METHODS AND PROTOCOLS

Importance of *codB* for new *codA*-based markerless gene deletion in *Gluconobacter* strains

**David Kostner · Björn Peters · Markus Mientus ·
Wolfgang Liebl · Armin Ehrenreich**

Abstract For the detailed molecular analysis, genomic modification, and application of acetic acid bacteria such as *Gluconobacter* in biotechnological processes, a simple markerless deletion system is essential. The available methods have either low efficiencies or their applicability is restricted to strains containing an *upp* mutation. We now developed a method based on counterselection by cytosine deaminase, encoded by the *codA* gene from *Escherichia coli*, in the presence of the fluorinated pyrimidine analogue 5-fluorocytosine (FC). The *codA*-encoded enzyme converts nontoxic FC to toxic 5-fluorouracil, which is channeled into the metabolism by the uracil phosphoribosyltransferase, encoded by the chromosomal *upp* gene of *Gluconobacter*. We found that the presence of *E. coli codB*, encoding a cytosine permease, was needed for a high efficiency of gene deletion. The system is applicable in wild-type strains because no preceding deletions are required. Based on the fact that a *codA* gene is absent and an *upp* gene is present in almost all acetic acid bacteria sequenced so far, the method should also be applicable for other genera of the *Acetobacteraceae*.

Keywords Markerless deletion system · Acetic acid bacteria · *Gluconobacter* · 5-fluorocytosine · *Cod* genes · Counter-selectable marker

Introduction

The group of acetic acid bacteria contains many genera with interesting biochemical characteristics. They are widely used in biotechnology. An important representative of the family is *Gluconobacter oxydans*, which is employed in several

D. Kostner · B. Peters · M. Mientus · W. Liebl · A. Ehrenreich (✉)
Lehrstuhl für Mikrobiologie, Technische Universität München,
Emil-Ramann-Strasse 4, D-85354 Freising, Germany
e-mail: aehrenr@mikro.biologie.tu-muenchen.de

biotechnological processes due to its ability to perform incomplete oxidations of a large variety of carbohydrates, alcohols, and sugar alcohols at relatively high substrate concentrations (Gupta et al. 2001). The oxidative fermentations allow the substitution of certain or multiple steps in industrial synthesis that cannot be achieved by organic chemistry with sufficient yield. For molecular analysis of the physiology of acetic acid bacteria and construction of strains tailored for biotechnological production purposes, an effective and easily applicable deletion system is important. So far, most of the mutants were constructed by inserting an antibiotic-resistance cassette into the genes of interest (Hölscher and Görisch 2006; Hölscher et al. 2007). Instead of a knockout system in which persisting resistance markers remain in the strain, accompanied by polar effects on the expression of downstream genes, there is a strong need for methods, which provide markerless in-frame deletions in acetic acid bacteria. This is also necessary because the available resistance cassettes that can be used for multiple knockouts are limited.

Typical markerless deletion systems are two-step methods, where a first step of vector integration at the target site is followed by a second step where a counter-selectable marker is employed to select for clones, which have lost the vector. Therefore, a good counterselection method is the key for an effective markerless deletion system. However, the *sacB* system that has been used in *Gluconobacter* for this purpose does not work satisfyingly (Gay et al. 1985; Hölscher et al. 2007). Commonly used approaches for counterselection in other organisms exploit genes involved in the purine or pyrimidine metabolism. For example, *upp* coding for a phosphoribosyltransferase, *pyrE/ura5* coding for an oratate phosphoribosyltransferase, or *hpt* encoding a hypoxanthine phosphoribosyltransferase have been used (Boeke et al. 1984; Boeke et al. 1987; Fabret et al. 2002; Keller et al. 2009; Pritchett et al. 2004; Wagner et al. 2012). All these systems are based on the fact that purine or pyrimidine analogues are converted to toxic compounds. Only cells lacking the gene for

the converting enzyme can survive the presence of the analogue. Plating of cells on media containing the analogue leads to a strong selection for clones, which have lost any chromosomally integrated copy of the gene for the converting enzyme. Therefore, parental strains used for genome modification must be devoid of the respective gene for purine or pyrimidine nucleotide biosynthesis. In a previous study by our group, the *upp* gene was utilized for the counterselection step (Peters et al. 2012). The uracil phosphoribosyltransferase encoded by this gene catalyzes the conversion of the pyrimidine analogue 5-fluorouracil (FU) to 5-fluorouridine-monophosphate (F-UMP) (Martinussen and Hammer 1994). F-UMP is then transformed to 5-fluorodesoxyuridine-monophosphate, which elicits a toxic effect by inhibition of thymidylate synthase, thereby blocking DNA repair and replication (Neuhard 1982). An *upp*-deficient strain of *G. oxydans* 621H and a deletion vector containing an *upp* gene were created for this system. The counterselection against this vector was done on media supplemented with FU. Despite its high efficiency, the need to work with a Δ*upp* strain is an important restriction that limits its application in the variety of acetic acid bacteria strains used in biotechnology.

In this study, we developed an alternative counterselectable marker based on *codA* and *codB* in order to address this problem and describe its utilization in a convenient markerless in-frame deletion system.

Materials and methods

Chemicals

Yeast extract and peptone were obtained from Carl Roth (Karlsruhe, Germany), 5-fluorocytosine (FC) from TCI Europe N.V. (Zwijndrecht, Belgium), kanamycin (Km) from AppliChem (Darmstadt, Germany), mannitol, glucose, cefoxitin, FU, and other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

Bacterial strains and culture methods

Escherichia coli Top10 was purchased from Invitrogen (Karslruhe, Germany) and grown in LB medium at 37 °C and 180 rpm on a rotary shaker (Sambrook et al. 1989). For plasmid selection, the medium was supplemented with 50 μg/ml Km. The strains *G. oxydans* DSM 2343 (ATCC 621H), DSM 3504, DSM 7145, *Acidomonas methanolica* DSM 5432, *Gluconacetobacter diazotrophicus* DSM 5601, *Acidiphilium multivorum* DSM 11245, *Granulibacter bethesdensis* DSM 17861 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), the strain *Acetobacter pasteurianus* LMG 1513 from BCCM/LMG Bacteria Collection (Gent, Belgium). The *G.*

oxydans strains were grown in mannitol medium containing 5 g/L yeast extract, 3 g/L peptone, and 50 mM mannitol at pH 6. For *G. diazotrophicus* and *A. pasteurianus*, the mannitol level was increased to 250 mM. *A. methanolica* was grown in glucose medium containing 5 g/L yeast extract, 20 g/L glucose, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, $MgSO_4 \times 7 H_2O$ 0.7 g/L, 0.5 g/L NaCl, 0.4 g/L $Ca(NO_3)_2 \times 4H_2O$ at pH 6.8, *G. bethesdensis* in 50 g/L glucose, 12.5 g/L $CaCO_3$, and 5 g/L yeast extract. *A. multivorum* was cultivated in 2 g/L $(NH_4)_2SO_4$, 1 g/L glucose, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \times 7 H_20$, 0.3 g/L yeast extract, and 0.1 g/L KCl at pH 3. The pH was adjusted using hydrochloric acid. The cultures were grown at 180 rpm on a rotary shaker at 30 °C. The selection conditions in growth media were 50 μg/μL for Km and 60 μg/μL for FC. Table 1 summarizes the strains used in this study.

DNA manipulations

Routine molecular biological procedures were done according to standard protocols (Sambrook et al. 1989). For preparation of plasmids, the AxyPrep (Union City, USA) plasmid miniprep kit was used. Genomic DNA from *G. oxydans* DSM 3504 and 621H was extracted with the Epicenter MasterPure DNA purification kit (Madison, USA). DNA purification was done with the Promega PCR clean-up system (Madison, USA). Restriction enzymes, DNA ligase, and alkaline phosphatase (FastAP) were obtained from Fermentas (Waltham, USA). PCR was done according to the manuals of the enzymes from Finnzyme (Vantaa, Finland). Phire polymerase was used for analytical reactions and Phusion polymerase for amplifications requiring proofreading. Oligonucleotides and sequencing services were provided by Eurofins MWG GmbH (Ebersberg, Germany). The fusion PCR technique was used to ligate the PCR products of flanking regions for the deletion vectors according to a long flanking homology (LFH) protocol (Peters et al. 2012; Wach 1996). Colony PCR was used to screen for mutants or to confirm integration of a deletion vector into the genome. Colonies were picked and dissolved in water. During PCR, the initial denaturation step was prolonged to 8 min. Enzyme-free cloning technique (Tillett and Neilan 1999) was used for the construction of the deletion vector pKOS6b. Table 2 summarizes the plasmids used for and constructed in this study.

Transformation of *Gluconobacter*

G. oxydans DSM 3504 was transformed via electroporation (Hall et al. 1992; Kallnik et al. 2010; Mostafa et al. 2002). Cells were grown to an OD_{600} of 0.9 in rich EP medium containing 80 g/L mannitol, 15 g/L yeast extract, 2.5 g/L $MgSO_4 \times 7H_20$, 1.5 g/L $CaCl_2$, and 0.5 g/L glycerol adjusted to pH 6. The culture was centrifuged at 4,000 rpm, 4 °C for 10 min, and washed three times in HEPES (1 mM, pH 7).

Table 1 Strains used in this study

Organism/strain	Features	Reference/source
Escherichia coli		
E. coli DH5α	F⁻, *endA1*, *hsdR17* (rk-mk+), *supE44*, *thi1*, *recA1*, *gyrA* (Nalr), *relA1*, D (*lacZYAargF*), U169, F80*lacZ*DM15	Hanahan (1983)
E. coli HB101	F⁻, *hdsS*20 (r-B, m-B), *supE*44, *ara*-14, *galK*-2, *lacY1*, *proA*2, *rpsL*20, *xyl*-5, *mtl*-1, *recA*13, KanR, oriColE1, RK2-Tra+, *mH*-1 carrying plasmid pRK2013	Boyer and Roulland-Dussoix (1969); Figurski and Helinski (1979)
E. coli K12 MG1655	F⁻, λ-, *rph-1*	
Acetic acid bacteria		
Gluconobacter oxydans 621H (DSM 2343)	Wild type, FCR, CefR	De Ley et al. (1984); Gillis (1980)
G. oxydans DSM 3504	Wild type, FCR, CefR	Motizuki et al. (1966)
G. oxydans DSM 7145	Wild type, FCR, CefR	De Ley (1961); Gossele et al. (1983); Mason and Claus (1989)
G. oxydans DSM 3504 Δ9570	Levansucrase deletion strain, FCR, CefR	This work
G. oxydans 621H Δ0854Δ0855	Polyol dehydrogenase deletion strain, FCR, CefR	This work
G. oxydans 621H pKOS6a2567flank	Strain with integrated pKOS6a in GOX2567 flanking region	This work
G. oxydans 621H pKOS6b2567flank	Strain with integrated pKOS6a in GOX2567 flanking region	This work
Gluconacetobacter diazotrophicus DSM 5601	Wild type, FCR, CefR	Yamada et al. (1997)
Acetobacter pasteurianus LMG 1513	Wild type, FCR, CefR	Gossele et al. (1983)
Acidomonas methanolica DSM 5432	Wild type, FCR, CefR	Urakami et al. (1989); Yamashita et al. (2004)
Acidiphilium multivorum DSM 11245	Wild type, FCR, CefR	Wakao et al. (1994)
Granulibacter bethesdensis DSM 17861	Wild type, FCR, CefR	Greenberg et al. (2006)

Cells were shock frozen in liquid nitrogen in 50 μL aliquots. The electroporation was carried out in cuvettes with 1 mm electrode distance from Peqlab (Erlangen, Germany) with 2.5 kV, 25 μF, and 400 Ω by a Gene Pulser apparatus from Bio-Rad GmbH (München, Germany). Fresh EP medium was added after the pulse cells were incubated on a rotary shaker over night for a better regeneration and were plated on mannitol plates containing kanamycin.

G. oxydans 621H was transformed by conjugation using triparental mating. *E. coli* Top10 was used as donor together with helper strain *E. coli* HB101 carrying plasmid pRK2013. *G. oxydans* and *E. coli* cells were grown to an OD_{600} of 0.9; 1 mL of donor and helper strain were mixed and washed with fresh LB, then 4 mL of *G. oxydans* culture was added and centrifuged. The resulting pellet was resuspended in fresh *G. oxydans* medium and placed on a mannitol-containing agar plate in a drop. After 24 h incubation, the cells were washed from the plate with fresh mannitol medium and the resulting cell suspension was plated on mannitol plates containing kanamycin and cefoxitin.

Table 2 Plasmids used in this study

Plasmid	Features	Reference/source
pAJ63a	Deletion vector *gusA* removed, *upp* integrated, KmR, FUS	Peters et al. (2012)
pKOS6a	Derivative from pAJ63a, *upp* removed, *codA* integrated, KmR, FCS	This work
pKOS6b	Derivative from pAJ63a, *upp* removed, *codBA* integrated, KmR, FCS	This work
pKOS6bΔ9570	Deletion vector based on pKOS6b for deletion of GLS_c9570 in *G. oxydans* DSM 3504, KmR, FCS	This work
pKOS6bΔ854Δ855	Deletion vector based on pKOS6b for deletion of GOX0854 and GOX0855 in *G. oxydans* 621-H, KmR, FCS	This work
pKOS6a2567flank	Integrative vector based on pKOS6a to study importance of codB for the deletion system in *G. oxydans* 621-H, KmR, FCS	This work
pKOS6b2567flank	Integrative vector based on pKOS6b to study importance of codB for the deletion system in *G. oxydans* 621-H, KmR, FCS	This work

Results

The *codA* gene of *E. coli* K12 was chosen as counter-selectable marker gene for the new strain-independent and markerless in-frame deletion system. To investigate the importance of *codB*, the second gene in the *cod* operon, we constructed one vector containing *codAB* and a second vector that includes only *codA*.

Construction of deletion vectors pKOS6a and pKOS6b

The construction of a *cod*-based deletion vector took pAJ63a as a starting point. Enzyme-free cloning techniques were used to remove the *upp* cassette and replace it with *codAB* genes, but retaining the *upp* promoter region. The primers pAJdUPP_F/F_ov and pAJdUPP_R/R_ov were used for the amplification of the vector and the primers codBA_F/codBA_F_ov and codBA_R/codBA_R_ov to amplify the *codAB* operon including 24 bp upstream sequence, using *E. coli* K12 chromosomal DNA as template (Fig. 1). The resulting vector pKOS6b was transformed in chemically competent *E. coli* Top10 cells and was completely sequenced. The sequence data was deposited in GenBank under the accession number JX885593.

A smaller vector, pKOS6a, lacking *codB* was constructed to investigate the role of *codB* for the functionality of the *cod*-based counterselection. The previously created pKOS6b served as a template for the amplification with the primers pKos6AmpFxho and pKos6aAmpRxho. The resulting PCR product was digested with *Xho*I and circularized with T4 ligase to pKOS6a. The vector was transformed in *E. coli* Top10 and verified by sequencing.

Construction of *G. oxydans* DSM 3504 Δ9570

A chromosomal deletion was constructed in strain *G. oxydans* DSM 3504 to examine the functionality of the generated vector carrying *codBA* as the counter-selectable marker. We choose GLS_C09570, a levansucrase, as the target gene. The flanking regions, covering approximately 900 base pairs (bp) upstream and downstream of the gene GLS_C09570, were amplified using the primers 09570_F_kpn/09570_FUSR and 09570_FUSF/09570_xba_R. Next, a LFH PCR was performed with primers 09570_F_kpn and 09570_xba_R to fuse the two fragments. The fused fragment and pKOS6b were digested with *Kpn*I and *Xba*I and ligated in the corresponding sites of a dephosphorylated pKOS6b vector. The resulting vector pKOS6bΔ9570 was transformed in chemical competent *E. coli* Top10 cells and verified by colony PCR and sequencing using the primers pk18MCS_F and pk18MCS_R/pk18MCS_2R.

The transformation of pKOS6bΔ9570 in *G. oxydans* DSM 3504 Δ9570 was done by electroporation. The

integration of the plasmid in the genome by a first recombination, which occurs randomly either up- or downstream of the target gene, resulted in several clones, which were verified using colony PCR with the genome-specific primer 09570check_F and the vector-specific primer pk18MCS_R (Fig. 2). All the clones were positive. They were grown in a medium, containing Km, and were plated on mannitol selection plates including 60 μg/mL FC. This results in around 50 clones per plate. Only those that had lost the vector by a second recombination were able to survive. This second recombination could either result in revertants or in the desired replacement of the target gene with the fused gene flanks of the deletion vector. After 3–5 days of incubation, the correct clones were identified by colony PCR and verified by sequencing, using the primers 09570check_F and 09570check_R. 9 out of 21 checked clones contained the desired deletion. The 1,317 bp deletion was also confirmed by Southern blot analysis using a probe targeting the upper flanking region (Fig. 3).

Construction of *G. oxydans* 621H Δ854Δ855

To prove the suitability of the method as a generalized deletion system that works for different *G. oxydans* strains, we created a Δ854Δ855 mutant lacking the polyol dehydrogenase in *G. oxydans* strain 621H. The construction and validation of the deletion vector was done as described above for pKOS6bΔ9570. The primers used are given in Table 3, and the insert was cloned in the *Eco*RI and *Xba*I sites of pKOS6b.

The deletion vector pKOS6bΔ854Δ855 was verified by PCR and sequencing and was transformed in strain 621H by conjugation using triparental mating. The rest of the procedure corresponds to the creation of the Δ9570 mutant in strain DSM 3504. The mutant had the expected phenotype of being unable to grow on mannitol due to its lack of polyol dehydrogenase. The validation of the in-frame deletion was done by PCR and Southern Blot (data not shown). The efficiency of gene deletion in strain 621H corresponded to the results for strain DSM 3504, demonstrating the suitability of the new deletion method for use in different *G. oxydans* strains.

Influence of *codB* on the efficiency of counterselection

Two vectors containing 1,094 bp of the downstream flanking region of the *G. oxydans* 621H gene GOX2567 were constructed. These constructs were able to integrate in the chromosome of *G. oxydans* 621H after transformation via a single recombination. The vector pKOS6a2567flank contained only the *codA* gene, while the vector pKOS6b2567flank contained the whole *codAB* operon. The minimal inhibitory concentration (MIC) of FC on both strains with chromosomally integrated *codA* and *codAB*, respectively, was determined in liquid cultures containing 50 μg/mL Km. After 3 days of
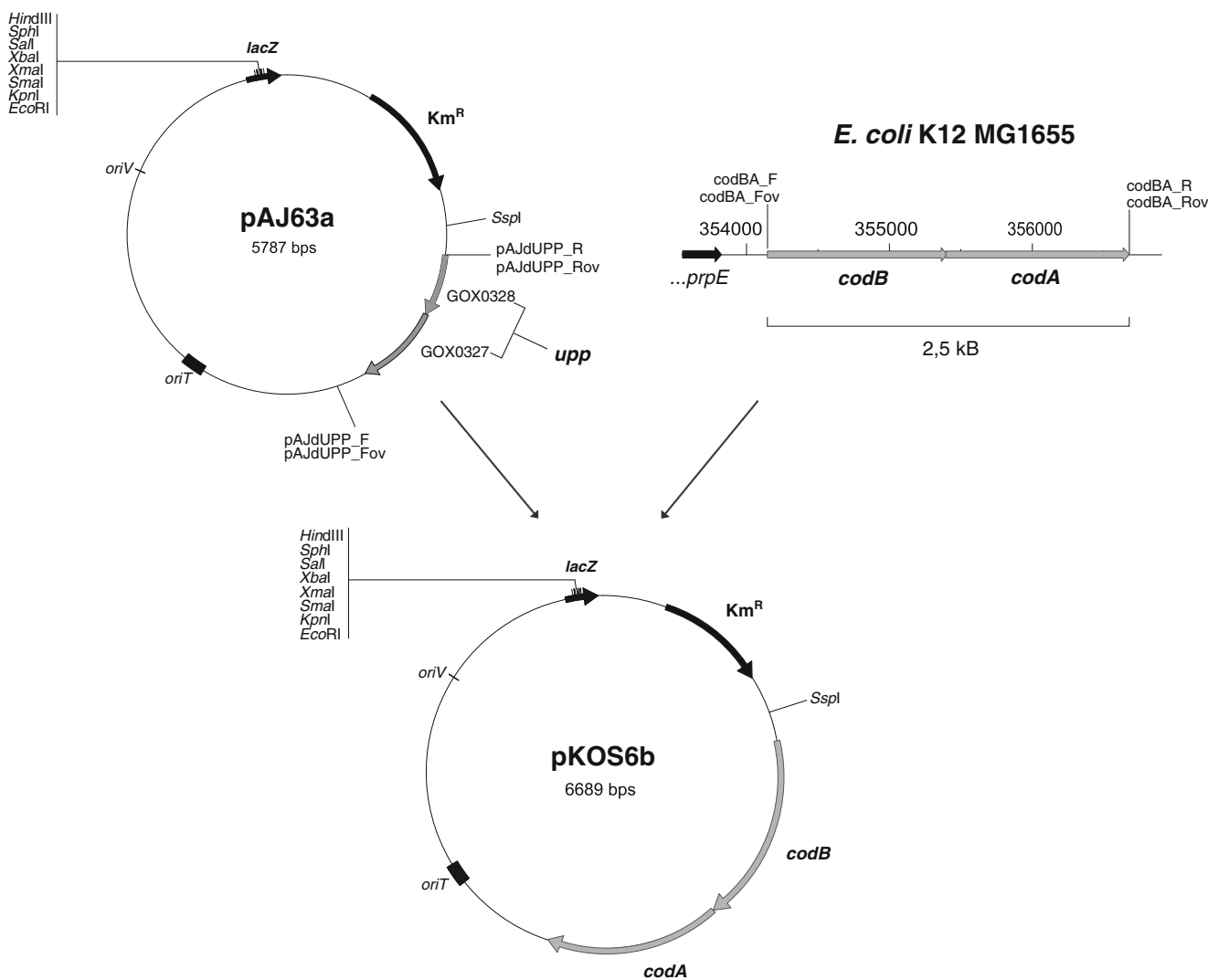
Fig. 1 Construction of deletion vector pKOS6b from pAJ63a. The *upp* cassette was replaced with the *codAB* operon amplified from *E. coli* K12 under maintenance of the *upp* promoter

growth, a MIC of 60 μg/mL FC was observed for the strain carrying both the *codA* and the *codB* gene, while a MIC of 500 μg/mL was observed for the vector lacking the *codB* gene (Table 4 below). This result clearly shows the importance of the cytosine permease gene *codB* for the efficiency of counterselection by FC.

Applicability in other strains of acetic acid bacteria

Due to the high effectiveness of the new deletion method in the two different *G. oxydans* strains DSM 3504 and 621H, we also examined the applicability in other species of acetic acid bacteria. We focused on strains with available genomic sequence data. In silico examination of sequence data revealed the presence of a uracil phosphoribosyltransferase gene (*upp*) in all of the regarded strains. A cytosine deaminase gene was identified by homology in *A. multivorum*, but the similarity to the *E. coli* K12 gene was low with an identity of 187 of 433 amino acids

(43.2 %). Growth experiments were done to define the MIC of FC and FU for the different strains. The resistance of the organisms towards FC and the sensibility towards FU are required for the functionality of the method described in this study. The growth studies were carried out in test tubes containing 5 mL of strain-specific media. FC and FU were added in various concentrations. All tested strains were able to grow after 24 h incubation even in the presence of FC concentrations up to 500 μg/mL. *A. multivorum* DSM 11245 showed normal growth in the presence of FC after 2 days of incubation. Furthermore, we found inhibition of growth by FU for every tested strain at concentrations over 60 μg/mL (Tables 4 and 5).

Discussion

Despite its usefulness for the introduction of deletions in *G. oxydans* 621HΔ*upp*, the system published by Peters et al.
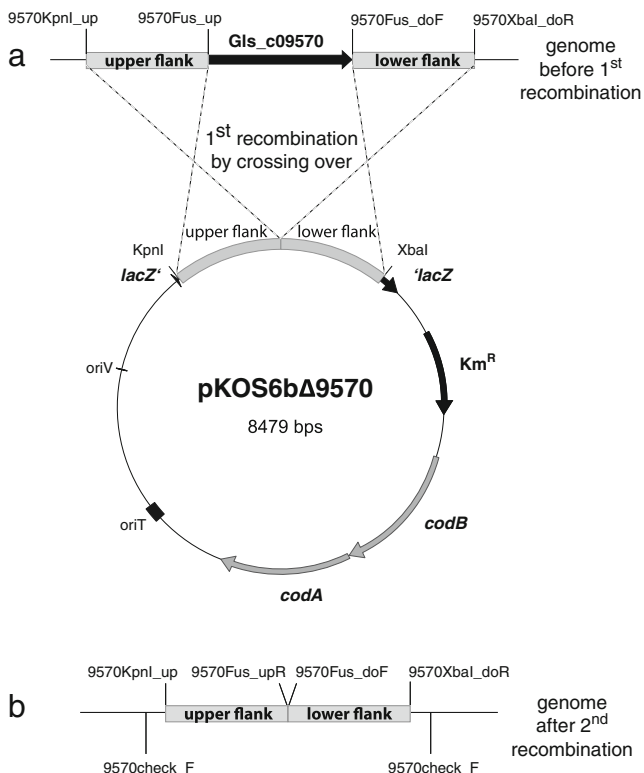
Fig. 2 Deletion of GLS_c9570 by integration via double crossing over and selection of mutants on 5-FC. The integration process is displayed in (**a**). **b** Shows the resulting markerless deletion mutant with lacking GLS_c9570 between the two flanking regions

(2012) has a crucial drawback; it cannot be used in wild-type strains. The elementary step for the development of a new strain-independent markerless in-frame deletion system was the choice of a reliable counter-selectable marker gene. To avoid the need to first introduce deletions in wild-type strains, it was fundamentally necessary to find a gene that works as counter-selectable marker but is naturally absent in *G. oxydans* and other acetic acid bacteria of interest. In contrast to other potential marker genes taken into consideration, this was the case for the *codA* gene of *E. coli* K12. The gene codes

for an *E. coli* cytosine deaminase and is arranged in an operon together with *codB*, which encodes a cytosine permease (Danielsen et al. 1992). The first attempt to use *codA* in a deletion system was made by Duraisingh et al. (2002) in *Plasmodium falciparum*. Unfortunately, however, the organisms did not develop any sensitivity towards FC, despite of the confirmed expression of *codA* in the cells. Several years later, a deletion system based on fungal cytosine deaminase delivered better results (Orr et al. 2012). Deletion systems based on *E. coli codA* were established in the genus *Rhodococcus* (van der Geize et al. 2008) and *Streptomyces* (Dubeau et al. 2009), indicating general applicability of the system for the *Actinobacteria*. Interestingly, however, the gene *codB* was never included in the deletion vectors, and its role was not investigated before this study. In our markerless deletion system for the acetic acid bacteria, the inclusion of *codB* was crucial. The *codB*-encoded cytosine permease obviously facilitates the entry of FC into the cell. The inhibition of growth in the presence of 500 μg/mL FC in *G. oxydans* 621H cells carrying the integrated vector pKOS6a2567 flank showed that with a vector containing *codA*, but lacking the cytosine permease *codB* gene, supplementation of high FC concentrations was needed to reach intracellular FC levels sufficient for growth inhibition. The presence of *codB* decreased the MIC to values under 60 μg/mL, and thus allows efficient counterselection. This suggests the general possibility to utilize FC counterselection in a broad range of bacteria by enhancing FC toxicity via functional expression of the *codB* in addition to *codA*, especially in microorganisms with a poor sensitivity for FC.

The parental vector pAJ63a was chosen because of its inability to stable replicate in *G. oxydans* strains (unpublished data). Such a suicide deletion vector is under strong pressure to integrate in the chromosome via recombination, if homologous regions are present as shown in other studies (van der Geize et al. 2008). This property ensures that clones with the ability to grow in the presence of Km are inevitably carrying the integrated vector.

Fig. 3 Southern-blot analysis of *G. oxydans* DSM 3504 Δ9570 mutant clones. *Eco*RI-digested chromosomal DNA from the wild type and three independent mutant clones were separated and hybridized with a probe consisting of 720 bp. The observed fragment sizes correspond nicely to the expected fragment sizes of 6,284 bp for the wild type and 4,967 for the mutant clones, confirming the markerless in-frame deletion
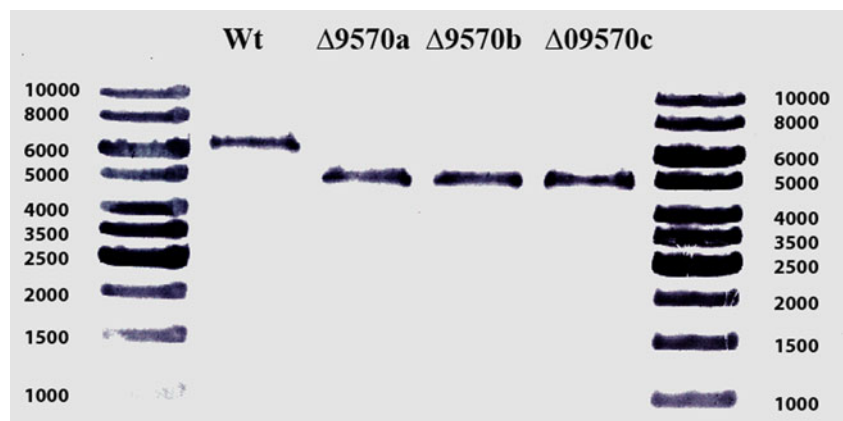
Table 3 Primers used in this study

Name	Sequence	Application
pAJdUPP_F_ov	CTCTCCTGGCGCACCATCGTCGGCTACAG	Amplification of pAJ63a for removal of *upp* cassette
pAJdUPP_F	ACCATCGTCGGCTACAG	
pAJdUPP_Rov	GGTTCTCCGGAAAATCTGAAAGGCGTCTTCTG	
pAJdUPP_R	AATCTGAAAGGCGTCTTCTG	
codBA_F	AAACAGCAAAAAGGGGGAAT	Amplification of codBA cassette for pKOS6b
codBA_F_ov	TTCCGGAGAACCAAACAGCAAAAAGGGGGAAT	
codBA_R	ATCCGGCATAAACTAAGCTC	
codBA_R_ov	GCGCCAGGAGAGATCCGGCATAAACTAAGCTC	
pKos6AmpFxho	AGCCTCGAGATGTGGAGGCTAACAGTGTC	Construction of pKOS6a
pKos6aAmpRxho	TCACTCGAGAATCTGAAAGGCGTCTTCTG	
09570_F_kpn	TCAGGTACCCTTGCTGCCGCTTACATCG	Amplification of upstream sequence of GLS_c9570
09570_FUSR	CTTTTTCATTTTCAGTTACGGGAGCCGATTTGCCCTCTC	
09570_FUSF	GAGAGGGCAAATCGGCTCCCGTAACTGAAAATGAAAAAG	Amplification of downstream sequence of GLS_c9570
09570_xba_R	CAGTCTAGATGCGGGTCTGAGTTTCAAG	
09570check_F	CCATCATGACCCTGCGTAAG	Confirmation of GLS_c9570 Deletion
09570check_R	CCTGACTGTTCCGTCTTTCC	
sldAB_xbaF	AGCTCTAGAATATGCCGAGAACGTGGTCC	Amplification of upstream sequence of GOX854-855
up_sldA_rev	CGGCCTCAGCCCCCGCTTTCTGGTGAAAGCCTCCAGAACT	
do_sldB_fwd	AGTTCTGGAGGCTTTCACCAGAAAGCGGGGGCTGAGGCCG	Amplification of downstream sequence of GOX854-855
sldAB_ecoR	TCAGAATTCGGTGCAGGTTCCAGCGAAAC	
sldABseq_F	CCATCATCTGCGCTTCCACC	
sldABseq_R	AGCCCGACCTTCACCTATCAG	Confirmation of GOX854-855 Deletion
pK18MCS_F	TTCCGGCTCGTATGTTGTG	pKOS6b screening primers
pk18MCS_R	GGGCCTCTTCGCTATTACG	
pk18MCS_2R	CAGCTGGCAATTCCGGTTC	

Table 4 Growth of different acetic acid bacteria in liquid medium in the presence of various 5-fluorocytosine (5-FC) concentrations

5-FC concentration (µg/ml)	0	30	60	120	240	500
Gluconobacter oxydans 621H	+	+	+	+	+	+
G. oxydans DSM 3504	+	+	+	+	+	+
G. oxydans DSM 7145	+	+	+	+	+	+
Gluconacetobacter diazotrophicus DSM 5601	+	+	+	+	+	+
Acetobacter pasteurianus LMG 1513	+	+	+	+	+	+
Acidomonas methanolica DSM 5432	+	+	+	+	+	+
Granulibacter bethesdensis DSM 17861	+	+	+	+	+	+
Acidiphilium multivorum DSM 11245	+	+*	+*	+*	+/−*	+/−*
G. oxydans 621H carrying del vect.pKOS6a 2567flank	+	+	+	+	+/−	−
G. oxydans 621H carrying del vect.pKOS6b 2567flank	+	+/−	−	−	−	−

+ Growth after one day of incubation, * growth was delayed but occurred after 2 days, − no growth after 4 days of incubation

In addition to the successful application in strain 621H, chromosomal deletions could also be achieved in *G. oxydans* DSM 3504. The deletion of GLS_C09570 in DSM 3504 was the first deletion ever described for this strain, for which previous deletion strategies did not work. The reliability of this new method was proven by numerous further deletions in strain DSM 3504 (data not shown).

Table 5 Growth of different acetic acid bacteria on various concentrations of 5-fluorouracil (5-FU)

5-FU concentration [µg/ml]	0	30	60	90	180
G. oxydans 621H	+	−	−	−	−
G. oxydans DSM 3504	+	−	−	−	−
G. oxydans DSM 7145	+	−	−	−	−
Gluconacetobacter diazotrophicus DSM 5601	+	−	−	−	−
Acetobacter pasteurianus LMG 1513	+	−/+	−	−	−
Acidomonas methanolica DSM 5432	+	−	−	−	−
Granulibacter bethesdensis DSM 17861	+	−	−	−	−
Acidiphilium multivorum DSM 11245	+	−	−	−	−

+ Growth after 1 day of incubation, − no growth after 4 days of incubation

The general presence of *upp* and the absence of an *E. coli*-like *codA* in almost all investigated acetic acid bacteria open a wide range of potential applications for this system. The growth analysis actually showed that the putative cytosine deaminase present in some strains, which differs at the sequence level substantially from *E. coli codA*, seems not able to convert the cytosine analogue efficiently.

In conclusion, we report the development of a new *codA*-based markerless deletion system for acetic acid bacteria by using the *codAB* genes from *E. coli* for counterselection. This offers the possibility to use this system for targeted genome modifications such as in-frame deletions, replacements, or insertions in wild type strains of different genera of acetic acid bacteria. Given the increasing number of genome sequences available for acetic acid bacteria and their increasing importance in biotechnology, this reliable deletion method will be instrumental to investigate and modify their genomes and to engineer genomes of acetic acid bacteria for improved biotechnological application.

References

Boeke JD, LaCroute F, Fink GR (1984) A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet 197(2):345–346

Boeke JD, Trueheart J, Natsoulis G, Fink GR (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol 154:164–175

Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41 (3):459–472

Danielsen S, Kilstrup M, Barilla K, Jochimsen B, Neuhard J (1992) Characterization of the *Escherichia coli codBA* operon encoding cytosine permease and cytosine deaminase. Mol Microbiol 6(10):1335–1344

De Ley J (1961) Comparative Carbohydrate Metabolism and a Proposal for a Phylogenetic Relationship of the Acetic Acid Bacteria. J Gen Microbiol 24:31–50

De Ley J, Swings J, Gossele F (1984) The genus *Gluconobacter*. In: N Krieg and J Holt (ed) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins Co., Baltimore, pp 267–278

Dubeau MP, Ghinet MG, Jacques PE, Clermont N, Beaulieu C, Brzezinski R (2009) Cytosine deaminase as a negative selection marker for gene disruption and replacement in the genus *Streptomyces* and other Actinobacteria. Appl Environ Microbiol 75(4):1211–1214

Duraisingh MT, Triglia T, Cowman AF (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. Int J Parasitol 32(1):81–89

Fabret C, Ehrlich SD, Noirot P (2002) A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Mol Microbiol 46(1):25–36

Figurski, DH, Helinski, DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA 76(4):1648–1652

Gay P, Le Coq D, Steinmetz M, Berkelman T, Kado CI (1985) Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J Bacteriol 164(2):918–921

Gillis M, De Ley J (1980) Intra- and intergenic similarities of the ribosomal ribonucleic acid cistrons of *Acetobacter* and *Gluconobacter*. Int J Sys Bacteriol 30:7–27

Gossele F, Swings J, Kersters K, Pauwels P, De Ley J (1983) Numerical Analysis of Phenotypic Features and Protein Gel Electrophoregrams of a Wide Variety of *Acetobacter* strains. Proposal for the Improvement of the Taxonomy of the Genus *Acetobacter* Beijerinck 1898, 215. Syst Appl Microbiol 4(3):338–368

Greenberg DE, Porcella SF, Stock F, Wong A, Conville PS, Murray PR, Holland SM, Zelazny AM (2006) *Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family Acetobacteraceae. Int J Syst Evol Microbiol 56(Pt 11):2609–2016

Gupta A, Singh VK, Qazi GN, Kumar A (2001) *Gluconobacter oxydans*: its biotechnological applications. J Mol Microbiol Biotechnol 3(3):445–456

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166(4):557–580

Hall PE, Anderson SM, Johnston DM, Cannon RE (1992) Transformation of *Acetobacter xylinum* with plasmid DNA by electroporation. Plasmid 28(3):194–200

Hölscher T, Görisch H (2006) Knockout and overexpression of pyrroloquinoline quinone biosynthetic genes in *Gluconobacter oxydans* 621H. J Bacteriol 188(21):7668–7676

Hölscher T, Weinert-Sepalage D, Görisch H (2007) Identification of membrane-bound quinoprotein inositol dehydrogenase in *Gluconobacter oxydans* ATCC 621H. Microbiology 153(Pt 2):499–506

Kallnik V, Meyer M, Deppenmeier U, Schweiger P (2010) Construction of expression vectors for protein production in *Gluconobacter oxydans*. J Biotechnol 150(4):460–465

Keller KL, Bender KS, Wall JD (2009) Development of a markerless genetic exchange system for *Desulfovibrio vulgaris* Hilden borough and its use in generating a strain with increased transformation efficiency. Appl Environ Microbiol 75(24):7682–7691

Mason LM, Claus GW (1989) Phenotypic Characteristics Correlated with Deoxyribonucleic Acid Sequence Similarities for Three Species of *Gluconobacter*: *G. oxydans* (Henneberg 1897) De Ley 1961, *G. frateurii* sp. nov., and *G. asaii* sp. nov. Int J Syst Bacteriol 39:174–184

Martinussen J, Hammer K (1994) Cloning and characterization of upp, a gene encoding uracil phosphoribosyltransferase from *Lactococcus lactis*. J Bacteriol 176(21):6457–6463

Mostafa HE, Heller KJ, Geis A (2002) Cloning of Escherichia coli *lacZ* and *lacY* genes and their expression in *Gluconobacter oxydans* and *Acetobacter liquefaciens*. Appl Environ Microbiol 68(5):2619–2623

Motizuki K, Takarazuka I, Kanzaki T (1966) Method for producing 2-keto-L-gulonic acid. US Patent Office 3,234,105

Neuhard J (1982) Utilization of preformed pyrimidine bases and nucleosides. In: Munch-Peterson A (ed) Metabolism of nucleotides nucleosides and nucleobases in microorganisms. Academic, London, pp 95–148

Orr RY, Philip N, Waters AP (2012) Improved negative selection protocol for *Plasmodium berghei* in the rodent malarial model. Malar J 11:103

Peters B, Junker A, Brauer K, Muhlthaler B, Kostner D, Mientus M, Liebl W, Ehrenreich A (2013) Deletion of pyruvate decarboxylase by a new method for efficient markerless gene deletions in *Gluconobacter oxydans*. Appl Microbiol Biotechnol 97(6):2521–30

Pritchett MA, Zhang JK, Metcalf WW (2004) Development of a markerless genetic exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new genetic tools for methanogenic archaea. Appl Environ Microbiol 70(3):1425–1433

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Tillett D, Neilan BA (1999) Enzyme-free cloning: a rapid method to clone PCR products independent of vector restriction enzyme sites. Nucleic Acids Res 27(19):e26

Urakami T, Tamakoa J, Suzuki KI, Komagata K (1989) *Acidomonas* gen. nov. Incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. Int J Syst Bacteriol 39:50–55

van der Geize R, de Jong W, Hessels GI, Grommen AW, Jacobs AA, Dijkhuizen L (2008) A novel method to generate unmarked gene deletions in the intracellular pathogen *Rhodococcus equi* using 5-fluorocytosine conditional lethality. Nucleic Acids Res 36(22):e151

Wach A (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast 12(3):259–265

Wagner M, van Wolferen M, Wagner A, Lassak K, Meyer BH, Reimann J, Albers SV (2012) Versatile genetic tool box for the crenarchaeote *Sulfolobus acidocaldarius*. Front Microbiol 3:214

Wakao N, Nagasawa N, Matsuura T, Matsukura H, Matsumoto T, Hiraishi A, Sakurai Y, Shiota H (1994) *Acidophilum multivorum* sp. nov., an Acidophilic Chemoorganotrophic Bacterium from Pyritic Acid Mine Drainage. J Gen Appl Microbiol 40:143–159

Yamada Y, Hoshino K, Ishikawa T (1997) The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci Biotechnol Biochem 61(8):1244–1251

Yamashita S, Uchimura T, Komagata K (2004) Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989. Int J Syst Evol Microbiol 54(Pt 3):865–870