model of calcification (*16*), zooxanthellae enhance the production of metabolic CO_2 by the host which cannot be removed and the carbon pool becomes diluted, leading to a depletion of the $\delta^{13}C$ record in skeletal carbonate. The $\delta^{13}C$ record in Fig. 1 suggests the CO_2 removal problem becomes particularly acute after *T. maxima* reaches sexual maturity and energy allocation between calcification and reproduction changes.

This isotopic evidence reveals (i) the existence of two distinctly different growth phases in a molluscan species that are associated with the onset of sexual maturity and (ii) that the onset of sexual maturity is reflected in the character of both the $\delta^{18}O$ and $\delta^{13}C$ records. Such pronounced changes have not been described before in mollusks (*2–4*), perhaps because the few temperate species studied to date reach sexual maturity comparatively early, in their first or second year of life. It appears that for *Tridacna* the energetic priorities change upon maturity when energies normally expended on calcification during summer months may be diverted to gametogenesis and spawning. Annual cycles in the $\delta^{18}O$ profile record a marked reduction in the growth rate of *T. maxima* at this point and reveal a life-span of several decades. The $\delta^{13}C$ profile also shows a marked change with the onset of sexual maturity suggesting that with the change in energy allocation comes an increased utilization of CO_2 from the host-zooxanthellae metabolic relationship. Furthermore, the overall carbon profile indicates that zooxanthellae have the effect of depleting the skeletal $\delta^{13}C$ record in mollusks that host symbionts.

These results may have implications for paleobiological studies because the same techniques may be used to interpret the records of fossil shells (*17*), such as fossil tridacnids (their fossil record begins in the Eocene), and to study changes in growth rate, longevity, and onset of sexual maturity, important factors in gauging the role of heterochrony as an agent of evolution (*18*). Analyses of the carbon records of fossil tridacnids in particular may reveal when the symbiotic relationship with the zooxanthellae began. Other fossil bivalve genera thought to host algal symbionts (certain rudists, inoceramids, and cardiids) may show similarly depleted $\delta^{13}C$ records. Such genera, which need to be in the euphotic zone, could be valuable paleodepth indicators in sedimentary sequences. Finally, because oxygen isotopic analyses of fossil *Tridacna* are used in investigations of ancient sea levels from raised coral terraces (*7*), it is important to understand that these organisms may or may not calcify during an entire seasonal range of paleotemperatures, depending on sexual maturity, and therefore that ontogenetic considerations are required in sampling of the shell for isotopic studies.

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Osmotic Adaptation by Gram-Negative Bacteria: Possible Role for Periplasmic Oligosaccharides

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The cyclic (1 → 2)-β-D-glucans produced by species of *Agrobacterium* and *Rhizobium* resemble the membrane-derived oligosaccharides of *Escherichia coli* in their periplasmic localization, intermediate size, and (1 → 2)-β-D-glucan backbones. The regulation of the biosynthesis of cyclic (1 → 2)-β-D-glucan by *Agrobacterium tumefaciens* is now shown to parallel the osmotic regulation of membrane-derived oligosaccharide biosynthesis in *Escherichia coli*. This result suggests a general role for periplasmic oligosaccharides in the osmotic adaptation of Gram-negative bacteria as ecologically diverse as enteric and soil bacteria.

OSMOTIC ADAPTATION IS A PROBlem for living cells of every kind because the plasma membranes of cells are freely permeable to water, but impermeable to most cytoplasmic solutes. Mammals have solved this problem by developing an elaborate system for the regulation of the osmolarity of the extracellular fluid in which cells are bathed, so that the osmotic differential across the cytoplasmic membrane is minimized. In contrast, many species of bacteria are capable of growth in environments of wide ranges of osmolarity. Very little is known about the molecular basis of osmotic adaptation in bacteria, but the importance of this problem is increasingly recognized and it is the subject of considerable interest and research (*1–4*). An understanding of the fundamental mechanisms of osmotic signaling and adaptation in bacteria may elucidate similar adaptations in higher plants, a topic of agricultural and economic importance (*4–6*).

The cytoplasm of bacteria, like that of other cells, contains essential constituents with a minimum total concentration of about 300 mos*M*. In a medium of low osmolarity, water will tend to flow into the

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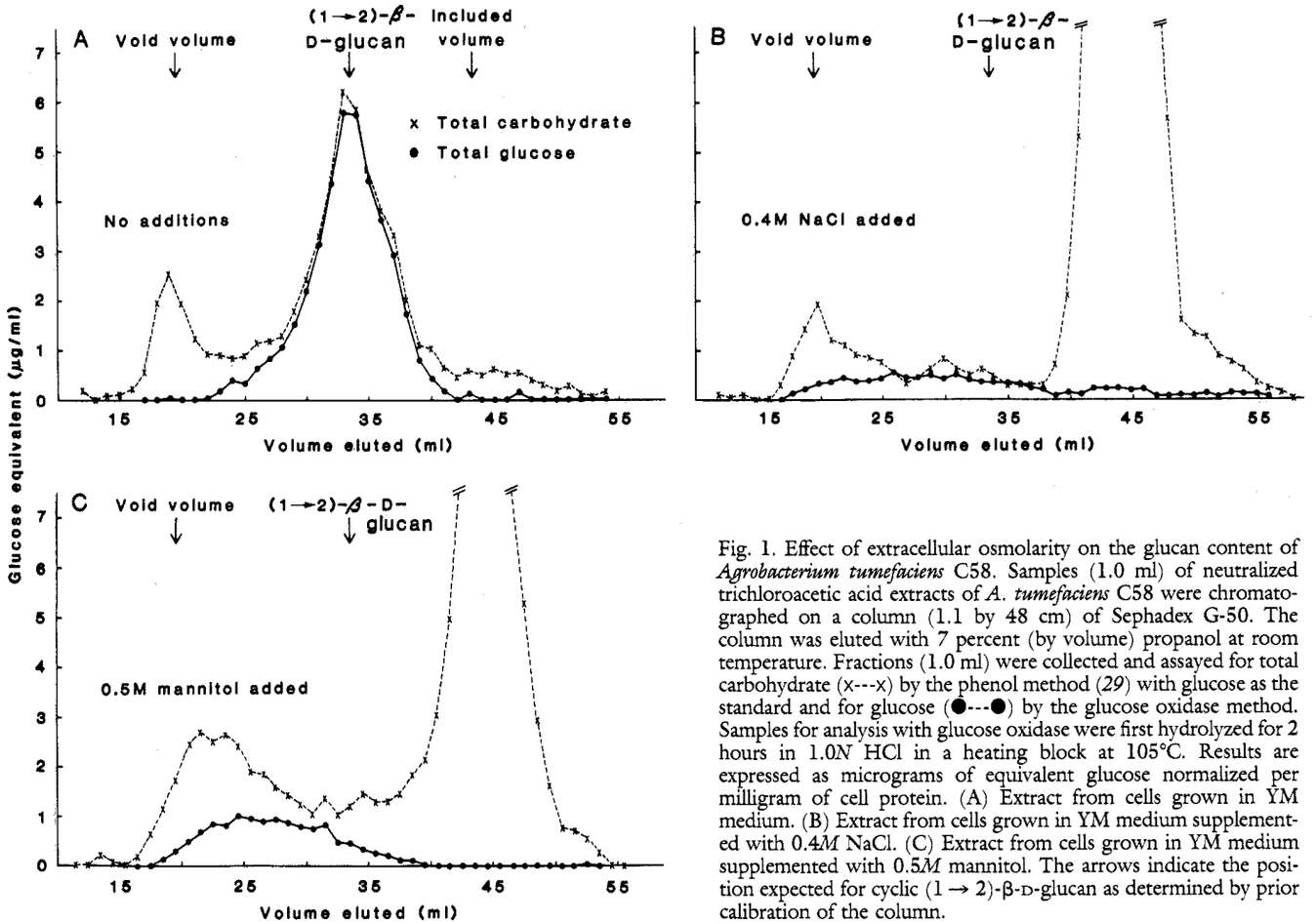

Fig. 1. Effect of extracellular osmolarity on the glucan content of *Agrobacterium tumefaciens* C58. Samples (1.0 ml) of neutralized trichloroacetic acid extracts of *A. tumefaciens* C58 were chromatographed on a column (1.1 by 48 cm) of Sephadex G-50. The column was eluted with 7 percent (by volume) propanol at room temperature. Fractions (1.0 ml) were collected and assayed for total carbohydrate (x---x) by the phenol method (*29*) with glucose as the standard and for glucose (●---●) by the glucose oxidase method. Samples for analysis with glucose oxidase were first hydrolyzed for 2 hours in 1.0*N* HCl in a heating block at 105°C. Results are expressed as micrograms of equivalent glucose normalized per milligram of cell protein. (A) Extract from cells grown in YM medium. (B) Extract from cells grown in YM medium supplemented with 0.4*M* NaCl. (C) Extract from cells grown in YM medium supplemented with 0.5*M* mannitol. The arrows indicate the position expected for cyclic (1 → 2)-β-D-glucan as determined by prior calibration of the column.

cell, causing it to swell. Adaptations required for the growth of bacteria under these conditions have been little studied. We now describe adaptations of Gram-negative bacteria to this form of osmotic stress.

Gram-negative bacteria are characterized by a complex envelope structure which includes two membranes. The cytoplasmic or inner membrane of the Gram-negative cell is separated from the outer membrane by a distinct aqueous compartment, the periplasmic space. In their fundamental study of the periplasmic compartment of *Escherichia coli* and *Salmonella typhimurium*, Stock *et al.* (*7*) reported that this compartment may represent 20 to 40 percent of the total volume of the cell. Although the outer membranes of these bacteria are permeable to hydrophilic molecules of molecular weight up to approximately 600 (*8*), Stock and co-workers (*7*) provided evidence that the periplasm is iso-osmotic with the cytoplasm and that a Donnan potential exits across the outer membranes of these bacteria. They concluded that the periplasmic space must contain bound anions amounting to about 150 to 200 microequivalents per gram (dry weight) of cells. In their inventory of the known constituents of the periplasmic space, a considerable fraction of the osmotically active solute and bound anion remained unidentified.

In 1982, Kennedy reported that the synthesis of a class of anionic periplasmic oligosaccharides within *E. coli*, the membrane-derived oligosaccharides (MDO), is osmotically regulated (*9*). The membrane-derived oligosaccharides of *E. coli* are composed of 6 to 12 glucose units linked by β-(1 → 2)- and β-(1 → 6)-bonds (*10*) and are variously substituted with phosphoglycerol, phosphoethanolamine, and *O*-succinyl ester residues (*11*). When cells of *E. coli* are grown in a medium of low osmolarity (about 30 mos*M*), MDO may represent as much as 5 to 7 percent (dry weight) of the cell, and may constitute a considerable fraction of the fixed anions in the periplasm. However, when *E. coli* is grown in a medium of high osmolarity (600 mos*M*), very little MDO is synthesized. The synthesis of these periplasmic oligosaccharides is, thus, a manifestation of osmotic adaptation in *E. coli*.

The surface carbohydrates produced by members of the two closely related Gram-negative genera, *Agrobacterium* and *Rhizobium*, have been studied because of the biological importance of these organisms and because of the possible involvement of these carbohydrates in the plant infection process. The well-characterized cyclic (1 → 2)-β-D-glucans produced by these bacteria appear to resemble the MDO of *E. coli* in several respects. (i) Previously, the cyclic (1 → 2)-β-D-glucans of *Rhizobium trifolii* have been shown to be localized within the periplasmic space (*12*). In the present study, we show that this is also true for *Agrobacterium tumefaciens*. (ii) The cyclic (1 → 2)-β-D-glucans are soluble oligosaccharides of similar size to MDO (*13–16*). (iii) Both MDO and the cyclic glucans possess a β-(1 → 2)-linked glucan backbone.

We now report evidence that the synthesis of periplasmic cyclic (1 → 2)-β-D-glucans by *A. tumefaciens* is osmoregulated in a manner strikingly similar to that of the MDO of *E. coli*.

Cultures (1 liter) of *A. tumefaciens* C58 (*17*) were grown at room temperature in YM medium of low osmolarity (Table 1). The cells were harvested by centrifugation and the cell-associated glucan was extracted with 1 percent (weight to volume) trichloroacetic acid (Table 1). The extract was chromatographed on Sephadex G-50 yielding one major glucose-containing peak in

Table 1. Effect of added solutes on cell-associated glucan content of *Agrobacterium tumefaciens* C58.

Additions to YM medium*	Cell-associated glucan† (μg glucose/mg cell protein)		
	Total	Neutral [cyclic (1 → 2)-β-D-glucan]	Anionic
No additions	37.4	18.3	19.1
NaCl (0.5*M*)	4.1	1.2	2.9
Mannitol (0.5*M*)	6.1		
K_2SO_4 (0.27*M*)	4.3		
$(NH_4)_2SO_4$ (0.27*M*)	5.5		
Lactose (0.4*M*)	11.4		
Sucrose (0.4*M*)	7.4		

*YM medium contained: K_2HPO_4 (5.74 m*M*), KH_2PO_4 (7.35 m*M*), $MgSO_4 \cdot 7H_2O$ (0.81 m*M*), NaCl (1.71 m*M*), $CaCl_2 \cdot 2H_2O$ (0.27 m*M*), monosodium glutamate (5.91 m*M*), mannitol (54.88 m*M*), and Difco yeast extract (0.1 percent, weight to volume) at *p*H 6.8. YM medium was supplemented by added solutes as indicated. Cells were harvested at a cell density of 0.20 to 0.42 mg of protein per milliliter. †Cell pellets were extracted at room temperature for 10 minutes with 1 percent (weight to volume) trichloroacetic acid in a final volume of 15 ml. Supernatants were removed after centrifugation at 3000*g* at room temperature for 20 minutes. The cell pellets were extracted a second time, and the supernatants were neutralized by the addition of NH_4OH, concentrated in a jet of air at 37°C, and analyzed on Sephadex G-50 as described in Fig. 1. Total glucan content refers to the total amount of glucose eluting in the peak containing cyclic (1→2)-β-D-glucan on Sephadex G-50 (see Fig. 1). In two experiments, this glucose-containing peak was further fractionated on DEAE-cellulose (as described in Fig. 2), and the amounts of neutral and anionic oligosaccharides (micrograms of glucose per milligram of cell protein) were determined. The neutral material, eluted in the void volume on DEAE-cellulose, was identified as cyclic (1→2)-β-D-glucan (see Fig. 2 and text).

the position expected for cyclic (1 → 2)-β-D-glucan (Fig. 1A).

Further fractionation of this G-50 peak by chromatography on DEAE-cellulose led to the separation of three fractions (Fig. 2). Fraction 1, not adsorbed on DEAE-cellulose, was identified as described below as the cyclic (1 → 2)-β-D-glucan characterized by other workers (*13–16, 18–21*). The amount of fraction 1 ranged from 13.6 to 19.9 μg of glucose equivalent per milligram of total cell protein. It accounted for 49 to 60 percent of the total glucose equivalent applied to the DEAE column. Glucose, measured by the glucose oxidase method after acid hydrolysis of samples, accounted for 91 to 100 percent of the total carbohydrate of this fraction. This glucan was further analyzed by permethylation (*22*) followed by reductive cleavage and acetylation (*23*). The only product detectable by gas chromatographic analysis (*24*) was 2-*O*-acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol. The absence of 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol indicated that the glucan was cyclic and composed of glucose linked solely by 1 → 2 glycosidic bonds. The optical rotation of a solution of glucan of 2.42 mg/ml in 50 percent (by volume) ethanol was −0.024°, corresponding to a specific rotation $[\alpha]_D^{24} = -9.92°$, characteristic of a β-glucan. Finally, the molecular weight distribution of these (1 → 2)-β-D-glucans was determined by fast atom bombardment mass spectrometry of the permethylated material. Fraction 1 was found by this means to be a mixture of seven cyclic species, containing 17 to 23 glucose residues, in good agreement with previous determinations of the size of cyclic (1 → 2)-β-D-glucans (*13–16*).

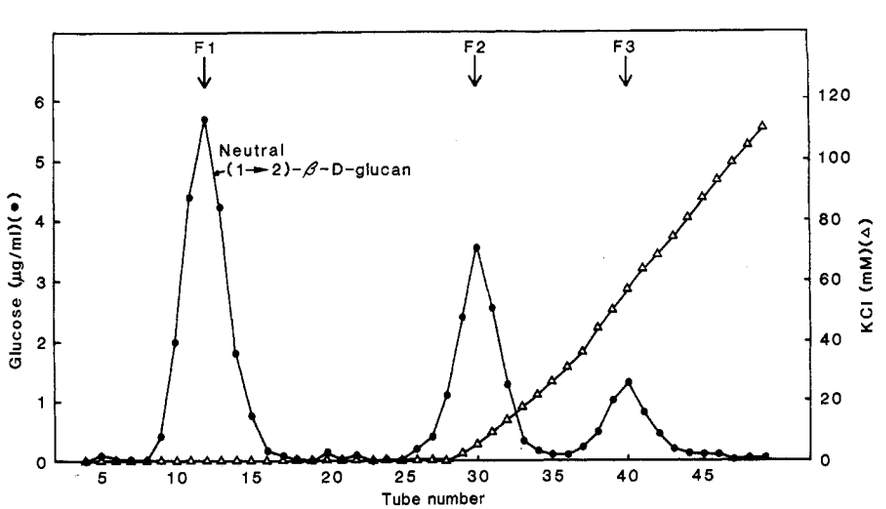

Fig. 2. Separation of glucans by DEAE-cellulose chromatography. Sephadex G-50 fractions containing cyclic (1 → 2)-β-D-glucan extracted from *A. tumefaciens* C58 grown in YM medium were pooled and concentrated. The concentrated sample (2 ml) was further fractionated on a column (1.1 by 24 cm) of DEAE-cellulose (Whatman DE-52). The column was eluted first at room temperature with 10 m*M* tris buffer (*p*H 7.4) in 7 percent propanol and then by a linear gradient of KCl (0 to 100 m*M*) in the same buffer. Fractions (2 ml) were collected and analyzed for glucose as described in Fig. 1. Results are expressed as micrograms of glucose normalized per milligram of cell protein. F1, F2, and F3 mark the peaks of fractions 1, 2, and 3 described in text.

Fractions 2 and 3 (Fig. 2) also contained glucose as the sole sugar, as determined with glucose oxidase after acid hydrolysis. These anionic glucans are about the same size as the cyclic (1 → 2)-β-D-glucans since they were contained in the same Sephadex G-50 peak (Fig. 1A). Their relation to the cyclic glucans requires further study.

The intracellular localization of the cyclic (1 → 2)-β-D-glucans and anionic glucans of *A. tumefaciens* was examined by the osmotic shock method of Heppel (*25*). When cells of *A. tumefaciens* C58 were subjected to osmotic shock under relatively mild conditions, about 72 percent of total cell-associated glucan was released, indicative of a periplasmic localization.

When cells of *A. tumefaciens* C58 were grown in YM medium supplemented with 0.4*M* NaCl, the amount of glucan recovered in the Sephadex G-50 peak was strikingly reduced (Fig. 1B) in comparison to the control (Fig. 1A). Further analysis (Table 1) revealed that both the neutral cyclic (1 → 2)-β-D-glucan and the anionic glucan fractions were reduced. Similar results were obtained with the addition of other salts or of sugars such as sucrose or mannitol (Fig. 1C and Table 1). This effect was independent of the chemical nature of the solute and thus appears to be dependent solely on the increased osmolarity of the medium.

The possibility was considered that cyclic (1 → 2)-β-D-glucan was released into the medium rather than retained by cells when *A. tumefaciens* C58 was grown in a medium of high osmolarity. When supernatants from cultured cells were analyzed for extracellular cyclic (1 → 2)-β-D-glucan, no significant difference was detected between cultures grown in YM medium or YM medium supplemented with 0.4*M* NaCl. For either condition, extracellular cyclic (1 → 2)-β-D-glucan content was approximately 5 μg per milligram of total cell protein (*26*).

Several lines of evidence suggest that periplasmic oligosaccharides may play a general role in the adaptation of Gram-negative bacteria for growth in low osmolarity environments. (i) Our study reveals that periplasmic oligosaccharide biosynthesis is osmoregulated in species as ecologically di-

verse as soil and enteric bacteria. (ii) Growth in very dilute environments may be accompanied by a limitation of available nutrients. The diversion of a significant fraction of the total cell mass toward the synthesis of periplasmic oligosaccharides under such conditions must be presumed to have adaptive value. (iii) It is estimated that 10 to 15 enzymes are required for the synthesis of the MDO of *E. coli*. At least some, and probably all, of these enzymes are synthesized constitutively, even by cells growing in media of high osmolarity (*27*).

With these considerations in mind, it is paradoxical that the *mdoA* mutation, which blocks the synthesis of MDO at an early stage in the assembly of the carbohydrate chains, is not associated with any distinct phenotype (*28*). It is now recognized, however, that there may be considerable redundancy in many processes localized in the cell-envelope of *E. coli*, as exemplified by the multiple transport systems for potassium, for iron, and for sugars. The function of any one of these multiple systems is difficult to detect until alternative systems have been genetically deleted. Perhaps the function of the periplasmic oligosaccharides will be seen more clearly if it becomes possible to recognize and eliminate alternative mechanisms for adaptation to low osmolarity.

The membrane-derived oligosaccharides of *E. coli* appear to be well suited to function in the maintenance of the osmolarity of the periplasm at a value close to that of the cytoplasm, as originally indicated by the study of Stock *et al.* (*7*). With molecular weights of about 2500, MDO are large enough to be retained by the pores of the outer membrane but not so large as proteins, which are less active osmotically per unit weight. The multiple substitution of MDO with anionic residues further enhances their osmotic effect through the binding of counterions and contributes to the Donnan potential across the outer membrane. The neutral cyclic (1 → 2)-β-D-glucans of *A. tumefaciens* may contribute to the osmolarity of the periplasmic compartment of this bacterium, as their size and periplasmic concentrations are comparable to that of the MDO of *E. coli*. Furthermore, the amounts of anionic oligosaccharides within *A. tumefaciens* were also greatly increased when cells were grown in a medium of low osmolarity (Table 1).

The fundamental mechanisms by which cells recognize and respond to changes in the osmolarity of their extracellular environment are unknown. Detailed study of effectors that modulate the activity of enzymes catalyzing the initial steps of periplasmic oligosaccharide biosynthesis may provide insight into this important problem.

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Heat Shock Elicits Production of Sexual Inducer in *Volvox*

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In the green alga *Volvox carteri*, heat shock had an unusual and adaptive effect mediated by induced production of a well-defined effector molecule. Females of this species normally reproduce asexually in the absence of a potent sexual inducer produced by mature sexual males, but they generated egg-bearing sexual daughters after a brief exposure to elevated temperatures. This response involved an "autoinduction" of sexuality, in which heat-shocked somatic cells made and released the sexual inducer, which then redirected development of the reproductive cells. Males, including a sterile mutant incapable of producing inducer in the usual manner, also produced the inducer in response to heat shock. The phenomenon probably is of significance in the wild, where *Volvox* reproduces asexually in temporary ponds in spring but becomes sexual and produces dormant, overwintering zygotes before the ponds dry up in the summer heat.

HEAT SHOCK, WHICH HAS SIMILAR effects on gene expression and development of thermotolerance in a wide range of organisms (*1*), has an additional, adaptively significant effect on gene expression in *Volvox carteri* f. *nagariensis*. The sexual inducer of this species, an approximately 30-kD glycoprotein (*2*), is one of the most potent biological effector molecules known. Males developing sexually, either in response to inducer made by another male (*3*) or because of a *sex*^{c}, or "constitutive sexuality," mutation (*4–6*), make and accumulate inducer in their sperm packets (*7*) and release it as sperm are released (*5, 7*). It is effective at $6 \times 10^{-17} M$, and one sexual male releases enough inducer to convert all the related males and females in a volume of 1000 liters from asexual to sexual reproduction (*8*). Because inducer is not made by sexual females (*3*) or the somatic cells of sexual males (*7*), it appeared that expression of the gene involved in inducer production was tightly linked to sperm development. However, the results reported here demonstrate that this is not the case: heat shock caused both asexual females and asexual

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