understand many more unknown queries about melanisation in cosmopolitan *Drosophila melanogaster.*

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Comparing selection schemes in BAC-recombineering method of tagging a novel *Drosophila* gene, *DmCSAS.*

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Abstract

Sialylation plays an important role in the *Drosophila* nervous system. However, the regulation of sialylation pathway remains poorly understood. We focused our analysis on a novel gene, CMP-Sialic acid synthetase (CSAS), that is predicted to be a key player of the pathway. In order to investigate its expression, we decided to introduce a tag sequence into the CSAS genomic locus within a BAC clone using recombineering strategy. We wanted to introduce the tag without any additional extraneous sequences in order to minimize the influence of the insert on the gene's function. We sought to modify existing recombineering protocols and test different selection and screening methods during recombineering. Our results confirmed the general utility of positive/negative selection approach using *rpsL*$^{+}$*-kana* marker. However, they also revealed the limitation of this strategy, as it did not allow unambiguously to identify recombinant clones, while resulting in enrichment rather than selection for desired recombineering events.

Introduction

Sialic acid is a nine carbon, negatively charged sugar that can be attached to the termini of carbohydrate modifications of glycoproteins and glycolipids. Sialylation is abundant and universally present in vertebrates. The sialylation of cell-surface molecules is involved in cell signaling, inflammatory response, immune response, and development, while sialylation defects have been linked to cancer and autoimmune diseases (Varki, 2008). Unlike vertebrates, invertebrate organisms, including *Drosophila*, appear to have a tightly controlled sialylation pathway limited to some cells and developmental stages (Repnikova *et al.,* 2010). *Drosophila* has homologues of most of the essential components of vertebrate sialylation pathway, which suggests that *Drosophila* can be used a model system to reveal mechanisms of sialylation in vertebrates. Recent studies revealed that *Drosophila* sialyltransferase (DSiaT), a key enzyme of the sialylation pathway, plays an important role in the regulation of neural transmission in the nervous system (Koles *et al.,* 2004; Repnikova *et al.,* 2010). The pattern of DSiaT expression is restricted to neurons within the CNS. At the same time, little is known about other components of *Drosophila* sialylation pathway, including enzymes functioning upstream of DSiaT, such as CMP-sialic acid synthetase (CSAS) that generates activated sugar donor for DSiaT-mediated sialylation. *Drosophila* CSAS protein was shown to have an unusual Golgi localization in cell culture experiments (Viswanathan *et al.,* 2006). However, the expression and function of CSAS *in vivo* have never been characterized. Detailed understanding of the expression of this protein could lead to a better grasp of the pathway itself and any interactions among the functional components within the pathway. Here we describe the generation of BAC construct with tagged CSAS gene using recombineering approaches.

Table 1. Oligonucleotide primers used in recombineering experiments.

Primer #	Primer name	Template used	Primer sequence (5'→3')
1	CSAS-kana-f	PL452	CGATCTGACTCTAGCCAAATACATCTTAAGTAGTGAAACAAAAACCGAGggtctgaagaggagtttacgtcc
2	CSAS-kana-r	PL452	CAAGATCAATGAACTTTACACTTTCTTTGATGAATAGCATATCTATTTCaagggttccgcaagctctagtc
3	CSAS-rpslkan a-f	pSK+RpsL-kana	CGATCTGACTCTAGCCAAATACATCTTAAGTAGTGAAACAAAAACCGAGtgggcggttttatggacagca
4	CSAS-rpslkan a-r	pSK+RpsL-kana	CAAGATCAATGAACTTTACACTTTCTTTGATGAATAGCATATCTATTTCgtgggcgaagaactccagcat
5	CSAS-gnmc-f	CSAS BAC	GAGTCCCATGACTGTGTTTTTGCCGCTAAGAGG
6	CSAS-gnmc-r	CSAS BAC	TGTGTGTCGCTCGGTTTGGCAGGACTTGCTTGG

Materials and Methods

The BAC clone for CSAS tagging (*CH322-158A02*) was obtained from BACPAC Resource bank at Children's Hospital Oakland Research Institute (CHORI). The clone includes 22.1 kb genomic insert containing *CSAS* locus. The *PL452* plasmid was obtained from Koen Venken (Baylor College of Medicine, Houston), *pSK+RpsL-kana* was purchased from Addgene. DY380 cells were obtained from NCI-Frederick Biological Resources Branch. Primer sequences used in recombineering experiments are shown in Table 1. Except for the modifications to protocols

discussed in the text, we used previously published recombineering methods described in (Venken *et al.*, 2008; Wang *et al.*, 2009).

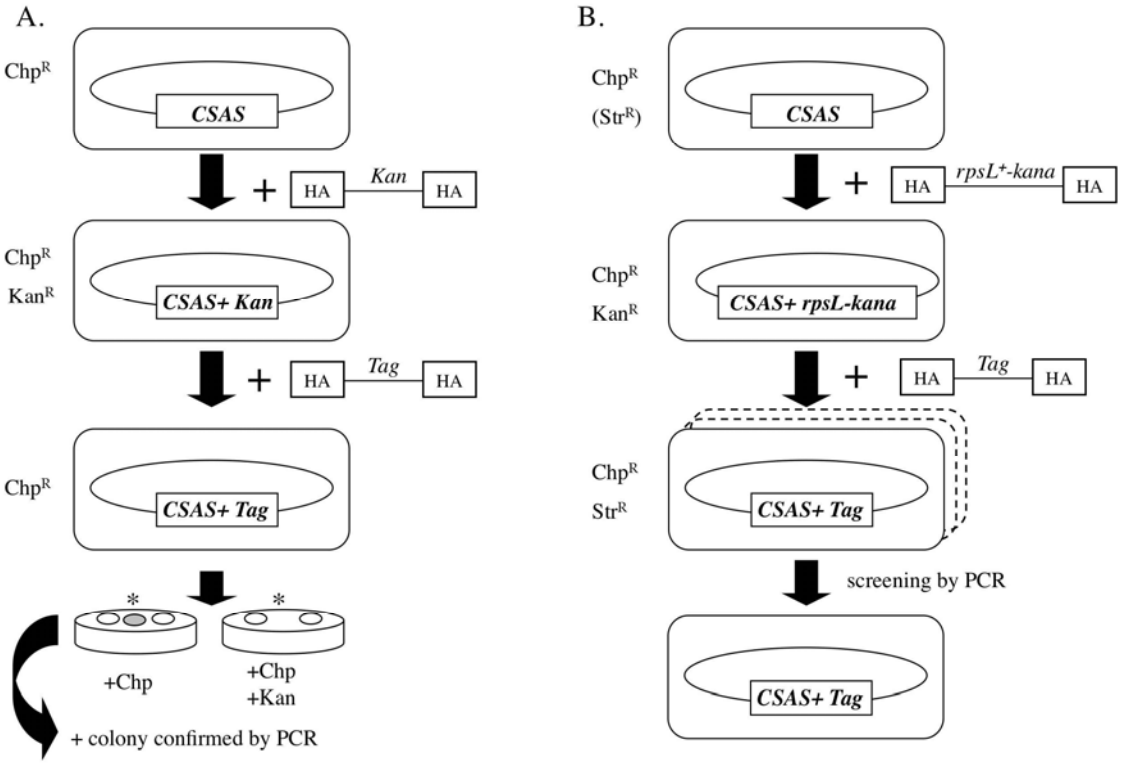

Figure 1. BAC recombineering-mediated tagging of *CSAS*. BAC constructs are shown inside DY380 cells containing λ-*cI857* repressor. A, A one-step positive selection strategy with replica plating. B, A two-step positive/negative selection strategy. In both cases inserts contained flanking homology arms (HA), with either selection or gene of interest sequences. The tag insert encodes 3FLAG protein tag. Chp^R, Kan^R, and Str^R indicate chloramphenicol, kanamycin, and streptomycin antibiotic resistance markers, respectively.

Results and Discussion

In order to study CSAS *in vivo* expression, we sought to generate a BAC-based transgenic construct that contains the entire *CSAS* gene along with a short sequence insert encoding the 3FLAG protein tag. Our rationale was based on the fact that BAC clones can contain gene loci including the majority of important regulatory elements that determine a spatiotemporal pattern of gene expression. The expression of genes included in transgenic BAC constructs was shown to reflect closely the endogenous expression pattern of these genes (Poser *et al.*, 2008; Venken *et al.*, 2008). Several methods for modifying sequences within BAC clones have been described, while most popular approaches commonly rely on a two-step recombineering strategy, with the first step including the introduction of desired changes along with an antibiotic marker (*e.g.*, kanamycin) to a genomic location. In the second step, this marker is removed by site-specific recombinase (*e.g.*, using Cre-Lox system) (Venken *et al.*, 2008). However, this strategy leaves behind some irrelevant insertions (*e.g.*, a LoxP site) that may affect gene functions. Several techniques that result in a "clean" introduction of a tag sequence or a mutation have been developed, including the *galK*

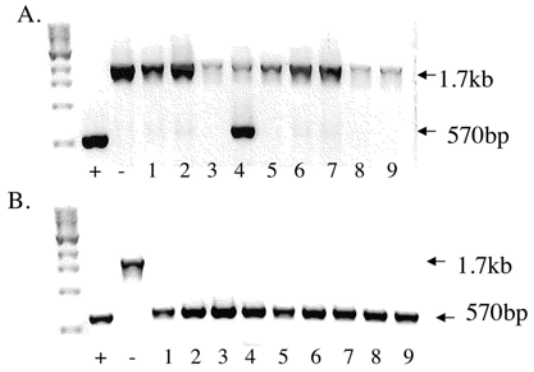

Figure 2. Confirmation of 3FLAG insert into CSAS BAC following recombineering. A, Colony PCR of selected colonies appearing to have lost streptomycin sensitivity. Most retained the selection cassette (~1.7kb). Positive clone is in lane 4 (570 bp insert). B, Individual colonies obtained from positive culture (lane 4) were confirmed by PCR to have 3FLAG insert. + and –, positive and negative controls for PCR amplification of the 3FLAG and selection cassette. The left lane is a DNA ladder.

positive/negative selections method and the *rpsL*$^+$*-kanamycin* scheme (Warming *et al.,* 2005; Wang *et al.,* 2009). The second step in these positive/negative selection schemes appears to be a bottleneck of the approaches that limits their efficiency and sometimes results in a low success rate. This problem arises from high frequency of false-positive colonies due to the loss of selection marker because of inactivating mutations (Wang *et al.,* 2009). Thus, we wished to modify the two-step protocol to simplify the scheme and decrease the background of false-positive clones. To this end, we decided to modify the second step of the procedure while leaving the first one essentially unchanged. Similarly to the *rpsL*$^+$*-kanamycin* scheme, our protocol started with introducing an antibiotic marker into the CSAS coding region that we wished to modify with tag-encoding sequence. Just the kanamycin marker alone was inserted into the 3' end of *CSAS* open reading frame. As described for other similar recombineering approaches (Venken *et al.,* 2008; Wang *et al.,* 2009), this step was very efficient and resulted in hundreds kanamycin-resistant clones. When analyzed by PCR, virtually all tested clones (>95%) included the desired insertion. The second step was designed to replace the antibiotic marker gene by a 3FLAG-encoding sequence (~70 bp). The efficiency of recombineering in a similar system without selection was estimated as 1.7×10^{-3}, or 1 out of approximately 600 induced DY380 cells electroporated with a targeting fragment including two 45 bp homology arms (Lee *et al.,* 2001). We reasoned that this frequency of targeting events should allow us to identify successful replacements of antibiotic marker with a tag-encoding sequence by comparing colonies on replica plates with and without antibiotic selection (Figure 1). To enhance the efficiency of recombination, we increased the length of homology arms to 300 bp. Using this approach, we screened approximately 7,000 individual colonies each replicated on two sets of plates, with and without kanamycin selection. We identified 3 colonies that lost the antibiotic marker; however, they all turned out to be false-positive as reveled by further PCR analysis. Thus, the efficiency of targeting in our case was estimated as at least an order of magnitude lower ($<1.4\times10^{-4}$) than in previously described experiments (Lee *et al.,* 2001). To compare these results to the efficiency of a two-step recombineering scheme with selection applied at both steps, we used the same homology arms as in our previous experiment while employing recombineering system with *rpsL*$^+$*-kana* cassette as a selection marker (Wang *et al.,* 2009). In order to isolate individual streptomycin-resistant colonies, we used several serial dilutions when plating electroporated cells during the second step of the procedure. In addition to 1:25 dilution recommended by the original protocol (Wang *et al.,* 2009), we used higher dilutions of 1:250, 1:625, or 1:2500. However, no positive results were obtained with higher dilutions. The colonies that had incorporated the tag-encoding insert must have lost the selection cassette containing *rpsL*$^+$ and, thus, they should be able to grow more robustly on the plates containing streptomycin. However, after 16-hour incubation, the time used in the original protocol, there was no distinguishable difference in size or morphology between colonies. After increasing the incubation time to 24 hours, a slight difference in colonies

could be detected on 1:25 dilution plates, but still no positive colonies were found on the plates with higher dilutions. This indicated that the efficiency of targeting events was rather low, which was consistent with conclusions from our previous attempt of targeting using no selection during the second step. By focusing on the 1:25 dilution plates, we found colonies that grew on the lawn and appeared larger and denser than surrounding areas, indicating that the selection step resulted in enrichment rather than a strict selection for desired recombineering events. These more dense areas were picked up and tested further by colony PCR. Out of 40 tested cultures, one positive clone carrying the tag insert was isolated (Figure 2A). In order to insure that the corresponding culture contained only one colony (the positive clone was picked from a lawn), cells from the colony PCR media were streak plated and grown overnight at 32°C, then retested by colony PCR again to confirm results (Figure 2B). Finally, the insert was sequenced using primers located outside of the region of the targeting sequence and found to include the correct insertion of 3FLAG-encoded sequence at the 3' end of the *CSAS* coding region (Figure 3). Our results from this experiment using the two-step selection scheme with *rpsL*$^+$*-kana* cassette were similar to previously published data (Wang *et al.,* 2009). The previous work estimated the efficiency ranged from 1-29% positive for the loss of the *rpsL*$^+$*-kana* cassette, but only <1-18% efficiency when considering the accuracy of inserted sequence (Wang *et al.,* 2009). Our two-step selection scheme resulted in 2% efficiency with no unwanted rearrangements as confirmed by sequencing.

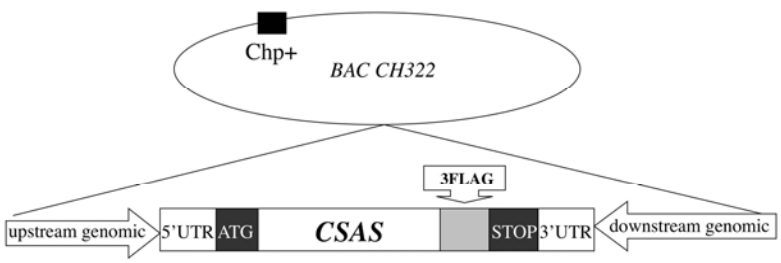

Figure 3. The CSAS BAC construct with 3FLAG-encoding insertion at the 3'-end of *CSAS* coding region. The insertion was confirmed by sequencing of the *CSAS* region of the clone encompassing the targeted region using primers #5 and 6 (Table 1).

In summary, we generated a BAC construct for transgenic expression of 3FLAG-tagged CSAS protein. This construct will be used to analyze CSAS expression *in vivo* and to investigate regulatory mechanisms of *Drosophila* sialylation. In our experiments, we compared two recombineering schemes for generating a BAC construct. We modified a two-step protocol by replacing the second negative selection step with the step based on comparing colonies on two sets of replica plates, with and without selection. This approach relies on a relatively high efficiency of targeting. However, despite the fact that the homology arms were increased in our approach to 300 bp, we found that the efficiency of targeting was significantly lower than the one previously reported for a similar recombineering system. This result can be potentially explained by a decrease efficiency of transformation of targeting fragment in our case, or by a peculiar property the DNA locus used in our experiments. Overall, our experiments indicated that a modified positive/negative selection scheme worked best when the efficiency of targeting is low. However, when the targeting efficiency is relatively high (> 0.1%) the scheme without selection at the second step may still provide some advantages, such as simplicity and speed.

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Preliminary data on the *Drosophila* fauna in the city of Tandil, Buenos Aires Province, Argentina.

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Introduction

It is said that the drosophilid fauna in temperate regions is better known than that of the tropical regions (Val *et al.,* 1981; Wheeler 1986). However, when we take into consideration what is known about the fauna of these insects in Argentina, we find that the published knowledge is precarious. The majority of the studies on drosophilids available there are centered on genetic and evolutionary questions of determined species (Barker *et al.,* 1985; Iriarte *et al.,* 2009; Soto *et al.,* 2010).

In temperate and cold regions the climatic factors have considerable influence on the drosophilid populations and limit the occurrence of many species. Because of this, Dobzhansky and Pavan (1950) stated that the number of species found dropped as they proceeded from the heat of the tropical areas to the colder regions. This indicates that it is a rare feature for a drosophilid to be adapted to life at low temperatures.

The objective of this study is to contribute to better knowledge of the Argentinean drosophilid diversity in Tandil city (located about 400 km south of Buenos Aires), an area never studied on this question up to the present.

Materials and Methods

Adult drosophilids were collected in Tandil city (37°19´S; 59°09´W), in the province of Buenos Aires, Argentina (Figure 1). This province is situated in the center-east of the country and is steppe-land mainly covered by herbaceous grass and known as the Pampas. A large part of the Pampas is at sea level, with the exception of two major mountain systems denominated Ventania and Tandilia. Tandil city itself is located in the Tandilia mountain foothills, in the southwest of Buenos Aires at about 200 meters above sea level. The climate in Tandil is temperate or humid meso-thermal with average annual temperature of 13.9°C, an average maximum of 20.1°C, and average minimum