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Cloning, expression and characterization of the ornithine decarboxylase gene from *Dictyostelium discoideum*

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ABSTRACT Ornithine decarboxylase (ODC) is a rate limiting enzyme in polyamine synthesis that decarboxylates ornithine to form the diamine putrescine. We report here the isolation, expression and characterization of a homolog of ODC from *Dictyostelium discoideum.* DdODC is conserved and shows sequence and structural homology with that from human. Both ODC transcript and protein are expressed at all stages of development and show high expression in prestalk/stalk cells. It is cytosolic and predominantly perinuclear in localization. Both overexpression of DdODC and putrescine treatment resulted in inhibition of cell proliferation.

KEY WORDS: *D. discoideum, ODC expression, putrescine*

Polyamines are ubiquitously present from bacteria to mammals (Tabor and Tabor 1985, Janne 2004) and regulate cell growth, proliferation, differentiation and cell death. The fast proliferating prokaryotes mostly contain putrescine and spermidine (Tabor and Tabor 1976) while the slow proliferating eukaryotes predominantly have spermidine and spermine (Fillingame 1975). A decrease in intracellular polyamines occurs during aging (Minois *et al*., 2011) but it still remains unclear whether this is a cause or consequence of aging. Addition of exogenous spermidine can extend lifespan in various animal models through epigenetic modifications, induction of autophagy and suppression of necrosis (Eisenberg *et al*., 2009).

Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyses the first and the rate limiting step of polyamine biosynthesis where L-ornithine is converted to putrescine (Heller *et al.,* 1976). It is strictly regulated during development (Heby and Persson 1990) and is involved in synthesis of spermidine and spermine. Decarboxylation of ornithine by ODC is the only pathway for *de novo* synthesis of polyamines in mammals and higher eukaryotes. The ability to strictly regulate cellular polyamine levels within a very narrow range is critical since extreme levels of polyamines (high or low) result in damaging effects, both on life of the cell and organism as a whole (Pegg and McCann 1982).

This study was undertaken to understand the role of putrescine, which is the most abundant polyamine found in *Dictyostelium discoideum* (Saran 1998). *D. discoideum,* a lower eukaryote has both unicellular and multicellular stages in its life cycle (Bonner 1944). Nutrient plays an important role and starvation triggers the vegetative amoebae to enter multicellularity. Vegetative amoeba grow and divide mitotically but upon starvation and in response to the chemoattractant, cAMP, they enter the developmental phase to ultimately form a fruiting body consisting of dead vacuolated stalk cells and viable spores. Since ODC is responsible for the formation of putrescine we characterized this enzyme. Harris and North (1982) did not find any significant ODC activity during development but Campagne and Lowik (1985) have reported high activity at the slug stage. Our earlier report (Saran 1998) has shown high putrescine levels during slug stage.

This is the first report on molecular cloning, expression and characterization of a putative ODC-like sequence from *D. discoideum*. We found it to be structurally conserved and predominantly expressed in the prestalk/stalk cells of multicellular structures. ODC fusion protein was localized in the cytosol in a large vacuole-like structure whose identity is still unclear. Both ODC overexpression (ODC^{OE}) and putrescine treatment slow growth. Putrescine treatment caused a block in the G2/M phase of cell cycle. Our results show putrescine to be more involved in cell proliferation than development.

Results and Discussion

Polyamines are essential for various processes of life but their role in either growth or development has been difficult to analyze

Abbreviations used in this paper: DdODC, Dictyostelium discoideum ODC; ODC, ornithine decarboxylase.

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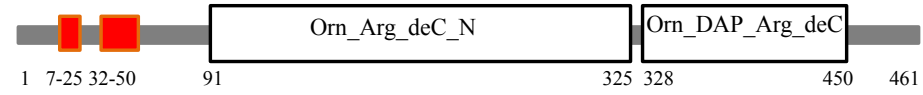

B

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D.dis       MTGTKRNGEEVVNENNNNNVAEETNKKAKVDESSTETTESTSCSLLSRCEKLDIVRKELD  60
H.sap       ------------------------------------------------MNNFGNEEFD
M.mus       ------------------------------------------------MSSFTKDEFD
                                                             .: ..*:*
D.dis       VKPWDQG---KVTIQELITSLLDKTDRDAFFVADVGVIIKQWQKWVKNLPNVKPYYAVKC  117
H.sap       CHFLDEGFTAKDILDQKINEVSSSDDKDAFYVADLGDILKKHLRWLKALPRVTPFYAVKC
M.mus       CHILDEGFTAKDILDQKINEVSSSDDKDAFYVADLGDILKKHLRWLKALPRVTPFYAVKC
             :  *:*   *  ::: *..: .. *:***:***:* *:*:  :*:* **.*.*:*****
D.dis       NPTVGVLRVLDALGTNYDCASRTEIESVLNLGVDPSRIIYANPCKQISALKFARAHNVKL  177
H.sap       NDSKAIVKTLAATGTGFDCASKTEIQLVQSLGVPPERIIYANPCKQVSQIKYAANNGVQM
M.mus       NDSRAIVSTLAAIGTGFDCASKTEIQLVQGLGVPAERVIYANPCKQVSQIKYAASNGVQM
            * : .:: .* * ** :****:***: *  ***  .*:********:* :*:*  . *::
D.dis       MTFDNLSELEKIEKFFPEAELVLRIAPDDSKSVMRFGSKFGVHIDDCNDLLEMAKEMNLK  237
H.sap       MTFDSEVELMKVARAHPKAKLVLRIATDDSKAVCRLSVKFGATLRTSRLLLERAKELNID
M.mus       MTFDSEIELMKVARAHPKAKLVLRIATDDSKAVCRLSVKFGATLKTSRLLLERAKELNID
            ****.  ** *: : .*:*:****** ****:* *:. ***. : .:  *** ***:*:.
D.dis       VVGVSFHVGSGCQSGDSYADALIMVKSVFDMAKKLNMELTLVDVGGGFTGSDDEKF--NA  295
H.sap       VVGVSFHVGSGCTDPETFVQAISDARCVFDMGAEVGFSMYLLDIGGGFPGSEDVKLKFEE
M.mus       VIGVSFHVGSGCTDPETFVQAVSDARCVFDMATEVGFSMHLLDIGGGFPGSEDTKLKFEE
            *:********** . :::.:*:  .:.****. :: :.: *:*:**** **:* *:  :
D.dis       FTKVIREKTAELFS--PNVKIIAEPGRYFAAQSHTLAVTVISKRSIKQEDNRQHPRRTSN  353
H.sap       ITGVINPALDKYFPSDSGVRIIAEPGRYYVASAFTLAVNIIAKKIVLKEQTGSDD-EDES
M.mus       ITSVINPALDKYFPSDSGVRIIAEPGRYYVASAFTLAVNIIAKKTVWKEQPGSDD-EDES
            :* **.    : *     *:********:.*.:.****.:*:*: : :*:  ..  . ..
D.dis       NMRQYNYYLADGVYGSFNNTKFDYAKVEPLLLKPSTK--QPTPCTLFGPTCDSIDVVLKD  412
H.sap       SEQTFMYYVNDGVYGSFNCILYDHAHVKPLLQKRPKPDEKYYSSSIWGPTCDGLDRIVER
M.mus       NEQTFMYYVNDGVYGSFNCILYDHAHVKALLQKRPKPDEKYYSSSIWGPTCDGLDRIVER
            . : : **: *******   :*:*:*: ** *  .   :   .:::*****.:* :::
D.dis       TQIPELKIGDWLYFQDMGAYTIASSSSFNGFCPPPVYYYNSIPEEELKNL---------- 461
H.sap       CDLPEMHVGDWMLFENMGAYTVAAASTFNGFQRPTIYYVMSGPAWQLMQQFQNPDFPPEV
M.mus       CNLPEMHVGDWMLFENMGAYTVAAASTFNGFQRPNIYYVMSRPMWQLMKQIQSHGFPPEV
             ::**:::***: *:::*** **:*::*:****  * :**  * *  :* :
D.dis       --------------------------------
H.sap       EEQDASTLPVSCAWESGMKRHRAACASASINV
M.mus       EEQDDGTLPMSCAQESGMDRHPAACASARINV
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Fig. 1. Domain architecture and multiple alignment of *Dictyostelium discoideum* ornithine decarboxylase (DdODC). (A) *Domain architecture of DdODC as predicted by* Pfam *programme. Small red bars represent low complexity regions within DdODC.* **(B)** *Multiple sequence alignment of full length ODCs from human (Hs), mouse (Mm) and* D. discoideum *(Dd). Red letters represent amino acids constituting Orn_Arg_deC-N domain and blue letters show Orn_DAP_Arg_deC domain. See text for details.*

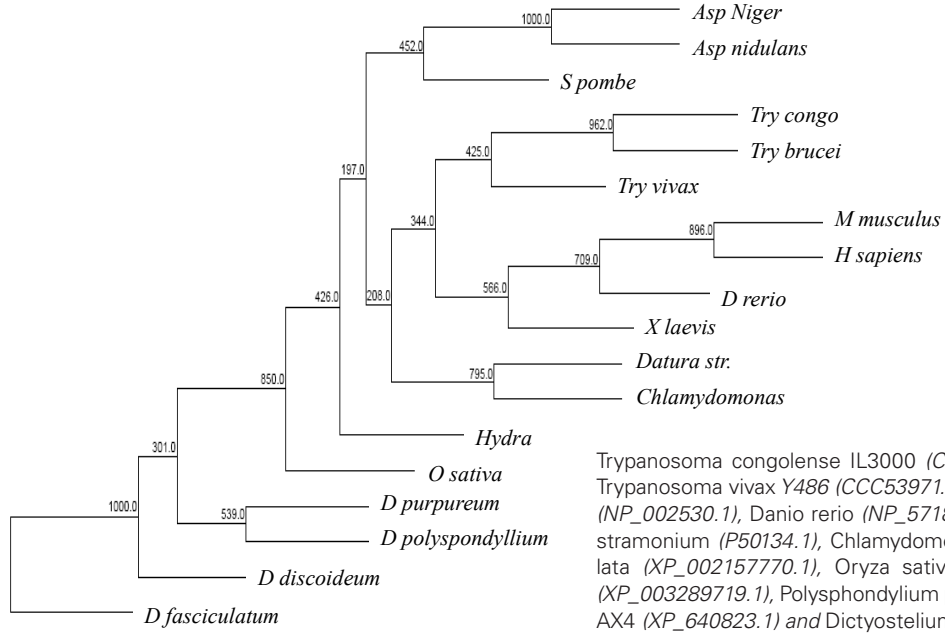

Fig. 2. *Dictyostelium discoideum* ornithine decarboxylase (DdODC) shares sequence homology with known ODC proteins. *Neighbor joining (NJ) phylogenetic trees with 1000 bootstrap replicates to create a consensus tree was drawn. All* Dictyostelium *species fall in one clade, the nearest homologue is plant* Oryza sativa *while human and mouse seems to be farthest. [*Aspergillus niger *(EHA20515.1),* Aspergillus nidulans *(CBF75254.1),* Schizosaccharomyces pombe *(NP_594665.1),* Trypanosoma congolense IL3000 *(CCD12316.1),* Trypanosoma brucei *(AAA30218.1),* Trypanosoma vivax *Y486 (CCC53971.1),* Mus musculus *(NP_038642.2),* Homo sapiens *(NP_002530.1),* Danio rerio *(NP_571876.1),* Xenopus laevis *(NP_001079692.1),* Datura stramonium *(P50134.1),* Chlamydomonas reinhardtii *(CAE46410.1),* Hydra magnipapillata *(XP_002157770.1),* Oryza sativa *(NP_001051890.1),* Dictyostelium purpureum *(XP_003289719.1),* Polysphondylium pallidum *(EFA85230.1),* Dictyostelium discoideum AX4 *(XP_640823.1) and* Dictyostelium fasciculatum *(EGG18477.1)].*

as these processes are intermingled. Here, we have chosen *Dictyostelium discoideum* as both growth and development could be studied independent of each other.

Isolation of ODC homologue in D. discoideum

D. discoideum ortholog of ODC (DdODC; gene id: DDB_G0281109) is located on chromosome 3 between coordinates 4069177 to 4070741. Based on amino acid sequence, the putative DdODC showed 49% similarity with *Xenopus laevis*, 45% with humans and 43% with *Aspergillus niger.* It has 3 exons and two introns with the genomic DNA length being 1565 bp. It is a single copy gene coding for 461 amino acids having a calculated mass of 51.7 kDa.

Domain architecture analysis shows the presence of two domains (Fig. 1A); ornithine and arginine decarboxylase (Orn_Arg_deC-N; 91-325 aa) and ornithine decarboxylase C-terminal sheet domain (Orn_DAP_Arg_deC; 328-450 aa). They are collectively known as group IV decarboxylases which are pyridoxal-dependent and act on ornithine, lysine and arginine. Sequences required for the catalytic activity and dimerization in human ODC (HsODC) are also found in DdODC. Similarly, the conserved lysine-69 residue required for attachment of pyridoxal-phosphate group and the stretch of three consecutive glycine residues proposed to participate in substrate-binding in HsODC (Fig. 1B) are also found. Glycine-387 residue essential for dimerization of monomers in HsODC is present at position 432. Sequence motif PFYAVKCN corresponding to position 64–71 of mammalian ODC containing lysine-69 residue is present at position 111-118 in DdODC except that phenylalanine is changed to tyrosine. Consensus sequence GPTCDGLD present in various eukaryotes is also found in DdODC (position 400-408) but has serine and isoleucine instead of glycine and leucine. Cysteine residue responsible for binding to difluoromethylornithine (DFMO) is present. Other conserved signature sequences/motifs like D(I/V)GGGF, FDCAS, EPGR, FNGF, and GAYT are also found though their functional significance is still unknown.

Evolutionary conservation analyses (Fig. 2) show all *Dictyostelium* species to

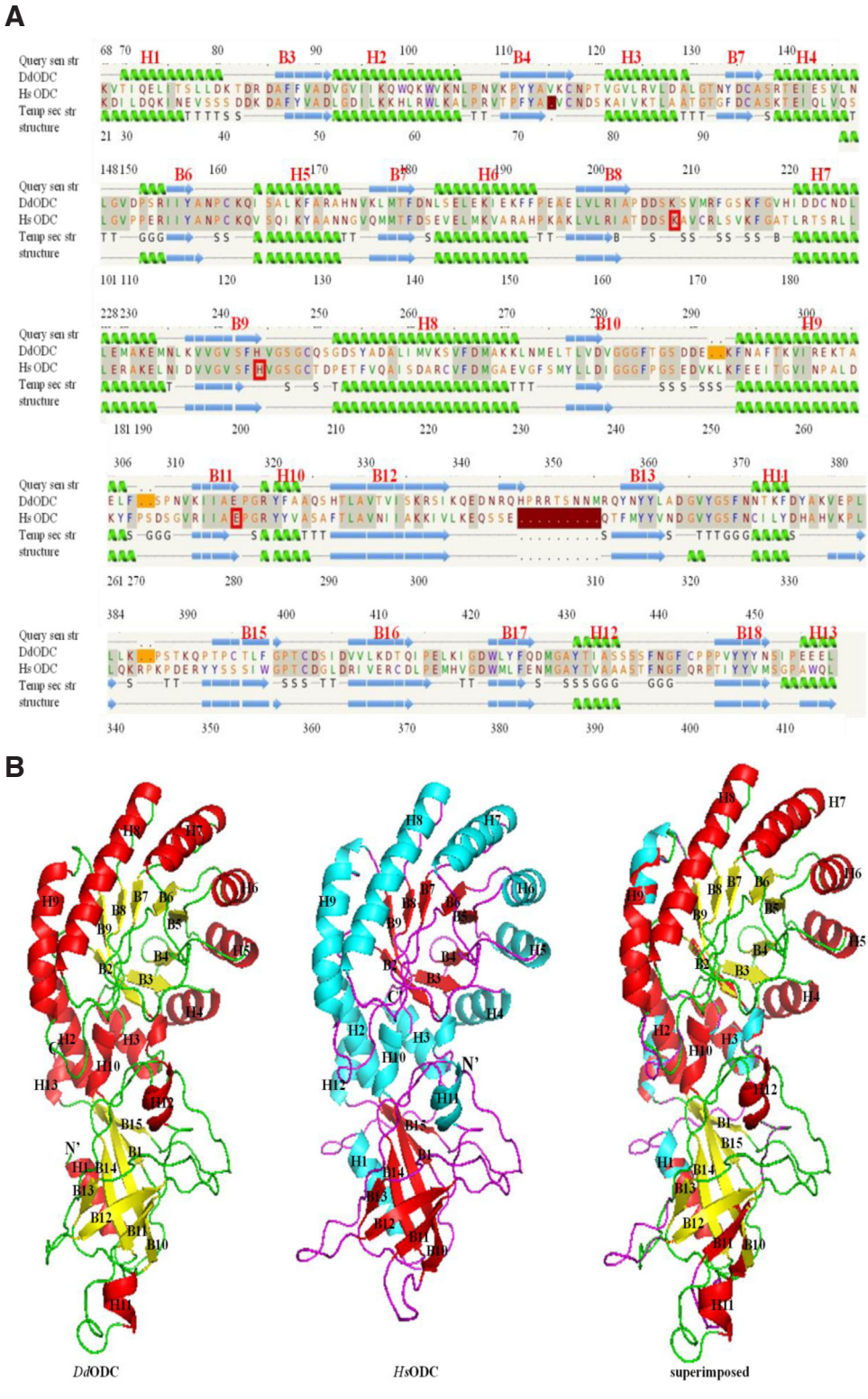

Fig. 3. Tertiary structure prediction of putative *Dictyostelium discoideum* ornithine decarboxylase (DdODC). (A) *Primary sequence alignment between human (HsODC) and* D. discoideum *(DdODC) ODCs. Secondary structure elements are labeled above the primary sequence; α-helices are identified with H, while β-strands are labeled with B. Numbering is based on HsODC. Secondary structure of DdODC was predicted by phyre2 software and compared to HsODC which showed best homology.* **(B)** *Tertiary structure of ODCs from* Dictyostelium, *human and superimposed structure of the same.*

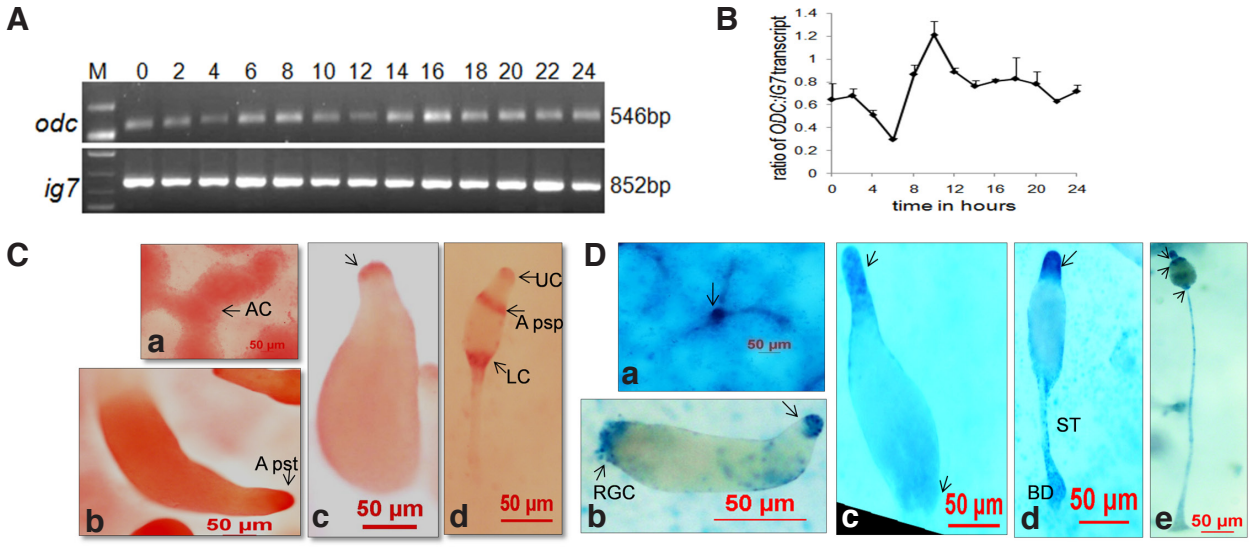

Fig. 4. Spatio-temporal RNA expression pattern of *Dictyostelium discoideum* ornithine decarboxylase (DdODC). (A) *Temporal expression pattern of* Ddodc *and its comparison to control* ig7 *as studied by RT-PCR. Time after starvation is indicated.* **(B)** *Relative expression as observed in (A) [n=3].* **(C)** In situ *hybridization by DIG-labeled* Ddodc *RNA probe.* **(D)** *Spatial expression pattern of putative* odc *promoter as detected by* ubi-LacZ. *Abbreviations: M, marker; a, aggregate; b, slug; c, early culminant; d, mid culminant; e, fruiting body; Ac, aggregate centre; Apst, anterior prestalk; UC, upper cup; LC, lower cup; Apsp, anterior prespore; RGC, rear guard cells; st, stalk; BD, basal disc).*

fall close to each other. The nearest homologue is the plant *Oryza sativa* while human and mouse sequences seem to be farthest.

Homology modeling

A comparative protein structure model was generated to study the degree of structural resemblance. Secondary and tertiary structures of the candidate protein were predicted by phyre2 (http://www.sbg.bio.ac.uk.phyre2) prediction server. Best homologous HsODC protein structure (c1d7kB) was used as template. Numbering of helices and sheets on the secondary structure (Fig. 3A) is based on that observed with HsODC (Almrud *et al.*, 2000). The homology was found to be nearly 100%. First two β-sheets, possibly present between amino acid 1 to 67 are not observed. A β-sheet comprising of 3 amino acid between β-sheets 12 and 13 is present in DdODC. Accordingly, β14-sheet present in HsODC comprising of 2 amino acid is absent in DdODC. High sequence homology and secondary structure prediction suggests that our query protein possibly is ODC which is also validated based on

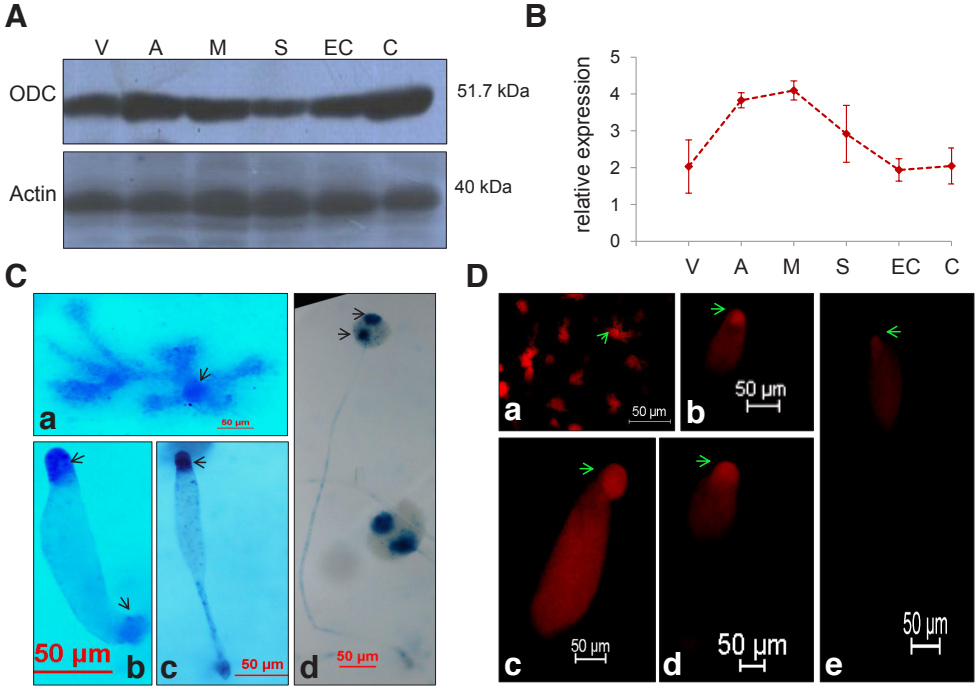

Fig. 5. Spatio-temporal protein expression pattern of *Dictyostelium discoideum* ornithine decarboxylase (DdODC). (A) *Temporal expression pattern of DdODC and its comparison to control actin as studied by Western hybridization.* **(B)** *Relative expression as observed in (A) [n=5].* **(C)** *Spatial expression pattern of ODC fusion protein as studied in Ax2/[*odc*]:*odc-LacZ. **(D)** *Immunofluorescence analysis in multicellular structures as studied using antibody against ODC. (V, vegetative; A, aggregate; M, mound; S, slug; EC, early culminant, C, culminant).*

free energy (Supplementary 1).

Predicted structure showed a confidence level of nearly 100%. Stereochemistry of the model prepared for full DdODC protein (Fig. 3B) is in agreement with most of the residues located in most favoured and additionally allowed regions of the Ramachandran plot (Supplementary 2). Residues present in the favoured regions are 90.1% and residues in the additional allowed region is 8.9%. 1.0% residues are in generously allowed region and 0% residues are in disallowed regions in the Ramachandran plot.

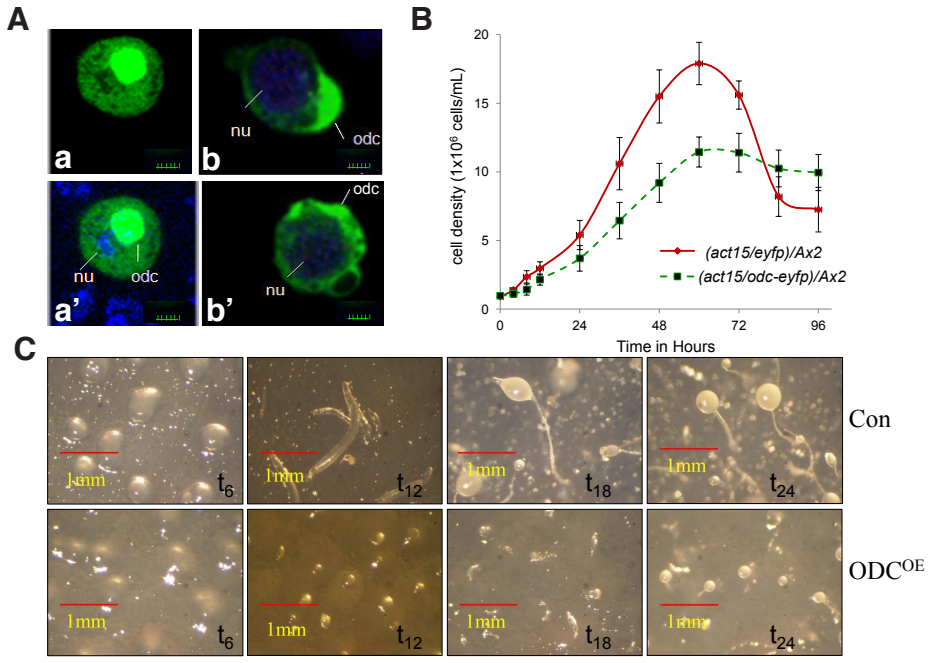

Fig. 6. Overexpression of DdODC. **(A)** *Subcellular localization of ODC-Eyfp. [Blue due to DAPI in nucleus; green is fusion protein]. a: fusion protein and a': colocalized with DAPI. b and b': two individual cells showing overlap.* **(B)** *Comparison of growth profiles of Ax2/[act15]:Eyfp and ODC^{OE} cells.* **(C)** *Comparison of developments of wild-type (Con) and ODC^{OE}. Time after starvation is indicated. [n=4].*

Expression analysis of DdODC

Expression of encoded mRNA and activity of the translated protein was found at all stages of development with higher levels during mound (t_{10}) (Figs. 4 and 5). RNA expression as studied by RT-PCR (Figs. 4 A and B), *in situ* hybridization (Fig. 4C) and fusion of *LacZ* to putative *odc* promoter (Fig. 4D), was found at all stages of development and in both cell types with higher levels in prestalk/stalk cells. Expression was high in slug tips corresponding to EcmA (anterior-most prestalk) cells and in fruiting body we found expression both in upper and lower cups and in anterior-most spore region. Minor discrepancies observed with results obtained in Fig. 4C and D could be attributed to the kind of products being visualized in both the cases.

Western analysis revealed similar results (Fig. 5 A,B). Protein expression as revealed by expression of ORF under its own promoter showed expression at all stages of development and higher levels in prestalk/stalk cells (Fig. 5C). Immunocytochemistry data revealed similar results (Fig. 5D).

Overexpression of DdODC inhibits cell proliferation and leads to mild developmental defects

Full length *odc* ORF was expressed under a constitutive promoter *actin15* as a fusion with enhanced yellow fluorescent protein (Eyfp) at C-terminal to make (Ax2[*act15*]:*odc-Eyfp*, (ODC^{OE}). The level of putrescine increased nearly 1.7 fold as compared to wild-type (data not shown). Eyfp (green) tagged DdODC was cytoplasmic with predominant intense perinuclear staining (Fig. 6A) showing high expression in vesicle like structure close to the nucleus and is in agreement with majority of earlier studies (Schipper and Verhofstad 2002). Notably, the perinuclear signal was very strong and did not overlap with lysotracker staining (data not shown). Role of ODC in the cytoplasm still needs to be elucidated but various studies indicate that polyamines may play essential roles in protein biosynthesis. Further analysis is still required to characterize the functions of ODC during cell-type differentiation.

ODC^{OE} cells grow slow and do not show a sharp decline phase (Fig. 6B). Development too was slow with mounds being formed at 8h after starvation as compared to 6h in case of wild-type. Slug formed at 18h as compared to 12h for wild-type. Maximum delay

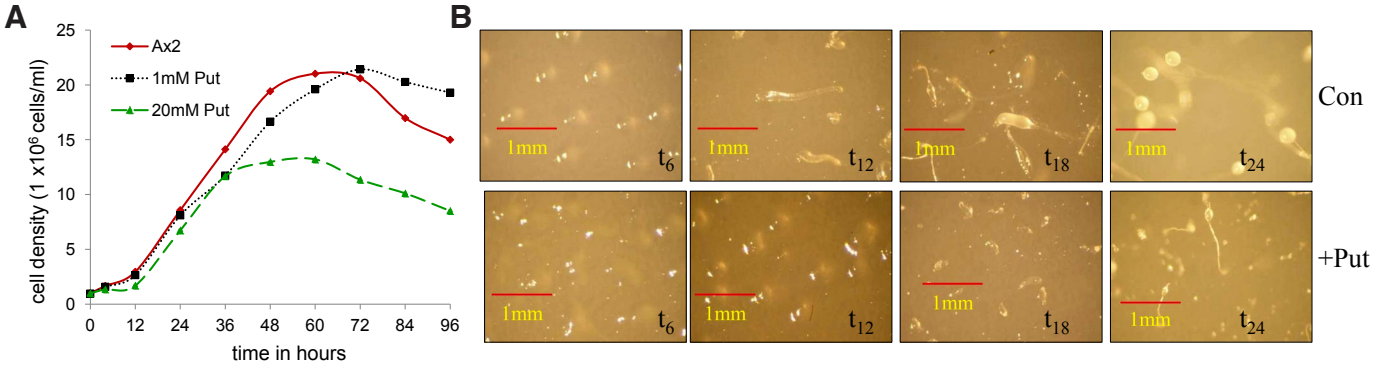

Fig. 7. Growth and development after addition of exogenous putrescine. (A) *Growth profile in presence of low (1 mM) and high (20 mM) concentrations of putrescine.* **(B)** *Development profiles of wild-type and putrescine treated cells developed in the absence of putrescine. [n=4-5].*

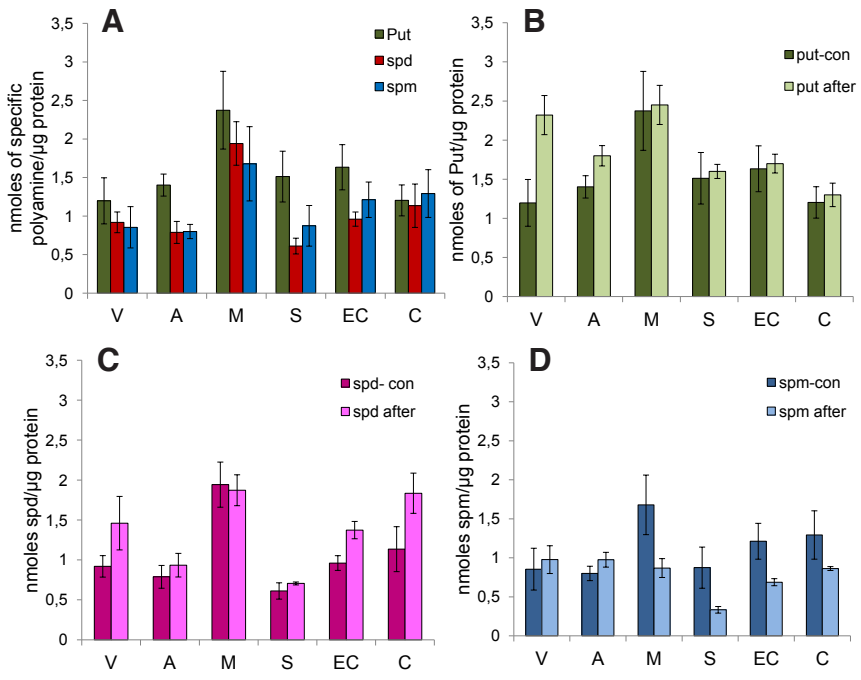

Fig. 8. Levels of various polyamines in control and after putrescine treatment during development. (A) *Levels of putrescine (Put), spermidine (Spd) and spermine (Spm) during development.* **(B)** *Levels of putrescine* **(C)** *spermidine and* **(D)** *spermine in control and after putrescine treatment during development. Cells were treated for 48 hours with 20 mM putrescine and allowed to develop in the absence of putrescine. (V, vegetative; A, aggregate; M, mound; S, slug; EC, early culminant; C, culminant). [n=6].*

was in forming slugs but time taken to form fruiting bodies was far less. Additionally, number of aggregates and sizes of the multicellular structures formed were smaller in comparison to wild-type (Fig. 6C).

Polyamines are essential for promoting cell growth but excessive accumulation of putrescine provokes apoptosis (Takao *et al.,* 2006). In case of *D. discoideum,* there is no apoptosis but autophagic cell death occurs during stalk cell formation (Swer *et al.*, 2014a, b). Polyamines increase Ca^{2+} accumulation in mitochondria; modulate mitochondrial permeability transition, and trigger cell death. In addition, elevation of cytoplasmic Ca^{2+} can lead to production of reactive oxygen species and vice-versa (Minois *et al.,* 2011). Oxidative stress could disrupt calcium homeostasis by promoting membrane lipid peroxidation and covalent modification of transporters (Jain and Shohet 1981; Kaneko *et al.*, 1994). Both oxidative stress and disruption of Ca^{2+} homeostasis appear to contribute to autophagy (Swer *et al.*, 2014b). We still need a clear understanding of calcium, ODC levels in the dying population which undergo autophagy in *D. discoideum*.

Exogenous putrescine inhibits growth

To address if the observed developmental defects were due to an increase in putrescine levels or of any other polyamines, we treated the cells with exogenous putrescine and monitored its effect both on growth and development. Lower concentrations of putrescine did not show any significant change in growth profile but at higher concentrations effect was similar to that observed with ODC^{OE} (Fig. 7A). Development had slowed down till slug formation taking nearly 6h extra in comparison to wild-type (Fig. 7B). Henceforth, development was near normal. Putrescine treatment formed smaller multicellular structures. Our unpublished results suggest that spermidine (4 mM) and spermine (0.35 mM) treatments further reduced the size and number of multicellular structures in comparison to putrescine treatment. Sizes of multicellular structures formed decreased as control>putrescine>spermidine>spermine treatments. Results confirmed that high putrescine levels were detrimental for cell proliferation in *D. discoideum* and brought about small developmental defects in comparison to other polyamines.

During normal development, high level of putrescine followed by spermidine and spermine at all stages of development is observed. Levels of all three were found to be highest at mound stage which coincides with the timing of initiation of cell-type gene expression. Level of spermine marginally increased over that of spermidine from migrating slug onwards, while the level of putrescine comparatively dropped reaching to near equal amounts by the time of culmination (Fig. 8A). To confirm if putrescine levels were important for the observed developmental defects, we treated the cells with exogenous putrescine (20 mM) for 48h and measured the levels of various polyamines at specific developmental stages and compared them to that observed with wild-type (Fig. 8). Results show nearly two-fold increase in putrescine levels in freshly starved cells (V), thereafter, levels decreased to control levels (Fig. 8B). An initial increase in the level of spermidine was observed and later a gradual increase over control after slug stage (Fig. 8C) was seen. Effect was just opposite for spermine levels (Fig. 8D) where we found a decrease over the control after mound stage till culmination. Results, thus confirmed putrescine to be largely involved in cell proliferation while spermine and spermidine in controlling development which prompted us to analyze their behaviour during cell-cycle progression (Fig. 9). The precise mechanism by which putrescine show growth inhibition remains to be elucidated but possibly by accumulation in G2/M phase of cell cycle. It is known that the expression profile of both, polyamines and cyclins/cdks show changes through cell-cycle but

TABLE 1

OLIGOS USED IN THIS STUDY

set	name	Oligo: 5′ to 3′
1.	*In situ*	FP-HindIII-5′- CATGAAGCTTTAGTATTAAGAATTGCACCAGATG -3′ RP-BamHI-5′-AGATGGATCCGTAATAATAAACTGGTGGTGGACA -3′
2.	RT-PCR-*odc*	FP-5′- GATGCGGATCCACAAAAAGAAATGGTGAAGAAGTTG-3′ RP-5′- GATCGGTACCTTCACTAAGATTATCAAATGTCAT -3′.
3.	RT-PCR-*ig7*	FP-5′-GGATTCTGCAAAATGGCAAC-3′ RP- 5′-GTCCTCTCGTACTAAAGGAAGG-3′
4.	ODC^{OE}	FP:(BamHI):5′- ACGCGGATCCAATCAATCTAAAACTATAAATTTAATATATAATG-3′ RP:(XhoI):5′- ACTACTCGAGTATAACCAATTGAAAAACCTGAAATTATATGAG-3′
5.	ODC-P	FP-XbaI- 5′- AGTCTCTAGAATAGATCAACCATTGAAATCACTTG -3′ RP-BglII- 5′- AGTCAGATCTGGTAGATTCAGTGGTTTCAGTTG -3′
6.	ODC-P-ORF	FP-BglII 5′- CTGAAGATCTCAATTAAATTTCCAAAAATGAGC-3′ RP-BglII 5′-ACTAAGATCTATAACCAATTGAAAAACCTGAAATTATATG-3′

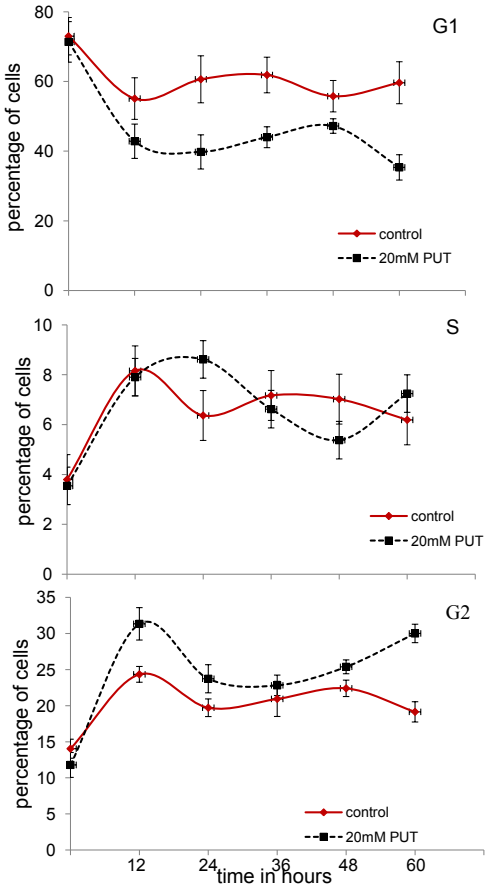

Fig. 9. Cell cycle analysis after addition of exogenous putrescine. *Percentage of cells in each phase of cell-cycle in treated and control cells. [n=4].*

their interaction remains to be defined.

In conclusion, we identified, cloned and characterized a novel ODC from *D. discoideum* genome. ODC expression was found at all stages of development with highest during initiation of differentiation. It was largely expressed in the dying cell population. We report that accumulation of putrescine inhibits cell proliferation but changes in developmental pattern are largely due to effective changes in spermidine and spermine levels. Our findings here advance our understanding of DdODC at the molecular level and permits future work in this area. It would be interesting to find the correlation between polyamines, calcium and ROS in bringing about autophagy in this organism.

Materials and Methods

Cell culture, growth and development

D. discoideum, Ax2 (axenic strain) cells (unicellular) were grown and developed according to Gosain *et al.,* (2012). The medium was supplemented with antibiotics as indicated.

Bioinformatic analyses of DdODC

Genomic DNA, cDNA and protein sequences of DdODC were obtained from dictyBase online resource (http://www.dictybase.org). Domain architecture of DdODC protein was deduced by Simple Modular Architecture Research Tool (http://SMART.embl-heidelberg.de). ODC orthologues were searched by Basic Local Alignment Search Tool (BLASTp) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), UniProt and dictyBase. Multiple alignments were performed using ClustalW2 at EBI server ((http://www.ebi.ac.uk/Tools/clustalw2/). PHYLIP package (Phylogeny Inference Package, version 3.68) was used to construct Neighbor joining (NJ) phylogenetic trees with 1000 bootstrap replicates to create a consensus tree.

RNA detection by in situ *hybridization and RT-PCR*

In situ hybridization: It was carried by the method of Gosain *et al.,* (2012). Probes were obtained by *in vitro* transcription of exonic region after cloning in commercially available pBluescriptII phagemid vector. RNA (genomic position 772-1529 bases) was synthesized from exonic region amplified by PCR using the primers set1 (Table 1) and cloned into HindIII/BamHI site of vector (supplementary 3). Construct pBSIISK+ (*odc* probe) was digested with HindIII to yield template for antisense probe synthesis by T3 RNA polymerase, while digestion with BamHI yield template for sense probe synthesis by T7 RNA polymerase. Sense and antisense probes were processed for hydrolysis due to their larger size (0.757kb).

RT-PCR: This was carried out by the method described in Gosain *et al.,* (2012). RT-PCR reactions were performed using gene specific primer set2 for *odc* and set3 for *ig7* (internal control) (Table1).

Protein detection by Western hybridization and immunocytochemistry

Western hybridization was essentially carried out as given in Swer *et al.,* (2014b) while immunocytochemistry was performed according to Alvarez-Curto *et al.,* (2007). Anti-ODC1 (#HPA001536) and anti-actin (#04-1040) antibodies were purchased from Sigma. The HRP conjugated secondary antibody was from Chemicon International (#AP307P) and was detected with Supersignal chemoluminescence kit (Pierce, USA) according to the manufacturer's instructions while the TRITC conjugated secondary antibody was from Sigma (#T6678).

Plasmid construction and transformation

p[*act15*]:*odc-Eyfp: Ddodc* gene was PCR amplified using primer set4 (Table1) from genomic DNA and constitutively expressed under *actin15* as a fusion protein with Eyfp at C-terminal (Gosain *et al*., 2012).

p[*odc*]:*ubi-LacZ*: 1104bp *odc* intergenic region from genomic DNA was PCR amplified using primer set5 (Table1). Product was digested with XbaI/BglII and ligated into vector upstream and in frame with *ubi-LacZ* open reading frame (ORF) sequence of p*EcmAO/63ubi-LacZ* vector, replacing *ecmAO* promoter to drive expressions of *ubi-LacZ* (ubiquitinated *ile-gal*).

p[*odc*]:*odc:LacZ*: Putative promoter with ORF region (2.51 Kb) of *Ddodc* was PCR amplified using primer set6. PCR product and *pEcmAO/LacZ* vector were digested with BglII and ligated in a non-directional cloning, replacing *ecmAO* promoter. Direction of insert was confirmed by internal digestion with XbaI which is present at 963 bp. Vectors were individually transformed in Ax2 cells and selected with 100μg/mL of G418.

X–gal staining of developing structures

Transformants were developed on treated dialysis membranes and processed for β-galactosidase staining as given in Swer *et al.,* (2014a).

Measurement of various polyamine levels

Polyamine was estimated according to Saran (1998) with slight modifications. Briefly, portions of perchloric acid extracts were dansylated with an equal volume of dansyl chloride (5mg/mL of acetone) in presence of saturating Na_2CO_3. Excess dansyl chloride was removed by addition of proline (1g/mL of water) and incubated in dark. Mixture was then extracted with benzene and the organic phase was collected and loaded on TLC plates. Chromatogram was developed with cyclohexane: ethyl acetate (5:4, v/v), dried, visualized and photographed using excitation wavelength 366nm and emission wavelength 495nm. Spots developed were quantified using spot densitometry program of Alpha imager. Values were obtained using standard curve prepared with different concentrations of mix and calculated on the basis of protein content.

Cell cycle analysis: Cell cycle analysis was carried out according to the protocol followed by Swer *et al.,* (2014b).

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