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A modified *Escherichia coli* protein production strain expressing staphylococcal nuclease, capable of autohydrolysing host nucleic acid

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Abstract

The large-scale production of recombinant biotherapeutics, particularly recombinant proteins, provides significant process and regulatory challenges to the biotechnology industry in order to meet the regulatory agencies stringent requirements in a cost-effective manner. Host cell derived nucleic acid causes problems from both a process and a regulatory perspective, as high molecular weight chromosomal DNA is responsible both for the viscosity of cell lysates, and it is a source of heterologous DNA sequences whose inclusion in the final product must be prevented. We have constructed a modified *Escherichia coli* JM107 expression host (JMN), containing a staphylococcal nuclease expression cassette, integrated into the host chromosome at the *dif* locus. The nuclease is expressed as a fusion to the *ompA* signal peptide, and is translocated to the periplasm of the cell, protecting the cytoplasmic nucleic acid present in the lysate. Results with this strain show that sufficient levels of nuclease activity are produced to completely auto-hydrolyse the host's chromosomal DNA to a size non-visible on 1% agarose gel, generating a markedly lower lysate viscosity. This provides a suitable methodology to remove heterologous DNA sequences early in the product stream and decrease lysate viscosity, improving the efficiency of downstream processing and product yield, whilst avoiding the addition of exogenous nuclease and its prohibitive costs at large-scale.

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1. Introduction

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The difficulties associated with the large-scale production of biotherapeutics provide a constant challenge to the biotechnology industry. The

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production of recombinant proteins using 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 (Di-Paolo et al., 1999; Food and Drug Administration (FDA, 1985, 1992); World Health Organisation (WHO, 1998)). Of particular concern is host cell derived nucleic acid, which causes problems from both process and regulatory perspectives.

A typical process for recombinant protein production involves the expression of the product in a high-density Escherichia coli fermentation culture, whereupon the cells are harvested and lysed, and the recombinant product purified from the resulting biomass. Cell lysis results in the liberation of high molecular weight (MW) host chromosomal DNA into the process stream and a marked increase in the level of viscosity of the lysate. From a regulatory perspective it is desirable to employ processes which act to remove host cell derived nucleic acid to prevent the inclusion of heterologous genetic sequences, which have the potential to introduce oncogenic or transforming, immunostimulatory, and antibiotic resistance encoding genotypes. FDA guidelines require nucleic acid contamination to be <100 pg per dose, proven by quantitative methodology sensitive to 10 pg (FDA, 1985, 1992).

The addition of exogenous nucleases, such as Benzoase[®] (Soren et al., 1990), a commercially available form of the extracellular nuclease of Serratia marcescens, provides a solution to these problems, but one that is prohibitively expensive at scale. An alternative to the addition of exogenous nucleases in downstream processing is to engineer a 'biological' solution by modifying the host strain. Such an approach has been demonstrated in the production of essentially RNA free plasmid DNA, by the integration of the bovine pancreatic ribonuclease encoding gene into an E. coli plasmid host's chromosome (Cooke et al., 2001). Boynton et al. (1999) described the construction of Pseudomonas putida strains for the production of Poly(3-Hydroxyalkanoates) by the chromosomal integration of the staphylococcal nuclease gene (nuc), which markedly reduced cell

lysate viscosity during processing. Expressing a heterologous nuclease in an endogenous manner via strain modification is a preferable approach at large-scale, as it avoids both the cost associated with the addition of exogenous recombinant enzymes, and any regulatory issues concerning the source of non-recombinant enzymes.

Staphylococcal nuclease (SNase, EC 3.1.31.3) is a Ca²⁺ dependent sugar-non-specific nuclease, capable of hydrolysing nucleic acids (DNA and RNA, single stranded, double stranded, circular and linear) to produce 3' oligo- and mononucleotide phosphates (Anfinsen et al., 1971), acting both exolytically on RNA and denatured DNA, and endolytically on native DNA. The enzyme shows a certain degree of sequence specificity, preferring adenine- and thymine-rich regions (Dingwall et al., 1981; Horz and Altenburger, 1981). The in vivo function of the enzyme is as an extracellular nuclease, presumably in the catabolism of nucleic acids as a source of metabolites for the bacteria. The properties of staphylococcal nuclease have been reviewed comprehensively (Anfinsen et al., 1971). The promiscuous nature of the enzyme makes it ideal for a bioprocessing application, as it is capable of hydrolysing chromosomal DNA, plasmid DNA and RNA.

Here we describe the construction of an E. coli protein expression strain, encoding an S. aureus (NCTC 8325) nuclease B (nucB) expression cassette. nucB is expressed as a fusion to the ompA signal peptide, which targets the protein to the periplasmic space during growth (Suciu and Inouve, 1996). This sequestration results in the production of the active nuclease, following cleavage of the ompA signal peptide, whilst protecting the host's cytoplasmic nucleic acid from the active (toxic) protein. The mature nuclease is then released during cell lysis, and subsequently acts to hydrolyse host nucleic acid. Such hydrolysis of the host's high MW chromosomal DNA minimises the regulatory problem of contaminating host nucleic acid without having to resort to the addition of exogenous recombinant enzymes and decreases the viscosity of the cell lysate, facilitating downstream processing. No deleterious effect upon recombinant protein products expressed by the host occurs due to the total absence of any

proteolytic activity demonstrated by the enzyme. We see the construction of this strain as another step towards the production of tailor-made expression hosts facilitating ease of purification for both biotherapeutics and recombinant products in general.

2. Materials and methods

2.1. Identifying the putative nuc ORF of S. aureus NCTC 8325

The *S. aureus* NCTC 8325 genome is currently being sequenced by the University of Oklahoma's Advanced Centre for Genome Technology (http:// www.genome.ou.edu/staph.html). The *nuc* gene of the Foggi strain of *S. aureus* (GenBank accession no. V01281) was used as a query sequence in performing a BLASTN comparison of the genome sequence data.

2.2. Bacterial strains, plasmids and media

S. aureus NCTC 8325 was used as a source of genomic DNA. The E. coli K-12 strain used as a plasmid host was JM107 [F'traD36 lacI^q $\Delta(lacZ)M15$ pro $A^+B^+/e14^-(McrA^-) \Delta(lac-proAB)$ thi gyrA96 (Nal^r) endA1 hsdR17($r_k^-m_k^+$) relA1 supE44] (Yanisch-Perron et al., 1985). Bacteria were grown under standard conditions in Terrific broth (Sigma), nutrient broth, or on either DNase or nutrient agar (Oxoid) at 37 °C, supplemented with antibiotic 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 (25 µg ml⁻¹).

pN1D274Ekan1 has been described previously (Cooke et al., 2001). The prokaryotic expression vector pKK223-3 was obtained from Amersham Pharmacia Biotech. pTP223 (Poteete and Fenton, 1984) was obtained from Keenan Murphy. pQR275 is pQE50 containing the *CYP105D1* gene, encoding the cytochrome P450 of *Streptomyces griseus* (Hussein et al., personal communication). pQR706 is pBluescript (Stratagene) containing a fragment of the *E. coli* chromosome encoding the transketolase gene and its native promoter (French and Ward, 1996).

2.3. Plasmid DNA isolation, manipulation and analysis, and other techniques

Plasmid DNA was isolated by alkaline/sodium dodecyl sulphate (SDS) lysis (Birnboim and Doly, 1979), using Qiagen kits. Restriction of DNA and other enzymic modification (New England Biolabs) was carried out under recommended conditions. Electrophoresis was performed using agarose and acrylamide gels in Tris–Borate EDTA (TBE) buffer, and gels stained with ethidium bromide. Plasmid constructs were transformed into CaCl₂ competent cells and clones identified by restriction analysis following plasmid isolation. All techniques used standard methodologies (Sambrook et al., 1989). DNA sequencing was performed by Oswel (http:// www.oswel.com).

2.4. Purification of S. aureus chromosomal DNA

A 100 ml nutrient broth culture of *S. aureus* was grown overnight, harvested by centrifugation (6000 × g; 6 min), and resuspended in 5 ml TE buffer. Lysostaphin, Pronase and RNase A (Sigma) were added to 50, 80 and 50 μ g ml⁻¹ respectively, and the lysate incubated (37 °C, 24 h). 7.5 ml of 5 M NaCl was added, 10 ml 100% ethanol layered on top of the mixture and the chromosomal DNA spooled onto a sterile glass rod and resuspended in 5 ml TE buffer.

2.5. PCR amplification and isolation of the nuc B encoding sequence of S. aureus NCTC 8325

The ~ 500 base pair (bp) region of the *nuc* gene encoding nucB (mature nuclease plus N-terminal 19 amino acid (aa) pro-peptide (Davis et al., 1977)) was amplified from chromosomal DNA by PCRs performed in 100 µl containing: ~ 15 µg template DNA, 0.5 µM primers, 200 µM dNTPs, 10 µl 10 × *Taq* polymerase buffer (1.5 mM final [MgCl₂]) and 1 unit *Taq* polymerase (Qiagen), overlaid with 50 µl mineral oil. The PCR was run for 30 cycles of 94 °C, 3 min (1st cycle only); 94 °C, 45 s; 60 °C, 30 s; 72 °C, 1.5 min (30 cycles); 72 °C, 10 min (last cycle only). Primers (synthesised by Amersham Pharmacia Biotech) were nucB(up) 5' <u>GAATTCGCTAGC</u>CAAACAGATAATGGCG-TAAAT 3' and nucB(down) 5' <u>GAAT-TCCCCGGGGTTATTGACCTGAATCAGCGT-</u> TGTC 3' (*Eco*RI, *NheI* and *SmaI* sites underlined). PCR products were gel purified using a QIAquick kit (Qiagen).

2.6. Cloning nuc B into the expression vector pKK223-3 and fusing to the omp A signal sequence

The nucB PCR product was cloned EcoRI-SmaI into pKK223-3 to produce pKKnucB. pKKnucB was digested with EcoRI and NheI, gel purified, and ligated with a 10 µM solution of six complementary oligonucleotides encoding the ompA signal peptide (5' AATTCAT-GAAAAAGACAGCTATC 3'; 5' GCGATTG-CAGTGGCACTGG 3'; 5' CTGGTTTCGCTAC-CGTAGCGCAGG 3': 5′ AATCGCGA-TAGCTGTCTTTTTCATG 3'; 5' AGCGAAAC-CAGCCAGTGCCACTGC 3': 5' CTAGCC-TGCGCTACGGT 3') to produce pKKompnucB.

2.7. Identification of nuclease expressing clones

The nuclease activity of clones was screened for on DNase agar supplemented with antibiotics as required, and 1.0 mM IPTG. After incubation plates were flooded with 2 M HCl precipitating the high MW DNA within the agar, producing clear halos around colonies displaying nuclease activity.

2.8. Construction of JMN

2.8.1. Chromosomal integration of the omp-nucB expression cassette

The P_{tac} -*ompnucB* expression cassette was excised from pKKompnucB by *Bam*HI restriction, and ligated into *Bcl*I linearised pN1D274E*kan*1 producing pDif::ompnucB with the nuclease expression cassette in the opposite transcriptional orientation to *kan* (Fig. 1).

The RecA⁺ strain JM107 was transformed with pTP223, which contains *tet*, the bacteriophage

lambda (λ) *red* recombination functions *bet* and *exo*, and the RecBCD-inhibiting λ gam (Murphy, 1998). Competent JM107[pTP223] were transformed with the 8.9 kb *Bam*HI fragment of pDif::ompnucB, and the cells recovered (18 h, 37 °C). JM107[pTP223]::ompnucB recombinants were selected by kanamycin resistance on nutrient agar (24 h, 37 °C). Recombinant clones were screened for nuclease activity, and pTP223 cured by growth in the absence of tetracycline. The chromosomal insertion of nucB was confirmed by PCR using the primers nucB(up) and nucB(down) (Section 2.5).

2.9. Expression of nucB in JM107[pKKompnucB] and JMN

JM107[pKKompnucB], JM107[pKK223-3], JM107 and JMN were grown overnight in 5 ml nutrient broth media containing ampicillin (plasmid containing cultures only), and 0.5 ml of this culture used to inoculate 50 ml of the same media in a 250-ml conical flask. At an optical density (OD) A_{600} of 0.4, expression was induced by 1.0 mM IPTG. Cultures were grown for a further 5 h. Equivalent cell numbers were harvested (25 ml culture OD $A_{600} = 1.5$). Periplasmic extracts were produced by a modified osmotic shock procedure (Tarragona-Fiol et al., 1992). Cells were resuspended in 250 µl extraction buffer, before inducing an osmotic shock by the addition of 250 µl water, and centrifuging (14000 rpm, 10 min) to separate the periplasmic and spheroplast species. Fifty microliter periplasmic samples were added to an equal volume of 2X SDS-PAGE loading buffer (0.1 M Tris-HCl (pH 6.8); 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol; 0.1%(v/v) β -Mercaptoethanol (added fresh)), 20 μ l analysed by 12.5% SDS page (Laemmli, 1970), and proteins visualised by staining with Brilliant Blue G250 (Sigma).

Western transfer to 0.45 μ M PVDF membrane (Pierce) was performed (3 h, 50 mA) in transfer buffer (48 mM Tris, 39 mM Glycine (pH 8.3); 0.037% (w/v) SDS; 20% (v/v) Methanol), the blot stained using Brilliant Blue G250, and stored at -20 °C prior to N-terminal sequencing



Fig. 1. (a) Genetic map of pDif::ompnucB. pDif::ompnucB is pN1D274Ekan1 (pN1 with $lacl^{qs}$ from pIQ and the kan gene from pUC4K (Section 2.3)), with the 840 bp BamHI P_{tac}-ompnucB expression cassette fragment inserted into the BclI site of $lacl^{qs}$. pDif::ompnucB has the nuclease expression cassette in the opposite transcriptional orientation relative to kan (Section 3.5). (b) 5' region of the nuclease expression cassette of pKKompnucB and JMN. The translated aa sequence is given in bold. The 15 aa ompA signal peptide is encoded by bp 266–329, the 19 aa pro-peptide by bp 330–387, and the 149 aa of the mature nuclease A protein by bp 388–833 (not shown). Transcription and translation associated sequences are highlighted. Restriction sites (underlined) are: BamHI GGATCC; Eco RI GAATTC; Nhe I GCTAGC.

(Alta Bioscience; http://www.bham.ac.uk/alta_bioscience).

2.10. Activity of nucB in periplasmic extracts of JM107[pKKompnucB] and JMN

Nuclease activity of periplasmic extracts was detected by incubating 1 μ l samples with 1 μ g high MW *S. aureus* chromosomal DNA (5 min, room temperature), supplemented with 2 μ l 10 × nuclease buffer (0.1 M Tris–HCl, pH 8.0; 0.1 M CaCl₂), in a total volume of 20 μ l. The quantity and size range of the remaining nucleic acid was visualised by 1% TBE agarose gel electrophoresis.

2.11. Auto-hydrolysis of chromosomal DNA in JMN lysates

Cultures of JMN and JM107 were grown, nuclease expression induced, and cells harvested as described in Section 2.9. Cell pellets were resuspended in 4.5 ml buffer P1 (Qiagen: 50 mM Tris–HCl (pH 8.0); 10 mM Na₂EDTA; 100 μ g ml⁻¹ RNase A) before the addition of 500 μ l 0.1 M CaCl₂. 500 μ l aliquots of cells were lysed by the addition of 50 μ l 10 × BugBusterTM (Novagen; http://www.novagen.com), vortexed, and incubated (20 min, room temperature), with repeated mixing at 5 min intervals to ensure thorough cell disruption and the quantitative release of chromo-

somal DNA. The control strain JM107 was lysed in the presence and absence of 25 units Benzonase[®] nuclease (Novagen). Nucleic acid was subsequently isolated from lysates by extracting with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1, Sigma), sampling 400 μ l of the aqueous phase, precipitating the nucleic acid species present (40 μ l 5 M NaCl and 800 μ l ice-cold absolute ethanol), and centrifuging (14 000 rpm, 10 min). The DNA pellet was washed with ethanol, re-centrifuged, dried, resuspended in 250 μ l EB buffer (Qiagen), and a 20 μ l sample analysed by 1.4% TBE agarose gel electrophoresis.

2.12. Growth curve, plasmid maintenance and stability of the chromosomal nuclease function of JMN in comparison to the parental strain JM107

of JM107[pQR275] Colonies and JMN[pQR275] were used to inoculate 5 ml of nutrient broth containing ampicillin, the cultures grown until mid-exponential growth phase, whereupon 50 µl was used to inoculate 200 ml of sterile Terrific Broth containing ampicillin in a 1-1 conical flask. These cultures were grown to an OD of \sim 5.0 A_{600} , before being used to inoculate the fermentation vessels. Batch cultures were grown in Applikon 71 (working volume 51) fermentation vessels fitted with three equally spaced top driven turbines. Medium comprising 4.8 1 Terrific Broth supplemented with Polypropyleneglycol (1 ml 1^{-1}), was sterilised in situ, before the aseptic addition of ampicillin using 0.2 µm filters (Gelman laboratory). Batch fermentation was performed to the following parameters: pH 6.9; DO₂ (dissolved oxygen setpoint) 20% of air saturation; temperature 37 °C. DO_2 was maintained by increasing the rate of stirring (1000 rpm maximum), and pH kept constant by titration with 5 M NaOH and 2 M HCl. Online monitoring and control was performed using the BIOWATCH software package (Applikon).

The vessel was sampled half hourly initially, then hourly over a 6 h period, and the OD A_{600} recorded to allow the calculation of specific growth rates (μ_{max}). Plating on selective and nonselective media allowed the stability of the chromosomal nuclease function (kanamaycin resistance), and plasmid maintenance (ampicillin resistance) of the modified strain to be determined in comparison to the parent strain JM107.

2.13. Protein expression using strain JMN

JM107[pQR706] and JMN[pQR706] were grown and induced as in 2.9, although post induction growth was for 12 h. Equivalent cell numbers (25 ml culture OD $A_{600} = 1.65$) were harvested (6000 g; 5 min), resuspended in 2 ml sterile water, and 100 µl subjected to SDS–PAGE as in 2.9 to visualise the level of transketolase protein expression.

3. Results and discussion

3.1. Identifying the putative nuc ORF of S. aureus NCTC 8325

A BLASTN search of the *S. aureus* NCTC 8325 genome using the Foggi strain *nuc* gene as a query gave a 98.86% identity (682/696 bp) to a putative ORF contained within sequencing contig 279 (295,784 bp). The corresponding protein sequences are identical apart from a conservative $41F\Delta Y$ within the signal peptide, and 206H ΔL which also occurs in the *S. aureus* V8 strain nuclease, and has been shown to have no effect on either structure or function (Cone et al., 1971; Cusumano et al., 1968). Thus we conclude that the NCTC 8325 strain's putative *nuc* ORF encodes SNase.

3.2. PCR amplification and isolation of the nuc B encoding sequence of S. aureus NCTC 8325

The ~500 bp nucB encoding region of the *nuc* gene of *S. aureus* NCTC 8325 was amplified by PCR. nucB is a processing intermediate of the protein comprising the mature 149 aa nuclease A, and a 19-residue N-terminal pro-peptide, but lacking the signal sequence for secretion (Davis et al., 1977). Previous work (Suciu and Inouye, 1996) has shown that the presence of the propeptide has a profound secretion enhancing effect when expressing the nuclease as an OmpA signal fusion in *E. coli*. The upstream oligo introduced a

silent triplet mismatch mutation changing the first codon of *nuc* B from TCA (Ser) to AGC (Ser), subsequently producing an *Nhe*I site following ligation to oligos encoding the OmpA signal peptide (Section 3.3).

3.3. Cloning nuc B into the expression vector *pKK223-3* and fusing to the omp A signal sequence

Following purification the PCR product was cloned *Eco*RI-*Sma*I into pKK223-3, generating pKKnucB. Restriction of pKKnucB with *Eco*RI and *Nhe*I allowed the insertion/ligation of oligos encoding the 21 aa OmpA signal peptide generating pKKompnucB. The sequence encoding the Nterminal Met of the signal peptide provides the ATG translation initiation codon, and the fused sequences are translated in-frame by virtue of the silent *Nhe*I site change which provides a strategy to replace the signal sequence (Fig. 1).



Fig. 2. 12.5% SDS-PAGE gel showing the expression of nucB in periplasmic extracts of JM107[pKKompnucB], JM107[pKK223-3], JMN and JM107 cultures. Periplasmic extracts of equivalent quantities of cells demonstrated the level of nuclease expression as follows: Lane (1) JM107[pKKompnucB], (2) JM107[pKK223], (3) JMN, and (4) JM107. The MW marker was Novagen Perfect ProteinTM Markers (10–222 kDa). The nuclease protein species is indicated (\Rightarrow).

3.4. Expression of nucB in JM107[pKKompnucB]

JM107[pKKompnucB] clones were screened for DNase activity on DNase agar supplemented with ampicillin and IPTG (data not shown). SDS-PAGE analysis of the proteins present in whole cell (data not shown) and periplasmic extracts of induced cultures of JM107[pKKompnucB] showed an additional highly prevalent species at $\sim 20 \text{ kDa}$ (nucB has a hypothetical MW of 19 kDa) compared to JM107[pKK223-3] (Fig. 2, lanes 1 and 2). The relative intensities of the overexpressed protein in periplasmic and whole cell samples indicated that the nuclease is efficiently targeted by the omp signal peptide. N-terminal sequence analysis of the first 8 aa of the expressed form of the nuclease yielded no signal other than the expected Ser Gln Thr Asp Asn Gly Val Asn of nucB, confirming that the pre-pro-peptide was being faithfully processed by the E. coli host, producing the mature, periplasmically sequestered nucB, as demonstrated with the Foggi strain nuclease (Suciu and Inouye, 1996). The efficiency of this sequestration ensures that no deleterious effects occur as a result of the host's cytoplasmic nucleic acid being exposed to the toxic nuclease activity of the enzyme.

3.5. Construction and characterisation of JMN: chromosomal integration of the nuclease cassette and expression of omp–nucB

We have immobilised the ~ 840 bp omp-nucB expression cassette of pKKompnucB within the E. *coli* JM107 chromosome using the bacteriophage λ recombination system Red (Fig. 1). The genotype JMN is $[(F'traD36 lacI^q)]$ $\Delta(lacZ)M15$ of $proA^{+}B^{+})/e14^{-}(McrA^{-})$ $\Delta(lac-proAB)$ thi gyrA96 (Nal^r) endA1 hsdR17($r_k^-m_k^+$) relA1 supE44 ÆhipA::nucB kan]. The cassette encodes the pre-pro-SNase (omp-nucB) under the control of P_{tac} and the ribosome binding site derived from pKK223-3. Recombination occurred between the insert cassette and homologous sequences flanking the core 28 bp sequence of the *dif* locus region of the E. coli chromosome. Previous work has shown the *dif* locus to be suitable for integrating additional sequences as the disturbance has a minimal

(if any) effect upon the stability of the host, providing that the integrity of the core sequence is maintained (Cooke et al., 2001; Leslie and Sherratt, 1995; Williams et al., 1998). This was ensured by introducing an identical sequence from pN1D274Ekan1 (Fig. 1). The presence of the integrated nucB expression cassette was confirmed by PCR (data not shown).

SDS-PAGE analysis of the proteins present in periplasmic extracts of induced cultures of JMN showed the presence of an additional species corresponding to the nuclease c.f. JM107 (Fig. 2, lanes 3 and 4). The nuclease was processed and sequestered in the same correct manner as when expressed from pKKompnucB. The level of nuclease expression by JMN was markedly reduced c.f. JM107[pKKompnucB], indicative of the reduction in copy number of the nuclease expression cassette from ~ 30 (pKKompnucB has the pBR322 replicon) to unity following the chromosomal insertion of a single cassette. DNase agar assays showed the induced JMN strain capable of producing nuclease activity, and a low level of uninduced nuclease activity corresponding to the basal level of transcription from P_{tac} under the leaky control of LacI^q (data not shown).

Analysis in 5 l cultures of JMN[pQR275] c.f. JM107[pQR275] showed that the modified strain was unaffected with respect to plasmid maintenance. Data showed that $93\% \pm 16$ of the JM107,

and $111\% \pm 14$ of the JMN population, displayed the amp^r phenotype indicative of the presence of the plasmid at the time of harvesting (8 h). $104\% \pm$ 5 of the JMN[pQR275] population displayed the kan^r phenotype at harvesting, indicating the stable inheritance of the chromosomal nuclease cassette.

Growth curve data and specific growth rates confirmed the lack of any significant deleterious metabolic burden or cytotoxic effect upon the modified strain JMN by expression of the nuclease $(\mu_{\text{max}} = 0.53 \text{ h}^{-1})$ c.f. JM107 $(\mu_{\text{max}} = 0.57 \text{ h}^{-1})$ (Fig. 3).

3.6. Nuclease activity of periplasmic extracts of JM107[pKKompnucB] and JMN

Agarose gel electrophoresis of high MW chromosomal DNA incubated with JM107[pKKompnucB] and JMN periplasmic extracts compared to DNA incubated in the presence of





Fig. 3. Growth curves of JMN[pQR275] (\blacktriangle) and JM107[pQR275] (\blacklozenge).

Fig. 4. 1% agarose gel showing the hydrolysis of high MW chromosomal DNA following incubation with periplasmic extracts of JM107[pKKompnucB] and JMN. High MW DNA was incubated with periplasmic extracts of equivalent quantities of cells (Sections 2.9 and 2.10 for detail) demonstrating nuclease activity as follows: Lane (1) No extract, (2) JM107[pKK223], (3) JM107[pKKompnucB], (4) JM107, and (5) JMN. The MW marker was λ DNA digested with *Pst*I.

JM107[pKK223-3], JM107 and no extract, demonstrated markedly differing levels of hydrolysis (Fig. 4). Lane 1 shows the characteristically high MW species of the 'no extract' chromosomal DNA control, compared to the MW marker, as do the control strains JM107[pKK223-3] and JM107 (lanes 2 and 4). E. coli periplasmic extract has a very low level of endogenous DNase activity, represented by a slight smearing of lower MW chromosomal DNA species, and a collection of protein- and other periplasmic species-DNA complex fluorescing species unable to permeate the gel matrix. The front edge of the chromosomal DNA band remains clean, rather than deteriorating as in the presence of significant levels of nuclease activity. By contrast, incubation of chromosomal DNA with periplasmic extracts from JM107[pKKompnucB] and JMN (lanes 3 and 5) showed total hydrolysis of the nucleic acid species-nothing was visible, clearly demonstrating that the activity and specificity of the nuclease are capable of hydrolysing high MW chromosomal DNA, presumably to 3' mono- and oligonucleotidephosphates (Anfinsen et al., 1971), even by the level of activity produced by the single copy expression cassette of JMN. For the hydrolysed species not to be visible on a 1% agarose gel, it would be expected that they would be of the order of ≤ 30 bp in length.

3.7. Auto-hydrolysis of host chromosomal nucleic acid by JMN

Cell samples of induced cultures of JMN and JM107 were lysed using the protein extraction reagent BugBusterTM (a mix of mild non-ionic detergents, which has no adverse effect on the nuclease) in the presence and absence of a Benzonase[®] supplement, and the nucleic acids present in the lysate were analysed by 1.4% TBE agarose gel electrophoresis (Fig. 5). The JM107 lysate (no Benzonase) produced high MW chromosomal DNA species, with no evidence of any hydrolytic activity, as well as some low MW RNA species. The presence of Benzonase resulted in the hydrolysis of the chromosomal DNA, resulting in only a lower MW smear < 800 bp. The exogenous nuclease also partially hydrolysed the RNA spe-



Fig. 5. 1.4% agarose gel showing the auto-hydrolysis of host chromosomal DNA by JMN. Nucleic acid extracts of cell lysates of: Lane (1) JM107, (2) JMN, and (3) JM107 supplemented with Benzonase[®] (1 μ l, 25 units). The MW marker was λ DNA digested with *Pst* I.

cies, as the corresponding band was of a lower intensity, with lower MW smearing. By comparison, the levels of nucleic acid extracted from JMN demonstrated that the modified strain was capable of producing enough nuclease activity to completely hydrolyse the high MW chromosomal DNA species present. It was also noted that the lysate viscosity of JMN was markedly decreased in comparison to that of JM107, presumably due to the auto-hydrolysis of the host's high MW DNA. This total auto-hydrolysis minimizes the level of host-derived nucleic acid present in the process stream, significantly improving the ease in which regulatory requirements are met, and produces an improving decrease in the visco-elasticity of the lysate.

3.8. Protein expression using strain JMN

Comparison of protein species present in induced cells of JMN[pQR706] and the parent strain JM107[pQR706] by SDS–PAGE provided an estimation that the modification of the strain by the additional nuclease expression cassette had not had any deleterious effect on the level of protein expression (Fig. 6).

3.9. Conclusions

We have constructed a modified protein expression host JMN, based upon E. coli JM107, which has an inducible staphylococcal nuclease expression cassette immobilised within the host chromosomal dif locus. Cultures of JMN express the nuclease as an ompA signal peptide-nucB fusion, which is translocated to the periplasmic space where the signal sequence is cleaved, generating nucB. The efficiency of the sequestration of the nuclease avoids the cytotoxic accumulation of hydrolytic activity during growth. The nuclease is released along with the host's nucleic acid at cell lysis, in sufficient levels to totally auto-hydrolyse the chromosomal DNA present in cell lysates, allowing the purification of essentially host DNAfree recombinant protein, and providing a marked decrease in the level of viscosity of the product



Fig. 6. 12.5% SDS–PAGE gel showing the expression of transketolase in JM107[pQR706], JMN[pQR706] and JM107[pUC18] cultures. Comparative whole cell samples of: Lanes (MW) MW marker, (1) JM107[pQR706], (2) JMN[pQR706], and (3) JM107[pUC18]. The transketolase protein species is indicated (\Rightarrow).

stream. This methodology provides a cost-effective solution to process and regulatory problems in the large-scale production of recombinant biotherapeutics.

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Abbreviations

λ	bacteriophage lambda
А	absorbance
aa	amino acid(s)
bp	base pair(s)
dNTP	deoxyribonucleoside triphosphate
EB	10 mM Tris-HCl (pH 8.5)
EDTA	ethylenediaminetetraacetic acid
EtdBr	ethidium bromide
FDA	Food and Drug Administration
g	relative centrifugal force
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase(s) or 1000 bp
MW	molecular weight
nucA	nuclease A
nucB	nuclease B
OD	optical density
ORF	open reading frame
р	plasmid
Р	promoter
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SNase	staphylococcal nuclease
TBE	tris-borate EDTA buffer
TE	10 mM tris-Cl (pH 8.0); 1 mM EDTA
WHO	World Health Organisation

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