Developmental effect of polyamine depletion in *Caenorhabditis elegans*

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Ornithine decarboxylase (ODC) catalyses the conversion of ornithine to putrescine, an obligate precursor to the polyamines spermidine and spermine. We reported previously that homozygous *odc-1 (pc13)* worms have no detectable ODC activity. Despite their inability to make polyamines, these mutant worms appear normal, but with a slight reduction in total brood size, when grown in complex medium that presumably contains polyamines. We now show that when ODC-deficient worms are transferred to polyamine-free medium, they show a strong phenotype. *odc-1* worms have two different fates, depending upon the developmental stage at which polyamines are removed. If the polyamines are removed at the L1 larval stage, the mutant animals develop into adult hermaphrodites that produce very few or no eggs. In contrast, if mutant larvae at the later L4 stage of development are transferred to polyamine-deficient medium, they develop and lay eggs normally. However, approx. 90 % of the eggs yield embryos that, although well differentiated, arrest at early stage 3. Either maternal or zygotic expression of ODC provides partial rescue of embryonic lethality. Supplementing deficient medium with the polyamine spermidine allows ODC-deficient worms to develop as on complex medium. Together, these findings suggest that ODC activity is most critically required during oogenesis and embryogenesis and, furthermore, that exogenous polyamines can override the requirement for ODC activity.

INTRODUCTION

Polyamines are ubiquitous and necessary for cell growth. Although micro-organisms or cultured cells from metazoans cannot grow without polyamines, growth resumes when they are provided exogenously [1,2,3]. Ornithine decarboxylase (ODC) catalyses the synthesis of putrescine (1,5-diaminobutane), a polyamine precursor. The polyamines spermidine and spermine are formed by the sequential enzymic addition to putrescine of one or two aminopropyl groups. ODC is highly conserved in structure among eukaryotes [4]. When ODC activity is perturbed, either genetically or pharmacologically, normal cell growth eventually ceases [2,5]. This growth inhibition can be overcome in single-cell organisms and cultured cells by simply adding polyamines to the culture medium. Thus cells appear to be indifferent to whether they produce or take up polyamines, as long as they are available. Is this conclusion, valid for micro-organisms and cultured metazoan cells, also true of multicellular organisms?

Some information relevant to this last question is available. Both biosynthesis and uptake contribute significantly to polyamine pools in rodents [6,7]. When pregnant mice are treated with difluoromethyl ornithine (DFMO), a specific suicide inhibitor of ODC, they resorb their embryos [8,9]. The temporal window of vulnerability to fetal wastage is confined to gestational days 7 and 8. This suggests that polyamine synthesis is important for specific developmental events and that uptake from exogenous sources (food, gut flora) may be insufficient. Such an experiment cannot determine whether termination of pregnancy is the result of maternal or zygotic ODC deficiency. *Caenorhabditis elegans* mutant *odc-1* (*pc13*) worms, referred to subsequently as *odc-1*, have no detectable ODC activity [4]. This mutation has only a minor phenotypic effect: a reduction in the number of progeny [4]. Several mechanisms could, in principle, account for the relatively benign effect of the mutation: mutant worms utilize a polyamine-biosynthetic pathway that does not require ODC, do not have a null *odc-1* allele, or obtain functionally adequate polyamine stores by uptake. Our previous experiments render unlikely, but do not preclude, the first and second of these mechanisms [4]. One way to investigate whether the mutant worms require and can use exogenous polyamines is to determine the consequences of depriving them of such an external source.

The development and genetics of *C. elegans* have been studied extensively, providing a favourable experimental context for understanding the consequences of polyamine deprivation [10,11]. These worms have two sexes, male and hermaphrodite. Progeny are produced either by self-fertilization (zygotes produced by union of hermaphrodite eggs and sperm) or by cross-fertilization (zygotes produced by union of hermaphrodite eggs and male sperm). Each adult hermaphrodite can lay about 300 eggs. Upon hatching, the animal goes through four larval stages (L1 through to L4) before becoming an adult. The completion of the life cycle takes 3 days under normal conditions at 20 °C.

We show here that depriving ODC-deficient worms of external polyamines leads to developmental disruption. When *odc-1* worms are transferred to polyamine-free medium as L4 larvae, their eggs arrest at early stage 3 of embryogenesis. If polyamines are removed from the medium earlier in development, at the L1 larval stage, development proceeds normally to adult stage, but eggs are not produced. Behaviourally, the adult animal is somewhat sluggish and has the appearance of being starved. Together, these findings suggest that ODC activity is most critically required during oogenesis and embryogenesis and, furthermore, that exogenous polyamines can override the requirement for ODC activity.

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethyl ornithine.

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MATERIALS AND METHODS

Preparation of polyamine-free bacteria

Escherichia coli HT289 (*spe*A, *spe*B, *spe*C, thr, leu), which is deficient in polyamine-biosynthetic enzymes [1], was grown overnight in M9 minimal medium [12] supplemented with 100 μg/ml threonine, 410 μg/ml leucine and 10 μM putrescine. The bacteria were pelleted and washed once with M9 in order to remove excess putrescine. The washed pellet was resuspended in M9 and used to inoculate M9 minimal medium supplemented with threonine, leucine and 8.5 μM cadaverine (1,5-diaminopentane). The cadaverine-supplemented bacterial culture was grown overnight at 37 °C.

Media for worm growth

Worms were routinely raised on NG agar plates (also referred to as rich medium) seeded with bacteria as described previously [13] with some modifications. *E. coli* HT289 was used as the food source. To establish polyamine auxotrophy of the *odc-1* strain, worms were transferred to plates containing 1.7 % (w/v) agarose in S-medium [13]. These plates, referred to as polyamine-deficient, were seeded with HT289 bacteria grown in cadaverine-supplemented M9 medium. Worms were incubated at 20 °C. To test for auxotrophy, polyamine-deficient plates were supplemented with the polyamine spermidine at a concentration of 10 μM, or with other concentrations where noted.

Worm strains

The *odc-1* strain has been described previously [4]. Bristol strain N2 was used as an isogenic wild-type control. Wild-type strain N2, *dpy-11*(*e224*) and *unc-42*(*e270*) used in this study were obtained from the *Caenorhabditis* genetics centre in Minnesota.

Analysis of the sterile adult and the embryonic-arrest phenotypes

Single L1 *odc-1* larvae were transferred from NG agar plates to polyamine-deficient plates or to identical plates supplemented with spermidine. Larval growth was examined every day. The rate of development of the mutant *odc-1* larvae was compared with that of the wild-type N2 strain. The adult worms were monitored for their ability to produce progeny. The mutant adults were further examined under Nomarski optics to determine the presence of eggs.

To assess the arrest phenotype of mutant embryos, single L4 larvae were transferred from NG plates to individual polyamine-deficient plates supplemented with 0 or 10 μM spermidine. The animals were allowed to lay eggs. Hatching was monitored. Any hatched animals were removed from the plates. The eggs that did not hatch after 24 h were scored as arrested. Some of the arrested embryos were examined under Nomarski optics to determine the extent of their development. Wild-type N2 strain was used for comparison.

Polyamine measurement

To measure polyamine pools, starved L1 larvae were transferred from rich medium plates to polyamine-deficient plates. The worms were allowed to develop to L4/adult stage and then washed off the plates in M9 buffer and pelleted. Worm pellets were stored frozen at -80 °C until used. The polyamine content of worm extracts was measured by HPLC as described previously [14].

Matings

Crosses were carried out by placing an individual L4 male with a single L4 hermaphrodite on an NG agar or S-medium agarose plate, as required. Success of mating was scored by the percentage of males produced in each cross, which is expected to be 50 % for cross-progeny and less than 0.1 % for self-progeny.

RESULTS

Identification of a defined medium lacking polyamines

We have previously described mutant worms [*odc-1* (*pc13*)] apparently devoid of ODC activity [4]. These worms could be auxotrophic for polyamines, but a medium deficient in polyamines is needed in order to test this. *C. elegans* is commonly grown in the laboratory on medium supplemented with *E. coli* [13]. Mutant bacteria that cannot make polyamines are themselves polyamine auxotrophs [1]. We screened a series of polyamine analogues to identify those that sustained growth of mutant *E. coli*, but could not themselves support the growth of mutant worms. Cadaverine (1,5-diaminopentane), an analogue of putrescine (1,4-diaminobutane) had this property. *odc-1* worms did not produce progeny on polyamine-deficient medium containing mutant *E. coli* raised on cadaverine, unless further supplemented with putrescine or spermidine (e.g. see Table 1). In contrast, wild-type worms grew normally on this medium without further supplementation with polyamines. We refer to defined medium supplemented with *E. coli* grown in this way as 'polyamine-free', even though, strictly speaking, it contains an unnatural diamine.

Mutant *odc-1* L1 larvae develop into sterile adults in polyamine-deficient medium

To investigate the effect of polyamine deficiency on the development of *odc-1* worms, L1 larvae were cloned on polyamine-free plates and their development was observed daily under a dissecting microscope. The development of the mutant worms

Table 1 L1 larvae develop into sterile adults in polyamine-deficient medium

L1 larvae were transferred from rich to polyamine-deficient plates and allowed to develop. The number of progeny produced by mutant worms was compared with that produced by wild-type worms. In addition, the effect of supplementing mutant worms with 1 μM spermidine was determined. Mutant adults were either sterile or produced very few eggs, which arrested as embryos under polyamine-deficient conditions; in contrast, mutants supplemented with polyamines produced progeny that were similar in number and viability to the wild-type. + spd, worms grown on spermidine-supplemented plates; n.d., not determined.

Experiment	1	2	3	4	5
Wild-type					
Gravid/total	5/5	8/8	7/7	10/10	5/5
Mean for eggs laid	172	n.d.	166	n.d.	135
odc-1					
Gravid/total	0/10	0/7	7/10	1/10	2/5
Mean for eggs laid	0	0	22	0.5	5
odc-1 + *spd*					
Gravid/total	7/7	5/5	8/8	n.d.	5/5
Mean for eggs laid	189	n.d.	192	n.d.	202

Table 2 Determination of polyamine pools in wild-type and mutant *odc-1* strains

Starved L1 larvae [wild-type (wt) and mutant] were transferred from NG plates to polyamine-deficient plates and allowed to develop. Worms were harvested at the L4/adult stage, and washed in M9 buffer. Extracts were prepared and the polyamine content was determined. Values shown are means ± S.D. for four independent experiments with wild-type strain, and two with *odc-1* strain.

	Polyamines (pmol/μg protein)				
Strain	Putrescine	Cadaverine	Spermidine	Aminopropyl cadaverine	Spermine
wt	11.55 (±1.17)	nd*	13.95 (±2.35)	16.66 (±2.06)	nd
odc-1	nd	nd	nd	26.40 (±0.43)	nd

* nd, not detectable at 0.05 pmol/μg protein

was compared with that of wild-type worms treated identically. The mutant worms completed larval development at about the same rate as wild-type. However, in contrast to wild-type, the mutant worms contained and laid few eggs or, indeed, none at all (Table 1). In three out of five experiments, a few eggs were apparent in the mutant animals (Table 1), but almost all of those laid failed to hatch. This implies that ODC activity facilitates development to the gravid adult stage, and that enzymic deficiency results in an incompletely expressed sterile phenotype. Also, the movement of adult mutant worms appeared somewhat sluggish. To confirm that the sterile adult mutant phenotype of the *odc-1* strain was indeed due to polyamine deficiency, mutant worms were grown on plates supplemented with either putrescine or spermidine. Animals grown on polyamine-supplemented plates produced eggs and progeny much like the wild-type animals (Table 1), demonstrating auxotrophy of the mutant worms and confirming that *C. elegans* requires polyamines for normal development.

To establish whether sterility was associated with a lack of polyamines, extracts were prepared from both *odc-1* and wild-type worms, and polyamines were measured. No putrescine or spermidine was detected in the mutant worms (Table 2). In contrast, the wild-type worms had levels of putrescine and spermidine more than 200-fold in excess of the minimal levels detectable. The spermidine homologue aminopropyl cadaverine, but not cadaverine, was present in both mutant and wild-type extracts; cadaverine was fully metabolized to aminopropyl cadaverine in this experiment, and partially so in other experiments, e.g. see Table 3, a process that takes place in both *E. coli* and the worms (supporting results not shown). In summary, L1 larvae deprived of polyamines become eggless adults.

Polyamines are required during embryogenesis

To determine whether the arrest phenotype was dependent upon the stage at which polyamines were withdrawn, we repeated the same experiment, but using L4 mutant larvae in place of L1 larvae. L4 larvae were individually transferred from rich medium to polyamine-deficient plates and allowed to lay eggs. Wild-type and polyamine-supplemented mutant worms were studied in parallel. Polyamine-starved mutant animals laid significantly fewer eggs than wild-type animals or polyamine-supplemented mutant animals (Figure 1). Additionally, most of the non-supplemented mutant embryos failed to hatch. A few mutant embryos (4–15%) proceeded further to the pretzel stage and some hatched. The hatched animals developed at the same rate as wild-type, but arrested as sterile adults. Therefore the *odc-1*

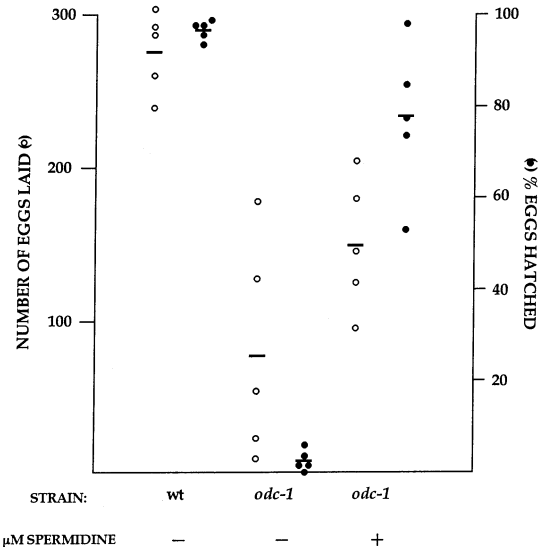

Figure 1 Polyamine deficiency results in embryonic arrest

Mutant L4 larvae raised on rich medium plates were transferred to polyamine-deficient plates supplemented with either 0 or 10 μM spermidine. Wild-type N2 strain, treated identically, served as the control. Worms were allowed to develop and lay eggs. Hatching was monitored. Embryos that did not hatch within 24 h were scored as arrested. Each data point represents the progeny of a single worm. (○) number of eggs laid, (●) percent eggs hatched. The horizontal bars indicate the group means.

mutation is completely penetrant but incompletely expressed. Supplementation of *odc-1* worms with polyamines mitigated, but did not fully eliminate, developmental arrest; approx. 80% of eggs hatched compared with almost 100% for wild-type (Figure 1).

The arrested embryos produced by polyamine-starved hermaphrodites were examined under Nomarski optics (results not shown). Nerve cells and gut granules were clearly seen. Twitching was observed in the mutant embryos, an indication that muscle cells were formed. Taken together, these data imply that the mutant embryos were generally well-differentiated and indicate that embryogenesis proceeded to at least the 550-cell stage in these embryos.

Two steps in *C. elegans* development are specifically sensitive to polyamine depletion

Depending upon when larvae were transferred from complex medium to the polyamine-deficient environment, the mutant worms had two different fates: L1 larvae developed into sterile adults, and L4 larvae laid eggs that usually failed to hatch. Two explanations are possible for these distinct outcomes: (i) the need for polyamines is most restrictive at these two steps or (ii) development stops whenever internal polyamine pools are sufficiently depleted, which happens at a different stage depending on whether external supplies are withdrawn at the L1- or L4-larval stage. To distinguish between these possibilities, we raised worms on different amounts of polyamines before transfer of L1 larvae to polyamine-deficient plates. If hypothesis (ii) is correct, we would expect different degrees of loading of polyamine pools to result in arrest at several different points. First, in order to determine the minimum polyamine requirement for proliferation, worms were raised on polyamine-deficient medium supplemented with 0.001, 0.01, 0.1 or 1 μM spermidine. Supplementation with 0.1 μM spermidine was the minimum polyamine concentration

Table 3 Preloading worms with varying concentrations of spermidine repletes polyamine pools in a dose-dependent fashion

Mutant worms were raised on polyamine-deficient plates supplemented with 0, 0.1, 1 or 10 μM spermidine until the L4/adult stage. Wild-type worms without supplementation were analysed in parallel. Worms were harvested and polyamines determined as before.

	Polyamines (pmol/μg protein)				
Strain	Spermidine supplement (μM)	Putrescine	Cadaverine	Spermidine	Aminopropyl cadaverine
Wild-type	0	39	< 1	53	49
odc-1	0	< 1	14	4	63
odc-1	0.1	< 1	12	5	49
odc-1	1	3	7	23	52
odc-1	10	17	< 2	27	45

sufficient to maintain growth of mutant worms for multiple generations (results not shown). We next preloaded worms with polyamines, using supplementation with 0.1, 1 or 10 μM spermidine, and then transferred their progeny, as L1 larvae, to polyamine-deficient medium. An average of nine, 55 and 89 eggs were laid by those worms whose mothers were raised on 0.1, 1 and 10 μM spermidine respectively; none of these hatched. We showed above that L1 larvae whose mothers were raised on complex medium developed into sterile adults when transferred to polyamine-deficient plates; these produced between 0 and 22 eggs [mean = 5.5, $n = 5$ (Table 1)]. This outcome suggests that biologically available polyamines present in rich medium are approximately equivalent to those present in polyamine-deficient plates supplemented with 0.1 μM spermidine. Direct measurement of the polyamine content of rich medium revealed 0.18 μM spermidine (and 0.93 μM putrescine), not inconsistent with this expectation.

We measured polyamine pools in extracts of worms raised on different concentrations of spermidine to determine whether we had indeed repleted them in a dose-dependent fashion. Table 3 shows that mutant worms raised on 1 μM spermidine had 5-fold more polyamines (putrescine plus spermidine) than those raised on 0.1 μM spermidine, and mutants raised on 10 μM spermidine had still higher levels of polyamines. This result is roughly in accordance with the number of embryos produced by the progeny of mutant hermaphrodites preloaded with these different concentrations of spermidine. A comparison of the data in Tables 2 and 3 suggests that the effectiveness of excluding polyamines from the diet of the mutant worms varied among experiments, but was sufficient to impose biologically effective starvation.

In order to determine the minimum concentration of polyamines sufficient to allow larvae to become gravid adults, we transferred L1 larvae raised on either polyamine-deficient medium supplemented with 0.1 μM spermidine or on rich complex medium to polyamine-deficient plates supplemented with 0, 0.01, 0.03, 0.1, 0.3 or 1 μM spermidine. Worms under each experimental condition grew to adult stage. However, worms transferred to plates supplemented with 0.01 μM or higher concentrations of spermidine became gravid; in contrast, worms transferred to polyamine-deficient plates grew to sterile adults independent of whether they were raised on plates supplemented with 0.1 μM spermidine or rich medium. This confirms that adequately repleting polyamine pools allows L1s to progress to gravid adult stage. Furthermore, a concentration of 1 μM spermidine provided full rescue, whereas lesser concentrations provided graded degrees of rescue (results not shown). This is consistent with the internal polyamine pools: polyamine levels in extracts from mutant worms raised on medium supplemented with 1 μM spermidine are similar to those from wild-type worms (Table 3). In conclusion, a polyamine requirement is most crucial to two steps: production of eggs by hermaphrodites and completion of embryogenesis. None of the various methods used to perturb polyamine pools succeeded in eliciting additional discernible aberrations of either morphology or development.

Homozygous mutant progeny of heterozygous hermaphrodites display incompletely penetrant zygotic lethality

Polyamine withdrawal from mutant worms results in developmental arrest, as described above. To determine whether the requirement for ODC activity is maternal or zygotic, we examined the progeny of heterozygous *odc-1* worms. If a maternal source of ODC mRNA, ODC protein or polyamines is sufficient, a homozygous *odc*-1 null mutation will have no effect in the first generation and the *odc-1* heterozygotes will segregate according to Mendelian genetics, yielding no deficiency of *odc-1* homozygous progeny. *odc-1* heterozygotes were obtained by crossing *dpy-11* males with *odc-1 unc-42* hermaphrodites. *dpy-11* and *unc-42* flank the ODC gene [4]; in this experiment, *unc-42* serves as a marker linked to *odc-1* and enables *odc-1* homozygous progeny to be readily scored as unco-ordinated (Unc) worms. Figure 2 shows that maternal ODC is not fully sufficient; there is about a 3-fold reduction in the frequency of Unc progeny of *odc-1 unc-42* heterozygotes compared with isogenic control *unc-42* heterozygotes. This result suggests that zygotic expression of ODC may be important to assure development of the worm embryo. Zygotic expression of ODC is not absolutely required for worm development, but its deficiency significantly reduces survival.

Zygotic expression of ODC mitigates embryonic lethality

We next asked the converse question: can zygotic expression of *odc-1* prevent the developmental consequences of maternal polyamine deficiency? To answer this question, we compared the outcome of mating homozygous wild-type males with homozygous *odc-1* hermaphrodites versus homozygous *odc-1* males with homozygous *odc-1* hermaphrodites. Wild-type males were also mated with wild-type hermaphrodites as an additional control. For each mating, a single hermaphrodite and one or more males were transferred to polyamine-deficient medium as L4 larvae. Because self-fertilization produces few males ($\approx$ 0.1%) and cross-fertilization produces about 50% males, the success of matings, and therefore of zygotic rescue, could be assessed by scoring the percentage of males produced in the F1 generation. Wild-type males mated with *odc-1* hermaphrodites produced a percentage of males in the F1 generation similar to that in the wild-type $\times$ wild-type cross, 30% in both, indicating that in these matings about 60% of the progeny were cross-

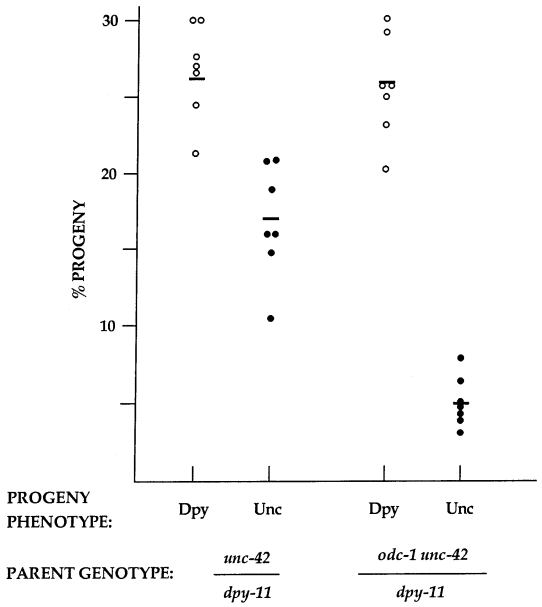

Figure 2 Homozygous progeny of heterozygous mutant mothers fail to complete development in polyamine-deficient medium

Seven ODC wild-type homozygous (+ + *unc-42*/*dpy-11* + +) or ODC mutant heterozygous (+ *odc-1 unc-42*/*dpy-11* + +) L4 larvae were transferred to polyamine-deficient plates (one per plate) and allowed to lay eggs. *dpy-11* and *unc-42* served as closely linked genetic markers that flank *odc-1*. The percentage of homozygous *dpy* (○) and *unc* (●) progeny produced by both strains was determined three days later. The mean numbers of progeny produced by ODC wild-type homozygous and ODC mutant heterozygous mothers were 166 (range 100–235) and 180 (range 140–201) respectively. The horizontal bars indicate the group means.

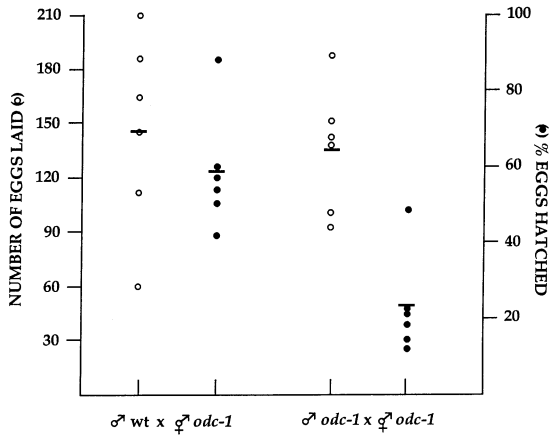

Figure 3 Wild-type males can partially rescue *odc-1* embryonic arrest phenotype

Mutant hermaphrodites were crossed with either wild-type or mutant males. Matings were performed on polyamine-deficient plates with one hermaphrodite and one to four males per plate. The number of eggs laid per hermaphrodite (○) and the percentage of eggs hatched (●) was determined. The horizontal bars indicate the group means.

progeny. In contrast, the *odc-1* male × *odc-1* hermaphrodite cross produced approx. 7% males. Cross-fertilization is thus much less frequent (about 14%) or, alternatively, *odc-1* is male-lethal. The latter interpretation is unlikely, as male progeny are as readily obtained in *odc-1* as in a wild-type background. The viable progeny produced by the cross wild-type male × *odc-1* hermaphrodite was significantly higher than that produced by the cross *odc-1* male × *odc-1* hermaphrodite (Figure 3). This result suggests that wild-type males can indeed provide rescue from embryonic lethality and confirms the importance of zygotic expression. However, the rescue was incomplete (approx. 59% of the eggs hatched in comparison with almost 100% for wild-type × wild-type cross); presumably rescue would have been more complete had the fraction of cross-progeny exceeded the 60% observed in this experiment. The number of eggs laid in the wild-type male × *odc-1* hermaphrodite cross (an average of 147) was similar to that of *odc-1* × *odc-1* cross (an average of 134), suggesting that ODC expression is not needed to start embryogenesis, but is needed to successfully finish this process.

ODC deficiency impairs male fertility

The above data, 14% cross-progeny versus 60% for wild-type, suggest that *odc-1* males are relatively infertile. To determine whether ODC deficiency indeed affects male fertility as well as that of hermaphrodites, we mated *odc-1* homozygous hermaphrodites with *odc-1* homozygous males. These matings, unlike those described in Figure 3, were performed on polyamine-rich medium (NG plates). As above, the number and fraction of male progeny was used to assess the efficiency of cross-fertilization. A control consisting of a wild-type to wild-type cross yielded the expected number of progeny (almost 300) and fraction of males (43–52%) among five independent matings. However, the male fraction was lower, and quite variable, when ODC-deficient males were mated with either *odc-1* or wild-type hermaphrodites (Figure 4). This result implies that ODC activity may be important for fertility of males even if, as here, the medium is replete with polyamines. The difference in number of progeny between *odc-1* male × *odc-1* hermaphrodite and *odc-1* male × wild-type hermaphrodite crosses is a reflection of a lower number of self-progeny produced by *odc-1* hermaphrodites in rich medium [4].

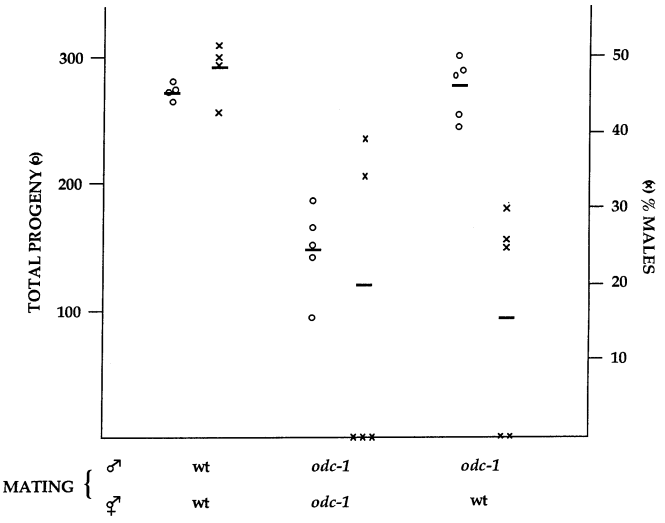

Figure 4 *odc-1* males do not mate efficiently on rich medium

odc-1 males were mated with either mutant or wild-type hermaphrodites. Mating of wild-type males × wild-type hermaphrodites was performed in parallel as a control. Crosses were performed by mating one male with one hermaphrodite per rich medium plate. The success of mating was determined by the percentage of males produced in the F1 generation. The horizontal bars indicate the group means.

DISCUSSION

Polyamines are ubiquitous in living organisms. Eukaryotic ODC activity is elaborately regulated in species ranging from yeast to humans, suggesting the utility of precise adjustment of polyamine pools [15]. It was therefore surprising that *C. elegans odc-1* mutants grew normally and showed no adverse effects, except for a slight reduction in progeny number. By establishing polyamine-free conditions for the growth of worms, we have demonstrated the dependence of the mutants on exogenous polyamines. This observation confirms our previous conclusions, made on the basis of genetic and biochemical evidence, that *odc-1* (*pc13*) is a null mutation and that ODC activity is a prerequisite for polyamine biosynthesis in *C. elegans*.

We found that polyamine depletion resulted in one of two fates, depending upon when it was imposed. Earlier depletion, at the L1 stage, resulted in animals that were morphologically adult but did not contain or lay eggs. Mutant worms transferred to polyamine-deficient medium at the L4 stage produced embryos that failed to hatch and arrested at about the 550-cell stage. Some embryos continued development beyond this stage: in some experiments as many as 20 % hatched and developed to the adult stage at approximately the same rate as the wild-type animals, except that the mutant animals never became gravid. This implies that either mutant embryos are heterogeneous in their polyamine stores, or development can proceed unimpeded if embryos stochastically escape stage-specific arrest. The L4 larval stage lasts about 10 h. The observed heterogeneity of outcome (Figure 1) may result from a more severe effect of imposing starvation earlier in L4.

Escapees traverse subsequent developmental landmarks unimpeded until they arrive at the next of the two arrest points identified here. This observation further supports the conclusion that polyamine depletion causes stage-specific defects. We tested additionally for the ability of *odc-1* worms to differentiate into an alternative L2 form (dauer form) in response to general nutrient starvation; this property was not discernibly altered by polyamine reduction.

ODC activity may be required to proceed efficiently beyond the 550-cell stage, or its deficiency earlier in embryogenesis may cause a phenotypic effect that only becomes apparent later. The *par* mutation exhibits such a phenotype: Par activity is required very early during embryogenesis to determine the cell-cleavage pattern [16], but *par* embryos arrest late and are differentiated. Wild-type males incompletely rescued the *odc-1* phenotype. This implies that *odc-1* does not produce a strict maternal effect and that zygotic expression of ODC promotes worm development. Conversely, *odc-1* heterozygotes produced homozygous mutant progeny, but with about a 3-fold reduction in number. Taken together, these results indicate that although zygotic expression of ODC promotes embryonic survival, it is not always sufficient for completion of embryogenesis. Apparently, either maternal or zygotic expression of ODC or polyamine supplementation can augment the yield of viable progeny.

A polyamine requirement for embryogenesis was not unexpected. When mice were treated with the ODC inhibitor DFMO during days 7–8 of pregnancy, the embryos were reabsorbed [8]; this period coincides with a transient rise of ODC activity in the pregnant uterus [9]. The contragestational effects of DFMO were further confirmed in rat and rabbit [9]. Lowkvist et al. [17] noticed two peaks in the activity of the ODC enzyme and polyamine levels in chicken embryos: one just before gastrulation and the other before early neurulation. Similar to the results in pregnant mice, inhibition of ODC activity and polyamine biosynthesis in early chicken embryos blocked their development at gastrulation [17]. In our previous experiments, we did not detect high levels of ODC activity in embryos, perhaps because we measured the activity of pooled unstaged embryos.

Different means and degrees of reducing polyamine pools yield graded effects. These are listed below in order of decreasing severity. (1) *odc-1* mutants show developmental arrest in polyamine-free medium, the arrest point depending on when polyamines are removed (Table 1 and Figure 1). (2) Either maternal or zygotic expression mitigates the effect of deprivation, but inefficiently. Maternal expression alone reduces the yield of viable progeny by about 3-fold compared with normal (maternal plus zygotic) expression (Figure 2). Zygotic expression alone (from the paternal allele) reduces the yield of viable progeny about 2-fold compared with maternal plus paternal expression (Figure 3). (3) In the absence of any ODC activity, polyamine supplementation (using rich medium, [4]) or spermidine supplementation of minimal medium (Figure 1) supports a yield of progeny of 70–80 % that of wild-type. (4) Heterozygosity for an *odc-1* mutation has no apparent effect; *odc-1* is haplo-sufficient in the worm.

None of the experimental manipulations of polyamines described here fully corrected the small but reproducible reduction of brood size in ODC-deficient worms. This could be due to failure to deliver polyamines at optimal times and amounts. Alternatively, there may be another mutation tightly linked to *odc-1*, despite the fact that the *odc-1::Tc1* strain used to generate *odc-1* (*pc13*) was out-crossed more than ten times [4]. Direct addition of polyamines to embryos cannot provide rescue (results not shown), but permeability barriers probably underlie this result. As with polyamine depletion at the L1 stage, a sterile adult phenotype is also observed in *spe* mutants [18,19,20]. This mutation is due to defects in sperm production and can be rescued by mating with a wild-type male. Since the *odc-1* phenotype could not be rescued completely by mating with wild-type males, the *odc-1* phenotype is not due solely to defective sperm.

odc-1 males do not mate efficiently on polyamine-rich plates. This phenotype is not completely penetrant; among individual crosses, some mutant males did not mate at all, some mated inefficiently, while others mated like wild-type. We do not know whether the fertility defect results from fewer sperm, defective sperm, or from the inability to copulate. It is striking that mutant worms, even when provided with access to exogenous polyamines, are moderately impaired in hermaphrodite fertility, but more severely defective in male fertility.

Polyamine pool control is complex and multilevel, employing mechanisms that depend on transcription, translation and degradation to regulate uptake, synthesis and catabolism [15]. What utility results from such elaboration of regulation? Transgenic mice that produce ODC in excess are phenotypically normal, but for impaired spermatogenesis [21]. Other transgenic mice that are activated in polyamine catabolism, resulting in accumulation of putrescine and acetylspermidine at the expense of spermidine and spermine, suffer predominantly from hair loss and hypoplasia of female reproductive organs [22]. Clearly, redundancy of control is present, but the adequacy of available regulatory mechanisms can be exceeded. Excluding polyamine biosynthesis, as in the *odc-1* mutant, produces a relatively modest effect on function and fecundity, at least for hermaphrodites. A much stronger phenotype is observed when polyamines are excluded from the environment, but this condition is difficult to impose in the laboratory and presumably rarely arises in nature. It appears likely that a fairly broad range of polyamine values is consistent with optimal biological function, and that

control systems act to preclude excursions outside these boundaries.

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