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Transformation of *Saccharopolyspora erythraea* by electroporation of germinating spores: construction of propionyl Co-A carboxylase mutants

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The introduction of plasmid DNA into germinating spores of an industrially improved strain of *Saccharopolyspora erythraea* was accomplished by electroporation. Various parameters affecting the efficiency of electroporation were examined. The most critical factor was the extent of spore germination. Electrocompetence was limited to a 4-h period following the initial emergence of the germ tube. Electroporation efficiencies as high as 2×10^5 CFU μg^{-1} of plasmid DNA were obtained using electrocompetent germlings. The optimal field strength was 12–14 kV cm^{-1} with a pulse duration of 15–20 ms. Electrocompetent germlings were stored at −80°C without a significant decrease in transformation efficiency. The utility of this protocol was demonstrated by isolating a propionyl-CoA carboxylase mutant through targeted gene disruption and replacement.

Keywords: electroporation; *Saccharopolyspora erythraea*; homologous recombination; propionyl-CoA carboxylase

Introduction

The manipulation of microbial biosynthetic pathways for the industrial production of biological compounds has historically been accomplished through directed selection or random mutagenesis and screening. Recently, microorganisms have been genetically engineered to provide increases in the yield and purity of antibiotic compounds [1,21]. This application of recombinant technology requires not only a fundamental understanding of the genes and gene products involved in the biochemical process, but also the technical protocols to genetically manipulate industrially improved microbial strains.

The erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea* contains more than 20 genes involved in the formation and modification of the macrolide backbone and in the biosynthesis and attachment of the two novel sugars desosamine and mycarose [9,22,23,26–28]. Genetic engineering of the polyketide synthase genes has led to the isolation of novel macrolide compounds and has provided some insight into the molecular structure of this enzyme complex [3–5,10–13,15,18]. Disruption of *eryG*, *eryK* and *eryG/eryK* has correlated these genes with biochemical function ([16,22], D Post, Abbott Laboratories, personal communication). These constructions also represent important source strains for the production of erythromycins C, B, and D, respectively. Characterization of null mutations in the sugar biosynthetic genes has improved our understanding of the formation of these unusual sugars and provided models for their biosynthetic route [8,23].

The introduction of DNA into *S. erythraea* wild-type strains has been accomplished by protoplast transformation techniques with efficiencies of 10^5–10^6 CFU μg^{-1} of replicating plasmid DNA [29]. The development of modified 'suicide vectors' which increased the frequencies of homologous recombination by utilizing unstable streptomycete replicons (eg pIJ702) was a significant advancement and made possible gene interruptions and replacements [27,28]. Unfortunately, many industrially improved strains of *S. erythraea* are recalcitrant to protoplast transformation. An alternative technique which was effective for introducing replicating plasmids into improved strains of *S. erythraea* (10^4 CFU μg^{-1} of plasmid DNA) was dependent upon ultrasound treatment of the mycelia to render them electrocompetent [7]. In an effort to improve transformation efficiencies further, we examined the different stages of the developmental life cycle for electrocompetency. We found that germinating spores (germlings) are capable of being transformed via electroporation. This observation confirms the reports by Tyurin *et al* [24,25] and extends the utility of this technique to *Saccharopolyspora erythraea*. We report here the optimal electroporation conditions for an improved strain of *S. erythraea* and the application of this method to generate high numbers of recombinant isolates. Additionally, to demonstrate the utility of this technique, we have isolated a propionyl-CoA carboxylase null mutant by disrupting the gene encoding the α-subunit of this enzyme.

Materials and methods

Bacterial strains and plasmids

S. erythraea strain CA340, an improved strain for erythromycin production from the Abbott Laboratories Culture Collection, was cultivated at 33°C. Spores were prepared by plating glycerol stock cultures onto ABB13 medium [7] and incubating them for 14 days. Thiostrepton and hygromycin were used at 15 and 90 μg ml^{-1}, respectively, for the selection of *S. erythraea* transformants.

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Minimal medium using propionate as the carbon/energy source consisted of the following (g L^{-1}): 3-*N*-morpholinopropane sulfonic acid (MOPS), 21.0 (pH 7.0); NaCl, 1.0; K_2HPO_4, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.05; thiamine HCl, 0.05; $MnCl_2 \cdot 4H_2O$, 0.001; ZnCl, 0.001; B_{12}, 0.01; d-biotin, 0.01; $MgSO_4 \cdot 7H_2O$, 1.0; $(NH_4)_2SO_4$, 2.0; propionic acid (sodium salt)), 5.0. For solid medium, $CaCO_3$ was added to a final concentration of 10 g L^{-1} and Noble agar at 15 g L^{-1}.

Escherichia coli DH5α (Gibco BRL, Gaithersburg, MD, USA) was used as host for plasmids and grown in LB medium at 37°C. Ampicillin, where appropriate, was used at 100 μg ml^{-1} final concentration.

The shuttle vector, pCD1, was a gift of C Dery (University of Sherbrooke, Quebec, Canada) and contains the *Streptomyces phaeochromogenes* pJV1 replicon (2.7 kbp *Sma*I fragment) [20] and the gene encoding thiostrepton resistance in addition to pUC19. The plasmid used for the electroporation optimization experiments, pMBE2, is a derivative of pCD1 in which the thiostrepton resistance gene has been replaced by a hygromycin resistance gene [7]. The integration vector, pJAY4, utilizes this unstable replicon to facilitate the isolation of recombinant strains.

Plasmid DNA was isolated from *E. coli* using Qiagen columns (Qiagen, Santa Clarita, CA, USA). Southern hybridizations were performed using a digoxigenin-labeled probe (Boehringher Mannheim, Indianapolis, IN, USA). DNA manipulations were performed as described in Sambrook *et al* [19].

Electroporation protocol

Approximately 10^{10}–10^{11} *S. erythraea* spores were recovered from confluent ABB13 plates and resuspended in 20 ml of SM (10 g L^{-1} soytone, 1.2 g L^{-1} MOPS (hemisodium salt)). The spore suspension was sonicated for 20 pulses using a Heat Systems-Ultrasonics, Model W-375 sonicator equipped with a tapered microtip at an output of 4.5, 50% duty cycle. This treatment generated a more uniform suspension of the spores and facilitated synchronous germination [14]. Spores were diluted into 800 ml of SM and allowed to germinate. Cells were harvested by centrifugation (12 000 × *g*, 10 min) and resuspended with 200 ml electroporation wash buffer (0.3 M mannitol and 3 mM HEPES buffer, pH 7.0). Cells were centrifuged as before and resuspended with 25 ml of wash buffer. Cells were finally resuspended in 0.8–1 ml of electroporation buffer (wash buffer containing 25% (w/v) PEG3350, Sigma, St Louis, MO, USA). Cells at this stage could be frozen quickly in a dry ice/ethanol bath and stored at −80°C. Plasmid DNA was mixed with 100 μl of electrocompetent cells, transferred into a chilled 0.1-cm gap cuvette (Bio-Rad, Hercules, CA, USA), and electroporated using a BTX (San Diego, CA, USA) cell manipulator T600 at a resistance setting of 720 Ohm. Immediately after the pulse, the cells were diluted with 0.9 ml of ABB1 (soytone, 10 g L^{-1}; yeast extract, 9 g L^{-1}; soluble starch, 10 g L^{-1}; cerelose, 15 g L^{-1}; MOPS, 50 mM pH 7.0), before plating on ABB13 plates. The cells were added to the plates in ABB1-soft agar overlays and allowed to recover overnight before being overlayed with antibiotic. Plates were scored after 7–10 days incubation at 33°C.

Propionate utilization

Ten milliliters of ABB1-grown cultures of *S. erythraea* strains were centrifuged and washed twice with equal volumes of sterile water before inoculating 50 ml of minimal medium containing propionate in 500-ml flasks. One-ml samples were taken from the cultures at intervals and the amount of propionate determined using a Perkin Elmer model 34485 gas chromatograph. To increase the sensitivity of detection, propionate in the samples was converted to propionic acid by the addition of an equal volume of 0.5 M HCl.

Results and discussion

The time course for germling electrocompetence showed a narrow window of optimal efficiency (Figure 1). This correlated with the initial emergence of the germ tube and diminished as the germ tube lengthened (Figure 2). Germination was sensitive to cell density and therefore the optimal time varied from preparation to preparation by several hours. Typically, when cell densities were below an OD_{600} of 0.2, approximately 50–70% of the spores germinated. Cell densities above an OD_{600} of 0.2 were inhibitory to spore germination. The degree of germination was best monitored microscopically. The optimal field strength for electroporation was 12 kV cm^{-1} (Figure 3). The time constants were approximately 15–20 ms. This high voltage and long time constant resulted in approximately 75% kill (Figure 3). The effect of the temperature during preparation and handling of the germlings prior to electroporation was examined and had no influence upon electroporation efficiencies (4–40°C). Prepared germlings could be stored at −80°C for a month without a significant decrease in efficiencies (data not shown).

To demonstrate the utility of this protocol for the isolation of mutants in improved strains of *S. erythraea*, we sought to isolate a propionyl-CoA carboxylase (Pcc) null mutant. The pathways which supply carbon for the erythromycin macrolide backbone have not been determined. To investigate the possible route of carbon flow from propionyl-CoA to methylmalonyl-CoA via Pcc, Donadio *et al*

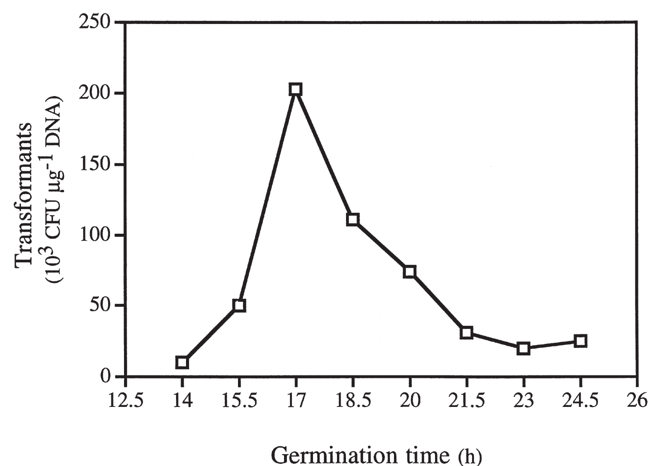

Figure 1 Transformation efficiency during spore germination. *S. erythraea* CA340 spores were harvested at the indicated time and subjected to electroporation with 100 ng of pMBE2.

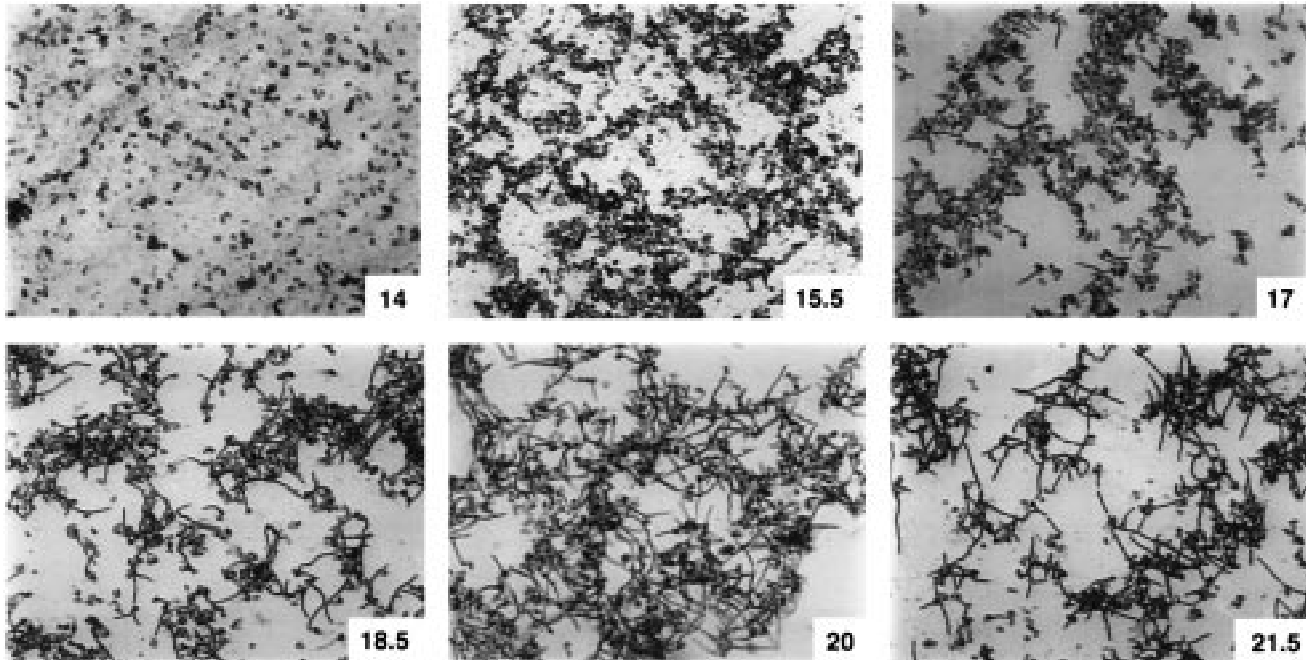

Figure 2 Photographs of spores during germination. Samples from the transformation experiment in Figure 1 were stained with crystal violet and visualized by brightfield microscopy. The sample time (h) is indicated in the lower right of each field. The average length of the population of germlings ranged from 2 μm to 5 μm.

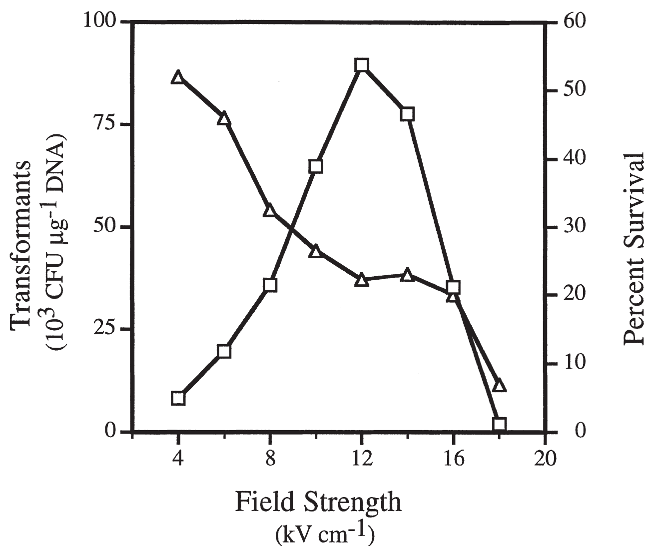

Figure 3 Effect of field strength on transformation efficiencies (□) and the survival of electroporated germlings (△). *S. erythraea* germlings were prepared as described in the Materials and Methods section. One hundred nanograms of pMBE2 were used in each transformation experiment. The results are an average of two experiments.

[2] isolated and sequenced the *pccB* gene encoding the β subunit of propionyl-CoA carboxylase from *S. erythraea*. The deduced amino acid sequence of the *S. erythraea* PccB is 65% identical to the PccB proteins from *Mycobacterium tuberculosis* and *Mycobacterium leprae* (GenBank Z95556, GenBank U00012, [6]). Disruption of the *pccB* gene in *S. erythraea* resulted in a loss of propionyl-CoA carboxylase activity. In *M. tuberculosis*, the gene encoding the α subunit of Pcc, *pccA*, is immediately downstream from the *pccB* gene (GenBank Z95556). Immediately downstream from the *S. erythraea pccB* gene is an open reading frame (designated *bcpA2*) with 73% amino acid identity to PccA from *M. tuberculosis* and *M. leprae* (GenBank Z95556, GenBank U00012, [6]). To definitively assign a function to the gene downstream from *pccB*, Donadio *et al* attempted to disrupt the *bcpA2* locus by replacing the genomic copy with a deleted version of the gene. However, in *S. erythraea*, there exist two nearly identical copies of this gene differing only in 8 bp out of 1746 bp (Figure 4a). One copy is immediately downstream from the *pccB* gene, *bcpA2*, and the other, *bcpA1*, is unlinked to this locus [17]. All seventeen of the mutants isolated by Donadio *et al* had recombined at the *bcpA1* gene leaving the *bcpA2* gene intact. One explanation considered for this result is that the *bcpA2* gene is essential for growth under the conditions employed in these experiments. The *bcpA1* mutants were unaffected in Pcc activity and no function could be assigned to this open reading frame. In order to reexamine the role of the *bcpA2* locus in *S. erythraea*, this gene was interrupted with a hygromycin resistance gene and cloned into the integration vector pJAY4 (Figure 4b). The pJV1 replicon contained in pJAY4 is unstable in *S. erythraea* and is lost rapidly during growth. Electroporation of germlings with this vector yielded transformation efficiencies of $4–8 \times 10^3$ CFU μg^{-1}. Twenty of these hygromycin-resistant transformants were then scored for resistance to both thiostrepton and hygromycin. All 20 of the initial transformants tested were resistant to both antibiotics. Upon further growth, nine of the 20 isolates lost resistance to both antibiotics, 10 were resistant to hygromycin and sensitive to thiostrepton, and one was still resistant to both. Colonies of the primary transformants likely include cells which con-

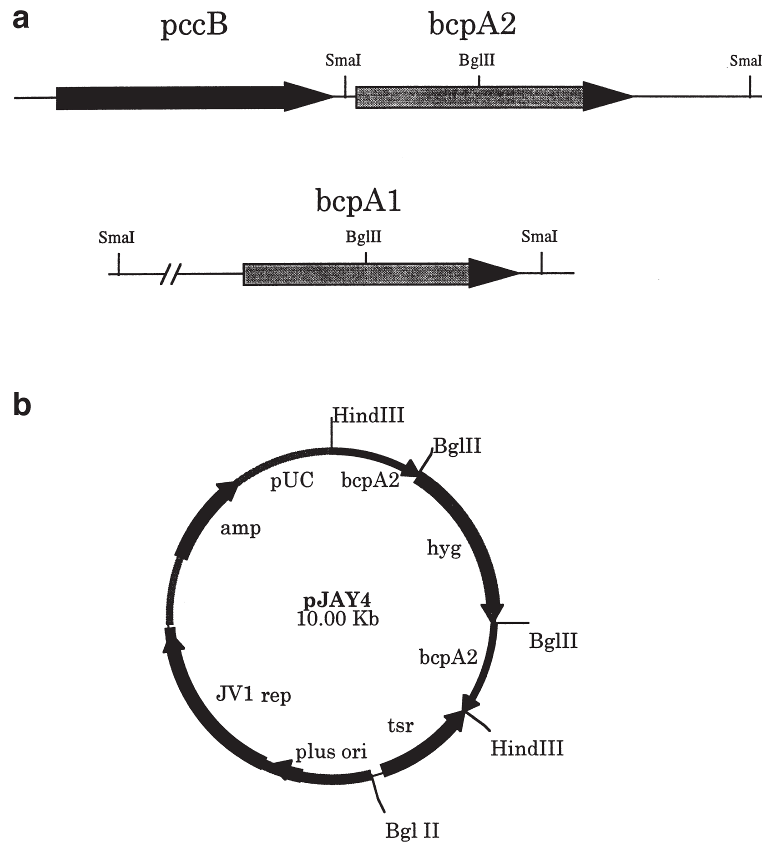

Figure 4 (a) Physical and genetic map of the two *bcpA* loci in *S. erythraea*. (b) Map of the integration vector pJAY4 used to interrupt the *bcpA* genes. The vector utilizes a portion of the pJV1 replicon to facilitate homologous recombination.

tain freely replicating and/or integrated plasmid DNA. The nine isolates sensitive to both antibiotics presumably lost the poorly replicating plasmid. The one isolate which maintained resistance to both antibiotics could be the result of a single crossover. The 10 isolates sensitive to thiostrepton and resistant to hygromycin had apparently undergone the desired second recombination event which resulted in the eviction of vector DNA leaving the hygromycin marker behind as a chromosomal insertion.

To confirm these results, Southern blots of several double crossover candidates and the single crossover isolate were hybridized with the *bcpA2* gene (Figure 5). The *bcpA1* and *bcpA2* genes are on 9.5- and 3.7-kbp *Sma*I fragments, respectively. Because the hygromycin resistance gene contains a *Sma*I site, the *Sma*I fragment containing the interrupted gene was cleaved into two smaller *Sma*I fragments while the unaffected locus remained intact. The results indicated that isolate J4–6 had been interrupted in *bcpA1* and isolates J4–7, J4–8, J4–16 had been interrupted in *bcpA2*. The isolate which maintained resistance to thiostrepton and hygromycin, J4–13, was indeed the result of a single crossover event in *bcpA1* as evidenced by the new junction fragments. Electroporation of *E. coli* with total DNA isolated from this strain did not yield any transformants, indicating the lack of detectable freely replicating plasmid.

To assess the effect of the gene disruptions on propionate metabolism, the mutants were scored for growth on minimal medium with propionate as the sole carbon/energy

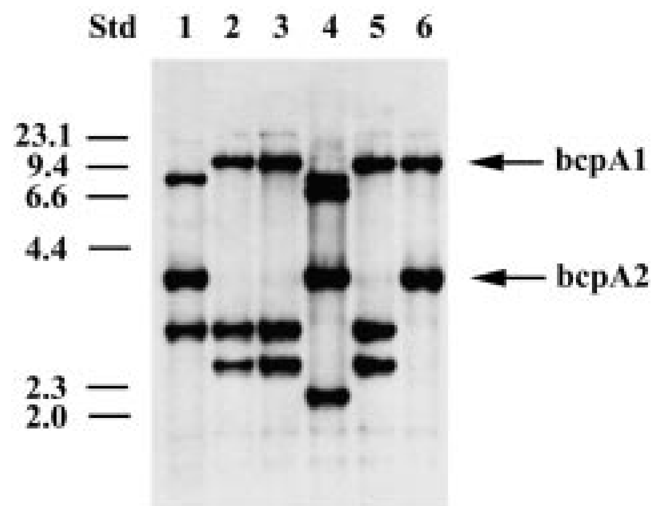

Figure 5 Southern hybridization analysis of *S. erythraea* recombinant isolates. Total DNA was purified from four random double recombinants and one single recombinant. Lanes 1–6 are isolates J4–6, J4–7, J4–8, J4–13, J4–16, and the parental strain CA340 digested with *Sma*I, respectively. The size standard was lambda DNA digested with *Hin*dIII. The hybridization probe was *bcpA2*.

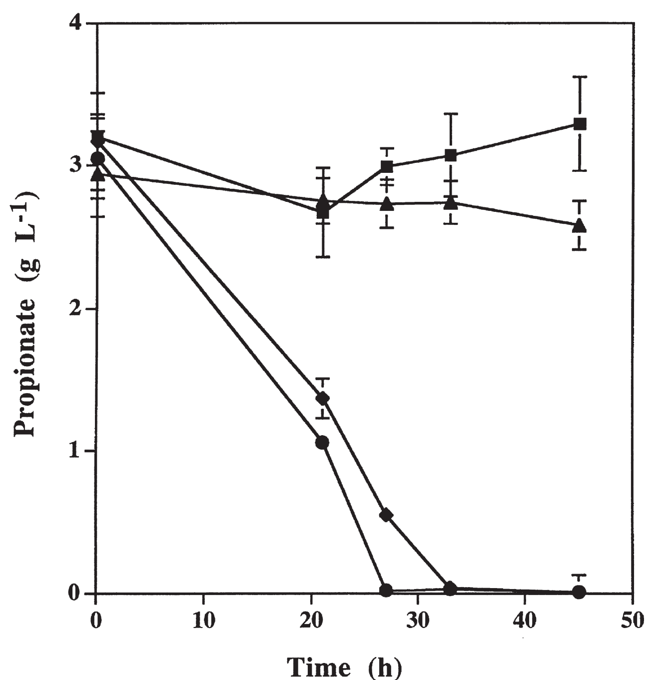

Figure 6 Propionate utilization by *bcpA* mutants. The parental strain (–◆–) is CA340. J4–6 (–●–) and J4–16 (–▲–) are double recombinants in which the *bcpA1* and *bcpA2* genes have been interrupted, respectively. The control (–■–) was an uninoculated flask containing minimal medium with propionate. Error bars are the standard error of duplicate samples.

source. The *bcpA1* mutant J4–6 and the parental strain CA340 showed normal growth on this medium. The *bcpA2* mutant (J4–16), however, did not grow on propionate plates. The *pccB* null mutant isolated by Donadio *et al* [2] also did not grow on this medium (S Geusz, Abbott Laboratories, personal communication). To confirm the plate phenotype, the ability of the mutants to use propionate in liquid medium was assessed. The parental strain CA340 and the *bcpA1* mutant rapidly consumed propionate while the *bcpA2* mutant did not (Figure 6). Based upon the propionate phenotype of the *bcpA2* mutant and the high level of homology to recently sequenced *pccA* genes, we propose that the gene which was tentatively named biotin containing protein A2 (*bcpA2*) be designated *pccA*.

The technique described and demonstrated here offers an alternative method for DNA transformation of industrially improved strains of *S. erythraea*. The transformation efficiencies represent a 10-fold increase over the previous sonication-dependent technique. This increase in efficiency facilitated the isolation of high numbers (10^3) of recombinant strains and allowed the isolation of a novel *pccA* null mutant.

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