Antisense oligonucleotides exhibit high potential for use as therapeutic agents. '10-23' DNAzymes are antisense molecules with a high chemical stability and catalytic efficiency. In the present study, we developed a phagemid containing a DNAzyme expression system regulated by two promoters. One of these promoters, pA1, promotes constitutive expression of Moloney murine leukemia virus reverse transcriptase (MoMuLV-RT). The other promoter, plac, regulates transcription of the RNA substrate from which MoMuLV-RT produces the DNAzyme by reverse transcription. The ftsZ DNAzyme was used to validate this expression system in the phagemid, named pDESCP. ftsZ DNAzyme expression altered the morphological pattern of Escherichia coli from a bacillary to filamentous form. In E. coli FtsZ is the primary component of the cell division apparatus, forming a structure known as Z-ring, which is the place of division. It is suggested that the DNAzyme ftsZ is decreasing the translation of this protein. Delivery of pDESCP into F+ strain of E. coli cells, using VCSM13, and the possible insertion of other DNAzymes into the cassette makes this phagemid an important prototype for phage therapy.

Keywords: '10-23' DNAzyme, DNAzyme vectors, Escherichia coli, phagemid transfection.

I. INTRODUCTION

Antisense oligonucleotides are short segments of DNA or RNA that can inhibit translation or the function of a given gene transcript. Antisenses are organized into three classes: antisense oligodeoxynucleotides (AS-ODN), interfering RNAs (irRNA), and nucleic acid enzymes (DNAzymes and ribozymes) [1]-[3]. Although used as potential therapeutic agents, the main problems related to antisense oligonucleotides are that they are easy targets for nucleases, immunogenic, and require appropriate delivery that directs them toward a specific cell type. Recent studies have focused on chemical modifications of these molecules to make them more chemically stable. However, increasing stability almost always causes reduced specificity/affinity for their respective targets [4].

'10-23' DNAzymes, which cleave RNAs, are more chemically stable and exhibit higher catalysis than the other antisense classes [2], [4], [5]. The first plasmid-based DNAzymes expression system was developed to interact with human cells in culture [6]. This system was improved during subsequent years [7]-[10], which also resulted in the development of the first vector for Escherichia coli [11]. Intracellular DNAzyme expression becomes necessary because it generates an adequate quantity of these molecules for a sufficient time to obtain a desired biochemical/physiological effect [4]. Plasmid vectors are tools that ensure this expression.

Recently, there has been a renewed interest in the use of phages as they are promising therapeutic agents. They can be used in the treatment of bacterial infections that are super- and multi-resistant to traditional antibiotics [12], [13]. Phage therapy began approximately 20 years before the practical application of penicillin (the first reported antibiotic) in the former Soviet Union and some eastern European countries [12], [14], [15]. However, the antibiotic era ended phage therapy. Recently, the FDA suggested a profile for therapeutic phages: they are obligatorily lytic, "non-transducing" agents that preferably recognize bacterial virulence factors and originate from natural environments [16].

The M13 phage is a non-lytic and non-lysogenic filamentous virus that infects E. coli after binding to the sex pilus of this bacteria [12], [17]. Wan and Goddard [17] and Lin et al. [18] report that if M13 were a lethal phage, it would be a promising weapon against the horizontal transfer of pathogenicity genes, including antibiotic resistance genes. Genes that confer multidrug resistance are highly disseminated from extraintestinal pathogenic E. coli (ExPEC) via conjugation. A modern system for phagemid DNAzyme
expression, which can be delivered by M13, was developed based on these tools.

II. MATERIAL AND METHODS

A. Bacterial Strains

The following bacterial strains were used in this study: E. coli BL21(DE3) (F ompT hsdS (r− m−) gal dcm (DE3)), E. coli DH5α (F φ80lacZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK− m−) phoA supE44 Δ thi-1 gyrA96 relA1), E. coli DH5α F’lc2ZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK− m−) phoA supE44 Δ thi-1 gyrA96 relA1/F’[proAB’ lacFZAM15 zf::Tn5 (Km+)], E. coli DH10b (F mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZAM15 ΔlacX74 recA1 endA1 araD139 Δ(arad-leu)7697 galU galK Δ phi80(Str+)supG), E. coli JM109 (endA1, recA1, gyrA96, thi, hsdR17 (rK− m−), relA1, supE44, Δ lac-proAB), [F’traD36, proAB, lacFZAM15]), E. coli K-12 MG1655 (F’λambdab-λIG- rfb-50 rph-1), E. coli TOPO 10 (F mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZAM15 ΔlacX74 recA1 araD139 Δ(arad-leu)7697 galU galK Δ phi80(Str+)supG).

B. Vectors and Helper Phage

pRT 30-2 is an expression vector for Moloney murine leukemia virus reverse transcriptase (MoMuLV-RT), kindly provided by Dr. Stephen Goff—Columbia University. This vector was used as a basis for constructing the pDESCP phagemid, which contains the ftsZ DNAzyme expression system developed in the present study. The pGEM®-T Easy vector (PROMEGA) was used to obtain the Ori fl gene region. The ftsZ DNAzyme cassette was chemically synthesized (GenOne, RJ-Brazil) and bound to pUC19 (pUC19-φlac-fzS). pRT30-2/Dz-fzS is a vector that contains the ftsZ DNAzyme expression system without the Ori fl region. VCSM13 (Stratagene) was the helper phage used to promote encapsidation of pDESCP and its subsequent delivery into E. coli.

C. Construction of pDESCP

Construction of this vector involved integrating two cassettes: one for MoMuLV-RT enzyme expression and the other for RNA transcript production to serve as a substrate for the synthesis of the ftsZ DNAzyme (developed by Tan et al. [11]) by reverse transcription. The cassette for ftsZ DNAzyme expression was designed and obtained by chemical synthesis (Genome Biotechnologies), and subsequently subcloned into pUC19, thus named pUC19-φlac-fzS. This, in 5’→3’ direction, has the following structures: CAP binding region, lac promoter, lac operator, a terminator of the reverse transcription for MoMuLV-RT, ftsZ DNAzyme sequence, a binding site for MoMuLV-RT, and a terminator of general transcription. HindIII sites were inserted at both ends of the cassette. Other sites for restriction endonucleases (EcoRI, EcoRV, XhoI, ApeI, XbaI, AvaII, SalI) were also strategically inserted throughout this cassette to facilitate future manipulations. The nucleotide sequence of this entire structure is shown in Fig. 1.

D. Effect of pDESCP in E. coli

The growth curves for five cultures of the DH5αF’lc2 strain transformed with the following vectors and cultured under the following conditions, respectively, were constructed to evaluate the effect of pDESCP on growth and/or viability of E. coli: pRT30-2 (1% glucose), pRT30-2 (1 mM IPTG), pDESCP (1% glucose), and pDESCP (0.1 mM and 1 mM IPTG). All of the cultures were incubated at 37 °C, in LB liquid medium, under agititation at 180 rpm, with absorbance measured every 2 h for 96 h.

E. Analysis of the ftsZ DNAzyme Expression System

DH5αF’lc2/pDESCP E. coli clones were grown in both LB liquid and solid culture media, both containing 100 μg/mL ampicillin, in the presence of an inducer (1 mM IPTG) or absence of activation (1% glucose) of ftsZ DNAzyme expression. Untransformed DH5αF’lc2 E. coli was used as a negative control. The cells were incubated at three temperatures: 32, 37, and 42 °C for 16 h. Growth in LB liquid medium occurred under continuous agitation at 180 rpm. After the incubation period, microscopic slides of all the cultures were prepared. The slides were heat-fixed, stained with safranin for 45 s, and observed under an optical microscope at 1,000 × magnification to phenotypically
analyze the colonies. *ftsZ* DNAzyme expression was determined by analyzing the presence or absence of these molecules together with total RNA, extracted by TRizol®, obtained from a clone grown in liquid medium containing 1 mM IPTG (modified from Mirochnitchenko et al. [19]).

**F. Transfection of pDESCP**

DH5αF’IQ/pDESCP *E. coli* cells were infected with a VC5M13 helper phage (adapted from Rader et al. [20]) to obtain the viral capsids of the M13 phage containing pDESCP. As the VC5M13 genome is strategically defective to packaging, the viral particles obtained were predominantly pDESCP. These particles were used to transfect the DH5αF’IQ and JM109 *E. coli* strains. The DH5α, DH10b, BL21(DE3), and K-12 strains were used as negative controls of the transfection.

**III. RESULTS**

**A. Construction of the Phagemid**

Construction of the pDESCP vector was confirmed by analyzing the restriction profile (Fig. 3). Lane 3 shows linearization of the pRT30-2 vector by *HindIII*. Digestion of pDESCP with *HindIII* liberated a single 1,048-bp insert (lane 4), as expected. This fragment corresponds to the *ftsZ* DNAzyme cassette (576 bp) fused to the Ori *fl* region (472 bp). The Ori *fl* bond within the *ftsZ* DNAzyme cassette was also confirmed via double-digestion of the vector with the EcoRI and XhoI enzymes (lane 5), releasing a 472-bp fragment. These results even corroborate the Ori *fl* amplicon, obtained from pGEM®-T Easy using Orifl1-F/Orifl1-R (lane 6).

**B. Analysis of *ftsZ* DNAzyme induction in *E. coli***

The growth curve (data not shown) demonstrated that the experimental controls, represented by DH5αF’IQ *E. coli* containing pRT 30-2/1% glucose, pRT 30-2/1 mM IPTG, and pDESCP/1% glucose, respectively, grew exponentially until approximately 4 h. After this time, they enter into a long stationary phase. The cultures containing 0.1 and 1 mM pDESCP/IPTG, respectively, also exhibited exponential growth until 4 h, subsequently entering into a long stationary phase. However, after 4 h there was a significant increase in optical density (OD) of these cultures, compared with the control cultures, indicating higher cell mass.

The *ftsZ* DNAzyme expression system induced in DH5αF’IQ *E. coli* resulted in altered morphology of this bacterium, from a bacillary to filamentous form (Fig. 4, boxes 9–12), compared to the control cultures (Fig. 4, boxes 1–4). Throughout each filamentous structure, the contrasting presence of two or more nucleoids was noted when the slides were micrometrically adjusted under the microscope. The cultures grown in solid medium exhibited more expressive filamentation than those grown in liquid medium (images not shown), regardless of the growth temperature analyzed (32, 37, and 42 °C). The micromorphology of the DH5αF’IQ/pDESCP clones grown in medium containing 1% glucose, and without IPTG, showed cells slightly more elongated than the control cultures (boxes 5–8). In some of these cells, it is also possible to observe the presence of one or more nucleoids.

Even based on pDESCP induced in DH5αF’IQ *E. coli*, it was possible to demonstrate the presence of what appears to possibly be a single-strand DNA through 3% agarose gel (data not shown). These qualitative data generate evidence that the *ftsZ* DNAzymes being properly produced, as all of the extracts obtained for single-strand DNA isolation were exhaustively treated with RNase A.
Fig. 4. Effect of pDESCP in DH5αF'Iq E. coli grown in LB agar: 1 - without vector and with 1% glucose; 2 - without vector and with 1 mM IPTG; 3 - with pRT 30-2 and 1% glucose; 4 - with pRT 30-2 and 1mM IPTG; 5, 6, 7, and 8 - with pDESCP and 1% glucose; 9, 10, 11, and 12 - with pDESCP and 1mM IPTG.

A - JM109

B - DH5αF'Iq

Fig. 5. Transfection in E. coli. A - JM109 E. coli: 1 - untransfected; 2, 3, and 4 - transfected. B - DH5αF'Iq E. coli: 1 - untransfected; 2, 3, and 4 - transfected.

C. Transfection of pDESCP

The pDESCP phagemid was properly transfected into JM109 and DH5αF'Iq E. coli, both F+ strains, from VCSM13. Micromorphological analysis of these bacteria confirmed that they became filamentous soon after transfection when they were grown in LB solid medium containing 1 mM IPTG (Fig. 5). Plasmid extraction performed in these cells demonstrated the presence of transfected pDESCP (results not shown). Non-producing strains of the F' sex pilus, DH5α, DH10b, BL21(DE3), and K-12 were unable to receive the phagemid and thus did not grow in pDESCP-selective medium (containing ampicillin). These findings demonstrate the efficiency of the delivery system and fisZ DNAzyme expression in F+ E. coli strains.

IV. DISCUSSION

pDESCP is an approximately 6-kb phagemid and is based on pRT 30-2. The latter was constructed from pKK 177-3 (Pharmacia, STO-Sweden), where the region comprised between the BamHI and HindIII sites presents the same basic sequence of the PvuII-HindIII region of the pKK vectors (the BamHI site was inserted with PvuII – Telesnitsky et al. [21]). Analysis of the enzymatic digestions performed with this vector allowed for confirming both insertion of the fisZ DNAzyme expression cassette and Ori fI (Fig. 3). The latter is positioned between the MoMuLV-RT and fisZ DNAzyme cassettes, as shown in Fig. 2.

Construction of pDESCP causes an RNA transcript to be released from the induced lac promoter. This transcript has a
binding site for MoMuLV–RT. For this enzyme to bind to this region, it should initially bind to an endogenous tRNA^ts of E. coli, which functions as a reverse transcription primer (Tan et al. [11]). pDESCP differs from the other DNAzyme expression vectors by exhibiting a more refined system. This is because the ftsZ DNAzyme exhibits expression independent of the MoMuLV–RT enzyme. Expression of this reverse transcriptase is regulated by pA1, a constitutive expression promoter, derived from the f1 phage (Telesnitsky et al. [21]). According to Prof. Stephen Goff (personal communication), pA1 is the most adequate promoter for the expression of this enzyme, as it can express adequate levels, compatible with its efficient catalytic activity. MoMuLV–RT expression by pA1 corresponds to ~4% of the total cellular proteins of E. coli [21].

pDESCP differs from the main DNAzyme vectors developed: pssXA, pssXB, pssXD, pssXE, and pssXGb. Of these, only pssXGb was constructed to act in E. coli, where the others were directed for human cells in culture. Transcription of a single cassette occurs in all of them, which contains both the reverse transcriptase sequence, which will be translated, and the sequence that leads to the DNAzyme of interest. A strong promoter is used in all vectors to ensure a high transcription rate. In 2010, Li et al. [5] reported the construction of a modern vector regulated by two promoters, pSDE01–DZ1. This vector was developed to act in human macrophages in culture (it does not work for bacteria). According to the authors, this type of vector is the most adequate model for DNAzyme expression, as it regulates the production of two transcripts that must be produced in different quantities: higher production of the transcript that leads to DNAzyme and lower expression of the reverse transcriptase. MoMuLV–RT has a relatively high catalytic processivity and endogenous RNase H activity that degrades the RNA substrate to the extent that it is retrotranscribed for the respective DNAzyme. Li et al. [5] speculated that excessive production of the reverse transcriptase enzyme could be toxic for cells.

The ftsZ DNAzyme used in this study exhibited a 10–23 catalytic motif and targeted the transcript of the ftsZ gene. The FtsZ protein is the prokaryotic tubulin homologue in eukaryotes. In E. coli, FtsZ is the primary component of the cell division apparatus, forming a structure known as a Z-ring, which is the division site. The Z-ring, associated with other cell division proteins, acts as a contractile ring that performs bacterial division [11], [22]. [23].

Tan et al. [11] demonstrated in vitro that the ftsZ DNAzyme they developed, expressed by pssXG, was able to cleave the transcript of the ftsZ gene at the proposed site. They found, in vivo, that expression of this DNAzyme in DH5α E. coli could alter the morphology of this bacteria, from a bacillary to filamentous form, with the presence of two or more nucleoids per cell. Tan et al. [11] also reported that these polyploid cells can grow; however, are more sensitive to lysis than normal cells. In the current experiments, induction of pDESCP triggered during the filamentation process of DH5αFlq E. coli primarily occurred in solid culture medium (Fig. 4). These findings corroborate those described by Tan et al. [11]; however, we could not find any possible sensitivity of these cultures to lysis, not even when they were grown in liquid medium. Growth curve analysis did not generate evidence that the DH5αFlq/pDESCP E. coli cultures, appropriately induced with IPTG, are more sensitive to lysis because of the ftsZ DNAzyme expression (results not shown). ftsZ DNAzyme expression based on pDESCP is mainly sustained by the filamentous phenotype observed. The likely presence of single-strand DNA, through 3% agarose gel (not shown), and increased D.O., observed in the growth curve of DH5αFlq/pDESCP cultures, also support the hypothesis that ftsZ DNAzymes are produced. In an important experiment performed by Stricker and Erickson [22], it was found that mutant ftsZ E. coli divided for approximately 180 min, followed by filamentation between 200 and 240 min. During this period, the cells were 6 to 12 times longer than the normal cells and further incubation of these bacteria resulted in slightly increased filamentation, followed by cell lysis.

The filamentous phenotype observed in the DH5αFlq (Fig. 4, boxes 9–12; Fig. 5B, boxes 2–4) and JM109 E. coli strains (Fig. 5A, boxes 2–4), both transformed and transfected with pDESCP, and suggested some disorganization in the division apparatus of these bacteria. The lack of, or possibly reduced, FtsZ protein at the intracellular level can thus result in the disorganization of the E. coli contractile ring, preventing it from dividing correctly. In boxes 5–8, of Fig. 4, some degree of elongation and more than one nucleoid can be seen in some DH5αFlq/pDESCP cells that were grown in medium containing 1% glucose. Therefore, because it is the lac promoter, it does not exclude possible residual expression in pDESCP/1% glucose.

An interesting finding described by Charbon et al. [24] is the importance of crescentin in the maintenance/stabilization of the elongated cell shape in the bacterium Caulobacter crescentus. Crescentin is a protein with similar functions to the intermediate filaments of eukaryotic cells, i.e., form a two-dimensional mesh under the plasma membrane inside these bacteria, able to support the form and their mechanical stress [24], [25]. According to Charbon et al. [24], crescentin extends along the length of the cell. These authors cultured an E. coli strain (CIJW2209) on a solid surface under conditions in which the ftsZ gene was not being expressed, whereas the crescentin gene was. It was found that as cells grow, becoming filamentous, the crescentin mesh slowly increased, suggesting that it acts as a mechanical support for the new dimensions of the cell. Elongation of the crescentin structure is bidirectional, occurring at both ends toward the center. When this filamentation process occurred in liquid culture medium, the protein mesh was responsible for occasional bending in the form of the bacteria, concentrating at the points of curvature.

Cabeen et al. [25] reported that when crescentin is ectopically synthesized in E. coli, a γ-Proteobacterium could form functional filamentous structures throughout the cell membrane, which is responsible for maintaining curvature during this bacteria's elongation. Despite the large evolutionary difference between E. coli and C. crescentus, this indicates that the link between crescentin and the cell wall is mediated by genetic components, such as MreB. MreB is a protein found in some bacteria and is considered as an actin homologue. However, crescentin does not bind to bacteria without MreB, and these bacteria should be closer from the evolutionary point of view than E. coli. Crescentin, or its equivalent form, has still not been found naturally occurring in E. coli. There is still much to be clarified regarding the E. coli cytoskeleton.

Because of the FtsZ protein's function and the results...
obtained in this study, we suggest that the ftsZ DNAzyme expression system is in fact functioning. However, we speculate that E. coli is creating some mechanism to support the tension imposed by its increased cell surface, to its detriment by reducing the cell division frequency, caused by reduced FtsZ. This does not eliminate that the maintained cell viability of E. coli in the filamentous state is possibly supplied by a two-dimensional protein mesh similar to crescentin [1, 24]-[28]. Moreover, it is important to note that much remains to be understood about the behavior of the FtsZ protein in E. coli, their interactions, and dynamics before and during bacterial division [23], [29], [30].

The efficiency of pDESCH transflection is fundamental as a biopharmaceutical prototype to this phagemid. The possibility of inserting new DNAzymes, via Apal, XbaI, AvrII, and Sall sites illustrates the probability of developing a more aggressive bactericidal biopharmaceutical. pDESCH has the potential to act as a tool for the reduced dissemination of virulence factors based on pathogenic E. coli (ExPEC - extraintestinal pathogenic E. coli), as this occurs exclusively via conjugation or horizontal transfer [31].

V. CONCLUSION

The DNAzyme expression vector operates with two promoters and, from the observed results, it is possible to suggest that the DNAzyme ftsZ is being expressed and causing a decrease in the translation of this protein, in the E. coli bacterial model. However, for the complete validation of this system, it is necessary to analyze the downregulation of the FtsZ protein. The delivery of pDESCH for F + strain of E. coli cells, using VCSM13, and the possible insertion of other DNAzymes in the cassette makes this phagemid an important prototype for gene silencing using DNAzymes.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the collaboration of Prof. Stephen Goff (Columbia University), Prof. José Odair Pereira (Federal University of Amazonas – UFAM), the Doctoral Program in Biotechnology at the UFAM, the Foundation for Research Support of Amazonas (FAPEAM), the Brazilian National Council for Scientific and Technological Development (CNPq), and the Coordination for the Improvement of Higher Education Personnel (CAPES).

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