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Epigenetic switches in *clag3* genes mediate blasticidin S resistance in malaria parasites

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Summary

Malaria parasites induce changes in the permeability of the infected erythrocyte membrane to numerous solutes, including toxic compounds. In *Plasmodium falciparum*, this is mainly mediated by PSAC, a broad-selectivity channel that requires the product of parasite *clag3* genes for its activity. The two paralogous *clag3* genes, *clag3.1* and *clag3.2*, can be silenced by epigenetic mechanisms and show mutually exclusive expression. Here we show that resistance to the antibiotic blasticidin S (BSD) is associated with switches in the expression of these genes that result in altered solute uptake. Low concentrations of the drug selected parasites that switched from *clag3.2* to *clag3.1* expression, implying that expression of one or the other *clag3* gene confers different transport efficiency to PSAC for some solutes. Selection with higher BSD concentrations resulted in simultaneous silencing of both *clag3* genes, which severely compromises PSAC formation as demonstrated by blocked uptake of other PSAC substrates. Changes in the expression of *clag3* genes were not accompanied by large genetic rearrangements or mutations at the *clag3* loci or elsewhere in the genome. These results demonstrate that malaria parasites can become resistant to toxic compounds such as drugs by epigenetic switches in the expression of genes necessary for the formation of solute channels.

Introduction

Plasmodium spp. parasites have a complex life cycle that includes several niches in two different hosts, humans and mosquitoes, but clinical symptoms of malaria disease are almost exclusively associated with cycles of asexual replication inside human erythrocytes. Intracellular parasitism has obvious advantages for many organisms, but it also poses important challenges. In the case of malaria asexual blood stages, the intraerythrocytic niche protects the parasite from immune attack, but this life style also implies that the parasite must develop a transport system to acquire nutrients that are not available inside the erythrocyte. It is well established that the membrane of erythrocytes infected with mature stages of *P. falciparum* (pigmented trophozoite and schizont stages) is permeable to numerous solutes that are not transported into non-infected erythrocytes, including ions and organic compounds such as sugars and amino acids, among many others. These new transport activities are collectively referred to as the new permeation pathways (NPPs) (Elford *et al.*, 1985; Ginsburg *et al.*, 1985; Saliba and Kirk, 2001; Desai, 2012).

The precise nature of the channel(s) that mediate solute uptake in infected erythrocytes remains unresolved (Staines *et al.*, 2007; Desai, 2012). Some authors postulated that a single channel type mediates NPPs (Kirk *et al.*, 1994; Desai *et al.*, 2000; Alkhalil *et al.*, 2004) and that this channel is likely to be parasite encoded (Alkhalil *et al.*, 2004; Baumeister *et al.*, 2006). This proposed channel, termed plasmodial surface anion channel (PSAC), combines transport of a broad spectrum of solutes with selectivity for structurally similar solutes (Desai *et al.*, 2000; Alkhalil *et al.*, 2004; Hill and Desai, 2010; Desai, 2012). However, others have suggested that several distinct channels with different electrophysiological properties contribute to the increased permeability of infected erythrocytes. These channels are mainly host-encoded endogenous erythrocyte channels activated by the parasite (Staines *et al.*, 2007; Bouyer *et al.*, 2011; Winterberg *et al.*, 2012). Regardless of whether the actual channel is encoded by the parasite or one or more endogenous

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channels are activated, a recent study using chemical inhibitors, linkage analysis of a genetic cross and transgenic parasite approaches unambiguously demonstrated that the parasite encoded proteins CLAG3.1 and CLAG3.2 (also named RhopH1/Clag3.1 and RhopH1/Clag3.2, ID PF3D7_0302500 and PF3D7_0302200) play a key role in the formation of active PSAC and are essential for the transport of numerous solutes into infected erythrocytes (Nguitragool *et al*., 2011). These proteins may constitute the actual channel, be part of the channel, or activate a channel formed by other proteins (Nguitragool *et al*., 2011; Desai, 2012). Follow up studies have demonstrated that CLAG3 proteins and PSAC play a critical role in nutrient acquisition and are essential for efficient parasite growth when the medium contains physiological concentrations of key nutrients (Pillai *et al*., 2012).

CLAG3 proteins are encoded by members of the *clag* gene family, which in *P. falciparum* consists of 5 different genes. The two *clag3* genes (*clag3.1* and *clag3.2*) are separated by only 10kb and share over 95% nucleotide sequence identity, whereas other members of the family (*clag2*, *clag8* and *clag9*) are more distantly related (Iriko *et al*., 2008). It remains unknown whether CLAG proteins other than CLAG3 participate in the formation of active PSAC. Of note, *clag3* genes show mutually exclusive expression, such that an individual parasite expresses only one of the two genes at a time. Initially described in parasites of 3D7 and HB3 genetic backgrounds (Cortés *et al*., 2007), mutually exclusive expression of *clag3* genes has been later confirmed by different laboratories in parasites of 3D7 genetic background (Comeaux *et al*., 2011; Crowley *et al*., 2011) and also in other genetic backgrounds (Nguitragool *et al*., 2011; Pillai *et al*., 2012). Together with *var*, *clag3* genes represent the only known example of this type of expression in malaria parasites (Guizetti and Scherf, 2013). The active or repressed state of *clag3* genes is regulated at the chromatin level, and clonally transmitted over several generations of asexual growth by epigenetic mechanisms (Cortés *et al*., 2007; Comeaux *et al*., 2011; Crowley *et al*., 2011). Transitions between the two states occur, albeit at low frequency, resulting in switches from expression of one *clag3* gene to expression of the other (Cortés *et al*., 2007; Comeaux *et al*., 2011; Crowley *et al*., 2011; Nguitragool *et al*., 2011; Pillai *et al*., 2012).

Among the solutes that require NPPs for transport across the infected erythrocyte membrane are some compounds that are toxic for the parasite, including diamidine compounds (Stead *et al*., 2001), bis-quaternary ammonium compounds (Biagini *et al*., 2003), the antibiotics fosmidomycin (Baumeister *et al*., 2011) and BSD (Hill *et al*., 2007), and the protease leupeptin (Lisk *et al*., 2008). Interestingly, *P. falciparum* parasites can acquire resistance to the antimalarial compounds BSD and leupeptin by alterations in PSAC activity, providing support to the idea that PSAC is encoded by the parasite (Hill *et al*., 2007; Lisk *et al*., 2008; 2010; Hill and Desai, 2010). These results establish that malaria parasites can acquire drug resistance by changes in the permeability of the infected erythrocyte membrane. While a mutation in *clag3.2* associated with leupeptin resistance has been identified (Nguitragool *et al*., 2011), no mutation associated with BSD resistance has been identified to date. Here we demonstrate that malaria parasites can acquire resistance to BSD by epigenetic changes in the expression of *clag3* genes. Parasites acquire resistance to low concentrations of the drug by switching from *clag3.2* to *clag3.1* expression, whereas resistance to higher drug concentrations involves simultaneous epigenetic silencing of both *clag3* genes, an unexpected expression pattern that had not been previously described. Our results imply that expression of alternative *clag3* genes results in different transport efficiency of PSAC and add epigenetic alterations to the list of mechanisms by which malaria parasites can become resistant to a drug.

Results

Resistance to BSD is associated with changes in clag3 expression

As part of our ongoing investigations on the rules that govern the mutually exclusive expression of *clag3* genes (N. Rovira-Graells, V.M. Crowley and A. Cortés, unpublished), we transfected *P. falciparum* parasites with the plasmid 3.2-1371-LH-bsdR, which contains a BSD resistance cassette (BSD deaminase gene under the control of a constitutive promoter) and the *clag3.2* upstream sequence driving the expression of a luciferase gene reporter (Fig. S1A). Transfected parasites were selected with 2.5 μg ml^{-1} of BSD to obtain a population of parasites stably maintaining the plasmid as an episome. For these experiments we used the 3D7 subclone 10G (Cortés, 2005), which predominantly expresses *clag3.2* and has *clag3.1* silenced (Cortés *et al*., 2007; Crowley *et al*., 2011; Rovira-Graells *et al*., 2012). Unexpectedly, we found that in the transfected and BSD-selected population the predominantly expressed endogenous *clag3* gene switched from *clag3.2* to *clag3.1* (Fig. S1B). To determine whether the switch was attributable to the episomal *clag3.2* promoter or it was related with BSD selection, we transfected 10G parasites with the BsdR plasmid, which contains the BSD resistance cassette but no gene reporter or *clag3* promoter (Fig. S1A). Upon selection of transfected parasites with BSD, expression of endogenous *clag3* genes was assessed at different times after transfection. Similar to the results with 3.2-1371-LH-bsdR, BsdR-transfected parasites progressively switched from *clag3.2* to *clag3.1*

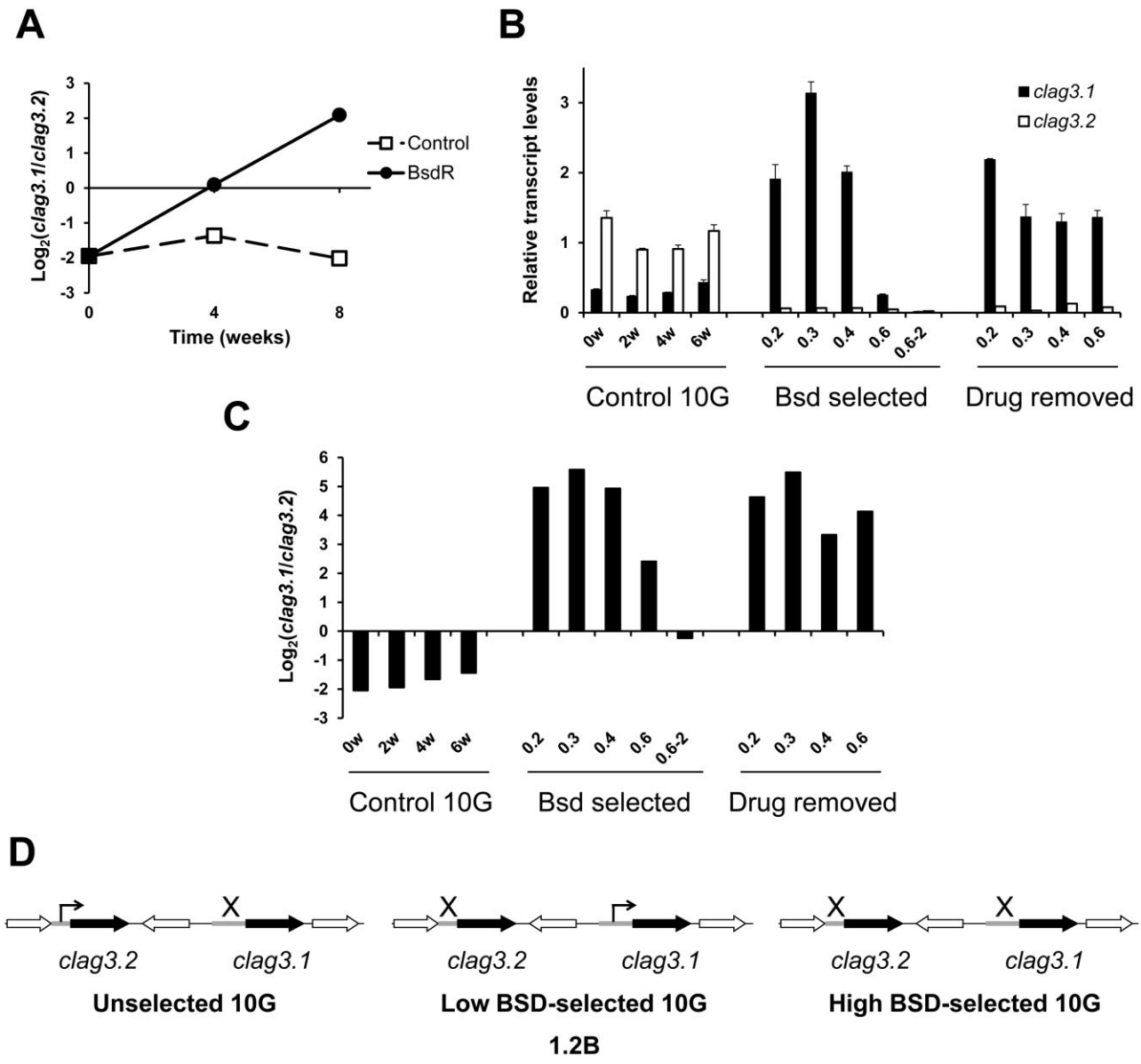

Fig. 1. Resistance to BSD is associated with changes in *clag3* expression.
A. Relative abundance of *clag3.1* and *clag3.2* transcripts in untransfected 10G parasite line or the same line transfected with the plasmid BsdR and maintained under BSD selective pressure. Values are the $\log_2$ of the expression ratio.
B. Transcript levels of *clag3.1* and *clag3.2* in unselected control 10G cultures at different times along the experiment (in weeks), in cultures selected with BSD at the concentrations indicated (in μg ml^{-1}) for 2 weeks (0.2 and 0.3) or 4 weeks (0.4 and 0.6), and in cultures maintained for two weeks in the absence of drug after selection with the concentration of BSD indicated. The culture 0.6–2 was sequentially selected with 0.6 μg ml^{-1} BSD for 4 weeks and then with 2 μg ml^{-1} for 2 weeks. Transcript levels are normalized against *rhoph2*, which has a similar time of expression to *clag3* genes along the asexual cycle. Values are the average of reactions performed in triplicate, with SD.
C. Relative transcript levels of *clag3.1* and *clag3.2*, expressed as the $\log_2$ of the expression ratios, in the same samples described in panel B.
D. Schematic representation of predominant *clag3* expression patterns in the parasite lines used in this study. An arrow indicates an active state whereas a cross indicates silencing. Low BSD refers to ≤ 0.4 μg ml^{-1} whereas high BSD refers to ≥ 0.6 μg ml^{-1}. The schematic is based on data from this study except for 1.2B (Cortés *et al.*, 2007; Crowley *et al.*, 2011).

expression (Fig. 1A). This switch was not observed in untransfected 10G parasites grown in parallel. These results indicate that BSD selection of transfected parasites can result in switches in the expression of *clag3* genes.

To address how BSD affects *clag3* expression in the absence of exogenous resistance markers, we selected untransfected 10G parasites with different concentrations of BSD ranging from approximately the BSD IC_{50} in 10G (see below) to a threefold higher concentration. In only 1 week (3–4 generations) we obtained parasite populations adapted to the two lower concentrations (0.2 and 0.3 μg ml^{-1}, 10G-0.2 and 10G-0.3 lines respectively), and

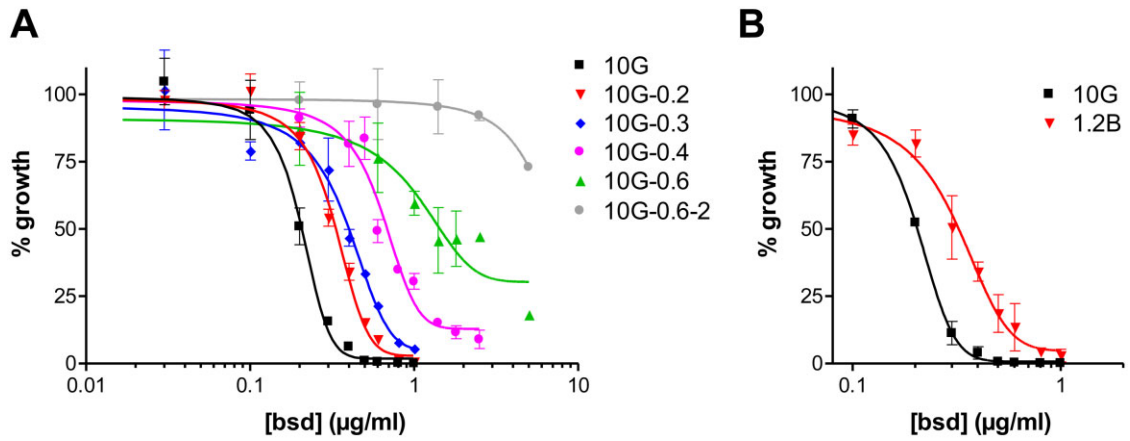

Fig. 2. BSD dose–response curves for parasite lines with different *clag3* expression patterns.
A. BSD susceptibility of 10G-derived parasite lines previously selected with different concentrations of the drug. Values are the result of a representative experiment performed in duplicate, with range.
B. Comparison of BSD susceptibility between the isogenic 3D7 subclones 10G and 1.2B, which spontaneously show different *clag3* expression patterns without having been selected with BSD. Values are the average of two independent experiments performed in duplicate, with range.

in about 2–3 weeks we also obtained cultures adapted to 0.4 and 0.6 µg ml^{-1} BSD (10G-0.4 and 10G-0.6 lines respectively). Expression patterns of *clag3* genes in untreated control 10G cultures remained stable throughout the experiment (Fig. 1B). In contrast, in cultures selected with 0.2 to 0.4 µg ml^{-1} BSD the majority of parasites expressed *clag3.1* instead of *clag3.2* (Fig. 1B), similar to the observations with transfected parasites. The ratio of *clag3.1* vs *clag3.2* expression changed over 100-fold (Fig. 1C). This result indicates that BSD selects for parasites expressing CLAG3.1 rather than the alternative CLAG3.2, implying that in this parasite line CLAG3.2 mediates more efficient uptake of BSD than CLAG3.1.

Selection with 0.6 µg ml^{-1} BSD not only altered the ratio of *clag3.1*-to-*clag3.2* expression but also resulted in reduced expression of the two *clag3* genes (Figs 1B, C and S2A). Next we challenged parasites adapted to grow under 0.6 µg ml^{-1} BSD with 2 µg ml^{-1} BSD for 2 weeks. 10G-0.6 did not require an additional adaptation period and was directly able to grow under this high drug concentration (10G-0.6-2 line). Expression of *clag3* genes was almost completely abolished in 10G-0.6-2 parasites (40-fold lower *clag3* expression than in control cultures, Figs 1B and S2A). All together, these results indicate that switching from *clag3.2* expression to *clag3.1* expression limits the entry of BSD at low concentrations, but resistance to higher BSD concentrations requires severely reduced expression of the two *clag3* genes, presumably compromising PSAC formation (Fig. 1D).

Stability of clag3 expression patterns in the absence of BSD pressure

We maintained the cultures adapted to the different BSD concentrations in the absence of drug for 2 weeks and measured their *clag3* expression. The *clag3* expression patterns of 10G-0.2, 10G-0.3 and 10G-0.4 did not change (Fig. 1B), in line with previous observations showing that *clag3.1* expression, as well as *clag3.2* expression, are stably transmitted expression patterns under culture conditions (Cortés *et al.*, 2007). On the other hand, 10G-0.6 parasites grown in the absence of drug resumed normal *clag3* expression (Figs 1B and S2A), indicating that severely reduced *clag3* expression imposes a growth disadvantage for the parasites. This idea is supported by the observation of a lower growth rate (measured in the absence of drug) in lines selected with higher drug concentrations (growth rate 5.33 ± 1.58 for 10G-0.6-2 compared with 8.99 ± 1.26 for unselected 10G, $P = 0.01$, $n \geq 4$).

clag3 expression patterns are associated with changes in BSD sensitivity

We compared BSD growth inhibition between the original 10G and the BSD-selected lines and found that BSD sensitivity decreased with increasing concentrations of drug used for selection (Fig. 2A and Table 1). Parasites

Table 1. BSD IC_{50} of parasite lines used in this study.

Parasite line	IC_{50} (in µg ml^{-1})
10G	0.20 (0.20–0.20)
10G-0.2	0.34 (0.32–0.35)
10G-0.3	0.43 (0.38–0.47)
10G-0.4	0.79 (0.69–0.88)
10G-0.6	1.64 (1.54–1.73)
10G-0.6-2	> 5 (> 5)
1.2B	0.31 (0.27–0.35)

Values are the average of two independent experiments performed in duplicate, with range.

selected with 0.2 to 0.4 μg ml^{-1} BSD showed progressive modest increases in their BSD IC_{50}. However, in 10G-0.4 substantial growth (almost 10% of growth in untreated cultures) was observed even at the highest BSD concentration tested (2.5 μg ml^{-1}). This may correspond to a subpopulation of highly resistant parasites in this selected culture. 10G-0.6 was highly resistant to the drug and 10G-0.6-2 was essentially insensitive even to high concentrations of BSD (Fig. 2A and Table 1), indicating that silencing of both *clag3* genes severely blocks the entry of this drug. Next we tested the BSD sensitivity of the 3D7 subclone 1.2B (Cortés, 2005), which is isogenic with 10G but spontaneously expresses *clag3.1* rather than *clag3.2* without having ever been selected with BSD (Cortés *et al*., 2007; Crowley *et al*., 2011). The subclone 1.2B was more resistant to BSD than 10G (Fig. 2B and Table 1). BSD susceptibility of 1.2B was similar to low BSD-selected 10G lines (10G-0.2 and 10G-0.3) that show the same *clag3* expression pattern as 1.2B (Figs 1D and 2 and Table 1). This result supports the idea that *clag3* expression, rather than other potential alterations arising during selection, is the main determinant of BSD sensitivity.

10G-0.6 cultures maintained in the absence of drug, which recovered normal *clag3* expression levels, only regained BSD sensitivity partially: growth of 10G-0.6 maintained for 3–4 weeks without drug was 61% inhibited by 1 μg ml^{-1} of BSD (range 59–62%, $n = 2$), which is higher than the inhibition of 10G-0.6 tested immediately after selection (34%, range 28–40%, $n = 2$) but similar to inhibition of 10G-0.4 (62%, range 55–69%, $n = 2$) that expresses normal *clag3* levels (*clag3.1*). However, growth of unselected 10G was invariably > 99% inhibited by this BSD concentration. These results indicate that a proportion of high-BSD resistant parasites remain in the 10G-0.6 population after several weeks without drug.

Expression of other clag genes in BSD-selected parasites

The participation of *clag* genes other than *clag3* in the formation of PSAC has not been determined. We measured expression of *clag2*, *clag8* and *clag9* in all BSD-selected cultures, and only *clag2* expression was reduced by selection with BSD, although expression levels did not correlate with the BSD concentration used for selection (Fig. S2A). However, *clag2* is the only member of the *clag* family besides *clag3.1* and *clag3.2* that shows clonally variant expression, and it is silenced in the 10G subclone (Cortés *et al*., 2007; Rovira-Graells *et al*., 2012) (note the different scale in the *clag2* panel in Fig. S2A, showing residual *clag2* expression only). To further assess the possible association between *clag2* silencing and BSD resistance, we selected with 0.3 or 0.6 μg ml^{-1} BSD the parasite subclone 1.2B, which is isogenic with 10G but has

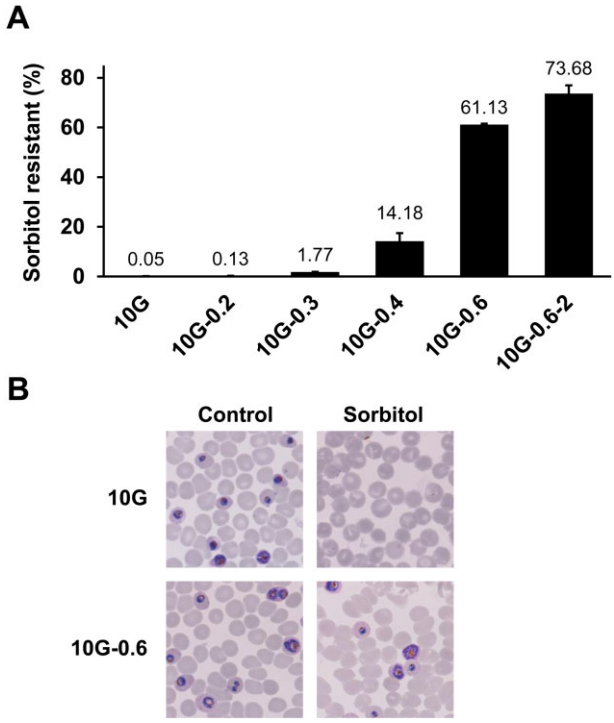

Fig. 3. Sensitivity to sorbitol lysis in parasite lines selected with different concentrations of BSD.
A. Values are the proportion of late-stage parasites (pigmented trophozoites and schizonts) resistant to lysis with 5% sorbitol, and are the average of two independent experiments, with range/2.
B. Representative fields of Giemsa-stained smears of 10G and 10G-0.6 treated or not with 5% sorbitol.

the *clag2* gene in an active state (Cortés *et al*., 2007; Rovira-Graells *et al*., 2012). *clag2* transcript levels were similar between control and BSD-selected 1.2B (Fig. S2B). Hence, since parasites can become resistant to BSD while expressing *clag2*, *clag8* and *clag9*, we conclude that these genes are unlikely to play a major role in the transport of BSD into infected erythrocytes in parasites of 3D7 genetic background, at least when the drug is present at moderate concentrations.

Resistance to BSD is paralleled by resistance to sorbitol lysis

Sorbitol transport into infected erythrocytes, which results in haemolysis, requires PSAC activity (Wagner *et al*., 2003; Nguitragool *et al*., 2011). Ring-stage infected erythrocytes are not lysed by sorbitol because functional PSAC is assembled at the onset of the pigmented trophozoite stage. We treated magnet-synchronized late stages (pigmented trophozoites and schizonts) of control and BSD-selected cultures with 5% sorbitol. The proportion of sorbitol resistant parasites was low in 10G-0.2 and 10G-0.3, but it was 14% in 10G-0.4 (Fig. 3). This is consistent

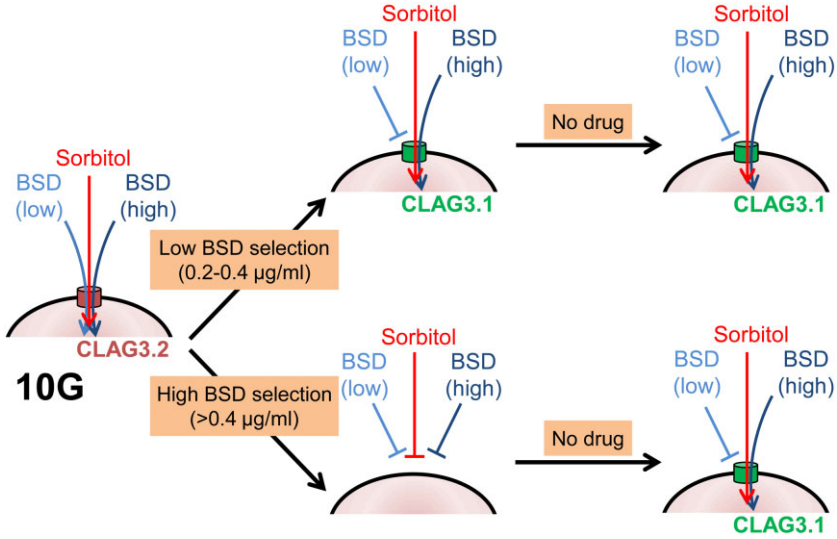

Fig. 4. Model for the acquisition of BSD resistance by epigenetic changes in the expression of *clag3* genes. The majority of parasites in the unselected 10G population express CLAG3.2 (red cylinder) and not CLAG3.1 (green cylinder). Culturing with BSD at low concentrations (up to 0.4 µg ml^{-1}) selects for pre-existing parasites within the population that had switched from *clag3.2* to *clag3.1* expression, which results in less efficient BSD transport and allows parasite survival at low BSD concentrations. However, higher concentrations of the drug select parasites with severely reduced *clag3* expression, which blocks uptake of the drug even at high concentration and also blocks sorbitol uptake. Whether residual *clag3* expression in these parasites allows the formation of a small number of active channels (not shown) is not known. This model represents the predominant expression patterns in each population, but cultures selected with 0.4 or 0.6 µg ml^{-1} BSD contain a small fraction of individual parasites with the high BSD and low BSD patterns respectively. Removal of drug from cultures selected with low BSD concentrations does not result in additional changes in *clag3* expression. However, removal of drug from cultures selected with high BSD concentrations results in recovery of *clag3* expression and a concomitant slow increase in sorbitol and BSD sensitivity, indicating that in the absence of drug parasites with both *clag3* genes silenced are selected against.

with the existence of a high BSD-resistant subpopulation in 10G-0.4 (Fig. 2A). Strikingly, over 60% of 10G-0.6 and over 70% of 10G-0.6-2 late stages were resistant to sorbitol lysis (Fig. 3), consistent with the large reduction in *clag3* expression observed in these parasite lines. Parasites selected with a high BSD concentration were also highly resistant to haemolysis of infected erythrocytes by a structurally unrelated PSAC substrate, the amino acid L-Alanine (data not shown). These results confirm impaired PSAC function in these parasites and the link between BSD resistance, *clag3* expression, NPPs and PSAC (Fig. 4). When 10G-0.6 parasites were maintained in the absence of BSD, they progressively lost resistance to sorbitol lysis (data not shown). By 4 weeks after drug removal, less than 20% of late stage parasites were resistant to sorbitol lysis. Together with the moderate increase in BSD sensitivity and the recovery of *clag3* expression observed after removing drug pressure, these results indicate that in the absence of BSD parasites with reduced total *clag3* expression are selected against. However, this negative selection is slow and a subpopulation of high-BSD and sorbitol resistant parasites remains after several weeks without drug. The expected reduction in *clag3* transcript levels associated with the presence of this subpopulation (e.g. 20% reduction) is below the accuracy limits of the transcript quantification method.

BSD resistance and switches in clag3 expression are not associated with large genetic rearrangements or mutations

The high level of similarity between *clag3.1* and *clag3.2* (95% identity in the ORF) can result in recombination events between the two genes (Iriko *et al.*, 2008; Pillai *et al.*, 2012). To exclude the possibility that the changes in *clag3* expression observed upon BSD selection are mediated by genetic rearrangements at the *clag3* loci, we analysed the genomic DNA of the selected lines by long PCR, but found no difference relative to the unselected line (Fig. S3A). We also excluded the possibility of a deletion affecting one or the two *clag3* genes, because qPCR analysis of the two genes revealed identical copy number relative to an essential gene between unselected 10G and 10G-0.6 (Fig. S3B). Furthermore, next-generation sequencing (NGS) of the complete genomes of unselected 10G and two of the adapted lines (10G-0.2 and 10G-0.6) did not reveal any genetic differences associated with BSD selection at the *clag3* loci (Fig. S4) or at other *clag* genes (data not shown). These results indicate that changes in *clag3* expression associated with BSD resistance are not mediated by major genetic rearrangements or mutations at the *clag3* loci. Furthermore, a genome-wide analysis of the NGS data for 10G, 10G-0.2 and 10G-0.6 did not reveal any

significant sequence differences associated with BSD selection (Table S2). Considering that we and others have previously demonstrated that expression of *clag3* genes is regulated by chromatin-based epigenetic mechanisms (Comeaux *et al*., 2011; Crowley *et al*., 2011), we conclude that epigenetic switches in *clag3* expression and not mutations are the main determinants of BSD resistance in our adapted lines.

Discussion

The PSAC plays an important role in the biology of *P. falciparum* by enabling the uptake of solutes that are necessary for parasite growth (Pillai *et al*., 2012). However, the presence of this broad-specificity channel in the surface of infected erythrocytes also poses a risk for the parasite, as it can allow the entrance of harmful solutes. Here we demonstrate that *P. falciparum* can evolve resistance to toxic compounds by altering the expression of *clag3* genes, which are necessary for the formation of functional PSAC. While parasites acquire resistance to low concentrations of BSD by switching from expression of one *clag3* gene to the other, resistance to high concentrations of the drug requires simultaneous silencing of both genes, resulting in severely affected PSAC activity (Fig. 4). Furthermore, the parasite subclone 1.2B, which is isogenic with 10G but spontaneously expresses CLAG3.1 instead of CLAG3.2 without having been exposed to BSD, is less sensitive to the drug than 10G, linking spontaneous switches in *clag3* expression with resistance to toxic compounds.

The results presented here have important implications for our understanding of the regulation and function of *clag3* genes: first, they imply that CLAG3.1 or CLAG3.2 confer different transport efficiency to PSAC, such that at least for BSD and in a 3D7 genetic background, CLAG3.2 determines more efficient transport than CLAG3.1. Whether CLAG3.2 generally provides more efficient solute transport, or each CLAG3 protein determines higher transport efficiency for different solutes, remains unknown. Of note, *clag3.1* and *clag3.2* are highly polymorphic and gene conversion events are common between these genes (Iriko *et al*., 2008), implying that CLAG3.1 and CLAG3.2 properties likely vary between parasites of different genetic backgrounds. A second important implication of our results is that mutually exclusive expression of *clag3* genes, such that in an individual parasite one and only one of the *clag3* promoters is active, is not strict and parasites with an unusual expression pattern can be detected under strong selective pressure. In contrast to previous studies that invariably showed one active *clag3* promoter in all parasites studied (Cortés *et al*., 2007; Comeaux *et al*., 2011; Crowley *et al*., 2011; Nguitragool *et al*., 2011; Pillai *et al*., 2012), here we describe a parasite line with the two *clag3* genes simultaneously silenced (10G-0.6-2). A previous study described a transgenic parasite line with a truncated *clag3.2* gene that did not express full-length *clag3* transcripts, but one of the *clag3* promoters was active even if it was controlling a truncated gene (Comeaux *et al*., 2011). The molecular mechanisms underlying mutually exclusive expression in malaria parasites remain a mystery (Guizetti and Scherf, 2013), but in the case of *clag3* genes it is possible that these mechanisms do not actively prevent simultaneous silencing of the two genes. Instead, the fitness cost associated with this *clag3* transcriptional state would keep the proportion of parasites that harbour it at very low levels within a population, making them detectable only under selective conditions that confer them a growth advantage. Our observation that parasites with severely reduced *clag3* expression regain normal *clag3* expression levels when cultured in the absence of drug, and the substantially lower growth rate of 10G-0.6-2 compared with unselected 10G, support this idea (Fig. 4). However, parasites with severely reduced CLAG3 levels still grow, albeit with reduced growth rate; whether residual expression of *clag3* genes when they are epigenetically silenced allows the formation of a small number of active channels sufficient for the uptake of some essential solutes is not known. Studies using CLAG3 inhibitors indicate that an essential role for CLAG3 is only revealed when using modified media with restricted concentrations of key nutrients (Pillai *et al*., 2012). A third important consideration is that our results provide a plausible explanation for the function of mutually exclusive expression in *clag3* genes. We propose that mutually exclusive expression of these genes plays a role in preventing access of undesired compounds present in the host plasma. In this scenario, changing metabolic or pharmacological conditions of the host would select parasites with *clag3* expression patterns that confer the best balance between efficient transport of necessary solutes and reduced permeability to toxic compounds. In the case of *var* genes, mutual exclusion is driven by immune evasion (Scherf *et al*., 2008; Deitsch *et al*., 2009), but given that mutual exclusion of *clag3* genes involves only two genes, we consider immune evasion a less likely driving force in their case.

Drug resistance in malaria parasites is usually mediated by genetic alterations, including point mutations or larger genetic alterations in genes encoding the target enzyme or transporters that pump the drug out of its site of action (Goldberg *et al*., 2012). However, the mechanism of resistance to several drugs remains largely unknown, including resistance to artemisinin derivatives (Cheng *et al*., 2012). The results of our studies with BSD, a drug that is not clinically used against malaria because of its high toxicity to human cells, provide a proof of principle demonstrating that in addition to genetic changes, para-

sites can develop drug resistance by transcriptional alterations transmitted by epigenetic mechanisms. Similar to genetic changes, epigenetic alterations are transmissible, but they provide the additional advantage for the parasite that they are easily reversible and hence confer flexibility to rapidly adapt to fluctuating conditions (Cortés *et al*., 2012; Rovira-Graells *et al*., 2012). It is currently unknown if other drugs are susceptible to development of resistance by transcriptional changes in *clag3* genes. Small hydrophobic compounds are expected to diffuse through lipid membranes and not require a channel to enter infected erythrocytes, suggesting that molecular size and hydrophobicity indexes such as the logP value could in principle be used to predict if a solute requires PSAC for its uptake (Lisk *et al*., 2010). However, experience has shown that no simple criteria can predict which solutes are transported via NPPs (Baumeister *et al*., 2011), as even some very hydrophobic compounds are known to use NPPs to enter infected erythrocytes (Stead *et al*., 2001; Biagini *et al*., 2003). Hence, only experimental validation will determine which drugs are susceptible to development of resistance by the mechanisms described here. In this regard, the parasite line 10G-0.6-2, which keeps the two *clag3* genes silenced, represents a valuable tool that should be incorporated into large drug screening efforts to determine which drug leads are susceptible to this mode of resistance, as 10G-0.6-2 may show reduced sensitivity to any toxic compounds that require PSAC activity to enter infected erythrocytes.

The pattern of adaptation to BSD observed here is consistent with BSD selecting for pre-existing parasites within the population (Fig. 4). Our previous work has established that genetically homogeneous *P. falciparum* populations are transcriptionally heterogeneous, such that some genes, including *clag3.1* and *clag3.2*, show clonally variant expression as an intrinsic property and are active in some individual parasites and silenced in others (Cortés *et al*., 2007; Rovira-Graells *et al*., 2012). 10G is a recently subcloned line, but it is expected that some individual parasites within the population have spontaneously switched from *clag3.2* to *clag3.1* expression or have silenced both genes after subcloning. We have previously proposed that epigenetic heterogeneity within isogenic parasite populations plays a role in the adaptation of parasites to changes in their environment by allowing selection of parasites with transcriptional patterns that confer more fitness (Cortés *et al*., 2012; Rovira-Graells *et al*., 2012). This bet-hedging adaptive strategy operates for immune evasion (Scherf *et al*., 2008; Deitsch *et al*., 2009) and for adaptation to periodical heat-shock mimicking cyclical malaria fever (Rovira-Graells *et al*., 2012), and here we demonstrate that it also plays a role in the adaptation of malaria parasites to the presence of toxic compounds.

CLAG3 proteins determine which plasma solutes access infected erythrocytes, the niche where malaria parasites spend most of their time and produce human disease. In spite of this key role in parasite biology, *clag3* expression dynamics in natural infections remains completely unknown. Understanding how *clag3* gene expression is altered in response to different host physiological conditions during the course of natural infections and determining to which drugs the parasite can develop resistance by changes in *clag3* expression should be considered urgent research priorities.

Experimental procedures

Parasite cultures

10G and 1.2B subclones of the 3D7-A stock of the clonal *P. falciparum* line 3D7 have been described and characterized before (Cortés, 2005; Cortés *et al*., 2007; Crowley *et al*., 2011; Rovira-Graells *et al*., 2012). Parasites were cultured in B+ erythrocytes at a 3% haematocrit under standard conditions, with Albumax II and no human serum. Cultures were synchronized by treatment with 5% sorbitol (unselected cultures or cultures selected with up to 0.3 μg ml^{-1} BSD) or by magnetic separation using Miltenyi Biotec CS columns (cultures selected with higher BSD concentrations containing an important fraction of parasites refractory to sorbitol synchronization). To prepare RNA for transcriptional analysis, synchronized cultures were harvested when the majority of parasites were at the schizont stage and a small proportion of schizonts had already bursted.

BSD selection, growth inhibition assays and determination of growth rates

BSD (Blasticidin S HCl) was obtained from Invitrogen. To select cultures for BSD resistance, the drug was initially applied to cultures at the ring stage. To determine BSD growth inhibition and IC_{50}, the parasitemia of synchronized ring stage cultures was measured by FACS and adjusted to 1%. In drug selected cultures, drug was removed 4 h before starting the assay. Cultures were grown in the presence of different concentrations of drug in duplicate wells of 96-well plates. After ~ 53 h, parasitemia was determined on Giemsa-stained smears by microscopy, blind-counting the number of infected erythrocytes in 1000 to 10 000 erythrocytes (depending on parasitemia). All experiments included a BSD dose–response assay with unselected 10G that demonstrated stable drug potency and consistency between assays performed on different dates. After LOG-transforming drug concentrations, data was fit to sigmoidal dose–response curves using GraphPad Prism (version 3) setting the maximum to 100 and the minimum to 0, with no weighting. To calculate growth rates, drug was removed from synchronized cultures at the ring stage 4 h before starting the assay. Parasitemia was adjusted to 1% and measured by FACS (initial parasitemia). After ~ 53 h, parasitemia was again determined by FACS or microscopy (final parasitemia) and the growth rate calculated as the ratio of final parasitemia/initial parasitemia. Growth rates are expressed as average ± SD and compared using unpaired Student's *t*-test.

Sorbitol sensitivity assays

To determine sorbitol sensitivity, late stage parasites were purified using magnetic columns. After adjusting parasitemia to approximately 10% by addition of uninfected erythrocytes, or after one additional complete asexual blood cycle, cultures were treated in parallel with 5% sorbitol in H_2O or with RPMI-HEPES (control) for 7 min at 37°C. After washing with RPMI-HEPES and resuspending in complete parasite culture medium, Giemsa-stained smears were prepared to determine parasitemia by microscopy. The proportion of sorbitol-resistant parasites was calculated by dividing parasitemia in sorbitol-treated samples by parasitemia in controls.

Genetic and transcriptional analysis

The *clag3* loci were analysed by long PCR of gDNA as previously described (Iriko *et al*., 2008). For RNA purification, culture pellets were collected in Trizol and RNA purified, DNase treated and reverse transcribed as described (Cortés *et al*., 2007). To exclude gDNA contaminations, parallel reactions were performed in the absence of reverse transcriptase. cDNAs were analysed by quantitative PCR in triplicate wells using PowerSYBR Green Master Mix (Applied Biosystems) as described (Crowley *et al*., 2011). Expression values, in arbitrary units, were calculated using the standard curve method (each 96-well plate contained an identical standard curve made with serial dilutions of 3D7 gDNA). The primers used are described in Table S1.

Next-generation sequencing

Genomic DNA libraries were prepared for multiplexed single-end Illumina TruSeq sequencing as previously described (Straimer *et al*., 2012). In brief, gDNA was sheared, size selected on a gel, and purified. NEBNext DNA Library Preparation reagents (NEB) were used to end repair, dA-tail, and ligate on NEXTflex DNA Barcodes (Bioo Scientific). Library quality and ligation efficiency was analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a Quant-iT dsDNA High-Sensitivity Assay Kit (Life Technologies). All libraries were determined to be of sufficient quality and quantity with no need for PCR amplification. The three individually barcoded libraries were multiplexed along with 25% PhiX control DNA and run on a single lane using the Illumina HiSeq 2500 Rapid Run (141 bp) system. For details on data analysis, please see Table S2 and Fig. S4.

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Note added in proof

During the revision of our manuscript an article was published by the Desai lab that provides additional support for some of our conclusions (Sharma *et al*., 2013). In that work, transcriptional analysis of FCB clone parasites selected with a high concentration of BSD (2.5 μg ml^{-1}) revealed that these parasites silenced expression of both *clag3* genes at the epigenetic level, as we have observed in our lines selected with ≥ 0.6 μg ml^{-1} of the drug.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used for qPCR analysis.

Table S2. Potential genetic changes between 10G, 10G-0.2 and 10G-0.6 as detected by Illumina next generation whole genome sequencing.

Fig. S1. Transfection experiments with plasmids containing BSD resistance markers.

A. Schematic of the 3.2-1371-LH-bsdR and BsdR plasmids. Luc refers to the luciferase ORF and bsd refers to the Blasticidin S deaminase ORF. The 3.2-1371-LH-bsdR plasmid was derived from the 3.2-1371-LH plasmid (Crowley *et al*., 2011) by inserting a *Sma*I-BglII fragment of the pHBupsC^{R} plasmid (Voss *et al*., 2006) containing the BSD deaminase cassette and rep20 repeats into unique *Sma*I-*BamH*I sites downstream of the luciferase expression cassette. To generate the BsdR plasmid, the luciferase expression cassette containing the *clag3.2* upstream region was removed by digestion with *Kpn*I and religation.

B. Transcript levels of *clag3.1* and *clag3.2* in unselected control 10G cultures and the same line transfected with the plasmid 3.2-1371-LH-bsdR and maintained under BSD selective pressure for over 2 months. Results were normalized against *rhoph2* expression as in Fig. 1B in the main text.

Fig. S2. Expression of *clag2*, total *clag3*, *clag8* and *clag9* in parasites selected with BSD.

A. Expression levels for *clag2*, total *clag3* (measured with primers that recognize a region that is identical between *clag3.1* and *clag3.2*), *clag8* and *clag9* were determined in the same samples as in Fig. 1B and C in the main text. Note the different scale of the *y*-axis in the *clag2* panel. Results were normalized against *rhoph2* expression as in Fig. 1B.

B. Transcript levels of *clag2* in 1.2B subclone selected for 2 weeks with 0.3 μg ml^{-1} BSD (1.2B-0.3), and then 4 weeks with 0.6 μg ml^{-1} BSD (1.2B-0.6), or grown in parallel in the absence of drug (1.2B). Expression levels in 10G are shown for comparison.

Fig. S3. Changes in *clag3* expression are not associated with genetic rearrangements at the *clag3* loci or altered copy number of *clag3* genes.

A. Long-PCR analysis of the *clag3* loci as previously described (Iriko *et al*., 2008). The combinations of primers in the two upper panels amplify the full unaltered *clag3.1* and *clag3.2* loci respectively. The primer combinations in the two lower panels would yield a PCR product only if recombination events had occurred between the two loci (Iriko *et al*., 2008).

B. qPCR analysis of genomic DNA with primers recognizing *clag3* genes. Values were normalized against the single copy essential gene seryl tRNA synthetase. Normalization against *rhoph2* yielded almost identical results (data not shown).

Fig. S4. Illumina next generation sequencing of 10G (unselected), 10G-0.2 and 10G-0.6 in the genomic region where *clag3* genes are located. Snapshot of the reads in the *clag3* region aligned to the 3D7 reference genome (v. 9.3), as viewed in the Integrative Genomics Viewer (IGV) (Robinson *et al*., 2011; Thorvaldsdottir *et al*., 2013). For each parasite line, the upper panel represents read coverage (scale 0-116, 0-276 and 0-163 for 10G, 10G-0.2 and 10G-0.6 respectively), whereas the lower panel shows the position of individual reads. Salmon and blue colours indicate reads corresponding to the two different DNA strands, whereas grey indicate non-unique reads. Non-unique reads corresponding to sequences that are identical between *clag3.1* and *clag3.2* were split between the two genes (a mutation occurring within non-unique regions in only one of the two genes would be expected to reach a maximum frequency of 50%). SNPs that reach a frequency above 20% are indicated in the coverage panels. The single mutation identified within the *clag3* region, which occurred in 10G-0.6, is indicated by an arrowhead. This mutation also occurred in control 10G and 10G-0.2, but it is not indicated in their coverage panels because the frequency of the alternative allele was slightly below the 20% threshold (19% in 10G and 18% in 10G-0.2, compared with 38% in 10G-0.6). Furthermore, this SNP is located next to a string of A's. Hence, this mutation is unlikely to be associated with BSD selection.