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Research paper

Overexpression of Ipe protein from the coliphage mEp021 induces pleiotropic effects involving haemolysis by HlyE-containing vesicles and cell death

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ABSTRACT

Lysogenic *Escherichia coli* K-12 harbouring the prophage mEp021 displays haemolytic activity. From a genomic library of mEp021, we identified an open reading frame (ORF 4) that was responsible for the haemolytic activity. However, the ORF 4 sequence contains four initiation codons in the same frame: ORF 4.1—ORF 4.4, coding for 83-a.a., 82-a.a., 77-a.a. and 72-a.a. products, respectively. The expression of the cloned ORF 4.3, or inducer of pleiotropic effects (*ipe*), reproduced the haemolytic phenotype in a native strain carrying the gene *hly*E^{+}, but not in the mutant *hly*E^{-} strain. The overexpression of Ipe induced several pleiotropic effects, such as the inhibition of cell growth and the deregulation of cell division, which resulted in a mixture of normal and desiccated-like cells: normal-filamentous, desiccated-like-filamentous bacilli, minicells etc. Other effects included abnormalities of the cell membrane, the production of vesicles containing HlyE, and finally, cell death. These events were analysed at the molecular level by microarray assays. The global transcription profile of *E. coli* K-12 strain MC4100, which expressed Ipe after 4 h, revealed differential expression of various genes, most of which were related either to cell membrane and murein biosynthesis or to cell division. The up-regulation of some of these transcripts was confirmed by qRT-PCR. Additional research is needed to determine whether these effects are directly related to Ipe activity or are consequences of the cellular responses to putative structural damage induced by Ipe.

1. Introduction

The temperate coliphage mEp021 belongs to a group of phages of unique immunity and was isolated from human faecal samples [1]. This coliphage possesses characteristics differentiating it from the lambdoid phages, as it is not recognised by antibodies against λ structural proteins, its DNA does not hybridise or recombine with λ DNA and it is not able to develop at 32 °C. As a prophage, it is not inducible by UV radiation. The only trait that mEp021 shares with lambdoid phages is the requirement for Nus factors, suggesting that this phage could have an antitermination mechanism homologous to that of lambdoid phages [1]. One feature that makes this phage group attractive for study is that ~20% (10 out of 48) of these prophages induce a haemolytic phenotype in their host *Escherichia coli* K-12 [2].

The haemolytic activity of *E. coli* K-12 involves the release of the bacterial protein HlyE (ClyA or SheA) via outer membrane vesicles [3,4]. The expression of *hly*E responds to specific environmental conditions, such as anaerobiosis or glucose starvation [5]. The regulation of *hly*E is driven by native transcriptional factors such as Fis, SlyA, Fnr, CRP and H–NS, or by foreign transcriptional factors as SlyA and HlyX, from *Salmonella enterica* serovar Typhimurium and *Actinobacillus pleuropneumoniae*, respectively [6–13]. HlyE release has been associated with the lytic activity of holins, such as Ehly1 and Ehly2 from the coliphages φC3888 and φC3208, respectively; Hol-1 from a *Xenorhabdus nematophila* phage; BlyA from *Borrelia burgdorferi* prophage φBB-1 or TcdE from *Clostridium difficile* pathogenicity region PaLoc [14–16]. It is well documented that phage-encoded proteins can directly affect other bacterial functions like cell division. DicB, from the Qin prophage, stimulates the MinC activity, resulting in the inhibition of cell division [17]. KilR and Kil,

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from the prophages Rac and λ, respectively, alter the Min proteins and produce filaments, thus decreasing cell viability [18,19].

In this work, we report that the overexpression of Ipe, which codes for a 77-a.a. peptide, induces a haemolytic phenotype through the release of HlyE-containing vesicles. In addition, the overexpression of Ipe produces alterations in cell division, forming not only normal-filamentous bacilli but even desiccated-like-filamentous bacilli or minicells, affecting cell viability. Using microarray analysis, we have confirmed that a high percentage of the deregulated genes are involved in cell division, murein and membrane biosynthesis. The contribution of prophage-expressed Ipe to the growth of the bacterial host and the possible structural damage caused when Ipe is overexpressed will be discussed.

2. Materials and methods

2.1. Bacteria, plasmids, bacteriophage and media

The relevant characteristics of the bacterial strains, plasmids and the bacteriophage mEp021 are shown in Table 1. Phage mEp021 was propagated in the *E. coli* W3110 strain. The *E. coli* TAP114 and DH5α strains were used for the transformation and the selection of recombinant plasmids. Strains MC4100 and CFP201, transformed with different plasmids, were used for the cell viability counts and growth assays. Luria-Bertani (LB), tryptone broth (TB) and TMG phage-dilution media were prepared as described by Silhavy et al. (1984) [20]. For haemolysis assays, trypticase soy agar was prepared according to the manufacturer's instructions, and aseptically prepared sheep blood was added to a final concentration of 4% for plates and 2% for slides. Competent cells were cultured in SOB media according to Hanahan [21]. Phosphate buffered saline (PBS) was used for resuspending the pellet containing the vesicles [22]. Restriction enzymes, Klenow polymerase, T4 DNA ligase and other enzymes used in plasmid construction were purchased from New England Biolabs Inc. Media were supplemented with 100 μg/ml of ampicillin (Amp), 1 mM of IPTG or 40 μg/ml of X-gal (Sigma–Aldrich), when it was required. All media were purchased from Difco® BBL®.

Table 1
Bacteria, bacteriophage and plasmids.

Strains, bacteriophage and plasmids	Genotype/relevant markers	Source/ reference
Bacteria		
W3110	F^- λ^- *rph*$^-$	[62,63]
TAP114	W3110 *lac*IQ Δ(*lacZ*)M15	[64]
MC4100	F^- *ara*D 139 Δ(*lac*IZYA-*arg*F)U169 *rps*L15 *rel*A1 *thi*A *flb*B5301 *deo pts* F25 *rbs*R	[65]
CFP201	MC4100 *she*A::Tn5-2.1	[66]
DH5α	*sup*E44 Δ(*lac*IZYA-*arg*F)U169 *deo*R (ϕ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	[21]
MC4100 (mEp021)	MC4100 with mEp021 prophage	This study
Plasmids		
pKQV4	Low copy vector, Amp^R	[23]
pGEM-3Zf (+)	Expression vector regulated by IPTG (Stratagene).	[67]
p3Z021-2	pGEM-3Zf (+) with 294 bp fragment of mEp021	This study
pEM-067	pGEM-3Zf (+) with ~3,000 bp fragment of mEp021	This study
pE021-4.1	pKQV4 with ORF 4.1	This study
pE021-4.2	pKQV4 with ORF 4.2	This study
pE021-4.3	pKQV4 with *ipe*	This study
pE021-4.4	pKQV4 with ORF 4.4	This study
pE021-4.3_M	pKQV4 with *ipe* (start codon ATG changed to ATT)	This study
pE021-4.3_His	pKQV4 with *ipe* (6xHis at C-terminus)	This study
Bacteriophage		
mEp021	A non-lambdoid bacteriophage	[1]

2.2. Bacteriophage propagation and phage DNA extraction

For phage propagation, 300 μl of an overnight (O/N) bacterial culture was infected using 3 turbid plaques. Adsorption was allowed for 15 min, and then 50 ml of LB broth was added. The cultures were incubated at 37 °C in a rotatory shaker at 200 rpm until cell lysis was observed. The DNA was extracted as described by Sambrook et al. [22]. Finally, the DNA pellet was resuspended in 50 μl of TE buffer.

2.3. DNA library and plasmid constructions and PCR

2.3.1. mEp021 genomic library and plasmid pE021-67 construction

For the mEp021 library construction, the phage DNA was partially digested with the restriction endonuclease *Sau*3A1, yielding 500–2000 bp fragments that were ligated to the *Bam*HI site of the vector pGEM-3Zf (+). The plasmid constructs were transformed into chemically competent TAP114 cells. Among others, the plasmid p3Z021-2, harbouring a 294 bp fragment, was obtained from this genomic library. For pE021-67 construction, a 3000 bp fragment obtained from mEp021 DNA digested with *Nde*I was first repaired with Klenow DNA polymerase and then ligated to the *Sma*I site of pGEM-3Zf (+) using T4 DNA ligase. The resulting constructs were transformed into the DH5α strain. The DNA library construction, PCR fragment amplification and plasmid construction were performed as described by Sambrook et al. [22].

2.3.2. Construction of ORF 4 derivative plasmids

Plasmid DNA purification was performed using the High Pure Isolation Kit (Roche), following the manufacturer's protocol. DNA sequencing was performed according to the standard protocol. Based on the 294 bp and 1450 bp sequences, different forward primers were designed for each ORF (ORF 4.1 Fw, ORF 4.2 Fw, *ipe* or ORF 4.3 Fw, ORF 4.4 Fw, ORF 4.3_M Fw and ORF 4.3_His Fw), but the reverse primer (ORF 4 Rev, Table S1) was the same for all of them, except for ORF 4.3_His. For this construct, six histidine codons were added. All of the forward primers contained an *Eco*RI restriction site, and all of the reverse primers contained a *Hind*III restriction site at their 5′ end (Table S1). The PCR reaction mix was prepared following the protocol for Platinum Taq DNA polymerase (Invitrogen®). Reactions were performed using a Gene Amp PCR System 2400 (Perkin Elmer) under the following conditions: a denaturing step at 94 °C/5 min, followed by 30 amplification cycles of 94 °C/60 s, 58 °C/60 s and 72 °C/30 s for each cycle, and a final extension step at 72 °C/2 min. The products were stored at 4 °C until their use. Each product was digested with *Eco*RI and *Hind*III to be cloned into the same restriction sites in the vector pKQV4 [23], generating the plasmids pE021-4.1, pE021-4.2, pE021-4.3, pE021-4.4, pE021-4.3_M and pE021-4.3_His (Fig. 3 and Table S1). It is noteworthy that pKQV4 is a low-copy-number plasmid and that each cloned ORF is translationally regulated under the Shine Dalgarno (SD) sequence from the vector (Fig. 3). The plasmid constructs were sequenced using universal primers (listed in Table S1), in an automatic sequencer Perkin Elmer™ ABI PRISM™ 310 (Applied Biosystems), at the sequencing facility of the Genetics and Molecular Biology Department (CINVESTAV-IPN, México).

2.3.3. Bioinformatic analysis

The 1450 bp and 294 bp sequences from pE021-67 and p3Z021-2, respectively, were used to identify open reading frames (ORFs) and SD regions using the software tools Vector NTI 5.1, DNA Strider 1.1 and the GeneMark: Heuristic Approach for Gene Prediction in