Technical Note

Efficient transformation of *Lactococcus lactis* IL1403 and generation of knock-out mutants by homologous recombination

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Lactococcus lactis IL1403 is a Gram-positive bacterium of great biotechnological interest for food grade applications. Its use is however hampered by the difficulty to efficiently transform this strain. We here describe a detailed, optimized electrotransformation protocol which yields a transformation efficiency of 10^6 cfu/μg of DNA with the two *E. coli* Gram-positive shuttle vectors pC3 and pVA838. The utility of the protocol was demonstrated by the generation of single- and double-knock-out mutants by homologous recombination.

Keywords: Electroporation / Gram-positive / Transformation efficiency / Single-crossover / Gene disruption

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Introduction

Lactococcus lactis IL1403 is a Gram-positive bacterium which is widely used in the food industry (De Vos 1996), but also as a model organism for molecular studies, because its genome has been sequenced recently (Bolotin *et al.* 2001) and its proteome has been extensively characterized (Guillot *et al.* 2003). Like many Gram-positive organisms, *L. lactis* is difficult to transform because of the rigid peptidoglycan layer which serves as a physical barrier for the entry of DNA (Beveridge 1981). With the development of electroporation, this barrier could be overcome in Gram-positive bacteria with varying efficiency. Electroporation introduces temporary holes into the cell wall and membrane by an electric discharge, thus allowing DNA to enter the cell (Potter 1988, Solioz and Waser 1990, Miller 1994, Drury 1994, Weaver 1995). The efficiency of transformation by electroporation is very strain and method specific and varies over a range of 10^2 to 10^8 colony forming unit (cfu) per μg of plasmid DNA (Luchansky *et al.* 1988). For example *Lactococcus lactis* subsp. *lactis* strains LM0232 and JK301 could reproducibly be transformed at an efficiency of only 10^3 cfu/μg (McIntyre and Harlander 1989a, 1989b), while transformation efficiencies of 10^6 to 10^8 cfu/μg were reported for *L. lactis* strain LM0230 (Powell *et al.* 1988, Holo and Nes 1995). Similarly, *L. lactis* subsp. *cremoris* could be transformed with an efficiency of 5.7×10^7 cfu/μg (Holo and Nes 1989b). These latter efficiencies approach those which can be obtained with *Escherichia coli* and open the door to most of the available genetic tools. However, there exist large differences in transformability, not only between species, but also between different strains of the same species. In particular, transformation of *Lactococcus lactis* IL1403 in excess of 10^4 cfu/μg could not be obtained in our and several other laboratories working with this organism (personal communication, Bojovic *et al.* 1991). This is in contrast to a report by Kim *et al.*, describing the electrotransformation of *L. lactis* IL1403 at an efficiency of 10^8 cfu/μg (Kim *et al.* 1996). It is not clear why this work could not be reproduced by us and others. Possibilities are that the IL1403 strain used by the Korean group differed from that used in other laboratories or that the high transformation efficiency was a specific property of the single vector, pGKV21, used in this study. We here describe a reproducible, efficient electrotransformation protocol for *Lactococcus lactis* IL1403, yielding 10^6 cfu/μg of plasmid DNA for two different *E. coli*-*L. lactis* shuttle vectors. The utility of the method was documented by the generation of gene knock-out and double knock-out mutants

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by homologous recombination with non-replicating *E. coli* vectors.

Materials and methods

Bacterial strains, plasmids and primers

Lactococcus lactis IL1403 was used for electroporation and for single cross-over recombination. *E. coli* DH5α and TOP10 (Invitrogen) strains were used in all the cloning steps for plasmid construction. The *E. coli*-Gram-positive shuttle vectors pC3 and pVA838 have been described previously (Solioz and Waser 1990, Macrina *et al.* 1982) and pCR-Blunt II-Topo™ vector was from Invitrogen. For electrotransformation, plasmid DNA was purified by CsCl density gradient centrifugation as described (Humphreys *et al.* 1975) and resuspended in 10 mM Tris-Cl, 1 mM EDTA, pH 7.5. Vector pCYZA27 containing the entire *L. lactis cop* operon was constructed by PCR amplification of a 3.9 kb genomic fragment of *Lactococcus lactis* IL1403, using primers dm01 and dm02 (Table 1), with TaKaRa LA Taq™ polymerase (Takara) and blunt-end ligation into the pCR-Blunt II-Topo™ vector (Invitrogen) as described by the manufacturer. For gene knock-out, the basal vector pSG1, which is unable to replicate in *L. lactis,* was constructed by PCR amplification of the erythromycin resistance gene of pVA838 with primer sg4 and sg5 and blunt-end ligation of the 1.8 kb product with the pCR-Blunt II-Topo vector. For disruption of the *copA* gene, a 1 kb internal *copA* fragment, cut from pCYZA27 with *Eco*RV and *Xba*I, was ligated with pSG1, cut with the same enzymes, resulting in pSG3. For disruption of the *copB* gene, an internal 1 kb fragment of *copB* was PCR amplified with primers sg7 and sg8 from genomic DNA of *L. lactis* IL1403 and ligated with pCR-Blunt II-Topo. From the resultant plasmid, pSG4, the *copB* fragment was excised with *Pst*I and ligated with *Pst*I-cut pSG1, resulting in pSG7. For double gene knock-out, pSG13 was constructed by PCR amplifying the chloramphenicol resistance gene of pC3 with primers sg14 and sg15, cutting the product with *Bam*HI and ligating the 1 kb fragment into pSG7, cut with the same enzyme. Cloning was performed by standard protocols as described by Sambrook *et al.* (1989). *E. coli* DH5α was transformed with the rubidium chloride method of New England Biolab. The routine isolation of plasmid DNA was performed with NucleoBond® Plasmid Purification Kits (Macherey-Nagel) according to the manufacturer's instructions.

Table 1. Primers used in this study.

Primer name	Sequence*
dm01	CCCAAGCTT**GGGCGGTTGCCGTCAATGAAGTG**
dm02	GCCTAGG**GCTTGCTGTCAGCATCCCTGTT**
fm19	**CCACCGGGTAAAGTTCACGGGAG**
sg4	CTGTCTCTTATACACATCT**TACCGCATTAAAGCTTG**
sg5	CTGTCTCTTATACACATCT**CGAGCGCTTAGTGG**
sg6	**TGCCGTTGCAGAGGCTGGTTATAAAG**
sg7	ACATGAATTC**TCCTTGGGCTAATAAACC**
sg8	ACATGAATTC**TGTGGAGCTTTCAGAAC**
sg9	**GCGTTGATTGAGACAATCAC**
sg14	TACGGGATCC**TTACCCTTAAGTTATTGGTATGAC**
sg15	GCGGATCC**CGACTAAAGCACCCATTAGTTC**
sg18	**TCACTATAGGGCGAATTGG**

* Primer sequences are in the 5′ to 3′ direction. The nucleotides in bold are complementary to the templates and underlined bases indicate restriction sites engineered into the 5' extensions of some primers.

Electroporation of *L. lactis* IL1403

Electroporation of *L. lactis* was performed as outlined in Table 2, using a Gene Pulser® from Bio-Rad, fitted with a 200 Ω (50 W) resistor, wired in series with the electroporation cuvette. This arrangement differs from the setup using a Bio-Rad pulse controller which allows to introduce various resistors in *parallel*. The cuvette had an electrode gap of 0.1 cm and was home-made as described previously. Capacitance was set to 25 μF. Plasmids pC3 and pVA838 were used for quantitative electroporation into *L. lactis* IL1403. *L. lactis* was grown at 30 °C in SGM17 media, which is M17 media (Terzaghi and Sandine 1975) supplemented with 1 mM $MgSO_4$, 0.5 M sucrose, 2% glycine, 1.5% glucose. SM17MC media for phenotypic expression was M17 media containing 0.5 M sucrose, to which 10 mM $MgCl_2$ and 2 mM $CaCl_2$ were added after autoclaving. Cells were plated either on M17, SR (containing per liter: 10 g tryptone, 5 g yeast extract, 200 g sucrose, 10 g glucose, 25 g gelatin, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, pH 6.8) or on BHIS media (38 g/l Brain Heart Infusion broth (Difco) supplemented with 1 mM MgSO4 and 1% glucose after autoclaving). Highest transformation efficiencies were obtained by plating on SR media. Erythromycin was used at 5 μg/ml and chloramphenicol at 15 μg/ml. Electroporation buffer was prepared either with EDTA (0.5 M sucrose, 10% glycerol, 50 mM Na-EDTA, pH 7.0) or without EDTA (0.5 M sucrose, 10% glycerol, pH 7.0) and was prepared and autoclaved on the day before use.

Gene knock-out and mutant analysis

To generate knock-out strains, *L. lactis* IL1403 was transformed with 1 to 2 μg of the corresponding plasmid DNA and cells plated on BHIS plates containing the corresponding antibiotic. For analysis, genomic DNA was isolated as described (Ausubel *et al.* 1995) and 0.5 μl was used for PCR. Knock-outs in Δ*copA* were confirmed

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Table 2. Transformation protocol.

Step	Operation
1	**Preparation of cells:** Inoculate 5 ml of SGM17 media with *L. lactis* IL403 from glycerol stock and incubate overnight at 30 °C in a closed tube.
2	Inoculate 50 ml of SGM17 with the 5 ml overnight culture and incubated at 30 °C overnight in a closed bottle.
3	Inoculate 450 ml of freshly prepared SGM17 with the 50 ml overnight culture and incubated at 30 °C in a closed bottle. Grow culture to an OD_{546} of about 0.4 (mid- to late-log phase) and collect cells by centrifugation for 10 min at 8000 × *g* at 4 °C.
4	Resuspended the pellets with 400 ml of ice-cold electroporation buffer without EDTA, using a 10 ml pipette, and centrifuge as before.
5	Resuspended the pellets in 200 ml of ice-cold electroporation buffer **with** EDTA and incubated at 4 °C for 15 min, followed by centrifugation as before.
6	Wash cells a third time with 400 ml of ice-cold electroporation buffer without EDTA.
7	Resuspend the final pellet in 4 ml of electroporation buffer at 4 °C. Flash-freeze aliquots of 120 to 500 μl in liquid nitrogen and store at −80 °C. These electrocompetent cells are stable for at least four weeks.
8	**Electroporation:** Thaw the electrocompetent cells on ice.
9	Flame the reusable electrodes with ethanol for sterilization and assemble them. Cool the electrode assembly (or a corresponding disposable cuvette) with liquid nitrogen until it gets frosted. Use the cuvette immediately when the frost thaws (e.g. near zero degrees).
10	**The following must be conducted as fast as possible:** Pipet 120 μl of electrocompetent cells into an Eppendorf tube on ice and add plasmid DNA. Gently mix by tapping and pipette the cells into the cold electroporation cuvette. Before triggering the discharge, draw 900 μl of ice-cold SM17MC media into an Eppendorf pipet tip. Electroporate at 1.3 kV, 200 Ω *serial* resistor, 25 μF and immediately wash the cells out of the cuvette into a fresh Eppendorf tube on ice with the 900 ml of cold SM17MC media ready in a pipet (not later than after 1 to 2 s after the pulse).
11	Incubate the electroporated cells on ice for 5 min.
12	Incubate the electroporated cells for 90 min at 30 °C to allow for phenotypic expression.
13	Plate the cells on either M17, SR or BHIS plates supplemented with 5 μg/ml of erythromycin or 15 μ/ml of chloramphenicol and incubate the plates overnight at 30 °C.

by PCR with primers fm19 and sg6 and those in *ΔcopB* with primers sg9 and sg18. PCR products were purified and digested with restriction enzymes as described (Sambrock *et al.* 1989). Double knock-outs were confirmed with the same primers. Commercial sequencing (Microsynth, Balgach, Switzerland) was used to verify the PCR products.

Results

Optimization of tansformation

Optimization of the transformation efficiency of *L. lactis* IL1403 required the adjustment of many parameters and Table 2 summarizes the transformation protocol we developed.

Most critical was the electrical circuit which was used and the parameters for electroporation. In contrast to previously published methods (e.g. Holo and Nes 1989c), a 200 Ω resistor was wired *in series* rather than in parallel with the electroporation cuvette, which resulted in longer pulse decay times, τ. It was found that τ-values of 20 to 40 ms were most efficient, requiring a voltage of 13 kV/cm. Other important factors were the growth media, the wash protocol for the cells, plasmid purity, cell density, expression media, and phenotypic expression times. High transformation efficiency was very dependent on the concentration of glycine in the growth media (Holo and Nes 1989a, Shepard and Gilmore 1995). It leads to replacement of D-alanine in peptidoglycan synthesis and the accumulation of modified peptidoglycan precursors accumulate. Since these are poor substrates in the transpeptidation reaction, a high percentage of muropeptides remains uncross-linked, which “loosens” the cell wall (Hammes *et al.* 1973). Concentrations of 2–3% glycine gave optimal results, while concentrations above 3% inhibited growth. I was crucial to harvest the cells in the mid-log to late-log phase of growth. The transformation efficiency was also strongly dependent on the handling of cells. Osmotic stabilization of the cells by adding sucrose to the buffers proved instrumental. Mechanical stress had to be avoided as much as possible and the cells had to be kept cold at all times. Finally, transformation efficiency was 20–30% higher when the cells were plated on SR plates compared to BHIS or M17 plates. Under the optimized conditions, transformation efficiencies of 10^6 cfu/μg could reproducibly be obtained. Fig. 1 shows the dependency of the transformation efficiency on the DNA concentration for the 6.3 kb *E. coli*-Gram-positive shuttle vector pC3. From 10 to 700 ng of plasmid DNA, the transformation efficiency was essentially constant, but dropped sharply at higher DNA concentrations. Transformation efficiencies ob-

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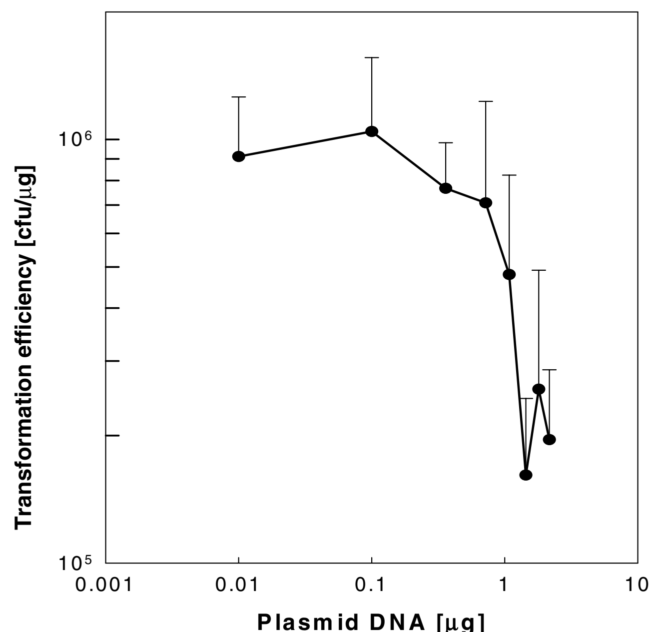

Figure 1. Dependence of transformation efficiency on amount of plasmid DNA. *L. lactis* IL1403 was transformed by the procedure given in Table 1 with varying amounts of pC3 and plated on SR plates containing 5 μg/ml erythromycin. All experiment were performed in a triplicates.

tained with the unrelated 9.2 kb *E. coli*-Gram-positive shuttle pVA838 were reduced in proportion to the larger size of this vector. The highest number of transformants in absolute terms (>10^6) was obtained with 1 to 2 μg of DNA, which were the conditions used for gene knock-out by homologous recombination.

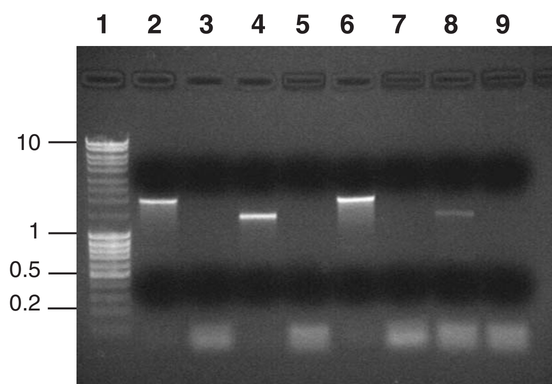

Figure 2. Genomic PCR products from knock-outs strain. Genomic DNA was PCR amplified as described under Materials and methods and the products resolved on a 2% agarose gel, stained with ethidium bromide. The PCR products of the following DNA-primer combinations are shown: *lane 1*, DNA standard (MassRuler, Fermentas); *lane 2*, Δ*copA*-fm19/sg6; *lane 3,* wild-type-fm19/sg6; *lane 4,* Δ*copB*-sg9/sg18; *lane 5*, wild-type-sg9/sg18; *lane 6*, Δ*copA*-fm19/sg6 of double knock-out; *lane 7*, wild-type-fm19/sg6; *lane 8*, Δ*copB*-sg9/sg18 of double knock-out; *lane 9* wild-type-sg9/sg18. The sizes of DNA markers are indicated in kb on the ordinate.

Gene knock-out by homologous recombination

To accomplish gene knock-out, we chose the strategy of Campbell-like plasmid integration by homologous recombination (Leenhouts *et al.* 1989, 1991). This results in the integration of the entire plasmid into the chromosome and, if the integration site is designed accordingly, inactivation of the desired gene. To this end, the pCR-Blunt II-Topo vector (Invitrogen) was used as a plasmid which cannot replicate in *L. lactis*. To provide antibiotic resistance markers which are suitable for *L. lactis*, the erythromycin and the chloramphenicol resistance genes of pC3 were cloned into this plasmid. As model genes, we inactivated the *copA* and *copB* genes, which encode putative copper ATPases and are approximately 2 kb long. DNA fragments of 1 kb and corresponding to the central regions of the *copA* or the *copB* genes were cloned into the modified pCR-Blunt II-Topo vectors pSG3 and pSG7, respectively. Following electroporation with these plasmids, only cells containing a plasmid copy integrated into the chromosome retained antibiotic resistance. Resistant cells were obtained at a frequency of approximately 10 per μg^{-1} of plasmid DNA. Chromosomal integration of the plasmid was tested by PCR amplification of genomic DNA, using a primer directed against the plasmid DNA and one directed against genomic DNA near the expected integration site. The expected PCR products were obtained, namely a 1.57 kb fragments for *ΔcopA* mutants and 1.96 kb fragments for *ΔcopB* mutants, respectively (Fig. 2). The PCR product of the *ΔcopAΔcopB* double knock-out strain was of low intensity for unknown reason, but DNA sequencing revealed 98% sequence identity with the predicted product. Correct integration was further confirmed by restriction digest analysis of the PCR products, which yielded the correct fragmentation pattern in all cases (Fig. 3). The integrations were apparently stable in the presences of antibiotics, as has been noted previously in other organisms (Leloup *et al.* 1997).

Discussion

We here describe a detailed, optimized electroporation protocol for *Lactococcus lactis* IL1403 to obtain a transformation efficiency of 10^6 cfu/μg DNA. This supports genetic engineering of this biotechnologically important strain by homologous recombination or by using targeted group II introns (Frazier *et al.* 2003). As proof-of-principle, we used the current transformation protocol to create single and double knock-out mutants in *L. lactis* IL1403 in two genes, *copA* and *copB*, by homolo-

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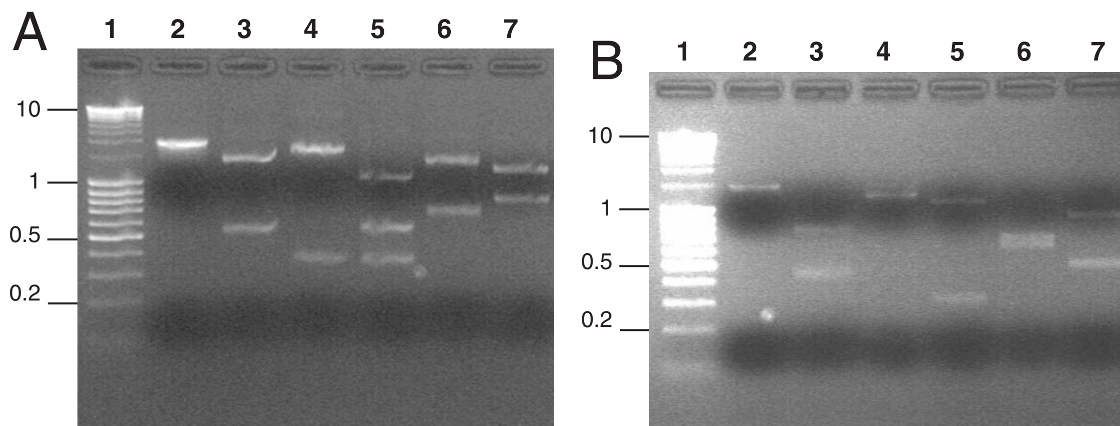

Figure 3. Restriction enzyme analysis of PCR products. (A) The PCR product Δ*copA*-fm19/sg6 (cf. Figure 2) was digested with restriction enzymes and resolved on a 2% agarose gel stained with ethidium bromide. *Lane 1*, DNA *standard, lane 2, undigested, lane 3, Eco*RV*; lane 4, Xba*I; *lane 5*, *Eco*RV and *Xba*I; *lane 6*, *Nsi*I; *lane 7, Cla*I. (B) The PCR product Δ*copB*-sg9/sg18 (cf. Fig. 2) was digested with restriction enzymes and resolved on a 2% agarose gel stained with ethidium bromide. *Lane 1*, DNA *standard, lane 2, undigested, lane 3, Hpa*I*; lane 4, Sph*I; *lane 5*, *Bst*EII; *lane 6*, *Van*911; *lane 7, Xba*I. The sizes of DNA markers are indicated in kb on the ordinate.

gous Campbell-like recombination with an *E. coli* plasmid unable to replicate in *L. lactis*. Use of this method has been wide-spread (Simon and Chopin 1988, Leenhouts *et al.* 1990, Biswas *et al.* 1993), but has, to our knowledge, never been applied to strain IL1403. The low transformation efficiency of IL1403 was previously overcome for genetic engineering experiments by the use of special thermolabile plasmids. These could carrying either an insertion sequence for unspecific integration (Maguin *et al.* 1996, Uguen and Uguen 2002), or a defined sequence for homologous recombination (Le Bourgeois *et al.* 1992, Sperandio *et al.* 2005). The high transformation efficiency to 10^6 cfu/μg of plasmid DNA reported here allows direct genetic engineering of IL1403 by homologous recombination using any *E. coli* plasmid carrying homologous *L. lactis* DNA. We obtained on average ten knock-out mutants per experiment, corresponding to an integration frequency of approximately 10^{-5}. This low integration frequency may be a specific characteristic of IL1403, as much higher integrations frequencies had been reported for other Gram-positive bacteria. A critical parameter for the integration frequency is the length of the homologous genomic DNA insert in the plasmid. Leloup *et al.* carefully studied the integration frequency as a function of insert size (Leloup *et al.* 1997). They found that the integration frequency in *Lactobacillus sakei* increased linearly with the insert size up to 10^{-3} at 1 kb of DNA. Larger inserts did not appear to significantly increase the integration frequency. We observe an integration frequency of only 10^{-5} with 1 kb inserts in *L. lactis* IL1403, suggesting that this strain has an intrinsically lower recombination frequency compared to other Gram-positive bacteria. Indeed, we were unable to effect integration into the *ytjD* gene with a 274 bp fragment, but could do so with a 379 bp fragment (Mourlane and Solioz, personal communication). Some of the critical parameters in transformation identified in this work, such as low temperature, osmotic stabilization, rapid transfers, and ohmically controlled discharge may be applicable to other hard-to-transform Gram-positive bacteria.

The efficient electrotransformation procedure described here features a 200 Ω serial resistor as a critical element. The protocol lends itself to genetic engineering of *Lactococcus lactis* IL1403 and possibly other Gram-positive bacteria refractory to efficient electroporation. It allows gene knock-out with standard *E. coli* vectors by Campbell like recombination, without the need for thermosensitive replicons. The two *E. coli*-Gram-positive shuttle vectors employed here, pC3 and pVA838, have not previously been used in *L. lactis* and appear well suited for genetic work in *L. lactis*.

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