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IS2-mediated overexpression of *kfoC* in *E. coli* K4 increases chondroitin-like capsular polysaccharide production

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Abstract Transposons are developing molecular tools commonly used for several applications: one of these is the delivery of genes into microorganisms. These mobile genetic elements are characterised by two repeated insertion sequences that flank a sequence encoding one or more orfs for a specific transposase that moves these sequences to other DNA sites. In the present paper, the IS2 transposon of *Escherichia coli* K4 was modified in vitro by replacing the sequence coding for the transposase with that of the *kfoC* gene that codes for chondroitin polymerase. KfoC is responsible for the polymerisation of the bacterial capsular polysaccharide whose structure is analogous to that of chondroitin sulphate, a glycosaminoglycan with established and emerging biomedical applications. The recombinant construct was stably integrated into the genome of *E. coli* K4 by exploiting the transposase from endogenous copies of IS2 in the *E. coli* chromosome. A significant improvement of the polysaccharide production was observed, resulting in 80 % higher titres in 2.5-L fed-batch cultivations and up to 3.5 g/L in 22-L fed-batch cultures.

Keywords IS2 · Capsular polysaccharide · Chondroitin · *E. coli* K4 · Chondroitin polymerase · GAG

Introduction

Different classes of active transposons have been observed in all genomes studied so far, including microorganims, plants and animals (also humans), with just few exceptions. Although their level of activity varies in the different species, they play key roles in the evolution of genomes generating variability by modifying genes and genomes from both structural and functional points of view.

Transposons are mobile genetic elements characterised by the presence of two terminal, repeated and inverted insertion sequences that flank one or more genes coding for a transposase, the enzyme that is specifically responsible for their mobility. IS2 is a 1.3-kb transposable element normally found in both the genome and in the F-plasmid of *Escherichia coli* strains (Ghosal et al. 1978). It is a member of the IS3 family that is characterised by an internal sequence coding for two orfs indicated as *OrfA* and *OrfB* flanked by imperfect inverted repeats, namely inverted left repeat (IRL) and inverted right repeat (IRR). *OrfA* codes for InsA, a 14-kDa protein that was shown to negatively regulate transposition (Hu et al. 1994). *OrfA* and *OrfB* code for a 46-kDa protein indicated as InsAB, via a −1 translational frameshift mechanism. InsAB is the transposase and, therefore, is responsible for the expression and transposition of IS2 (Hu et al. 1996). Lewis and Grindley (1997) demonstrated, in fact, that the overexpression of InsAB increases the frequency of transpositional recombination and the formation of two transpositional products and figure-eight molecules that are processed into IS2 minicircles.

An IS2 insertion sequence is located within region 2 of the gene cluster responsible for capsular polysaccharide (CPS) biosynthesis of *E. coli* K4 (Ninomiya et al. 2002). The latter belongs to the serological group II of K antigens organised into three regions. Regions 1 and 3 include the *kps* genes, common to all group II members. They are primarily involved in the transport of the polymer into the periplasm and outside the cell wall (Smith et al. 1990). The genes coding for enzymes that direct the synthesis and assembly of the final polysaccharide are found in region 2, the serotype-specific region. Besides three

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genes of unknown function (*kfoB*, *kfoG*, *kfoD*), region 2 is comprised of *kfoE* that is responsible for the addition of fructose residues (Trilli et al. 2011), *kfoA* coding for a UDP-glucose 4-epimerase, *kfoF* coding for a UDP-glucose dehydrogenase and the well-characterised *kfoC* gene coding for chondroitin polymerase (Ninomiya et al. 2002; Suzuki 2008). The insertion sequence, IS2, is located between the *kfoC* and *kfoD* genes.

E. coli K4 is a natural source of a capsular polysaccharide whose structure is closely related to that of the glycosaminoglycan (GAG) chondroitin sulphate (CS). As a matter of fact, they share a common carbon backbone, chondroitin, formed by repeated disaccharide units of *N*-acetyl-D-galactosamine (GalNAc-β 1:4 linked) and glucuronic acid (GlcA-β 1:3 linked) and differ in the presence of fructose and sulphate residues. CS has a central role in the extracellular matrix of vertebrates since it boosts the biosynthesis of connective tissue components and it inhibits enzymes that degrade cartilage (Imada et al. 2010). For this reason, CS is widely used for the treatment of osteoarthrithis. However, recent literature has also investigated attractive potential applications of this GAG in the formulation of skin substitutes, cancer prevention and vaccine development (Achur et al. 2008; Wang et al. 2006; Pothacharoen et al. 2006).

In order to guarantee an unlimited source of CS and bypass the current animal-based extraction procedures, numerous attempts to increase the levels of CPS produced by wild-type *E. coli* K4 have focused on the improvement of fermentation and purification strategies from simple batch processes on a 12-L scale (Manzoni et al. 1996) to the development of high cell density cultivation followed by purification of the polysaccharide through membrane ultrafiltration strategies and ethanol precipitation (Cimini et al. 2012; Schiraldi et al. 2012). Furthermore, a natural CS equivalent was obtained by chemical sulphation of the purified chondroitin as described by Bedini et al. (2011). Yields were further improved by single overexpressions of two genes involved in the biosynthesis and regulation of CPS production, namely *kfoC* and *rfaH*. In the first case, even though KfoC was shown to be crucial in the improved production of polysaccharide, the use of a vector expression system was found to be unsuitable for fermentation processes due to its instability in the unconventional genetic background of *E. coli* K4 (Cimini et al. 2010a, b). In the second case, the employment of an integration cassette for *rfaH* overexpression resulted in high yields (5.3 g/L) of CPS in DO-stat 2-L fed-batch processes (Cimini et al. 2013). Recently, the *kfoE* gene was deleted from the genome of *E. coli* K4 in order to produce a defructosylated polymer that is more similar to the target active principle (Trilli et al. 2011).

The presence of transposons clearly impacts the host genome where they can exert deleterious effects by disrupting genes or by negatively influencing gene regulation or even promoting damaging recombinational events (Hedges and Deininger 2007; Callinan and Batzer 2006). However, besides the established primary role in genome evolution, they represent an important tool for cell engineering. They have long been used in molecular biology for several purposes. For instance, transposon-based vectors are useful for integrating genes into different mammalian cell types. In fact, the sleeping beauty transposition element was used for transfecting hematopoietic cells in a stable and effective manner (Izsvak et al. 2009) and also for generating induced pluripotent stem cells (Muenthaisong et al. 2012). Several applications have also been reported which regard the use of transposons for the random mutagenesis of both prokaryotes and eukaryotes (Hayes 2003) or for the identification of functional genes (Lewenza et al. 2005; Shin et al. 2006; Wright et al. 2001). Tn5-based mutagenesis was in fact used as a tool to identify essential genes in *E. coli* for the construction of a minimal bacterial genome (Yu and Kim 2008). Several authors have also described the use of transposons for the creation of transcriptional or translational fusions for the study of differentially regulated genes (Lewenza et al. 2005; Buchan et al. 2008).

Transposons therefore represent a novel and potentially interesting approach for the engineering of *E. coli* K4. In the present paper, we describe the modification of an IS2 element of *E. coli* K4 and its use for the generation of a recombinant strain overexpressing the endogenous gene *kfoC* that is involved in capsular polysaccharide biosynthesis. A significant enhancement of CPS production was obtained, demonstrating the efficacy of the engineering method and confirming the critical role of KfoC, as previously demonstrated (Cimini et al. 2010a, b). The stability of the engineered strain was verified not only by repeated growth cycles in shake-flasks but also through batch and fed-batch experiments in 2.5- and 22-L fermenters. These results are central towards pilot-scale and large-scale production processes.

Materials and methods

Medium

The standard medium used for all shake-flask and fermenter production studies consisted of a defined salt medium (KH_2PO_4 2 g/L, K_2HPO_4 9.7 g/L, $Na_3C_6H_5O_7$ 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, $MgCl_2$ 0.1 g/L) supplemented with glycerol (10 g/L) as the main carbon source and neutralised soy peptone (1 g/L) as an additional nitrogen source (Oxoid). Luria-Bertani (LB) medium was used during transformation experiments. Transformed cells were grown on LB medium supplemented with kanamycin (50 μg/mL). The wild-type strain used in all experiments was *E. coli* K4

serotype O5:K4:H4 (CCUG 11307), purchased from the Culture Collection of the University of Gothenburg.

Materials

Genomic DNA, plasmid DNA and RNA were isolated using the Qiagen DNeasy kit and the QIAamp DNA tot, the Qiagen miniprep kit and the Qiagen RNeasy kit (Qiagen, Valencia, CA), respectively, according to the manufacturer's instructions. Restriction endonuclease digestions, DNA ligations, SDS-PAGE and agarose gel electrophoresis were performed using standard techniques (Sambrook and Russel 2001).

Construction of the cassette for *kfoC* overexpression: IRL-*kfoC*-IRR

The strategy used for the construction of the transposon containing *kfoC* is shown in Fig. 1. The *kfoC* gene and the IRL region belonging to the IS2 insertion sequence were amplified from *E. coli* K4 chromosomal DNA. *kfoC* was amplified with primers 5′-kfoC (Cgg gAT CCC gAT gAg TAT TCT TAA TCA AgC) and 3′-kfoC (CgA gCT Cgg CCA gTC TAC ATg TTT ATC) containing the *Bam*HI and *Sac*I linker sites, respectively. Primers irla (5′-TAg ACT ggC CCC CTg AAT CTC C-3′) and irlb (5′-Cgg gAT CCT CCA ATg ACT AgT CTA AAA ACT Ag-3′) were used for the amplification of the IRL sequence from IS2. Irlb contains the linker site for *Bam*HI. Polymerase chain reaction (PCR) was carried out with Expand High fidelity PCR System (Roche, Monza, Italy) according to the manufacturer's protocol.

The complementary fragments, containing the *Sac*I linker site, IRR (5′-Tgg ATT TgC CCC TAT gTT TCC AgA TAC CTg TTA TCA CTT AAA gCT-3′) and anti-IRR (5′-TTA AgT gAT AAC Agg TAT CTg gAA ACA TAg ggg CAA ATC CA-3′) that correspond to the IRR region of the IS2 transposon, were directly synthesised by Roche.

DNA fragments were recovered from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Restriction endonucleases were purchased from New England Biolabs, and ligases were purchased from Invitrogen (Carlsband, CA). Nucleotide sequencing of all cloned PCR fragments was carried out at BMR Genomics (Padova, Italy) to verify that the sequences were correct.

The assembled fragment containing the *kfoC* gene flanked by the ends of IS2 rensponsible for transposition was amplified with primers irla and irrb (5′-Tgg ATT TgC CCC TAT gTT TCC Ag-3′) and cloned into the XL cloning vector (Invitrogen, CA) following the manufacturer's instructions. This generated plasmid pCP04.

Construction of BK4071

Transformation

For the construction of all strains, *E. coli* K4 electrocompetent cells were prepared according to the instrument instructions and transformed through electroporation using a Bio-Rad Gene Pulser (2-mm cuvettes, 2.5 kV, 200 Ω, 25 μF). *E. coli* K4 was transformed with the vector pCP04 and plated on LB plates supplemented with 50 μg/mL kanamycin to select for positive clones. Three clones were analysed in shake flask, as described in the following sections, for the production of K4 polysaccharide. The best performer identified here as *E. coli* K4-pCP04_1 was selected for the following experiments.

Transposition assay

Strain *E. coli* K4-pCP04_1 was grown in a shake flask in order to induce the transposition event causing integration of the modified transposon (IRL-*kfoC*-IRR) into the genome of *E. coli* K4. Flasks with 200 mL of medium in the presence and absence of kanamycin (50 μg/mL) were inoculated with a starting concentration of 0.04 g_{cdw}/L of cells and incubated at 37 °C at 200 rpm in a rotary shaker. Three consecutive 24 h experiments were performed for a total of 72 h of growth. After 8, 24, 32, 48, 56 and 72 h, samples of biomass were collected for the extraction of genomic and plasmid DNA. Samples of broth were plated on LB with and without antibiotic at all time points to follow the loss of recombinant plasmid over time. Three of the colonies grown after 72 h on plates without antibiotic were randomly selected to perform the following shake-flask experiments.

Shake-flask experiments

Shake-flask experiments were performed in order to evaluate the effect of *kfoC* gene overexpression on the production of K4 polysaccharide. Before each experiment, cells from 20 % (*w*/*v*) glycerol stock preparations were streaked on agar plates and grown overnight (o/n) at 37 °C. Single colonies were then used to inoculate pre-cultures that were incubated o/n at 37 °C in shaking conditions.

The medium used for all shake-flask production studies is described in the "Medium" section.

For all experiments, 200-mL cultures of wild-type and recombinant *E. coli* K4 were grown in 1-L baffled flasks, keeping a 1:5 medium/air volume ratio, at 37 °C and 200 rpm in a rotary shaker incubator (model Minitron, Infors, Bottmingen, Switzerland). Samples were withdrawn during the course of the experiment to analyse polysaccharide

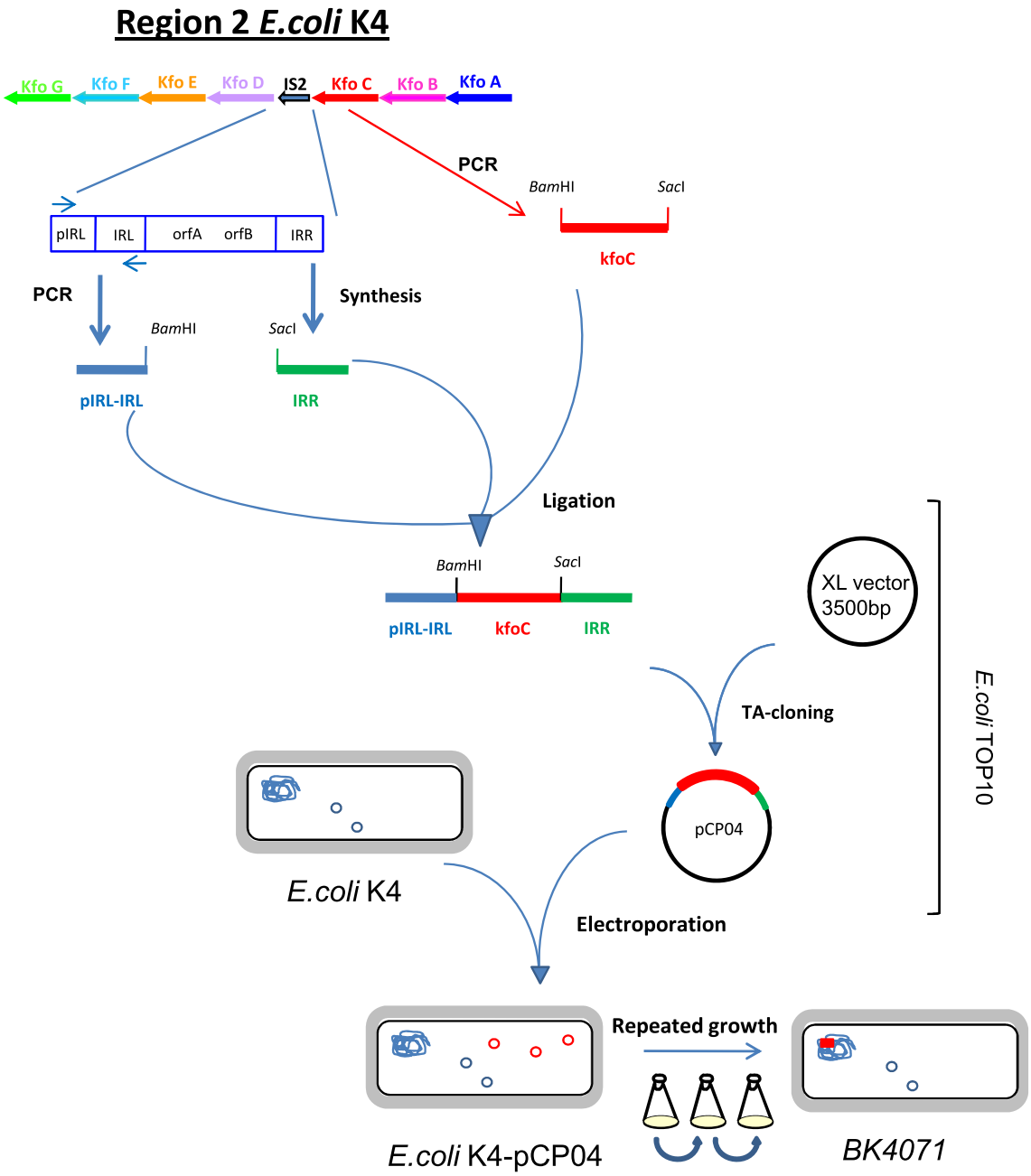

Fig. 1 Schematic representation of the engineering strategy used for the construction of BK4071

production. Every shake-flask experiment was repeated at least four times.

Evaluation of strain stability

In order to test the stability of the recombinant strain, repeated shake-flask experiments consisting of a total of 150 h of growth were performed in the same conditions described above. The final titre of polysaccharide produced was analysed every 24 h before restarting the culture. Experiments were performed in triplicate.

Analysis of mRNA expression level

Semi-quantitative reverse transcriptase PCR was performed on *E. coli* K4 and on the BK4071 recombinant strain to evaluate the increase of the *kfoC* mRNA pool in time during growth. For this purpose, the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase was used (Invitrogen Carlsband, CA). RNA was treated with DNase using the DNA-free kit following the supplied protocol (Ambion Inc Austin, TX) before reverse transcription and amplification. The 16S rRNA gene was used as a control to normalise data.

Fermentation experiments

Batch (2.5 L)

Fermentation experiments were carried out as biological triplicates in a Biostat CT reactor (Sartorius Stedim; Melsungen, Germany) with a 2-L working volume. A constant pH of 7.5 was maintained via automated addition of 30 %*v*/*v* NH_4OH and 30 %*v*/*v* H_2SO_4.

Seed cultures were prepared by inoculating a single colony into 200 mL of medium in 1-L baffled shake flasks. The flasks were incubated o/n at 37 °C and 200 rpm. During incubation, 5-mL samples were withdrawn from the reactors at regular time intervals for the determination of substrates and extracellular metabolites and for polysaccharide quantification.

For batch experiments, the concentration of dissolved oxygen (DO) was maintained above 20 % with varying airflow rate (1–1.5 L/min) and stirring rate (200–800 rpm) according to oxygen demand. The basic medium used for all experiments was the one described in the "Medium" section; however, experiments in which the medium was modified by the addition of 9.5 mg/L of arginine and 45 mg/L of glutamic acid, or by the addition of 0.1 g/L of sucrose, or by the use of 1.5 g/L of soy peptone were also performed.

Fed batch (2.5–22 L)

For fed-batch experiments, cells were grown in the semi-defined medium containing glycerol and soy peptone. After the batch phase, when the DO spiked indicating C source exhaustion, a concentrated nutrient solution (360 g/L glycerol and 36 g/L soy peptone for experiments in the 2.5-L fermenter and 480 g/L glycerol and 48 g/L soy peptone for experiments in the 22-L fermenter, plus inorganic salts 20-fold concentrated) was fed to the culture in the following growth phase. The feeding rate was between 0.5 and 3 g/L h.

Quantification of CPS

Broth samples were collected from shake-flask and fermentation experiments and centrifuged at 1,700×*g*. Supernatants were then ultrafiltered on 10-kDa centrifugal filter devices (YM-10 Centricon, Millipore, Bedford, MA, USA) at 5,000×*g* and concentrated to 1/10th of their initial volume. The retentate was twice diafiltered and then analysed for the content of K4 polysaccharide.

Capillary electrophoresis analysis was performed on a Beckman-Coulter HPCE instrument (P/ACE MDQ, Palo Alto, CA) equipped with a diode array detector and a UV lamp, using an uncoated fused-silica tube (70 cm in total length, 60 cm in effective length, 50 μm in I.D.) at 25 °C. Separation and quantification of the K4 and K4 defructosylated polysaccharides were performed according to the previously described method (Restaino et al. 2009).

kfoC gene copy number determination

The number of copies of *kfoC* present in the genome of BK4071 was established by real-time PCR. The total DNA of the wild type (wt) *E. coli* K4 and of the recombinant strain was extracted by using the QIAamp DNA Mini kit (Qiagen). The *dsx* gene was used as an internal control since only one copy of the gene was present in the genome of *E. coli* strains (Hahn et al. 2001). The primers used for the amplification of *kfoC* and *dsx* are reported in Table 1. PCR amplification was performed in an iQ5 instrument (Bio-Rad, CA). Amplification was carried out in 25-μL volume containing 5 μL DNA, 12.5 μL of iQ Syber Green Supermix (Bio-Rad, CA) and 0.5 μL of each primer at a concentration of 0.4 μM. After incubation at 95 °C for 3 min, amplification proceeded with 40 cycles of 95 °C for 10 s and 62 °C for 1 min. Tenfold serial dilutions of the purified *kfoC* and *dsx* fragments were used for the construction of calibration curves in which the copy *n*°/*n*° of molecules was related to Ct values according to Whelan et al. (2003) and Lee et al. (2006). The ratio between the number of amplified *kfoC* and *dsx* molecules in the wt and in the recombinant strain indicated the number of *kfoC* gene copies in BK4071. Two biological samples each were analysed in triplicate.

Results

Strain construction

E. coli K4 was initially modified by the addition of the pCP04 plasmid containing a modified IS2 element in which *OrfA* and *OrfB* were replaced by *kfoC* (Fig. 1). The gene was therefore flanked by IRL and IRR, required for the transposition, and it was located downstream of the P_{IRL} promoter. Repeated shake-flask experiments on the standard medium without antibiotic supplementation led, after 72 h, to the complete loss of the recombinant plasmid and to the integration of the modified transposon into the genome of *E. coli* K4, yielding a strain that does not require antibiotic selection. An overview of the engineering strategy used is given in Fig. 1.

Loss of the XL vector was verified by plating the cells on the medium supplemented with kanamycin and by extraction of plasmid DNA verifying the absence of the recombinant vector. Although no plasmid bands were visible on agarose gels, specific primers (M13 Forward and M13 Reverse, furnished by Invitrogen) were used as a further control for the amplification of eventual plasmid residues; however, no band was obtained (data not shown).

Table 1 Gene copy number determination by real-time PCR in strain BK4071

Gene target	Primers (5′→3′)	Fragment length	Efficiency (%)	Slope	Estimated copy number
kfoC	Fw: ttttccctgccgcacgat Rv: ttcggtctgttgaaggagcaatgg	81	100.4	−3.31	1.76±0.063
dsx	Fw: tccacgccgtgcctaaatttgatc Rv: ttcgcacaaccagtcgccaaag	101	92.9	−3.50	–

The *dsx* gene was used as an internal control to normalise the *kfoC* gene copy number since it is present only once in the genome of *E. coli* strains. The wt *E. coli* K4 was used as the "calibrator" strain

In order to verify that the integration of the modified IS2 element occurred by transposition and not by homologous recombination, two primer pairs, namely M13 Forward/irla and M13 Reverse/irrb, were used to amplify the total DNA of BK4071 and no band was obtained, indicating the absence of plasmid fragments from the genome of BK4071 and, therefore, confirming the transposition event (data not shown).

Shake-flask experiments

E. coli K4 –pCP04 was grown in shake-flasks on the standard medium containing glycerol and soy as main carbon and nitrogen sources, respectively, and the titre of capsular polysaccharide was analysed to determine whether it was affected by the presence of additional pIRL-controlled *kfoC* copies (Table 2). Following integration of the modified insertion sequence (IRL-*kfoC*-IRR) and elimination of the XL vector, the resulting strains were also analysed in shake flasks in the same conditions. All of the three randomly selected colonies grown in parallel showed an increase of CPS production compared to the wild type after 24 h of growth; however, the best clone that demonstrated a significant 1.8-fold increase was chosen for following experiments and designated as BK4071 (Table 2). By comparing the titre of CPS pre (strain *E. coli* K4 –pCP04)- and post (strain BK4071)-integration, a decrease was observed, probably due to a lower number of additional copies of the cassette present in the integrative strain. In order to verify the genetic stability of BK4071, repeated growth experiments in shake-flasks were also performed showing that after three cycles, each lasting 24 h, the production of biomass and associated CPS remained unaltered (data not shown).

In comparison to the wild type, BK4071 also showed a 1.7-fold higher $Y_{K4/X}$ of 94±7 mg_{K4}/g_{cdw}, thereby demonstrating that the additional *kfoC* copy enhances CPS production without affecting the yield of biomass (Table 2).

The abundance of *kfoC* transcripts in the wt and recombinant strain was determined at three time points during growth. Results demonstrate an increased expression of the gene by 20 and 60 % after 6 and 24 h of growth, respectively, whereas expression is similar to that observed in the wt strain after 8 h of growth (Fig. 2).

Table 2 Summary of growth, K4 CPS production and relative yields obtained in shake-flask experiments from the reference *E. coli* K4 and the recombinant strains

Strain	Biomass (g_{cdw}/L)	μ_{max} (h^{-1})	K4 CPS (mg/L)	$Y_{K4/x}$ (mg/g_{cdw})
E. coli K4	2.24	0.88	124	55
E. coli K4 –pCP04_1	2.49	0.75	302	121
BK4071	2.34	0.76	221	94

The concentration of capsular polysaccharide was measured in the broth at the end of the experiment after 24 h of growth. $Y_{K4/x}$ indicates milligrams of K4 CPS produced per gram of dry cells. μ_{max} indicates the maximum specific growth rate. The values are averages of at least four separate experiments with a standard deviation below 10 %

Batch experiments in 2.5-L bioreactors

The physiology of BK4071 was studied in 24 h batch experiments in the standard medium (SM), in order to compare the

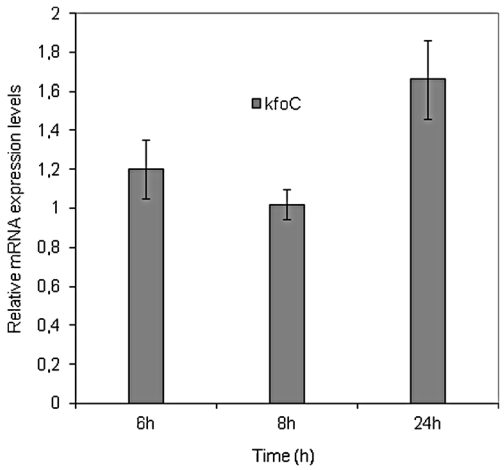

Fig. 2 Analysis of *kfoC* gene expression in *E. coli* K4 and BK4071 in shake-flask experiments. Histogram representing the fold overexpression of the gene under investigation in the recombinant strain BK4071 compared to the wt *E. coli* K4

strain performance to that obtained by the wt *E. coli* K4, as well as in SM supplemented with either glutamic acid and arginine or sucrose in order to stimulate growth. Results are summarised in Table 3. The addition of the two sources of amino acids unexpectedly inhibited growth, whereas the final titre of polysaccharide was only slightly reduced; therefore, a high $Y_{K4/X}$ was obtained. Both the concentration of biomass and CPS were slightly lower when sucrose was added to the SM, whereas the increased initial concentration of soy peptone in the broth (1.5 vs 1) mainly stimulated biomass production without affecting the synthesis of CPS. Compared to experiments performed with the wt *E. coli* K4, the best results were obtained in the SM without any supplementation. BK4071 showed a 40 % increase in the final titre of CPS over wild-type levels. Moreover, the maximum concentration of product (425 mg/L) was reached after only 8 h of growth, greatly improving process productivity.

Fed-batch experiments on 2.5- and 22-L scales

BK4071 was analysed in fed-batch fermentations on 2.5- and 22-L scales. In both fermentations, agitation speed and oxygen supply were modified according to the strain's metabolic demand in order to keep the pO_2 always above 20 % of saturation. The final production of biomass and K4 polysaccharide was higher in the process performed on the higher scale culminating in 3.47 g/L of K4 CPS after 32 h of growth (Fig. 3). However, the ratio of K4 CPS produced to biomass did not vary in the two processes, as indicated by a $Y_{K4/X}$ value of 0.22 g/g. This discrepancy might be attributed to small differences in the feeding strategy; in fact, a residue (1–2 g/L) of carbon source was always present in the broth for experiments performed in the 22-L bioreactor, whereas no accumulation of carbon source was observed during processes performed in the 2.5-L fermenter. A summary of the results obtained with the wt and recombinant strains during fed-batch experiments on the 2.5- and 22-L scales is reported in Table 4.

kfoC gene copy number determination

Real-time PCR amplifications of the total DNA from samples of cultures of BK4071 and of the wt *E. coli* K4 were performed to compare the number of copies of *kfoC* present in each strain. The *dsx* gene was used as an internal single-copy control gene. Standard curves for each gene were constructed in order to relate the Ct cycle to the *n*° of copies/molecules of the target gene as described in the "Materials and methods" section. The ratio between the number of *kfoC* and *dsx* molecules in the wild-type strain was used as a calibrator. The number of copies of *kfoC* in BK4071 was obtained by dividing the *kfoC*-to-*dsx* ratio in BK4071 by that of the calibrator. As reported in Table 1, the obtained value is equal to 1.76± 0.06, probably indicating the presence of one additional gene copy, introduced with the modified insertion sequence, compared to the wt *E. coli* K4.

Discussion

The goal of obtaining microbially derived chondroitin sulphate was addressed in our recent studies of the development of fermentation and engineering strategies to maximise the

Table 3 Summary of growth, K4 CPS production and relative yields obtained in batch fermentation experiments from the wt *E. coli* K4 and the recombinant strain BK4071

Strain	Medium	Biomass (g_{cdw}/L)	μ_{max} (h^{-1})	K4 CPS (mg/L)	$Y_{K4/x}$ (mg/g_{cdw})
E. coli K4	10 g/L glycerol, 1 g/L soy peptone	3.4/3.8[a]	0.84	310/100[a]	91.1/26.1[a]
BK4071	10 g/L glycerol, 1 g/L soy peptone	1.8/2.3[a]	0.97	390/425[a]	216.6/184.7[a]
E. coli K4	10 g/L glycerol, 1.5 g/L soy peptone	4.0	0.83	330	82.5
BK4071	10 g/L glycerol, 1.5 g/L soy peptone	3.7	0.79	396	107.0
E. coli K4	10 g/L glycerol, 1 g/L soy peptone, 9.5 mg/L arginine, 45 mg/L glutamic acid	3.0	0.82	252	84.0
BK4071	10 g/L glycerol, 1 g/L soy peptone, 9.5 mg/L arginine, 45 mg/L glutamic acid	1.5	0.91	267	178
E. coli K4	10 g/L glycerol, 1 g/L soy peptone, 0.1 g/L sucrose	3.6	0.79	290	90.6
BK4071	10 g/L glycerol, 1 g/L soy peptone, 0.1 g/L sucrose	2.1	0.94	355	169.0

Experiments were conducted in a 2.5-L bioreactor where growth on different media was analysed. The concentration of capsular polysaccharide was measured in the broth at the end of the experiment after 24 h of growth. $Y_{K4/x}$ indicates milligrams of K4 CPS produced per gram of dry cells. μ_{max} indicates the maximum specific growth rate. Values are averages of at least four separate experiments with a standard deviation below 10 %

[a] Values of biomass and CPS production obtained after 8 h of growth and resulting yield

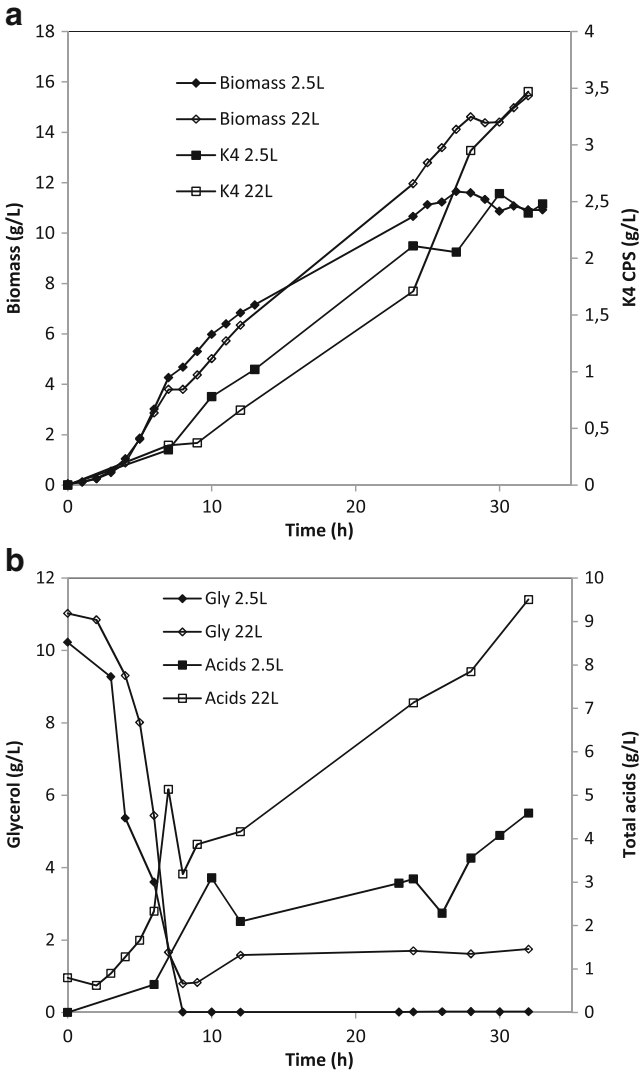

Fig. 3 Time course of fed-batch experiments in 2.5- and 22-L fermenters with strain BK4071. **a** Production of biomass and K4 CPS. **b** Consumption of glycerol and production of total acids

capacity of *E. coli* K4 to produce CS-like capsular polysaccharide (Cimini et al. 2012, 2013; Restaino et al. 2013). A plasmid-based expression system was recently used for the homologous overexpression of the *kfoC* gene in *E. coli* K4 (Cimini et al. 2010a). *kfoC* codes for a glycosyltransferase that is responsible for the polymerisation of the nascent chains of capsular polysaccharide by the alternate addition of glucuronic acid and *N*-acetyl-galactosamine to the growing end of the polymer. Data from our previous shake-flask experiments demonstrated that increased intracellular levels of the enzyme have a significant impact on improving polysaccharide production since a two fold enhancement of the final titre of CPS was achieved. However, the strain was extremely unstable, and in order to reproduce the results on a larger scale (2.5-L fermenter), the constant maintenance of selective antibiotic pressure throughout the batch process was crucial (Cimini et al. 2010a).

Currently, more effort is being addressed towards the modification of chromosomal DNA through systems that do not require selective pressure with antibiotic supplementation. Because our previous results were unsuitable for scale up to industrial applications, this work has exploited the potential of increasing the *kfoC* copy number by using a different engineering strategy and expression system that employed an endogenous copy of the insertion sequence IS2. IS2 is a member of the widespread family of IS3 insertion sequences although it differs from most members of this family in the asymmetry of the transposition mechanism of minicirle formation, in which IRR is the exclusive donor and IRL is always the target (Lewis et al. 2001). The cluster of genes that is responsible for the biosynthesis of CPS in *E. coli* K4 is characterised by the presence of an IS2 element between the *kfoC* and *kfoD* genes. A modified transposable expression cassette was obtained by constructing a new element in which *Orfs A* and *B*, of the native IS2 insertion sequence, were replaced by the *kfoC* gene. In this construct, *kfoC* was flanked by the two inverted repeats and preceded by the pIRL promoter. A carrier plasmid (pCP04) was then transformed into wt *E. coli* K4. We posited that during extended growth of this transformed strain, the IRL::*kfoC*::IRR cassette would be integrated into the host chromosome. After 72 h of growth, the delivery vector was expelled and the engineered cassette was integrated into the genome of *E. coli* K4. According to real-time PCR experiments, only one copy of the modified IS2 element was transposing in the genome of *E. coli* K4, and the additional *kfoC* copy in the recombinant strain induced a 1.8-fold increase of the CPS compared to the wild type (Table 2); moreover, the strain proved its stability by producing the same final amount of polysaccharide after several generations in shake-flasks.

Liu et al. (2009) increased the production of hyaluronic acid (HA) by 34 % through the cultivation of *Streptococcus zooepidemicus* in the presence of arginine together with cysteine and lysine. They observed that the depletion of such essential amino acids was followed immediately by the

Table 4 Summary of growth, K4 CPS production and relative yields obtained in fed-batch fermentation experiments in 2.5- and 22-L bioreactors from the wt *E. coli* K4 and the recombinant strain BK4071

Strain	Scale (L)	Biomass (g_{cdw}/L)	K4 CPS (g/L)	$Y_{K4/x}$ (g/g_{cdw})	Reference
E. coli K4	2.5	15.3	1.4	0.09	Cimini et al. (2010b)
BK4071	2.5	11.8	2.6	0.22	This manuscript
E. coli K4	22	10.9	1.4	0.13	This manuscript
BK4071	22	15.9	3.5	0.22	This manuscript

The concentrations of biomass and capsular polysaccharide reported are the maximal obtained during the process. $Y_{K4/x}$ indicates grams of K4 CPS produced per gram of dry cells. Values are averages of three separate experiments with a standard deviation below 10 %

end of the exponential phase. Since HA is produced by *S. zooepidemicus* as a growth-associated product, they extended the exponential phase via addition of three key amino acids and therefore improved the HA yield. The effect of the presence in the standard medium (glycerol-soy) of glutamic acid, arginine and sucrose on the production of K4 polysaccharide was analysed in the present study in 2.5-L batch fermentations. Surprisingly, the final concentration of CPS in the broth was lower compared to that obtained in the medium without supplementation. Growth was promoted for BK4071 and for the control strain by a higher concentration of soy peptone that however did not reflect in higher CPS yields. We previously showed that fructose supplementation increased the amount of CPS produced from *E. coli* K4 (Restaino et al. 2013). The addition of 0.1 g/L of sucrose, a cheaper source of fructose, however, did not produce the same results. Sucrose catabolism is in fact an extremely variable and poorly described feature among *E. coli* strains (Sabri et al. 2013). The highest titre of CPS was obtained on the SM medium without any supplementation, and compared to the wt strain, a great improvement of the process productivity was observed since K4 CPS production peaked after only 8 h of growth.

Strain performance was evaluated in fed-batch experiments on 2.5 and 22-L scales. An exponential feeding profile well suited BK4071. The $Y_{K4/X}$ of the processes on the two scales was conserved, demonstrating strain stability and the consistency of the results. The fed-batch performed in the 22-L fermenter, however, resulted in a higher final titre of CPS probably due to the slightly different feeding profile and to the changed reactor configuration (H:D 3:1 vs 2:1) in which the air bubble residence time was longer, thus improving oxygen mass transfer.

Imre and co-workers (2011) modified the IS30 transposon for the expression of a fusion transposase and observed that the modified construct retained the target recognition ability of the wt transposase. However, a higher frequency of integration into new target sites, not preferred by the latter, was also described. The integration and expression of the modified IS2-*kfoC* transposable cassette in the genome of *E. coli* K4 was not necessarily predictable; the accidental activation or repression of portions of the recipient cell's genome that are adjacent to the integration site could in fact have occurred. Our results here, however, have showed that the engineering of IS2 produced an appropriate tool for the overexpression of the *kfoC* gene. Expression of *kfoC* in this construct generated a 2.5-fold increase in the production of capsular polysaccharide in fed-batch experiments in a 22-L scale compared to experiments performed with the wt strain, showing the suitability of the system for further investigations in pilot-scale production levels. This represents a first step towards the biotechnological production of chondroitin and chondroitin sulphate for nutraceutical and pharmaceutical applications.

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