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Purification of essentially RNA free plasmid DNA using a modified *Escherichia coli* host strain expressing ribonuclease A

G.D. Cooke [a,c], R.M. Cranenburgh [b,d], J.A.J. Hanak [b], P. Dunnill [a], D.R. Thatcher [b], J.M. Ward [c,*]

[a] *The Advanced Centre For Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK*

[b] *Cobra Therapeutics Ltd, The Science Park, University of Keele, Keele, Staffordshire ST5 5SP, UK*

[c] *Department of Biochemistry and Molecular Biology, University College London, Darwin building, Gower Street, London WC1E 6BT, UK*

[d] *Division of Molecular Genetics, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*

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Abstract

Regulatory agencies have stringent requirements for the large-scale production of biotherapeutics. One of the difficulties associated with the manufacture of plasmid DNA for gene therapy is the removal of the host cell-related impurity RNA following cell lysis. We have constructed a modified *Escherichia coli* JM107 plasmid host (JMRNaseA), containing a bovine pancreatic ribonuclease (RNaseA) expression cassette, integrated into the host chromosome at the *dif* locus. The expressed RNaseA is translocated to the periplasm of the cell, and is released during primary plasmid extraction by alkaline lysis. The RNaseA protein is stable throughout incubation at high pH ($\sim$12–12.5), and subsequently acts to hydrolyse host cell RNA present in the neutralised solution following alkaline lysis. Results with this strain harbouring pUC18, and a 2.4 kb pUC18Δ*lacO*, show that sufficient levels of ribonuclease (RNase) activity are produced to hydrolyse the bulk of the host RNA. This provides a suitable methodology for the removal of RNA, whilst avoiding the addition of exogenous animal sourced RNase and its associated regulatory requirements.

Keywords: Chromosomal integration; Periplasm; RNase; Gene therapy

Abbreviations: λ, Bacteriophage lambda; *A*, absorbance; aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; CJD, Creutzfeldt–Jakob disease; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; EMEA, European Agency for the Evaluation of Medicinal Products; EtdBr, ethidium bromide; FDA, Food and Drug Administration; IPTG, iospropyl β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; OD, optical density; p, plasmid; P, promoter; RNase, ribonuclease; RNaseA, bovine pancreatic ribonuclease; SDS, sodium dodecyl sulphate; TBE, tris–borate EDTA buffer; TE, 10 mM Tris–HCl, 1 mM EDTA buffer; TSE, transmissible spongiform encephalopathies; WHO, World Health Organisation.

* Corresponding author. Tel.: +44-20-76792242; fax: +44-20-76797193.

E-mail address: ward@biochemistry.ucl.ac.uk (J.M. Ward).

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1. Introduction

The difficulties associated with the large-scale production of biotherapeutics provide a constant challenge to the biotechnology industry. However, it is critical to bring bioproducts to market quickly and within budget, especially for patented products. Processes based on recombinant DNA technology require extensive characterisation in order to meet international regulatory agency requirements for biotherapeutics, concerning both product- and process-derived impurities and contaminants (FDA, 1996; Middaugh et al., 1998; DiPaolo et al., 1999).

Plasmid borne genes are such a biotherapeutic, promising a new generation of DNA vaccines, tumour therapy, and gene replacement therapy (Liljeqvist and Stahl, 1999; Mountain, 2000). One of the challenges associated with such technology is the development of large-scale processes for the production of cost-effective plasmid DNA that is capable of providing the required levels of purity, potency, efficacy, and safety (FDA, 1996; Prazeres et al., 1999; Varley et al., 1999). The introduction to patients of plasmid or host nucleic acid sequences that are potentially oncogenic, immunogenic, or that encode antibiotic resistance genes, is of particular concern (Williams et al., 1998). As such, host RNA contamination of a recombinant therapeutic product must be minimised, particularly for therapies that require multiple patient dosing (DiPaolo et al., 1999). The work described here focuses on a biological method for the removal of RNA contamination of plasmid DNA by host cell engineering.

Methods of removing bulk RNA from plasmid lysate preparations include selective precipitation, typically with polyethylene glycol and/or metal salts, ammonium acetate, filtering through diatomaceous earth, and heat treatment (Higgins et al., 1976; Horn et al., 1995; Ferreira et al., 1999) and are covered by several patents (Marquet et al., 1995; Horn et al., 1996; Chen and Ruffner, 1998).

An alternative method of removing RNA is the use of bovine derived pancreatic ribonuclease A (RNaseA, EC 3.1.27.5) (McClung and Gonzales, 1989), an endoribonuclease favoured for small-scale plasmid isolation in molecular biology. This is a highly effective method of removing the host RNA released during/after lysis, due to the robust nature of the active protein, which continues to be stable throughout incubation at high pH. RNaseA is a monomeric protein of 124 amino acid (aa) residues, which catalyses the degradation of single stranded RNA via a transphosphorylation mechanism. Initially, a 2′,3′-cyclic phosphate intermediate is formed, which is then hydrolysed, producing a 3′-phosphate product. This hydrolysis serves to break down large molecular weight RNA species into smaller oligoribonucleotides, which have suitably different physicochemical properties compared with plasmid DNA, and can thus be easily separated further down the process stream.

The use of RNaseA is, however, not without complication. Urgent questions arose concerning the introduction of bovine- (animal-) derived products and by-products entering the food chain, or being used in the production of therapeutics, following the report by the national health authorities in the United Kingdom of new variant Creutzfeldt–Jakob disease (CJD) (Will et al., 1996). Subsequently, the conclusions and recommendations of the surveillance report of the World Health Organisation (WHO) consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies (TSE) (WHO, 1997), recommended that the use of bovine derived materials should be avoided entirely where possible. The FDA and the European Agency for the Evaluation of Medicinal Products (EMEA) now have guidelines indicating that the use of animal sourced materials should be avoided in the production of biotherapeutics. The substitution of bovine RNaseA with recombinant RNaseA is one option, but would be expensive given the quantities required upon scale-up for commercial manufacture.

Monteiro et al. (1999) have described the use of endogenous nuclease activity remaining in lysates of the non-nuclease deficient *Escherichia coli* strain WK6, achieving a 40% reduction in the level of RNA. However, the nuclease activity is not RNA specific, and the super-coiled plasmid

DNA is also hydrolysed, resulting in the conversion of the DNA to open circular (so called nicked), and linear forms. This is not desirable given the FDA guidelines concerning the requirement of homogenous, super-coiled plasmid DNA for successful drug approval (1996).

Here, we describe an approach involving genetically engineering the host cell to enhance the large-scale purification of essentially RNA free plasmid DNA, via the production of a modified *E. coli* host strain encoding a chromosomal RNaseA expression cassette. Pre-RNaseA is expressed and targeted to the periplasm during growth, this sequestration protects the cytoplasmic RNA from the active protein. The mature, active RNaseA, is then released during lysis, to hydrolyse host RNA. This methodology removes RNA early in the process operation, avoiding additional precipitation stages that decrease product yield, and without having to resort to the addition of either animal-sourced, or recombinant enzymes. We see this as a step towards the production of tailor-made expression strains facilitating ease of purification for both biotherapeutics and recombinant products in general.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The *E. coli* K-12 strain used as a plasmid host was JM107 [F'Δ*traD36 lacI*^{q}Δ (*lacZ*)*M*15 *proA*$^{+}$ *B*$^{+}$/e14^{-}(McrA^{-}) (*lac-proAB*) *thi gyrA*96 (Nal^{r}) *endA*1 *hsdR*17($r_k^- m_k^+$) *relA*1 *supE*44] (Yanisch-Perron et al., 1985). Bacteria were grown under standard conditions in nutrient broth, on nutrient agar (Oxoid), or in Terrific Broth (Gibco BRL) supplemented with glycerol (4 ml l^{-1}), at 37°C, supplemented with antibiotics as appropriate: ampicillin sodium salt (100 μg ml^{-1} in liquid media and 500 μg ml^{-1} in agar plates), kanamycin sulphate (50 μg ml^{-1}) and tetracycline hydrochloride (20 μg ml^{-1}).

Plasmids used in this study included the construct pQR163 described previously (Tarragona-Fiol et al., 1992), which comprises a bovine cDNA sequence encoding the pre-RNaseA protein, and an additional 5′-cistron encoding a hexapeptide, cloned into pKK223.3 (Pharmacia) under the control of the *tac* promoter. pN1D274E*kan*1 is pN1 (pUC18 containing a 5.5-kb fragment of the *E. coli* genome, including the *dif* locus region, inserted into the *Bam*HI site of the polylinker, Leslie and Sherratt, 1995) with *lacI*^{qs} from pIQ (Chang et al., 1994) inserted between two *Sty*I sites, and a *Pst*I fragment containing the *kan* gene from pUC4K (Pharmacia) cloned into an *Nsi*I site (Fig. 1).

pUC18Δ*lacO* was derived from pUC18 by restriction with *Pvu*II, yielding two products, a 322-bp fragment containing *lacZM15*, $lacO_1$ and $lacO_3$ operator sequences, and the remaining backbone fragment of the plasmid (2364 bp). The larger fragment was isolated by gel extraction, and re-ligated using T4 DNA ligase.

2.2. Plasmid DNA isolation and analysis

Plasmid DNA was isolated by alkaline/sodium dodecyl sulphate (SDS) lysis (Birnboim and Doly, 1979), using commercially available kits (Qiagen). Preparative-scale plasmid purification (500 ml) was carried out by means of alkaline/SDS lysis followed by isopropanol precipitation, and CsCl/ethidium bromide (EtdBr) density gradient centrifugation. Digestion of plasmid DNA with restriction enzymes and other enzymic modifica-

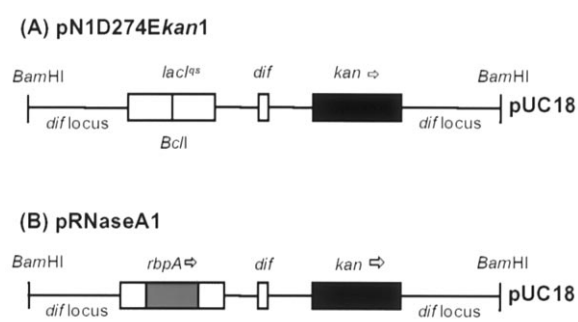

Fig. 1. Genetic maps of pN1D274E*kan*1 and pRNaseA1. (A) pN1D274E*kan*1 is pN1 with *lacI*^{qs} from pIQ and the *kan* gene from pUC4K (see Section 2.1). (B) pRNaseA1 is pN1D274E*kan*1 with the 775 bp *Bam*HI P_{tac}-*rnaseA* expression cassette inserted into the *Bcl*I site of *lacI*^{qs}. pRNaseA1 has the insert in the same orientation as the *kan* (see Section 2.3).

tion (New England Biolabs and GibcoBRL) was carried out under conditions recommended by suppliers. Electrophoresis was performed using either 1 or 1.5% (w/v) agarose gels in tris–borate EDTA (TBE) buffer. Plasmid constructs were transformed into $CaCl_2$ competent cells and clones of the correct structure identified by restriction analysis following plasmid isolation. These and all other techniques were carried out using standard methodologies (Sambrook et al., 1989).

2.3. Construction of JMRNaseA — chromosomal integration of the RNaseA expression cassette

The P_{tac}-*rbpA* (*rbpA*, RNaseA from Bovine Pancreas) was excised from pQR163 as a 775-bp fragment by *Bam*HI restriction, and ligated into *Bcl*I linearised pN1D274E*kan*1 (non-methylated plasmid was prepared from *E. coli* GM2163, which is *dam*$^-$). This process destroys both the *Bam*HI and *Bcl*I sites, producing two constructs containing the RNaseA insert in each orientation. pRNaseA1 (insert in the same orientation as *kan*, Fig. 1) was used for this investigation.

The RecA$^+$ strain JM107 was transformed with pTP223, which contains *tet*, the λ red recombination functions *bet* and *exo*, and the RecBCD-inhibiting λ gam (Murphy, 1998). Electro-competent JM107[pTP223] were transformed with the 8.3 kb *Bam*HI fragment of pRNaseA1, and incubated overnight at 37°C. JM107[pTP223]::*rbpA* recombinants, produced by a double recombination event occurring at the areas of *dif* locus homology, were selected on nutrient agar by kanamycin resistance. Recombinant clones were confirmed by sensitivity to ampicillin, and pTP223 was cured by growth in the absence of tetracycline. The chromosomal insertion of *rbpA* was confirmed by polymerase chain reaction (PCR) using specific primers designed to the RNaseA gene — 5′-GCAGCAGC-CAAGTTTGAGCGGCAG 3′ and 5′-GCACG-TACGGGTTTCCCTCACAAG 3′, which flank a 340 bp-region of the insert. Single colony transformants were picked, resuspended in 100 μl of sterile water, and 1 μl used as a source of template chromosomal DNA. PCR was performed in 20 μl, containing 1 μl DNA, 1 μM of each primer, 250 μM of each deoxyribonucleoside triphosphate (dNTP), 1 U *Taq* polymerase, and 2 μl 10 × *Taq* polymerase buffer, overlaid with 50 μl mineral oil. The PCR was run for 26 cycles of 95°C, 4 min (first cycle only); 95°C, 1 min; 55°C, 2 min; 72°C, 1 min (25 cycles); 72°C, 2 min (last cycle only).

2.4. Growth curve and plasmid yield of JMRNaseA in comparison to the parental strain JM107

Well defined colonies of JM107[pUC18Δ*lacO*] and JMRNaseA[pUC18Δ*lacO*] were picked from freshly streaked nutrient agar plates containing ampicillin, and ampicillin and kanamycin, respectively, and used to inoculate 5 ml of nutrient broth containing ampicillin, in a 20-ml universal bottle. These cultures were grown at 37°C in an orbital shaker (200 rpm) until mid-exponential growth phase, whereupon 4 ml of culture was used to inoculate 200 ml of sterile Terrific Broth containing ampicillin in a 1 l conical flask. These cultures were grown to an optical density (OD) of ~ 7.0 A_{600}, before being used to inoculate the fermenters. Batch cultures were grown in Applikon 7 l (working volume 5 l) fermentation vessels, fitted with three equally spaced top driven turbines. Medium comprising 4.8 l Terrific Broth supplemented with glycerol (4 ml l^{-1}), was sterilised in situ, before the aseptic addition of ampicillin using 0.2-μm filters (Gelman laboratory). The vessels were inoculated using the cultures described, and batch fermentation performed to the following parameters, pH 6.8; dissolved oxygen setpoint 50% of air saturation; temperature 37°C. Throughout growth, DO_2 was maintained at 50% by increasing the rate of stirring (1000 rpm maximum), and pH kept constant by titration with 5 M NaOH and 2 M HCl as required. Online monitoring and control was performed using the Biowatch software package (Applikon). The vessel was sampled regularly and the OD A_{600} recorded to allow the calculation of specific growth rates.

Plasmid DNA was isolated from comparative cell samples ($150/OD_{600}$ ml) taken at the fermentation end-points using Qiagen Maxi plasmid purification kits (Qiagen Ltd). Quantitative plas-

mid yields of the modified and parental host strains were subsequently calculated by ultraviolet spectrophotometry (absorbance at 260 nm), using a Beckman DU-600 spectrophotometer.

2.5. Expression and activity of RNaseA in E. coli JMRNaseA

JMRNaseA containing different plasmids was grown in 200 ml of media containing ampicillin, in 2-l shake-flasks at 37°C, and growth was monitored by measuring absorbance (A) at 600 nm. When cultures were in early exponential growth ($A_{600} \sim 0.4$), RNaseA expression was induced by the addition of iospropyl β-D-thiogalactopyranoside (IPTG) to 2.0 mM. Cultures were then left to grow overnight (approximately 20 h in total).

Comparative samples (~ 1.5 ml) of culture were lysed (Birnboim and Doly, 1979), using mini-prep reagents (Qiagen), according to the protocol provided, but without the addition of RNaseA to the lysis buffer (P1). The relative levels of RNA in the cleared lysates of the JMRNaseA plasmid host were compared with those of JM107 prepared in the presence and absence of the Qiagen RNaseA supplement. To reduce salt concentrations, and thus facilitate electrophoresis, 400-μl lysate samples were precipitated with 2.5 volumes of 100% ethanol, and resuspended in 50-μl 10 mM Tris–HCl; 1 mM EDTA (TE) buffer. The levels of RNA in 10-μl samples were compared on 1.5% agarose gels containing 0.6 μg ml^{-1} ethidium bromide and the relative levels of RNA estimated by band intensity, using the UVIBand software package (v97.04, UVITech).

3. Results and discussion

3.1. Construction and characterisation of JMRNaseA

We have immobilised a 775 bp RNaseA expression cassette from pQR163 within the genome of *E. coli* JM107 using the bacteriophage λ recombination system Red. The genotype of JMRNaseA is [(F'*traD36 lacI*^{q} Δ(*lacZ*)*M*15 *proA*$^+$*B*$^+$)/e14$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA*1 *hsdR*17($r_k^- m_k^+$) *relA*1 *supE*44 Æ*hipA*::*rbpA*::*kan*]. Essentially, this cassette comprises a cDNA sequence encoding pre-bovine pancreatic RNaseA preceded by a cistron encoding a hexapeptide, under control of P_{tac}, and other associated regulatory sequences required for efficient transcription and translation. Recombination occurred between the regions flanking the insert cassette, and homologous sequences, which flank the core 28-bp sequence of the *dif* locus region of the *E. coli* chromosome. The regions flanking the cassette were introduced by cloning the *rbpA* fragment into pN1D274E*kan*1. Previous work has shown the *dif* locus to be a suitable site for integrating additional sequences into the genome, as the disturbance has a minimal (if any) effect upon the stability of the host, providing that the integrity of core sequence is maintained (Leslie and Sherratt, 1995; Williams et al., 1998). The core *dif* sequence was maintained by introducing an identical additional site from within pN1D274E*kan*1 (Fig. 1). The presence of the integrated *rbpA* cassette was confirmed by PCR.

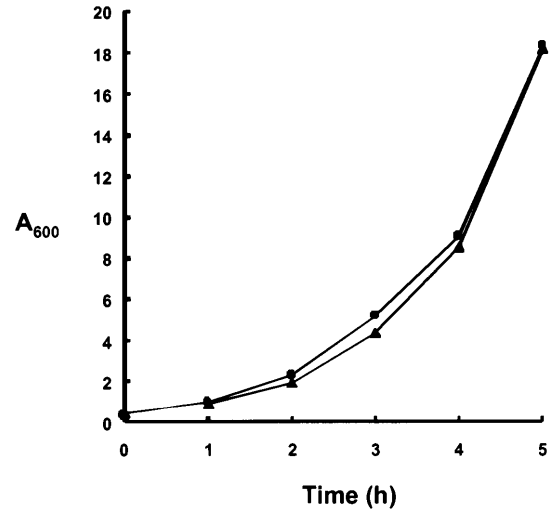

Fig. 2. Growth curves of JMRNaseA[pUC18Δ*lacO*] (●) and JM107[pUC18Δ*lacO*] (▲).

Growth curve analysis in 5-l fermentations of induced cultures of JMRNaseA[pUC18Δ*lacO*] compared with JM107[pUC18Δ*lacO*] showed that the modified strain was unaffected in comparison to the parent strain (Fig. 2). From these data,

μ_{max} values were calculated for the modified and parental strains, and found to be identical (0.77 h^{-1}), indicating that expression of RNaseA did not exert a significant metabolic burden, and was not toxic to the cell.

Quantitative comparison of plasmid levels purified from JMRNaseA and the parent strain JM107 showed yields of 1.6 and 1.1 μg per OD_{600}, respectively, indicating that the expression of RNaseA had not compromised the capacity of the strain to act as a plasmid expression host.

3.2. Induction of RNaseA expression in cultures for plasmid preparation

RNaseA expression was induced by the addition of IPTG at early log phase (OD $A_{600} \sim 0.4$), to a final concentration of 2.0 mM. After growth had continued overnight (~ 20 h, final OD $A_{600} \sim 2.5$), comparative samples of cultures were lysed and the relative levels of RNA analysed following precipitation and electrophoresis (Fig. 3).

The lysis of JM107[pUC18Δ*lacO*] in the presence and absence of 10 μg ml^{-1} RNaseA supplement provided positive and negative controls to the hydrolysis of the host RNA. The lysate of JM107[pUC18Δ*lacO*] contained no visible RNA following lysis in the presence of the RNaseA supplement, while in the absence of any RNaseA, a large characteristic smear was displayed on the gel in the region corresponding to the molecular size of RNA species.

JM107[pQR163] lysates had essentially no visible RNA following lysis, regardless of induction. The uninduced level of RNaseA activity from pQR163 corresponds to the basal level of transcription from P_{tac} under the leaky control of $LacI^q$. As pQR163 is based upon the pBR322 replicon, and thus has a copy number of ~ 30 per cell, this level of basal expression must be sufficient to completely hydrolyse the RNA present in the lysate samples.

Likewise, lysates of non-induced cultures of JMRNaseA[pUC18Δ*lacO*] contained significantly reduced levels of RNA. As JMRNaseA contains only a single copy of the RNaseA expression cassette derived from pQR163, this gives a clear indication of the low levels of expression required to bring about a significant decrease in the levels of RNA in lysates. No remaining RNA was visualised in the lysates of induced cultures of JMRNaseA[pUC18Δ*lacO*].

Uninduced cultures of JMRNaseA harbouring the complete pUC18 showed a higher level of basal expression, as slightly less RNA could be seen in comparison to the lysate of uninduced JMRNaseA[pUC18Δ*lacO*], which we attribute to the action of the $lacO_1$ and $lacO_3$ operator sequences of pUC18 titrating $LacI^q$ from the operator site regulating the RNaseA expression cassette of the host. In JMRNaseA[pUC18Δ*lacO*] the modified plasmid, with the operator sequences removed, prevent this LacI repressor titration effect.

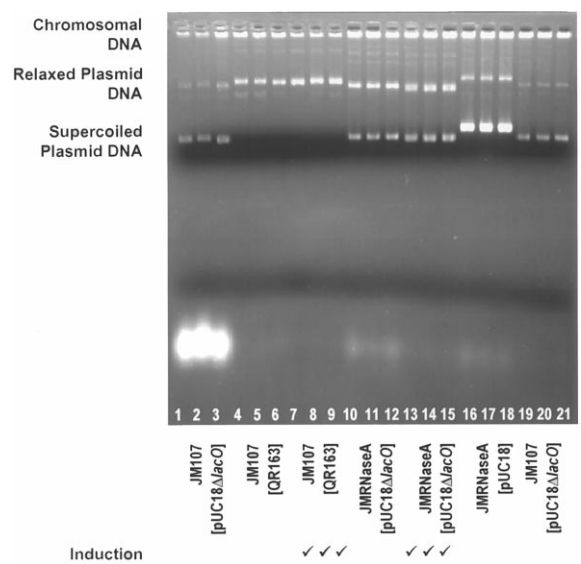

Fig. 3. Analysis of RNA levels in cell lysates. Agarose gel (1.5%) of ethanol precipitated cell lysates. The gel was photographed in two sections (upper region containing DNA, and the lower region containing RNA) so that optimal exposure times could be used. Results are shown in triplicate as follows, lanes (1–3) JM107[pUC18Δ*lacO*], no RNase control; (4–6) JM107[pQR163] uninduced; (7–9) JM107[pQR163] induced; (10–12) JMRNaseA[pUC18Δ*lacO*] uninduced; (13–15) JMRNaseA[pUC18Δ*lacO*] induced; (16–18) JMRNaseA[pUC18] uninduced; (19–21) JM107[pUC18Δ*lacO*] + 10 μg ml^{-1} Qiagen RNaseA supplement.

3.3. The significance of JMRNaseA in plasmid isolation for gene therapy

The RNA specific nuclease activity of this modified *E. coli* strain provides ideal methodology to remove RNA following alkaline lysis in the large-scale isolation process of any plasmid species. As no additional incubation period is required following lysis, there is no opportunity for remaining endogenous nuclease activity to act upon the plasmid DNA (c.f. the procedure of Monteiro et al., 1999). With prolonged incubation times endogenous nuclease activity will result in a much higher proportion of the plasmid DNA found in the nicked, and even linear forms.

The efficient removal of RNA from the process stream also has a significant effect on the final chromatographic stages of purification. Purifying plasmid DNA on anion exchange matrices is problematic due to the hydrodynamic volume of the macromolecule limiting the systems available to use. Capacities of traditional chromatographic matrices designed for proteins are typically 1000-fold lower for plasmid DNA, therefore, it is of paramount importance to maximise this by the removal of any other polyanionic species present, such as RNA.

3.4. Other implications of this work

This expression strain could also be used for the production of recombinant species other than plasmid DNA, for example proteins with possible therapeutic application, allowing RNA free primary extraction to be carried out at the start of a purification process. Due to the difficulties associated with regulatory issues of recombinant technology, other forms of modified expression strains may provide cost-effective solutions to process problems in the large-scale production of biotherapeutics. An ultimate goal will be to have 'biological solutions' within the host strain, which can replace or circumvent some of the physical steps in the purification of biomolecules.

3.5. Conclusions

We have constructed a modified plasmid expression host, based upon *E. coli* JM107, which has an inducible RNaseA expression cassette immobilised within the host chromosomal *dif* locus.

Cultures of JMRNaseA harbouring plasmids express pre-RNaseA, which is translocated to the periplasmic space, where the signal sequence is cleaved, generating mature RNaseA. The active RNaseA does not contact the cytoplasm during growth due to this sequestration. The mature RNaseA is released at cell lysis during primary plasmid extraction, in sufficient levels to hydrolyse RNA present in cell lysates, allowing the purification of essentially RNA free plasmid DNA.

This methodology provides a convenient alternative to other techniques described previously that are often impractical for larger-scale processes and may lead to the instability of the plasmid DNA in the process stream.

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