However, a large number of circular patches of clinopyroxenite xenoliths varying in size from < 1 mm to 5 mm in a tholeiitic basalt flow are now reported from Mumbai. These 'campus xenoliths' occur in a basalt flow seen within the campus premises of the Indian Institute of Technology (IIT), Powai (lat. 19°8′N; long. 72°56′E; Figure 1 *b*). These xenoliths comprise clinopyroxenites with polygonal, rounded or nodular outlines (Figure 3 *c* and *d*). Pyrometamorphic texture, which is conspicuous in Kutch xenoliths, is not observed in the campus xenoliths where their contact with the host basalt is sharper. In some cases the pyroxenes are arranged in a radiating pattern (Figure 3 *d*). Innumerable minute opaque inclusions, arranged in concentric circles, are seen in the pyroxene grains (Figure 3 *d*). Spinels, which are very conspicuous in Kutch xenoliths, are totally absent in the campus xenoliths. Twinning is quite common in the pyroxenes of these xenoliths (Figure 3 *c* and *d*) but absent in those of Kutch. The $2V_z$ and $Z \wedge c$ of the clinopyroxenes vary between 32–60° and 38–40°, respectively, which correspond to augites and ferroaugites. Though kink bands have been reported in several xenoliths[1,11,12], such bands are not observed in either the Kutch or the campus xenoliths.

The basalt flow hosting the campus xenoliths is medium-grained, holocrystalline, aphyric, subophitic and contains plagioclase laths, pyroxenes and opaques. Olivine occurs occasionally as subhedral grains and is altered to iddingsite. Thus based on the texture and mineralogy, it is apparent that the Kutch xenoliths are accidental while the campus xenoliths are cognate.

The occurrence of three compositionally different types of xenoliths (spinel peridotites, spinel lherzolites and clinopyroxenites) implies that either the mantle below Kutch is zoned or layered and contains these types of rocks which were picked up by the alkali olivine basalt extrusive or the clinopyroxenites are the products of reaction between peridotite (olivine and orthopyroxene) and carbonated alkaline magma at temperatures between 1050 and 1100°C and 17 kb pressure[13]. Considering the proximity of the xenolith-bearing flows to the carbonatite province of Gujarat, it is quite likely that the Kutch clinopyroxenite xenoliths got formed due to the later process. The campus xenoliths, on the contrary, seem to represent cumulates of clinopyroxenes in the magma chamber which were picked up and brought up by a post-pyroxenite pulse of eruption. Detailed investigation on the mineral chemistry and whole rock isotope geochemistry of the Kutch and campus xenoliths is presently going on in order to decipher their petrogenesis.

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Cloning and characterization of the *Plasmodium falciparum* adenylosuccinate synthetase gene

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Parasitic protozoa lack the *de novo* purine biosynthet pathway and rely exclusively on the salvage pathwa for their purine nucleotide requirements[1]. Purine sa vage enzymes are therefore potential chemotherape tic targets. This paper reports the cloning and deduc amino acid sequence of *Plasmodium falciparu* adenylosuccinate synthetase (PfADSS), an enzyn involved in purine salvage. PfADSS exhibits 67 homology with that of the human enzyme. On expr ssion in *E. coli*, enzymatically active ADSS was pr duced as deduced by functional complementatic analysis. The PfADSS activity was shown to be inh bited by hadacidin, a known competitive inhibitor this enzyme.

ALMOST all protozoan parasites lack the biosynthet machinery required for the *de novo* synthesis of purines

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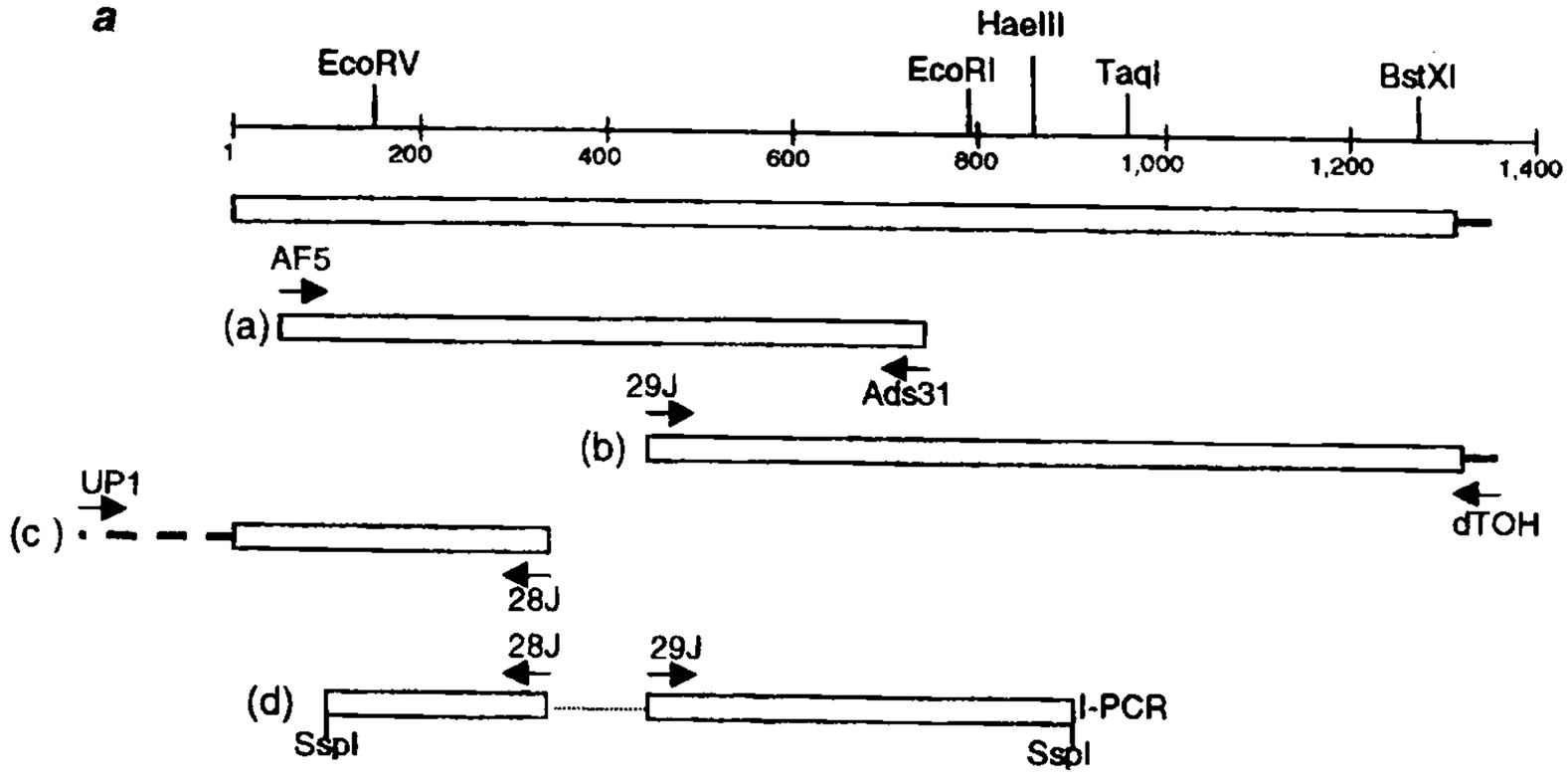
Nucleotide sequence reported in this paper is available in the Ge Bank[TM] data base under the accession number AF095282.

The malaria parasite, *Plasmodium falciparum*, during its intraerythrocytic stages depends only on the purine salvage pathway for its supply of purine nucleotides[2]. The major purine base available to the intraerythrocytic parasite is hypoxanthine[2,3]. Depletion of hypoxanthine by addition of xanthine oxidase to *in vitro* cultures of *P. falciparum* results in the death of the parasites[3]. Further, inhibition of *P. falciparum* adenosine deaminase with 2′-deoxycoformycin also brings about the death of the parasites[4], indicating that hypoxanthine is the major purine precursor that the parasite utilizes. Hypoxanthine is converted to inosine monophosphate (IMP) by the parasite enzyme, hypoxanthine guanine phosphoribosyltransferase (HGPRT)[5]. IMP is the main precursor for adenosine monophosphate (AMP) and guanosine monophosphate (GMP)[6]. The IMP to AMP conversion pathway involves the enzymes adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase[6,7]. The mature human erythrocyte lacks the adenylosuccinate pathway[7]. Earlier studies have shown that the ADSS inhibitor, hadacidin has anti-

a



b

AF5	5′ CCAGAGCTCCAATGGGG(AT)GA(TC)CA(AG)GG(AT)AA 3′
ADS31	5′ ACAGGATCCGT(TA)AC(AG)(TA)A(TA)GG(AG)TA(TAG)GT(TA)CC 3′
28J	5′ ACAGGATCCCAAATAATATATGTGCTTTATTTG 3′
29J	5′ ACAGGATCCAGAATAGGTATAAGATTAGG 3′
DTOH	5′ CGCAAGCTTCTCGAG$(T)_{17}$
UP1	5′GTAAAACGACGGCCAGT 3′

Figure 1. ***a***, Schematic representation of the *P. falciparum* ADSS gene restriction map and location of the PCR fragments. 5 unique restriction sites are shown. Open rectangles represent the ORF and the thick, dark line the 3′-noncoding region; ***b***, List of oligonucleotide PCR primers used to obtain the *P. falciparum* ADSS gene. Primers AF5 and ADS31 are degenerate. Degenerate bases are enclosed in parenthesis. *Method*: Fragment (a) was generated by PCR using T9/106[17] *P. falciparum* genomic DNA as template with AF5 and ADS31 as primers. PCR conditions for each cycle were denaturation at 94°C for 30 sec, followed by annealing at 40°C for 40 sec and extension at 73°C for 1 min and 30 sec. PCR was carried out for a total of 30 cycles. Fragment (b) was obtained by RTPCR. 15 µg of total RNA isolated from a trophozoite-rich *P. falciparum* (T9/106) culture using standard protocols[22] was reverse transcribed using oligo $d(T)_{18}$ and AMV reverse transcriptase. Reverse transcribed product corresponding to 1.5 µg of total RNA was subjected to PCR using the primers 29J and dTOH. The PCR conditions were 35 cycles of amplification with each cycle of amplification consisting of denaturation at 95°C for 30 sec; annealing at 45°C for 45 sec and extension at 73°C for 3.5 min. Fragment (c) was generated as follows. 20 µg of T9/106 *P. falciparum* genomic DNA was digested with *Eco*RI and *Sau*3AI and Southern blotting yielded a 2.5 kb fragment when probed with ^{32}P-labelled pADSS700. The 2.5 kb fragment was ligated to *Eco*RI, *Bam*HI digested plasmid pBluescript II KS(+). PCR on the ligation reaction with the primers 28J and a vector derived primer UP1, yielded a 1.1 kb fragment which was Southern positive with labelled pADSS700 as probe. This fragment was cloned into the *Sac*I, *Bam*HI site of plasmid pBluescript II KS(+) to obtain the 400 bp fragment (c). The sequence in segment (d) was obtained by inverse PCR. *Ssp*I digest of *P. falciparum* genomic DNA (375 ng) was self-ligated in the presence of 434 µM ATP and 30 mM KCl with 5U of T_4 DNA ligase in a volume of 100 µl. T_4 DNA ligase was heat inactivated and the reaction mixture was precipitated with 3 volumes of ethanol, using 10 µg of glycogen as carrier. The circularized fragments were digested with 5U of *Ava*II, precipitated, redissolved in 50 µl water and the entire quantity was used for PCR carried out with the primers 28J and 29J in a final volume of 100 µl. The conditions used were denaturation at 95°C for 30 sec, annealing at 40°C for 1 min and extension at 73°C for 2 min, for a total of 40 cycles. As fragment (d) was obtained by inverse PCR, the missing segment between the primers 28J and 29J is indicated by a dotted line.

parasitic activity and inhibits the formation of AMP from IMP[5]. HGPRT of *P. falciparum* and of other protozoan parasites is being pursued as a drug target[9-11]. Recently, the sequence of *P. falciparum* adenylosuccinate lyase was reported[15]. This dependence of *P. falciparum* on the salvage of purines makes this pathway an attractive target for drug development. In this paper, we describe the molecular cloning and the deduced amino acid sequence of *P. falciparum* adenylosuccinate synthetase (PfADSS). The PfADSS gene was sub-cloned into the expression vector pTrc^{99A}[16] and shown by functional complementation in a *purA* deficient *E. coli* strain to express enzymatically active protein.

Comparison of the amino acid sequence of ADSS from both eukaryotic and prokaryotic organisms showed three major blocks of identity. Three degenerate oligonucleotide primers, with *P. falciparum* codon bias[17], that encode the sequence in these blocks of homology were tested in polymerase chain reaction (PCR) using parasite genomic DNA as template. Genomic DNA was isolated from an *in vitro* culture of the *P. falciparum* clone T9/106 (ref. 18). Figure 1 lists the primers used and illustrates the strategy involved in cloning the *P. falciparum* ADSS gene. PCR with primers AF5 and ADS31 yielded an amplified product of the expected size, i.e. 692 bp. The DNA sequence of the cloned 692 bp (pADSS700) fragment yielded an ORF of 211 amino acids that was very similar to a homologous region of ADSS from other organisms (data not shown).

Using the sequence data from pADSS700, two oligonucleotides 28J and 29J (Figure 1) were synthesized. The complete 3′ end of the *P. falciparum* ADSS gene was obtained by reverse transcription – polymerase chain reaction (RTPCR) using total parasite RNA isolated from a trophozoite rich *in vitro* culture of the strain T9/106. cDNA synthesized using $d(T)_{18}$ was subjected to PCR using the primers 29J and dTOH (Figure 1). Agarose gel analysis of the PCR product yielded a 914 bp fragment along with two smaller fragments of 550 and 300 bp. All three PCR amplified fragments were found to be Southern positive (data not shown) when probed with the 692 bp ADSS gene fragment (pADSS700). The 914 bp amplified fragment was cloned into pBluescript II KS(+) (ref. 19) to obtain (p3′ADSS) and sequenced. This fragment yielded an ORF of 289 amino acids which exhibited 65% similarity with a homologous region of ADSS from other organisms. A 3′ untranslated stretch of 38 bp, including a polyA tract consisting of 19 adenines was present in p3′ADSS.

A sub-library of 2.5 kb-sized fragments of *P. falciparum* genomic DNA digested with *Eco*R1 and *Sau*3A1, identified Southern positive with pADSS700 as probe (data not shown), was constructed in pBluescript II KS(+)[19]. Screening of this library by colony hybridization and with pADSS700 as probe did not yield any positive colonies. However, PCR with the above ligation reaction mixture as template and 28J and UP1 (a vector derived primer) (Figure 1) as primers yielded a 1.1 kb Southern

```
PF      ------------------QIKNVDKGNVVAILGAQWGDEGKGKIIDMLSEYSDITCRFNGGANAGHTISVNDKKYALHLLPCGVLYDNNISVLGNGMVI  81
DD      MA--------------------------SIIIGSQWGDEGKGKLVDILSQQFDVVARCQGGANAGHTIVVDGKKIALHLIPSGILNEKASCILGNGMVI
SC      ----------------------------VNVVLGSQWGDEGKGKLVDLLVGKYDIVARCAGGNNAGHTIVVDGVKYDFHMLPSGLVNPNCQNLLGNGVVI
HU      MAFAETYPAASSLPNGDCGRPRAAGGNRVTVVLGAQWGDEGKGKVVDLLVGKYDIVARCAGGNNAGHTIVVDGVKYDFHMLPSGLVNPNCQNLLGNGVVI
EC      --------------------------GNNVVVLGTQWGDEGKGKIVDLLTERAKYVVRYQGGHNAGHTLVINGEKTVLHLIPSGILRENVTSIIGNGVVL
                                          * ********* * *        *  ** *****      *   *  * *          *** *

PF      HVKSLMEEIES--VGGKLLD----RLYLSNKAHILFDIHQIIDSIQE---TKKLKEGKQIGTTKRGIGPCYSTKASRIGIRLGTLKN-----FENFKNMY 167
DD      HLPTFFKEVQG--LQDKGIN-YKGRLFVSDRAHLVFDLHQMIDAMKEAELS-NGTSNDSIGTTKRGIGPCYSSKASRGGLRVCDLYSP-----EHFRKTF
SC      HVPSFFKELET--LEAKGLKNARSRLFVSSRAHLVFDFHQVTDKLRELELSGRSKDGKNIGTTGKGIGPTYSTKASRSGLRVHHLVNDQPGAWEEFVARY
HU      HLPGLFEEAEKNVQKGKGLEGWEKRLIISDRAHIVFDFHQAADGIQEQQ--RQEQAGKNLGTTKKGIGPVYSSKAARSGLRMCDLVSD----FDGFSERF
EC      SPAALMKEMKE--LEDRGIPV-RERLLLSEACPLILDYHVALDNARE-----KARGAKAIGTTGRGIGPAYEDKVARRGLRVGDLFDK-----ETFAEKL
              *                    **  *'      * *   *   *              ***  **** *  *  * * * *   *          *

PF      SKLID----HLMDLYNITEYDKEKELNLFYNYHLKLRDRIVDVISFMNTNLENN-KKVLIEGANAAMLDIDFGTYPYVTSSCTTVGGVFSGLGIHHKKLN 262
DD      TRLVE----NKHKRFGSFEYDVEAELKRYQEFAEMLKPFVIDSVYYLNQAFKDG-KKVLIEGAQSTMLDLDFGCYPYVTSSASSVGGACTGLGISPNKVV
SC      KRLLE----TRRQRYGDFEYDFEAKLAEYKKLREQLKPFVVDSVVFMHNAIEAK-KKILVEGANALMLDIDFGTYPYVTSSNTGIGGVLTGLGIPPRTID
HU      KVLAN----QYKSIYPTLEIDIEGELQKLKGYMEKIKPMVRDGVYFLYEALHGPPKKILVEGANAALLDIDFGTYPFVTSSNCTVGGVCTGLGMPPQNVG
EC      KEVMEYHNFQLVNYYKAEAVDYQKVLDDTMAVADILTSMVVDVSDLLDQA-RQRGDFVMFEGAQGTLLDIDHGTYPYVTSSNTTAGGVATGSGLGPRYVD
                        *   *                *                    ***    ** * * ** ****    **   * *

PF      LVVGVVKSYLTRVGCGPFLTELNNDVGQYLREKGHEYGTTTKRPRRCGWLDIPMLLYVKCINSIEMINLTKLDVLSGLEEILLCVNFKNKKTGELLEKGC 362
DD      TQIGVVKAYTTKVGSGPFPTEQNDHVGDSLRKAGSEFGTTTGRPRRIGWLDAVVLRYTSMINDFTRLNLTKLDVLSDFEEIKIGVDYKYK--GETIKS--
SC      EIYGVVKAYTTRVGEGPFPTEQLNENGEKLQTIGAEFGVTTGRKRRCGWLDLVVLKYSTLINGYTSLNITKLDVLDTFKEIPVGISYSIQ--GKKLDL--
HU      EVYGVVKAYTTRVGIGAFPTEQDNEIGELLQTRGREFGVTTGRKRRCGWLDLVLLKYAHMINGFTALALTKLDILDMFTEIKVGVAYKLD--GEIIPH--
EC      YVLGILKAYSTRVGAGPFPTELFDETGEFLCKQGNEFGATTGRRRRTGWLDTVAVRRAVQLNSLSGFCLTKLDVLDGLKEVKLCVAYRMPDGREVTTT--
           *  * * * ** * * **     *  *   * * * ** * ** ****          *        **** *    *

PF      YPVEEEISEEDEPVYGKFSGWKEDISTCNEFDELPENAKKYILAIEKYLKTPIVWIGVGPNR-KNMIVKKNFNLN 436
DD      FPASLETLAQCEVVYESFPGWKCDLSHVTEYDQLPIQAKNYIKRIEELVGVPIVYIGVGVER-KNLIERKE--LI
SC      FPEDLNILGKVEVEYKVLPGWDQDITKITKYEDLPENAKKYLKYIEDFVGVPVEWVGTGPAR-ESMLH-KE--IK
HU      IPANQEVLNKVEVQYKTLPGWNTDISNARAFKELPVNAQNYVRFIEDELQIPVKWIGVGKSR-ESMIQ-----LF
EC      -PLAADDWKGVEPIYETMPGWSESTFGVKDRSGLPQAALNYIKRIEELTGVPIDIISTGPDRTETMILRDPFDA-
         *         *  *    **              **  *  *   **     *       *  *
```

Figure 2. Comparison of ADSS amino acid sequences from *P. falciparum* (PF) (this paper), *Dictyostelium discoidium* (DD)[26], *Saccharomyces cerevisiae* (SC)[27], human (HU)[25] and *Escherichia coli* (EC)[28]. The different binding sites proposed for the substrates and cofactor and at the monomer–monomer interface are given in bold characters. Residues found identical in all sequences are marked by an asterisk. Multiple alignment was carried out using the program CLUSTAL V[38].

positive fragment. This fragment was digested with *Sac*I (site adjacent to UP1 in the vector pBluescript II KS(+)) and *Bam*H1, ligated again with the plasmid pBluescriptII KS(+) and transformed into the *E. coli* strain DH5α. On screening of the transformants with ^{32}P labelled pADSS700 as probe, only one positive colony was obtained. Plasmid isolated from this colony contained only a 400 bp insert instead of the expected 1.1 kb. Sequencing of this clone yielded an additional 72 bp at the 5′ end of the *P. falciparum* ADSS gene corresponding to 24 amino acids at the amino terminus of the enzyme. However, this did not yield the initiator methionine. Despite repeated attempts, a clone of the entire 1.1 kb fragment containing the 5′ end of the *P. falciparum* ADSS gene could not be obtained. Ligation of the 1.1 kb 5′ADSS gene fragment into pBluescriptII KS(+) led to unstable recombinant plasmids and subsequent loss of insert in the transformants. Similar difficulties have been reported in the cloning of the *P. falciparum* calmodulin, glucose 6-phosphate dehydrogenase and other genes[20,21].

*Ssp*1 digested *P. falciparum* genomic DNA was circularized by self-ligation, restricted with *Ava*II and subjected to inverse PCR[22] using the primers 28J and 29J. This PCR yielded a 654 bp DNA fragment that was Southern positive on probing with labelled pADSS700 (data not shown). However, sequencing of this fragment yielded only 147 bp downstream of the sequence in pADSS700. Additional strategies tried, involved (i) screening of a λgt11 *P. falciparum* cDNA library with ^{32}P labelled pADSS700 and (ii) PCR using the library DNA as template along with a vector-derived primer and a gene specific primer. However, these again did not yield the 5′ segment of the PfADSS gene.

The 1311 bp coding region of the *P. falciparum* ADSS gene has 72% A + T content, characteristic of other genes from this parasite[23]. The *P. falciparum* ADSS gene codes for 436 amino acids. The *Pyroccocus* ADSS[24] containing only 338 amino acids is the shortest while the human enzyme[25] has 455 amino acids. Hence, the *P. falciparum* ADSS described in this paper is probably short of only 5–10 amino acids at the amino terminal end of the enzyme. The parasite ADSS exhibits highest homology with the ADSS from *Dictyostelium discoideum* (70%)[26] followed by the yeast[27] and human[25] enzymes showing a similarity of 67%. With the *E. coli*[28] enzyme, *P. falciparum* ADSS showed 62% homology. An alignment of *P. falciparum* ADSS with prokaryotic and eukaryotic ADSS is shown in Figure 2. The high degree of positional identity seen with PfADSS supports the fact that the *P. falciparum* gene sequence reported in this paper codes for an enzyme with adenylosuccinate synthetase activity.

In a GTP (guanosine triphosphate) consuming reaction, ADSS catalyses the addition of aspartic acid to inosine monophosphate, thereby generating adenylosuccinate[29]. Structural studies along with site-directed mutagenesis on *E. coli* ADSS highlight the role of several residues in the interaction with both GTP and the substrates IMP and aspartate or in the interaction between the monomers of the homodimer[30–34]. The active site residues involved in catalysis and binding to GTP, aspartate and IMP are well conserved in PfADSS (these segments are highlighted in bold in Figure 2). The aspartate binding[31] segment occurs between residues 302 and 313. The highly conserved gly-

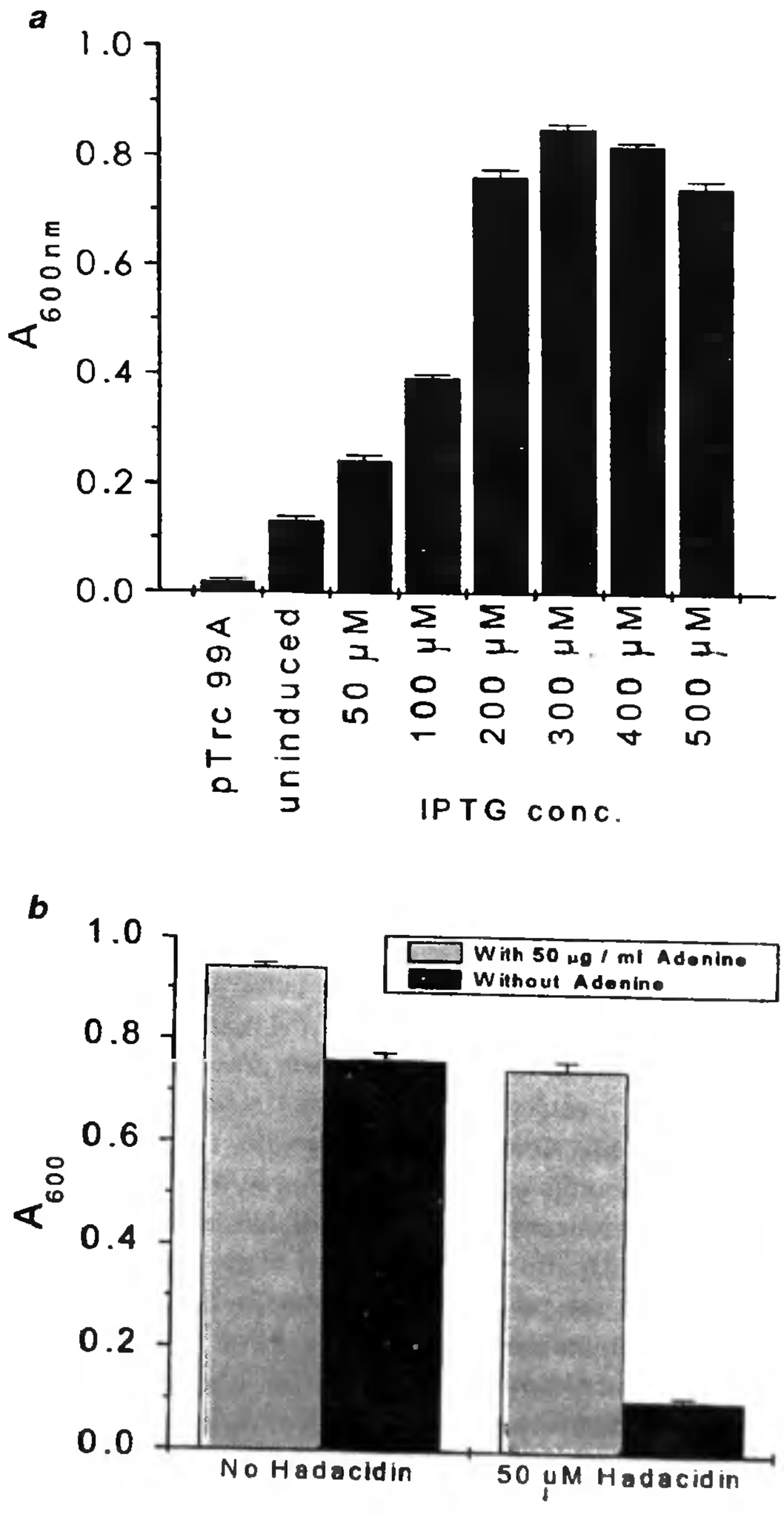


Figure 3. *a*, Growth of pTADS7/H1238 in M9 minimal medium at various concentrations of IPTG. M9 minimal medium containing 40 µg/ml of threonine, 40 µg/ml arginine and 100 µg/ml ampicillin was inoculated with 1% of overnight culture of pTADS7/H1238 grown in LB with antibiotic and washed with M9 medium. OD_{600} was recorded after 24 h of growth at 37°C. pTrc99A/H1238 grown under similar conditions was used as control. Data in this figure represent the average of three independent experiments each done in duplicate. *b*, Inhibition of pTADS7/H1238 growth in minimal medium by hadacidin. Conditions used were as in (*a*) with adenine and hadacidin added to the medium at the indicated concentrations. IPTG was added to a concentration of 200 µM.

cine (residue 303) in this segment is replaced by a lysine in the PfADSS sequence.

The GTP-binding site comprises a phosphate-binding loop and a guanine-specific binding region[30]. The glycine-rich phosphate binding loop, typical of all nucleotide binding proteins[35] and located between residues 12 and 31 is completely conserved in PfADSS. The guanine-specific binding region TKLD (residues 332–335) is also fully conserved. Residues 132–157 that participate in GTP and IMP binding also interact with the other monomer in the homodimer. Arginine 149, which interacts with the phosphate of IMP is conserved in PfADSS. In the *E. coli* ADSS crystal structure[30,31], this segment makes contacts with residues 41–49 and 219–243 of the other monomer. The sequence of both these segments is again completely conserved in PfADSS. The presence of these conserved segments which participate in catalysis along with an overall high degree of homology strongly indicate that this *P. falciparum* gene codes for an enzyme with adenylosuccinate synthetase activity.

The PfADSS gene was subcloned into the expression vector pTrc99A[16] to obtain the plasmid pTADS7. A *pur*A deficient *E. coli* strain, H1238 (ref. 36) (*arg*I61, *arg*F58, *thr*25, *pur*A54, *ton*A49), was transformed with the construct pTADS7. Figure 3 *a* shows the growth of pTADS7/H1238 in minimal medium at different concentrations of isopropyl β-D-thiogalactopyranoside (IPTG). Induction of the PfADSS gene which is under the control of the *Trc* promoter in pTADS7 produces functionally active ADSS as seen by increase in growth of cells with increase in IPTG concentration (Figure 3 *a*). Effect of hadacidin, an analogue of aspartate and a competitive inhibitor of ADSS[37], was monitored using the complementation assay. IPTG-induced cultures of pTADS7/H1238, upon treatment with 50 μM hadacidin, showed greater than 85% suppression of cell growth (Figure 3 *b*). Recovery of cell growth was seen when adenine was added to cells treated with hadacidin (Figure 3 *b*), as AMP formation is now brought about by bacterial adenine phosphoribosyltransferase. This indicates that the mode of action of hadacidin is by inhibition of the parasite ADSS proving that the parasite gene codes for an enzyme with adenylosuccinate synthetase activity.

In the intraerythrocytic parasite, the purine base hypoxanthine is actively salvaged and converted into IMP by the enzyme HGPRT[1–5]. IMP is the key precursor for AMP formation[7]. The two enzymes involved in the formation of AMP from IMP are ADSS and ADSL[7,15]. In this paper, we have reported the sequence of PfADSS, an enzyme essential for parasite survival. The expression construct, pTADS7, produces a functionally active enzyme. The genetic complementation assay described could be used to screen for novel parasite ADSS inhibitors. Purification of the hyper-expressed PfADSS followed by structural studies will aid in identification of features specific to the parasite enzyme. We are currently in the process of carrying out enzymatic analysis on PfADSS for its detailed characterization as a chemotherapeutic target.

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An agar plate assay to detect cell wall active antifungal agents

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A rapid economical plate bioassay to detect inhibitors of fungal cell wall was developed using two Sabouraud agar plates seeded with *Candida albicans*. One of them was supplemented with 0.8 M sorbitol, an osmo-protectant which prevented the lysis of *C. albicans* cells by cell wall active agents. In the absence of sorbitol, an antifungal agent gave a zone of inhibition and a reduced hazy zone in the sorbitol-supplemented plate indicated the antifungal agent to be active against the cell wall. When broth samples of 550 fungal isolates were screened, one of them giving a hazy zone was identified to be papulacandin. Thus, the newly developed plate bioassay is suited for high-throughput screening for novel fungal cell wall inhibitors.

ALL over the world, fungal infections are steadily increasing in frequency and clinical importance. This trend has been attributed to the growing number of immunocompromised patients either due to aggressive treatment for illnesses or due to infection with Human Immunodeficiency Virus (HIV)[1,2]. Even more disturbing is the growing number of reports of resistant fungi against commonly used azole antifungal agents[3]. Hence there is an urgent need for new antifungal drugs with new modes of action to combat the expanding spectrum of fungal infections. The cell wall (CW) of fungi, being unique and essential for survival and not present in mammalian cells, presents an attractive target for new antifungals[2,4]. Also, agents acting on new targets are likely to circumvent specific resistance mechanisms to existing drugs[4].

A whole cell *Candida albicans* screen to identify inhibitors of fungal CW synthesis and assembly based on osmotic support and morphological characteristics of the cells has been described earlier[5]. Damage to essential CW components by any CW active agent will lyse the cells, but they will continue to grow if a suitable osmo-protectant like sorbitol is present in the medium.

Combining this rescue of growth with sorbitol and morphological characteristics of the cell formed the basis of the Sorbitol Protection And Morphology (SPAM) assay[5]. This method involves the determination of minimum inhibitory concentration (MIC) values of the test samples by broth microdilution method in 96 well plates using Yeast Nitrogen Base (YNB) and YNB supplemented with 0.8 M sorbitol. These plates are checked for growth in each well after 2 and 7 days of incubation. CW active antifungal agents gave higher MIC in the presence of sorbitol after 7 days as cells continued to grow in the medium due to osmotic protection.

This paper describes an agar plate bioassay which is a modification of the SPAM broth assay. It is a simple method mainly for the detection of CW-active antifungal agents. The assay consists of two bioassay plates of Sabouraud agar seeded with *C. albicans*, one of which is supplemented with 0.8 M sorbitol as an osmo-protectant. Any sample showing a reduced and a hazy zone of inhibition in the sorbitol-supplemented plate, resulting from osmotic protection, as against a clear zone of inhibition in the control plate due to lysis of *C. albicans* cells, was considered to have a CW-active antifungal agent.

For bioassay, the inoculum was prepared by growing *C. albicans* ATCC 10231 in Sabouraud broth overnight and adjusting the OD to 1.0 at 600 nm (using Spectronic Genesys 5, Milton Roy). One hundred micro-liters of this inoculum was added to 40 ml per plate of molten and cooled Sabouraud agar and Sabouraud agar containing 0.8 M sorbitol to prepare two bioassay plates of 15 cm diameter. Twenty-five wells of 6 mm diameter were punched in each plate and 50 µl of the test sample and appropriate solvent controls were added to each well and the plates were incubated at 37°C for 18 h.

The assay was validated using mulundocandin, a known CW-active agent[6,7] as a positive control. Antifungal agents, viz. amphotericin B, azoles, 5 fluorocytosine and terbinafine, all of which have modes of action other than inhibition of fungal CW synthesis[2] were used as negative controls (Table 1).

A clear zone was observed for mulundocandin in the Sabouraud agar plate whereas in the presence of sorbitol, it was hazy and smaller (Figure 1). The zone sizes were proportionate to the concentration of mulundocandin in

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