid Transfer

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Abstract

Horizontal gene transfer (HGT), the transmission of genetic material to a recipient that is not the progeny of the donor, is fundamental in bacterial evolution. HGT is often mediated by mobile genetic elements such as conjugative plasmids, which may be in conflict with the chromosomal elements of the genome because they are independent replicons that may petition their own evolutionary strategy. Here we study differences between type 3 fimbriae encoded on wild type plasmids and in chromosomes. Using known and newly characterized plasmids we show that the expression of type 3 fimbriae encoded on plasmids is systematically different, as MrkH, a c-di-GMP dependent transcriptional activator is not needed for strong expression of the fimbriae. MrkH is required for expression of type 3 fimbriae of the Klebsiella pneumoniae chromosome, from which the fimbriae operon (mrkABCDF) of plasmids is believed to have originated. We find that mrkABCDFs of plasmids are highly expressed via a unique promoter that differs from the original Klebsiella promoter resulting in fundamental behavioral consequences. Plasmid associated mrkABCDFs did not influence the swimming behavior of the host, that hereby acquired an exceptional phenotype being able to both actively swim (planktonic behavior) and express biofilm associated fimbriae (sessile behavior). We show that this exceptional phenotype enhances the conjugal transfer of the plasmid.

Introduction

Two distinct general behavioral states are currently recognized in most bacteria: a planktonic state and a sessile state, with the latter initiating biofilm formation. Biofilms are densely packed
bacterial communities, encased in a self-produced polymeric matrix, and typically associated with a surface or interface. Bacteria in the planktonic state are normally not associated with other cells or surfaces, and they are actively motile, if capable [1]. Central to the regulatory control of the shift between the sessile and the planktonic state, in members of Enterobacteriaceae and many other Bacteria, is a system that directs and responds to the secondary messenger cyclic-di-GMP (3',5'-cyclic diguanylic acid). Lowered levels of c-di-GMP stimulate a planktonic state, while high levels induce the biofilm phenotype [2].

C-di-GMP is synthesized via diguanylate-cyclases (DGCs) containing GGDEF domains that catalyze the conversion of $2 \times GTP$ into c-di-GMP. The removal of c-di-GMP is mediated by phosphodiesterases (PDE) that contain EAL or HD-GYP domains. EAL and HD-GYP PDEs hydrolyze c-di-GMP into pGpG and $2 \times GMP$, respectively. The intra-cellular level of c-di-GMP increases or decreases, via DGC and PDE activity, respectively, as a response to the physiological state of the cell and to external clues in the immediate environment [3], such as temperature [4] and electron acceptor availability [5], but also to signals such as quorum sensing autoinducers produced by bacteria [6–8]. Proteins bind c-di-GMP specifically in order to facilitate such responses and therefore typically mediate a shift towards the planktonic or the sessile cell state, in accordance with the intra-cellular levels of c-di-GMP [2].

A key factor in sessile biofilm formation and virulence of Klebsiella pneumoniae is the expression of type 3 fimbriae, which are thin protein appendages that protrude from the cell exterior and mediate adherence to both biotic and abiotic surfaces [9, 10]. Type 3 fimbriae are recognized as a virulence factor specifically associated with urinary tract infections (UTIs), where the ability to bind abiotic surfaces can extend complications in UTIs associated with foreign bodies such as catheters [11]. Type 3 fimbriae are chromosomally encoded by the mrkABCDF operon, which is expressed via the $P_{mrkA[Kp]}$ promoter located 204 bp upstream (transcriptional start site) of the $mrkA$ gene [10]. Type 3 fimbriae are typical chaperon-usher type fimbriae: MrkA is the major fimbrial subunit, MrkB a chaperon protein [12, 13], MrkC an usher translocase, MrkD the fimbrial tip adhesin [14, 15], and MrkF has a putative assembly/stability function [16]. The regulatory genes $mrkHI$ and $mrkJ$ are located immediately downstream, but transcribed convergently, of the $mrkABCDF$ operon [17]. MrkH is a c-di-GMP-dependent transcriptional activator of $P_{mrkA[Kp]}$, controlling the expression of the $mrkABCDF$ operon. MrkH binds the secondary messenger c-di-GMP via a PilZ domain, which prompts MrkH to bind upstream of $P_{mrkA[Kp]}$, thereby greatly enhancing the activity of transcription of this otherwise very weak promoter, and boosts the expression of type 3 fimbriae [17]. MrkI is a transcriptional regulator that also positively activates transcription of both $mrkHI$ and $mrkABCDF$. The molecular function of MrkI is less well resolved [18]. MrkJ is a PDE that catalyzes degradation of c-di-GMP via its EAL domain. MrkJ therefore negatively regulates type 3 fimbriae formation indirectly by reducing the local c-di-GMP pool [19].

The $mrkABCDF$ operon is not only found on the chromosome of K. pneumoniae but also encoded on the chromosome of various other members of Enterobacteriaceae, in addition to a number of conjugative plasmids [20]. The $mrkABCDF$ cassette has previously been identified on plasmids of the IncX1 group where the operon is likely to originally have been mobilized by a composite transposon, Tn6011, from the chromosome of K. pneumoniae onto an IncX1-type plasmid [21].

Here we examine the difference between type 3 fimbriae encoded on plasmids versus chromosomes, as little is known about the potential underlying differences. The understanding of biofilm-associated factors encoded on plasmids is currently inadequate as most research typically focuses on conserved biofilm-associated factors encoded by the chromosomes of various model bacteria. While such studies have generated important knowledge about the chromosomally encoded biofilm systems of a diverse array of bacteria, the chromosome of bacteria
is normally complemented by a variety of extra-chromosomal elements such as plasmids, many of which transfer horizontally. Importantly, it has previously been shown that conjugative pili of incF plasmids enhance biofilm formation [22, 23], suggesting that promoting biofilm formation could be linked to the transfer success of plasmids. Here, we illustrate that type 3 fimbriae encoded on plasmids enhance the horizontal transfer rate by enforcing biofilm formation and thus cell-to-cell interactions, while leaving the host motile, further ensuring a high rate of encounters between donors and recipients. We suggest that this dual faceted phenotype thrives due to fundamental changes in the expression of the \textit{mrkABCDF} operon of plasmids compared to that of chromosomes.

**Results and Discussion**

**Most \textit{E. coli} strains capable of excessive biofilm formation did so via plasmid encoded traits**

A small strain library consisting of 75 veterinary \textit{E. coli} isolates was screened for biofilm formation (CV assay). 7 of these isolates were found to be especially good at forming biofilms on the polystyrene surface of the microtiter plates. The threshold for “especially good biofilm formers” was set at an absorbance of OD\textsubscript{590} > 1.0, which was approximately 10 times as much as \textit{E. coli} MG1655 cells under the provided conditions. As the ability to form biofilm varied markedly among the veterinary strains, we hypothesized that the traits that enabled the hyper-biofilm forming behavior could be encoded on plasmids. Plasmids from the 7 biofilm-forming strains were therefore isolated and transformed into cells of \textit{E. coli} GeneHogs\textsuperscript{™}. 6 of the transformants showed a substantial increase in biofilm formation, suggesting that the biofilm-promoting traits of these 6 strains were encoded on plasmids. Performing gel-electrophoresis based on the plasmid DNA from the 6 strains indicated that only one plasmid was present in each strain. To avoid redundant sequencing, each of the plasmids was “fingerprinted” by restriction endonuclease digestion using \textit{EcoRI} and \textit{NcoI} followed by gel electrophoresis. Of the 6 plasmids analyzed, band patterns revealed that 3 of the 6 plasmids were unique. These three plasmids were hereafter fully sequenced using the 454 pyrosequencing platform and gaps between contigs were closed by Sanger sequencing. Two of the plasmids, pIS15\textsubscript{43} and pIS04\textsubscript{68}, were found to encode type 3 fimbriae and are presented here. The 3\textsuperscript{rd} plasmid did not encode type 3 fimbriae, but other potential biofilm-promoting genes. Further characterization of this plasmid will be presented elsewhere.

**Nucleotide sequencing of plasmids pIS15\textsubscript{43} (IncX1) and pIS04\textsubscript{68} (IncR)**

Plasmid pIS15\textsubscript{43} (Fig 1) is a 42804 bp long conjugative plasmid showing high resemblance to other \textit{IncX1} plasmids, particularly pOLA52 (NC\_024961), which has been thoroughly described [24, 25]. Annotation revealed 56 putative open reading frames (ORFs). Genetic load regions [26] included a beta-lactamase (\textit{bla}) gene associated with a \textit{Tn3} transposon and the type 3 fimbriae encoding \textit{mrkABCDF} cassette flanked by IS (insertion sequence) elements.

Plasmid pIS04\textsubscript{68} (Fig 1) is a 67973 bp large circular plasmid with 76 putative ORFs. No conjugation or mobilization elements were identified on pIS04\textsubscript{68}. pIS04\textsubscript{68} is a composite/truncated plasmid composed of 4 regions that each resemble other plasmids published in GenBank (i:pKP1780, ii:pYR1, iii:pOLA52 & iii\textsubscript{i}p1658/97). (i) The bulk of backbone elements are associated with \textit{IncR} plasmids (64.3–68 Kbp & 1–8.2 Kbp) including: the replication initiation protein (RepB), the stabilization toxin/antitoxin system (\textit{vagCD}), the DNA breaking/joining resolvase (ResA), the partitioning locus (\textit{parAB}), the DNA repair system (\textit{umuCD}) and the
reverse transcriptase (RetA). These backbone elements of pIS04_68 showed especially high nucleotide and structural resemblance with those of IncR plasmid pKP1780 [27] (DQ449578).

(ii) A 14.4 Kbp large region (29.8–44.2 Kbp) encoded both backbone and load ORFs otherwise associated with IncX1 plasmids (Fig 1), which include: type 3 fimbriae (mrkABCDF), a partitioning locus (parFG), a putative DNA invertase (pIS04_68_35), a putative degenerate EAL domain containing protein (xeal) and a gene cluster proposed to be involved in gene expression modulation (gem). This region includes putative genes encoding: DNA topoisomerase III (topB), a gene expression modulator (hha), and a transcriptional regulator (h-ns) [24].

(iii) One genetic load region (11.9–25.3 Kbp) was dominated by putative antibiotic resistance genes: streptomycin resistance (strAB), antibiotic biosynthesis monooxygenase (ydjA), tetracycline resistance (tetABCD) and beta-lactamase (bla). A similar region has been found on plasmid pYR1 [28].

(iii) The 4th region (46.6–62.9 Kbp) encodes conserved hypothetical ORFs with putative stability functions including: DNA repair ATPase (pIS04_68_62), endonuclease (pIS04_68_63), methyltransferase (pIS04_68_64) and Lon protease (pIS04_68_66). This region is highly similar to regions of p1658/97 [29].

**Unique mrkA promoter on plasmids enable high mrkABCDF expression independently of MrkH**

To date, 9 plasmids that carry the mrkABCDF cassette have been fully sequenced and made available in GenBank. It has been described how the mrkABCDF cassette found on plasmids...
outside of \textit{K. pneumoniae} is believed to have originated from a close relative of \textit{K. pneumoniae}, where the \textit{mrkA} cassette was mobilized from its chromosome onto a plasmid via an IS composite transposon. This was mainly recognized due to a highly conserved positioning of IS elements upstream, and to a lesser degree downstream, of the \textit{mrkA} cassette \cite{21}. The same consistency of IS element positioning flanking the \textit{mrkA} cassettes was found in both pIS15\_43 and pIS04\_68.

Sequence analysis of all published \textit{mrkA} carrying plasmids revealed that the \textit{mrkH} and \textit{mrkJ} genes were not present on any of these plasmids. Also, the MrkH box \((\text{GATCTATCAATG})\) and an AT-rich UP-element, both known to be important for MrkH mediated transcription induction \cite{17}, were not identified in any of the plasmids. Further analysis revealed conserved differences in the nucleotide sequence of the \textit{mrkA} promoter region of the plasmids (P\textsubscript{mrkA}[P]) originating from non-\textit{Klebsiella} hosts when compared to the \textit{mrkA} promoter region of the \textit{K. pneumoniae} chromosome (P\textsubscript{mrkA}[Kp]) (Fig 2). The distance from the start codon of \textit{mrkA} to the transcriptional start site of the \textit{mrkA} promoter is identical in both \textit{K. pneumoniae} and plasmids (197 bp) and so is the -10 box of both P\textsubscript{mrkA}[Kp] and P\textsubscript{mrkA}[P]. The -35 box of P\textsubscript{mrkA}[P] is, however, different from P\textsubscript{mrkA}[Kp]. Comparative analysis suggests that changes to the spacer region of P\textsubscript{mrkA}[P] in addition to the -35 box (Fig 2) could lead to changes in the promoter activity of P\textsubscript{mrkA}[P] compared to P\textsubscript{mrkA}[Kp].

To study the properties of P\textsubscript{mrkA}[P] and P\textsubscript{mrkA}[Kp], each promoter was ligated in front of \textit{lacZ} on plasmid pRS415 to create transcriptional fusions. The fusion constructs were hereafter transformed into \textit{E. coli} GeneHogs\textsuperscript{+}. P\textsubscript{mrkA}[P] amplification was done using pOLA52 as the template and spanned the region from -55 to +151 relative to the transcriptional start site. \textit{K. pneumoniae} C3091 was used as the template for P\textsubscript{mrkA}[Kp]. Expression profiles of each promoter fusion were assessed in LB medium at 37°C (Fig 3). The P\textsubscript{mrkA}[Kp]-\textit{lacZ} fusion showed low β-galactosidase activity with a minor peak just before the onset of the exponential phase. Much higher β-galactosidase activities were observed with the P\textsubscript{mrkA}[P]-\textit{lacZ} fusion, where the

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Fig 2. Nucleotide sequence alignment of \textit{mrkA} promoters of plasmids from non-\textit{Klebsiella} hosts (P\textsubscript{mrkA}[P]) and \textit{K. pneumoniae} (P\textsubscript{mrkA}[Kp]). Residues that do not align with P\textsubscript{mrkA}[Kp] are indicated in red, which are all associated with IS1 elements (red lining). Blue background highlights conserved P\textsubscript{mrkA}[Kp] components and green background denotes the unique -35 box found in P\textsubscript{mrkA}[P]. TSS: transcriptional start site. P\textsubscript{mrkA}[P] is predicted to be a stronger promoter than P\textsubscript{mrkA}[Kp] due to an optimized -35 box and a 2 bp longer spacer region between the -10 box and -35 box.

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activity increased steadily until the end of the exponential phase, illustrating a strong expression profile. This indicates that the IS1 associated -35 box, in addition to the optimized 17 bp spacer between the -35 and -10 box, found on the plasmids, changed the efficiency of the promoter dramatically compared with the P_{mrkA[Kp]} promoter in hosts like E. coli where no MrkH is present [17]. We found, in agreement with previous studies, that P_{mrkA[Kp]} is a weak promoter when not induced by MrkH [17]. The P_{mrkA[P]} did, however, not require MrkH induction for efficient expression, suggesting that the mrkABCDF operon of the plasmids is not controlled and regulated in sync with other biofilm genes of the host.

**mrkABCDF plasmids from non-Klebsiella hosts do not encode mrkHI or mrkJ**

Fig 3 shows that the mrkABCDF operons found on non-Klebsiella plasmids were expressed without induction by MrkH. We were therefore interested in understanding whether the mrkABCDF operon of plasmids in general is not associated with regulatory genes mrkH, mrkI, or mrkJ.

Comparative analysis of available and completely sequenced replicons encoding the mrkABCDF operon revealed a large variety in the genetic architecture of putative mrkH-like (mrkH\_L), mrkI-like (mrkI\_L), and mrkJ-like (mrkJ\_L) genes flanking the mrkABCDF operon. Genes were classified as mrkH\_L if the translated amino acid sequence holds a putative PilZ-type (cl01260) c-di-GMP binding motif and is classified as a putative transcriptional regulator. mrkI\_L codes for putative proteins that have a LuxR motif (cl17315) and are transcriptional regulators. mrkJ\_L genes encode putative proteins that hold an EAL-domain (cl00290). Fig 4 illustrates that 9 out of 10 chromosomally encoded mrkABCDF operons were flanked by putative mrkH\_L, mrkI\_L and/or mrkJ\_L genes, whereas only one Klebsiella plasmid (pKPN\_262) encoded an intact mrkJ\_L ORF. The type 3 fimbria operon of pKPN\_262 is, however, not intact as mrkA and half of mrkB is missing likely due to disruption by a transposable element. The one exception among chromosomes was in the Enterobacter cloacae strain UCICRE 5 where only mrkAB and the beginning of mrkC were identified as part of a putative genomic island and flanked by IS elements and phage derived sequence, possibly transferred to this location by HGT. Fig 4 suggests that regulatory genes mrkJ, mrkI and mrkJ are not selected for on plasmids in concert with mrkABCDF, possibly because these genes do not regulate P_{mrkA[p]} activity.
Fig 4. Genetic map of the mrkABCDF operon and flanking putative regulatory elements encoded on all fully sequenced representative replicons available in GenBank. Red: mrkABCDF genes. Orange: mrkH and mrkHL genes. Purple: mrkJ and mrkJL genes. Green: mrkI and mrkIL genes. Blue: putative genes encoding incomplete EAL domains. Dark gray: IS/transposon elements. Light gray: phage associated genes. (*) Plasmids pKPN_CZ and pKPN_262 have been isolated from Klebsiella hosts while all other plasmids originate from non-Klebsiella hosts. doi:10.1371/journal.pone.0162390.g004

Fig 5 is an unrooted phylogram tree based on the nucleotide sequence of the PapD_N (pfam00345) and PapD_C (pfam02753) domains of the mrkB genes. The tree is based on the same strains as were presented in Fig 4. This shows that the mrkABCD operons found on plasmids are closely related to those of K. pneumonia and that the mrkABCD operons encoded by chromosomes demonstrate higher diversity than those associated with plasmids, supporting the notion that the plasmid mrkABCD operons have mobilized recently from a K. pneumoniae-like chromosome. Interestingly, mrkB of E. cloacae UCICRE 5 is grouped with both the plasmids and K. pneumoniae, suggesting that this could be a genomic island that might have been mobilized from a K. pneumoniae-like chromosome. The low diversity among the plasmid encoded mrk genes is possibly linked to the lack of regulatory activator MrkH on the plasmids and the strong MrkH independent expression observed from PmrkA[p], suggesting a plasmid specific selection for the highly expressed PmrkA[p] mrkABCDF cassette that does not need activation by MrkH.

The PmrkA[p]mrkABCDF cassette enables abundant biofilm formation but does not influence motility

Motility and biofilm formation are opposing phenotypes, and bacteria such as E. coli either swim or form biofilm [3]. We, therefore, tested whether E. coli cells carrying an mrkABCDF encoding plasmid were also less motile, thus embracing a typical biofilm phenotype, or if the host would have an unusual phenotype, both expressing biofilm fimbriae and being actively motile. First, we tested if the promoter activities of PmrkA[Kp] and PmrkA[P] would directly correlate with low and high biofilm formation, respectively. Vector constructs based on the low copy number plasmid, pLOW2 [30], ligated with the PmrkA[Kp] mrkABCDF or PmrkA[P] mrkABCDF.
mrkABCDF fragment were transformed into *E. coli* (GeneHogs™ and MG1655) and tested for biofilm formation in the crystal violet (CV) assay (Fig 6a). Biofilm formation was strongly induced only when *mrkABCDF* was expressed via the P*mrkA*[P] promoter (see also Fig 7) as opposed to the P*mrkA*[Kp]→*mrkABCDF* construct, which made as little biofilm as the vector control (pLOW2 without *mrkABCDF* insertion). The swimming motility of the *E. coli* hosts was not influenced by the expression of the intact P*mrkA*[P]→*mrkABCDF* cassette of plasmid pOLA52 (pOLA52-*oqxB::KAN^R^), compared with pOLA52 where *mrkC* has been disrupted by transposon insertion (pOLA52-*mrkC::KAN^R^) (Fig 6b). We observed the same for cells of both *E. coli* GeneHogs™ and MG1655. Hence, no difference in swimming motility was found, illustrating that the P*mrkA*[P]→*mrkABCDF* cassette of wild type (WT) plasmids enforces an unusual phenotype of the hosts expressing both biofilm and planktonic phenotypic traits.

Putative genes that are found on multiple *mrkABCDF* plasmids were identified as candidates that can be linked to functions that might influence biofilm and/or planktonic behavior of *E. coli* (excluding the *mrkABCDF* cassette). These putative genes are part of the *gem* region (*hha* & *h-ns*) or *xead* (Fig 1). Hha and H-NS have been shown to interact and thereby participate in the regulation of a number of genes influencing both biofilm formation and motility functions of *Enterobacteriaceae* [31–35]. *xead* holds a putative degenerate c-di-GMP EAL domain and was therefore also investigated.

A number of vector constructs were created and tested in the CV, swimming motility and Congo red (CR) assays in order to assess if the aforementioned genes (*hha*, *h-ns* and *xead*) affect host biofilm formation or motility. Congo red binds to curli fimbriae and cellulose, which are expressed from chromosome encoded genes during *E. coli* biofilm formation [36]. *E. coli*
colonies therefore become red in the CR assay if curli fimbriae and/or cellulose is expressed. Fig 7 summarizes the results of these experiments. The overall conclusion is that none of the putative genes xeal or those of the gem region influenced the biofilm or planktonic motile behavior of the host under the tested conditions. The only deviation from this was that less CR was bound when xeal was highly expressed by an L-rhamnose inducible promoter. Although this is a potentially interesting observation, further analysis is required to clarify if this was an artifact of overexpressing Xeal, or if Xeal influences curli fimbriae and/or cellulose formation. Utilizing the other constructs that also hold xeal, no distinction was found between biofilm formation in the CV assay and the swimming motility assay. We therefore conclude that none of the putative genes that were annotated as potential biofilm or motility modulators (hha, h-ns and xeal) encoded on plasmids pOLA52, pIS15_43 and pIS04_68, influenced the performance of the hosts within the detection limits of the conditions provided here.

In order to corroborate the above findings we generated transcriptomes of E. coli MG1655 in exponential phase and compared transcription profiles in cultures with and without pIS15_43 (two biological replicates were made of each). Data is summarized in S3 and S4 Tables. Genes were considered differentially expressed if there was at least a 2-fold change in expression supported by P-value < 0.05.

First, the transcriptome data was used to verify that the P_mrkA(p) promoter prediction earlier (Fig 2) was correct (S1 Fig), which was done by mapping reads against the upstream region of mrkA. We found that transcription started around 194-204bp upstream of mrkA. The only likely P_mrkA(p) promoter for transcription to start this far upstream of mrkA is the one shown in Fig 2.

Overall, no consistent patterns of transcription indicated that biofilm or motility functions in general were up or down regulated differentially in plasmid bearing cultures compared to plasmid free ones, other than the plasmid encoded mrkABCDF operon. For example, the generated dataset suggests no significant differences in transcription for curli fimbriae (csg), colonic acid (wza/cps), cellulose (bcs), type 1 fimbriae (fim), flagellum (flh, fli, flg–except flgB), chemotaxic (che), GGDEF and/or EAL domain protein genes (dos, rtn, yahA, yaiC, ycdT, ycdT, ycgF, ycgG, yciR, ydaM, ydvV, YdeH, ydiV, yeaL, yeaL, yeaP, yedQ, yegE, yfeA, yfgF, yfiN, yhdA, yjH, yhjK, yjcC, ylaB, yliE, yliF, yneF, yoaD) or PilZ domain protein genes (ycgR, bcsA). Four
non-conjoined genes that potentially are associated with biofilm and/or motility phenotypes were, however, found to be differentially expressed: flu, flgB and hdfR were less expressed in plasmid bearing cultures, 12.6, 2.4 and 2.7 fold less, respectively. flu encodes antigen 43, which has been shown to cause aggregation and inhibition of motility in E. coli [37]. flgB encodes a flagella component of the cell-proximal portion of the basal-body rod. Interestingly, flgB has been reported to be up-regulated in biofilms compared to cells in suspension [38]. hdfR encodes a H-NS dependent transcriptional regulator of the flhDC operon, which is the major regulator of flagella assembly and function [39]. However, the flhDC operon was not expressed differently in plasmid and plasmid free cultures. plaP, encoding a putrescine low affinity permease, was expressed 3.5 fold more in plasmid carrying cultures. Deletion of plaP has been shown to abolish swarming motility when all other predicted putrescine transporters were also deleted [40]. Given that the differential transcription of flu, flgB, hdfR and plaP, in theory, would lead to opposing biofilm/motility phenotypes makes it difficult to draw a consistent picture of how to interpret the significance of these observations. Taking together the lack of conjoined differential gene expression of biofilm or motility genes, revealed by the transcriptome data, with the phenotypes established in the previous sections (Fig 7), we deduce that the plasmid did not change the host behavior effectually besides enabling biofilm formation via plasmid encoded type 3 fimbriae.

Conjugative plasmid transfer is enhanced by both motility and type 3 fimbriae

It seems that mrkABCDF encoding plasmids act by enforcing a change in key behaviors of their hosts while bypassing regulation. We therefore hypothesized that the unusual motile and...
fimbriae expressing phenotype is connected with the horizontal transmission success of the plasmid replicon. Transfer frequencies were compared between a conjugative plasmid with an intact mrkABCDF cassette (pOLA52-oqxB::KAN<sup>R</sup>) and a non-functional cassette (pOLA52-mrkC::KAN<sup>R</sup>). Both a motile (MG1655) and a non-motile (MG1655 ΔflhD) E. coli donor strain were used in order to imitate the more typical motile+/fimbriae- (MG1655//pOLA52-mrkC::KAN<sup>R</sup>) and motile-/fimbriae+ (MG1655 ΔflhD//pOLA52-oqxB::KAN<sup>R</sup>) phenotypes, but also the unusual motile+/fimbriae+ (MG1655 ΔflhD/pOLA52-oqxB::KAN<sup>R</sup>), type 3 fimbriae negative plasmid. Each transfer frequency was different from the other (Error bars denote ±SEM, n = 3).

In accordance, our transcriptome data shows that type 3 fimbriae (mrk), the conjugative machinery (pilx, taxAB) and flagella associated genes (flh, fli, flg) were all expressed during the
late exponential phase in cells harboring the plasmid (S4 Table, average RPKMs: mrk: 337, pilx: 18, taxAB: 28, flh: 17, fli: 17 & flg: 16). This supports the notion that the host acquires an exceptional phenotype, that both expresses biofilm and planktonic behaviors due to the acquisition of the mrkABCDF encoding plasmid, and that these phenotypes, transcription profiles, and elevated horizontal transfer rates of mrk-encoding plasmids are linked temporally.

Concluding remarks

Transitioning between a planktonic and biofilm state is an example of phenotypic plasticity of bacteria. Phenotypic plasticity is typically directly connected to the fitness of organisms, as it enables a swift response to environmental change [42]. Mutation driven adaptation can lead to fixation of specific phenotypes and thereby reduce the phenotypic plasticity of bacteria [43]. Such fixation is known to occur when conditions in the local environment become more static, because selection then may favor a specific fixed phenotype and not plasticity [44].

Here we show that plasmid-encoded mrkABCDF enable bacteria to produce biofilm while retaining its ability to swim. Plasmid-encoded mrkABCDF genes are highly expressed without MrkH activation, which is a fundamental difference between mrk-operons on plasmids and chromosomes. mrkABCDF encoding plasmids, therefore, enforce a reduction in behavioral plasticity as the host is “locked” in a sticky biofilm-type behavior. This, however, is fundamentally different from mutations in the chromosome that induce the biofilm phenotype because the type 3 fimbriae are acquired by E. coli via HGT. Therefore, an entirely novel function is acquired by the E. coli host, which may increase the fitness of the host under specific selective pressures. Also, the phenotype that the plasmid imposes via the strongly expressed mrkABCDF operon was shown to enhance the horizontal transfer success of the plasmid and may thus be, at least in part, a plasmid selfish trait.

The long controversy of whether plasmids are molecular parasites or functional modules that only persist when complementing the host is therefore also in this case complex, as this is likely to primarily be determined by the level of local selective pressures and the degree of phenotypic plasticity that makes the host most fit. Plasmid encoded antibiotic resistance and toxin/antitoxin systems have been the focus of most research that examines the evolutionary dynamics of plasmid transfer, but these systems represent a very small fraction of the genes that are encoded on MGEs. Here we illustrate that factors such as fimbriae encoded by plasmids can also change the fundamental behavior of the host bacterium and argue that such factors are evolutionary successful not only because the host may become more fit in environments where adhesion is a selective advantage, but also because the horizontal transfer success of the plasmid is enhanced.

Materials and Methods

Bacterial strains and plasmids

The veterinary Escherichia coli strain-library used for the initial screen was kindly provided from the Danish integrated antimicrobial resistance monitoring and research program, DANMAP. 25 E. coli isolates were used from swine, cattle or poultry, respectively, and each isolate originated from a different animal (DANMAP 2008, [45]). All bacterial strains and plasmids are listed in S1 Table (supporting information). All bacteria used in this study were grown on Luria-Bertani (LB) agar (1.5%) medium at 37°C or in LB broth at 37°C at 250 rpm, unless otherwise stated. The following concentrations of antibiotics were used: Ampicillin (AMP); 100 μg/mL, Chloramphenicol (CHL); 50 μg/mL, Kanamycin (KAN); 50 μg/mL, Nalidixic acid (NAL); 100 μg/mL, Rifampicin (RIF); 100 μg/mL and Tetracycline (TET); 10 μg/mL.
Crystal violet biofilm assay

Biofilm formation was quantified using a modified version of the Calgary assay previously described [46–48] using peg-lids (TSP) from NUNC. Incubation was done overnight at 37°C for 24h and staining was done with 1% crystal violet (CV). OD$_{590}$ was used to measure CV absorbance on an EL 340 microplate reader (Bio-Tek Instruments).

DNA manipulation techniques

Plasmid DNA was purified using the Plasmid Mini AX kit and genomic DNA was obtained using the Genomic Mini kit (A&A Biotechnology, Poland). PCR reactions were performed according to the standard protocol provided by Thermo scientific (Phusion Hot Start High-Fidelity DNA polymerase F540). Primers are shown in S2 Table. PCR fragments were extracted from agarose gels using QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany). Enzymatic restrictions were performed according to standard procedures as stated by the manufacturer (New England Biolabs, Ipswich, USA). T4 DNA ligase was used for all ligation reactions following the provided protocol (New England Biolabs, Ipswich, USA). Electroporation with electro-competent *E. coli* Top10 and GeneHogs cells (Invitrogen) was done using a Gene Pulse apparatus (Bio-Rad). *E. coli* cells were made chemically competent and heat-shock transformed following standard protocols [49].

Plasmid purification, sequencing and assembly of plasmids pIS04_68 and pIS15_45

Plasmid purification, sequencing and assembly was performed following the same steps as previously described [21]. *E. coli* GeneHogs transformants with either pIS04_68 or pIS15_43 were selected for on LB supplemented with AMP. pIS04_68 and pIS15_43 were sequenced on the GS sequencer FLX high throughput platform (454 Life Sciences, Branford, CT, USA). Single strand DNA libraries were constructed according to Roche protocols. Sequence assembly was performed with Newbler 2.6 (454 Life Sciences, CT, USA) and Consed [50]. Final assembly of contigs was done by Sanger sequencing (Macrogen, Korea) via PCR fragments that were made to span the gaps between the contigs. Primers are shown in S2 Table (supporting information). Accession numbers of pIS15_43 and pIS04_68 are NC_024961 and NC_024960, respectively.

Annotations and sequence analysis

Annotation was done using Glimmer V3.02 [51] and CLC main workbench V6.9.1 (CLC Bio). General sequence analysis was done with the CLC main workbench software accompanied by Artemis [52], NCBI BLAST tools [53], BPROM (Softberry) and IslandViewer [54]. MEGA v.6.0.6 [55] was used to construct a Maximum likelihood phylogram, and Figtree v1.4.2 was used for visual representation. Accession numbers: CP007734, EU682505, NZ_KI535506, NC_013850, NC_018106, NC_021066, NC_009792, NC_020064, NC_017910, NC_010378, NC_019256, NC_019390, NC_013503, NC_016036, NC_019013, CP007530, and CP009450.

Conjugative transfer experiments

Spontaneous RIF$^R$ and NAL$^R$ mutants were produced from the *E. coli* MG1655 [56] strain: 1 mL ON culture was spun down (5000 × g, 5 min), then re-suspended in 100 μL LB broth and spread on LB agar medium with RIF$_{100}$ or NAL$_{100}$. Single colonies were picked after ON incubation at 37°C and re-streaked multiple times to ensure pure clones. Purified plasmids pOLA52-*oqxB::KAN$^R$ and pOLA52-*mrkC::KAN$^R$ were transformed into the MG1655 RIF$^R$ and MG1655 Δ*flhD* mutant strains. The four transformants were used as donors and the
MG1655 NAL\textsuperscript{R} strain was used as the recipient in all conjugative transfer experiments. ON cultures of donor and recipient strains were adjusted to OD\textsubscript{600} = 0.4, washed twice in LB broth, then adjusted to OD\textsubscript{600} = 0.2 and left on ice for the duration of the mixing steps. Donor and recipient cultures were mixed to a ratio of 1:4 and incubated without any shaking at 37°C for 20h. Serial dilutions of the mating mixtures were hereafter prepared. Selective plating was done on LB agar medium using the following antibiotics: Transconjugants: NAL\textsuperscript{100} + KAN\textsuperscript{50}. MG1655 RIF\textsuperscript{R} donor: RIF\textsuperscript{100} + KAN\textsuperscript{50}. MG1655 Δ\textit{flhD} donor: CHL\textsuperscript{20} + KAN\textsuperscript{50}.

Plasmid constructs and promoter fusions

Promoter fusions $P_{\text{mrkA[Kp]}}$-$\text{lacZ}$ and $P_{\text{mrkA[P]}}$-$\text{lacZ}$ were constructed by PCR amplifying the $\text{mrkA}$ promoter region using \textit{K. pneumoniae} C3091 and pOLA52, respectively, as DNA templates. All primers are shown in S2 Table. Promoter fusions were transformed into competent \textit{E. coli} Top10 cells. Constructs pLOW2-$P_{\text{mrkA[Kp]}\text{mrkABCDF}}$, pLOW2-$\text{xegal}$, pLOW2-$\text{gem}$, pLOW2-$\text{xegal}$-gem, pLOW2-$\text{xegal}$-$P_{\text{mrkA[P]}}$-$\text{mrkABCDF}$ and pLOW2-$\text{gem}$-$\text{xegal}$-$P_{\text{mrkA[P]}}$-$\text{mrkABCDF}$ were made using primers as specified in S2 Table. pOLA52 or pIS15\textsubscript{43} was used as a DNA template. pLOW2 based constructs were transformed into \textit{E. coli} GeneHogs, Top10 and MG1655 strains. The Expresso\textsuperscript{R} Rhamnose Cloning and Expression System, C-His (Lucigen\textsuperscript{R}, Wisconsin, USA) was used to construct pRham-$P_{\text{rham}}$-$\text{xegal}$, an L-rhamnose inducible $\text{xegal}$ expression plasmid, following the guidelines provided by the manufacturer. Importantly, the $\text{xegal}$ insert included a stop codon in order to avoid translation of the C-terminal His\textsubscript{10} tag region. pIS15\textsubscript{43} was used as a DNA template. Control plasmids, pRham-$\Delta$His\textsubscript{6} and pRham-$\Delta P_{\text{rham}}$His\textsubscript{6} were made by PCR amplifying the pRham vector, then digesting with BamHI followed by ligation for re-circularization of the vector without the His\textsubscript{6} tag and the L-rhamnose inducible promoter plus His\textsubscript{6} tag, respectively. pIS15\textsubscript{43}-\text{xegal}:KAN\textsuperscript{R} was constructed using the lambda red approach employing vector plasmids pUC4K \cite{57} and pUCP-18-RedS \cite{58}.

$\beta$-galactosidase activity assay

$\beta$-galactosidase activity was assayed as described elsewhere \cite{59–61}. ON cultures of \textit{E. coli} harboring pRS415-$P_{\text{mrkA[Kp]}}$-$\text{lacZ}$ and pRS415-$P_{\text{mrkA[P]}}$-$\text{lacZ}$ were diluted 1:1000 in LB-broth supplemented with AMP and grown at 37°C, 250 rpm to an OD\textsubscript{600} of approximately 1.8. Samples were taken continuously over a period of 7.5h.

Congo red assay

10 \muL of ON culture was spotted on LB-agar plates without NaCl, with 0.5\% NaCl and with 1.0\% NaCl. The plates were supplemented with 40 \mug/mL Congo red (CR)(Sigma) and 20 \mug/mL Coomassie brilliant blue (Sigma). Incubation was done at 30°C for up to 4 days. \textit{Pseudomonas aeruginosa} PA14 and \textit{P. aeruginosa} PA14 $\Delta$\textit{pel} were used as positive and negative controls, respectively.

Swimming motility assay

The swimming motility assay was performed both in LB medium with 0.3\% agar and in medium composed of 1\% tryptone and 0.3\% agar. 2 \muL of ON culture was deposited into the agar-medium using a pipette tip. Plates were incubated at 37°C for 8h or 16h.

RNA isolation, library construction, sequencing and data normalization

Total RNA was isolated from exponentially growing cultures (OD\textsubscript{600} = 0.4) using RNeasy Mini Kit (Qiagen). No plasmid loss was observed in cultures with pIS15\textsubscript{43} although AMP\textsubscript{100} was
not added. This was tested by CFU counts on LB agar with and without AMP$_{100}$ after the cultures had been in stationary phase for approximately 2h. (LB: $3.1 \times 10^7 \pm 5.0 \times 10^6$ CFUs, LB AMP$_{100}$: $3.0 \times 10^7 \pm 5.2 \times 10^6$ CFUs, n = 3). Ribosomal RNA was reduced using the Ribo-Zero rRNA Removal Bacterial Kit (Epicentre Biotechnologies) according to the manufacturer’s protocol. The mRNA-enriched fraction was used as a template for preparation of indexed RNA-seq libraries using the ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies). The individual libraries were pooled and sequenced on a MiSeq platform using 2x75 PE v3 sequencing kit (Illumina). Obtained reads were trimmed and normalized with the CLC Genomic Workbench 7.5.1 (CLC). Briefly, reads were trimmed removing adapter sequences and discarding those of low quality using “trim sequences” tool (settings: ambiguous limit = 2, quality limit = 0.05). Afterwards, reads were depleted of rRNA sequences of the strain MG1655 (NC_000913). Next, un-mapped reads were mapped to the reference genome MG1655 and plS15_43 plasmid (NC_024961) using the “map reads to reference tool” with the same settings as above. Finally, the genome-mapped and rRNA-depleted reads were normalized to the total number of 1.8 million reads for each replicate by random picking. RNA-seq analysis was performed in CLC Genomic Workbench 7.5.1 using “RNA-seq Analysis” tool with RPKM as the expression value. The differential expression experiment was set as two-group comparison with two replicates each (total nr of samples = 4). Group comparison of differentially expressed genes (DGE) was run on non-transformed data using a negative binominal model and exact testing as implemented in edgeR [62]. The false discovery rate (FDR) control was done according to Benjamini and Hochberg [63] and FDR-corrected p-values were considered for differentially expressed genes.

**Supporting Information**

S1 Fig. RNA-seq. reads mapped against the upstream region of *mrkA* (plS15_43) verifying the predicted location of promoter $P_{mrkA(p)}$.

(DOCX)

S1 Table. Strains and plasmids used in this study.

(DOCX)

S2 Table. Primers used in this study.

(DOCX)

S3 Table. Transcriptome data analysis of differential gene expression comparing plasmid harboring (plS15_43) and plasmid free *E. coli* MG1655 cultures.

(XLSX)

S4 Table. Transcriptome data analysis of gene expression levels in plasmid harboring (plS15_43) *E. coli* MG1655.

(XLSX)

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References


