Obtaining cell proliferation for chromosome preparation in gill tissue culture of the oyster *Crassostrea gigas*

Michel Cornet

Laboratoire d'Océanographie Biologique, CNRS UMR 5805, Université Bordeaux I, Avenue des Facultés, 33405 Talence Cedex, France

(E-mail: cornet@biocean.u-bordeaux.fr)

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Abstract

The present results were obtained in the course of the adjustment to the oyster *Crassostrea gigas* of a tissue culture technique recently developed for the mussel *Mytilus edulis*. With respect to the protocol originally described, the effects of two modifications are reported: (1) replacement of chick embryo extract by chicken serum for medium enrichment, and (2) achievement of cultures in rotating tubes (roller drum) in place of stationary condition. Paradoxical results were obtained: whereas taken separately, each modification exerted a negative effect which is statistically significant, combined, they exerted a high positive effect representing a three-fold increase of the mean metaphase spread number per slide (i.e. 71.5). Hypotheses are proposed to explain the mechanisms involved. It is suggested that the two additives work differently and that cultures with chick embryo extract enriched medium could not withstand the condition generated by the roller drum. Conversely, cultures performed with chicken serum enriched medium would be in a better physiological state and the roller allow to obtain a cell proliferation after only six days of incubation.

Introduction

A short-term culture method for chromosome preparation from somatic tissues of adult mussel *Mytilus edulis* has been recently introduced (Cornet, 1993, 1995). Briefly, primary cultures were initiated from small pieces (explants) of mussel mantle by placement in an artificial growth medium fortified with chick embryo extract (4–8%). After 6–7 days of incubation at 18 °C in plastic tubes, cultures were stopped and explants used to prepare slides on which metaphase chromosomes were present. Hence, with this method, a reliable cell proliferation was obtained.

In response to a request related to an important local aquaculturing activity – oyster farming in the Bay of Arcachon – the adjustment to *Crassostrea gigas* of the culture technique originally developed for *Mytilus edulis* was considered. It became, indeed, more and more evident that a thorough knowledge of shellfish genetics at the chromosomal level should be

regarded as one of the basic requirements for the understanding of many aspects of aquaculture (Colombo et al., 1990; Beaumont, 1994) and of water contamination (Moore et al., 1986; Hummel and Patarnello, 1994).

Since 1960, attempts to grow oyster cells or tissues have been already reported (e.g. Vago and Chastang, 1960; Perkins and Menzel, 1964; Li et al., 1966; Tripp et al., 1966; Wallis, 1972; Ieyama et al., 1979; Brewster and Nicholson, 1979; Sami et al., 1991; Kleinschuster and Swink, 1992; Wen et al., 1993; Domart-Coulon et al., 1994; Le Deuff et al., 1994; Renault et al., 1995). Most of the procedures described, however, were not designed for chromosome preparation and were not followed by cytogenetical applications, even in the cases where mitosis were observed (e.g. Wen et al., 1993). As a consequence, available data about chromosomes of *Crassostrea gigas* were only obtained from investigations achieved either with the squash technique or with the direct

method of preparation (see Macgregor and Varley, 1983), both using mitosis occurring in tissues of living animals (Ahmed and Sparks, 1967; Ieyama and Inaba, 1974; Thiriot-Quiévreux and Ayraud, 1982; Thiriot-Quiévreux, 1984; Thiriot-Quiévreux et al., 1988; Thiriot-Quiévreux and Insua, 1992).

When used just as it was, our culture technique gave poor results with oyster tissues. Modifications were, therefore, necessary to make it functional. We first established (unpublished results) that when mantle was replaced by gill tissue and chick embryo extract concentration was raised up to 12–13%, a better efficiency was obtained. In such conditions, cell divisions were observed but the mitotic index remained rather low (ca. 20 metaphases per slide in average). Further attempts to improve the culture efficiency were then undertaken or are currently in progress. In the present paper, two modifications which gave unexpected results are described and discussed. These changes are concerned with the serum used to fortify the culture medium (chicken serum in place of chick embryo extract) and the technique of incubation of the culture tubes (dynamic condition in place of stationary condition).

Materials and methods

Maintenance of animals

Oysters (45–60 mm shell length) were collected at Cap Ferret, in the Bay of Arcachon (French Atlantic coast), in April and May 1997. In the laboratory, they were reared in 5 l capacity aquaria of natural aerated sea water maintained at 18 °C and under a daily photoperiod regime of 14 h light followed by 10 h of darkness. Half of the water was renewed every two days. Animals were fed with an individual daily ration of 0.05 ml of 'Liquifry marine', a liquid artificial food intended for filter feeders. Oysters were kept no more than 17 days before processing.

Cell culture

The techniques used for culturing, harvesting and slide preparation were identical to those described previously for *Mytilus edulis* (Cornet, 1993, 1995). Tissues were processed as follows. After dissecting out, gills were washed in three changes (20 min each) of antibiotic-antimycotic enriched medium and rinsed in sterile serum-free medium. Gills were then cut into 4 mm² explants. Cultures were performed in plastic

culture tubes containing 3 ml of medium and 2–4 gill explants. The medium was composed of Eagle's Basal Medium (Sigma) and inorganic salts (see medium A in Cornet, 1993), buffered at pH 7.50 with sodium bicarbonate and HEPES, and supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). This technique allows a good maintenance of the pH without the necessity for a CO₂ incubator. For experiments, the medium was fortified either with 12–13% chick embryo extract (Eurobio), as in the original method, or with 12–13% chicken serum (Eurobio). The culture tubes were incubated at 18 °C for 6 days, either in stationary condition, as in the original method, or in dynamic condition by placing in a roller drum (0.25 r.p.m.).

Six series of cultures were performed according to the above protocol. Each series was constituted of 4 tubes containing a variable number of explants: 2 in the 2nd and 3rd series, 3 in the 1st, 4th and 5th series, and 4 in the 6th series. Within a series, each tube corresponded to one of the four following combinations: (1) chick embryo extract without roller; (2) chicken serum without roller; (3) chick embryo extract with roller; (4) chicken serum with roller. In each series explants came from either only one specimen (3rd and 6th series) or two specimens (1st, 2nd, 4th and 5th series). Therefore, a total of ten oysters was used. Table 1 gives the repartition of specimens, treatments and explant numbers with respect to culture series.

Chromosome preparation

Harvesting included blocking cell divisions at metaphase using colchicine (50–60 µg/ml final concentration) for 1 h 50, treatment with a hypotonic solution (0.6% tri-sodium citrate) for 45 min, and fixation in ice-cold 3:1 methanol-acetic acid (four changes for a minimum of 2 h). For slide preparation, explants were dissociated with 45% acetic acid and the resulting cell suspension was dropped onto warm slides (42±0.5 °C). Preparations were stained in 3% Giemsa in phosphate buffer, pH 6.8, for 20 min, rinsed with distilled water and air dried.

Data analysis

Since only one explant was used to prepare one slide and assuming all explants had the same volume and the slide preparation technique was always identical, the mitotic activity induced by the various treatments was evaluated by scoring all the metaphase spreads present on the slides. Metaphase numbers were then

Table 1. Gill tissue culture of the oyster *Crassostrea gigas*: summary of materials studied and experimental methods used for comparisons of the four treatments. sp. no.: oyster specimen number; CEE: medium containing chick embryo extract; CS: medium containing chicken serum; ROL: culture tubes placed in a roller drum. The asterisk indicates a microbial contamination

Culture series	Culture tube				Explants per tube
	1	2	3	4	
1	sp. no. 1		sp. no. 2		3
	CEE without ROL	CS without ROL	CEE with ROL	CS with ROL	
2	sp. no. 3		sp. no. 4		2
	CEE without ROL	CEE with ROL	CS without ROL	CS with ROL	
3	sp. no. 5				2
	CEE without ROL	CEE with ROL	CS without ROL	CS with ROL *	
4	sp. no. 6		sp. no. 7		3
	CEE without ROL	CS without ROL	CEE with ROL	CS with ROL	
5	sp. no. 8		sp. no. 9		3
	CEE without ROL	CEE with ROL	CS without ROL	CS with ROL	
6	sp. no. 10				4
	CEE without ROL	CEE with ROL	CS without ROL	CS with ROL

Figure 1. Effect of four culturing conditions on cell proliferation in explants of gill tissue of the oyster *Crassostrea gigas*. Culture medium was fortified either with chick embryo extract (CEE) or chicken serum (CS) and culture tubes were incubated either with a roller drum (ROL) or without. Each bar represents the number of metaphases scored on one slide. Asterisks indicate cultures contaminated by microorganisms.

Figure 2. Estimation of cell proliferation, expressed as the mean metaphase number, with the four treatments tested, and results of the Kruskal-Wallis test between the original technique of culture (chick embryo extract without roller) and the three other experimental methods. CEE: medium containing chick embryo extract; CS: medium containing chicken serum; ROL: culture tubes placed in a roller drum. Interval estimates are 95% confidence limits. Kruskal-Wallis χ^2 : *: $p < 0.05$; ***: $p < 0.001$. For $k_1 = 1$, then $\chi^2_{0.95} = 3.84$, $\chi^2_{0.99} = 6.63$ and $\chi^2_{0.999} = 10.8$.

compared statistically by means of the non-parametric Kruskal-Wallis test (Sokal and Rohlf, 1981).

Results

Sixty eight slides – 17 for each treatment – were analysed. Overall, they provided 1857 metaphase spreads. Individual slide results are shown graphically in Figure 1 and the means and their confidence limits are given in Figure 2. It can be observed that a large variation occurs both within and between treatments. The most obvious is the markedly increase which appears in five series out of six when the combination chicken serum plus roller was used. The two abnormal metaphase numbers (3 and 16) recorded in the third series

came from a single tube in which a microbial contamination was detected, resulting in a dramatic decrease of mitosis. As they were not representative, those two values were discarded in further analyses.

The degree of significance of these differences was estimated using a Kruskal-Wallis χ^2 for the two types of variation (within and between treatments). First, the χ^2 was computed to test the null hypothesis that metaphase spread numbers were homogeneous among all series, excepted the third one because one treatment was missing. It revealed that the differences between series was statistically not significant ($\chi^2 = 4.42$, $df = 4$, $p > 0.05$).

Second, the χ^2 was computed to test the null hypothesis that two series of compared values came from the same population, in other words, the modification

tested did not give any change in mitosis production. Thus, when cultures were achieved with the original technique, i.e. chick embryo extract without roller, an average of 23.4 metaphase spreads per slide was obtained. If chick embryo extract was replaced by chicken serum, all other culture conditions being unchanged, an average of 13 metaphase spreads per slide was obtained and the statistical analysis showed the decrease was significant ($p < 0.05$) (Figure 2). When chick embryo extract was retained but tubes were placed in the roller, the average of metaphase spreads per slide fell to 8.6; in this case, the statistical analysis revealed that the decrease was highly significant ($p < 0.001$) (Figure 2). Finally, if chick embryo extract was replaced by chicken serum and if tubes were placed in the roller, the average rose up to 71.5 mitosis, that is a high increase of the cell proliferation in comparison with the above situations. The statistical analysis showed this tripling of metaphase spread number per slide was highly significant ($p < 0.001$) (Figure 2).

As shown in Figure 3, metaphases obtained from gill explants grown with the medium fortified with chicken serum and placed in the roller were of sufficiently good quality for cytogenetical applications. As that was still reported for *Mytilus edulis* (Cornet, 1993), about 20% of the metaphases were not complete. Since such losses of chromosomes were found whatever the species and method (direct or indirect) used to obtain mitosis, we believe this is rather a technical problem arising during slide preparation (tissue dissociation with acetic acid and dropping the cell suspension onto hot slides) than an aneuploidy related to the culturing conditions.

Discussion

The two modifications tested herein were initially chosen for the following reasons. First, we were thinking about replacing chick embryo extract in order to reduce the cost of the culture technique. Since the superiority of media enriched with additives of bird origin in culturing invertebrates tissues – especially molluscan tissues – is established for many years (Jones, 1966), an alternative for chick embryo extract was chicken plasma or chicken serum. The latter which is commercially available and much more inexpensive than chick embryo extract was retained. Second, the roller drum was initially design to improve the collect of mitotic cells which proliferate primarily

at explant periphery. In stationary condition, explants attach to tube walls and cells migrated out forming a monolayer which was partly broken when explants were removed from the tubes, before slide preparation. Assuming mitosis were present in this monolayer, the roller was expected to avoid the loss of mitotic cells by preventing explants from adhering.

The experiments undertaken allowed the comparison of the two additives, i.e. chick embryo extract vs. chicken serum, and of the two culture techniques, i.e. static tubes vs. rotating tubes. Results show that, whereas taken separately, each modification exerts on cell proliferation a negative effect which is statistically significant, combined, the modifications exert a high positive effect representing a three-fold increase of the mean metaphase spread number per slide.

Those paradoxical observations can be explained, at least in part, only if it is assumed that chick embryo extract and chicken serum were not involved alike as medium enrichment factors. Although the composition of both additives is not fully defined, it is likely they differ qualitatively and quantitatively. Originating from an embryonic organism, chick embryo extract may contain much more components (e.g. growth factors, hormones) needed to enhance cell proliferation whereas chicken serum which originates from an adult organism may rather improve basic metabolic activity without a necessarily instant high cell proliferation. Those assumptions are supported by the fact that the use of chick embryo extract has been advised for preparing growth media, i.e. media having a composition which favours cell multiplication, while chicken serum has been advised for preparing maintenance media, i.e. media which permit prolonged survival of tissues by furnishing the requirements necessary for low growth rate (Penso and Balducci, 1963; Jayme et al., 1997).

Thus, there would be two manners for obtaining cell multiplication in cultures of invertebrate tissues: (1) by an early stimulation of the tissues by factors of embryonic origin (short-term cultures), and (2) by providing components intended to enhance the physiological state, leading to a later cell proliferation following a relatively long lag phase in which cell division and nucleic acid synthesis are arrested (long-term cultures). In the former case, however, survival of tissues would remain more precarious and the system would be more unstable than in the latter. The greater instability of cells in quick proliferation phase was attributed to the higher metabolism producing toxic material accumulation (e.g. lactate and ammonia)

Figure 3. Mitotic metaphase spread of *Crassostrea gigas*, with a diploid number of $2n = 20$ chromosomes, obtained from gill explant culture. Culture medium was fortified with chicken serum and culture tubes were incubated in a roller drum. Bar scale: $5\ \mu\text{m}$.

in the case of closed culture systems (Jayme et al., 1997; Kadouri and Spier, 1997). This might explain why, in stationary culture condition, replacement of chick embryo extract by chicken serum would result in a decrease of cell proliferation after only six days of growing.

Conversely, it is less obvious to understand the role of the roller drum when combined with either additives. We think the results of this study could be attributable to the cell metabolism related to oxygen uptake rate. In animal cell culture, dissolved oxygen tension has been found to be one of the most critical parameters (Palomares and Ramirez, 1996). With closed culture systems, oxygen is provided only by surface aeration. In the culture tubes used in our experiments, however, the surface area available for transfer is small (less than $1.8\ \text{cm}^2$ for 3 ml of medium), and oxygen may become rapidly a limiting factor (Spier, 1997). Moreover, poor mixing can result in local heterogeneities affecting the culture performance (Ozturk, 1996). Agitation of the cell container is one of the most efficient means for maintaining oxygen supply (Griffiths, 1990). Here, the function of the roller is therefore not only to prevent explants from adhering to the tube walls but also to favour O_2/CO_2 exchanges between the different compartments of the culture system:

gas phase $\leftrightarrow$ culture medium $\leftrightarrow$ tissues ,

the result being an acceleration of cell metabolism. If this hypothesis is exact, culturing efficiency is expected to be improved by maintaining explants in suspension. It is likely the case when the roller is associated with chicken serum which would allow tissues kept *in vitro* to be in good physiological condition. The roller would enhance this action that results in a significant increase of the mitotic activity. In contrast, tissues grown with chick embryo extract enriched medium might be in a poor physiological state and not able to withstand new conditions generated by the roller, leading to a drastic decrease of the mitotic activity. It might seem the roller is inconsistent with the precarious metabolic state of tissues grown with chick embryo extract enriched medium, since it produces stressful environmental condition for the culture

Conclusion

The above technique provides a way for obtaining a workable quantity of mitotic metaphase cells. While the mechanism of how the combination chicken serum plus roller affects cell proliferation is still unknown, it is not necessary for practical implementation of the culture technique as a tool for obtaining metaphase spreads and, therefore, the method described herein is routinely used in our laboratory.

However, even if a thorough biochemical analysis of the mechanisms involved was far beyond the limits of this work, it could be of great value to examine the hypothesis suggested above by studying the effects of variations of both additive concentrations as well as the cell cycle kinetics, in stationary and dynamic conditions. Additional experiments would allow further improvements of the *in vitro* system and help in the design of experimental models for cytogenetical studies as well as bioassay systems for viral and cytotoxic compounds studies.

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References

- Ahmed M and Sparks AM (1967) A preliminary study of chromosomes of two species of oysters (*Ostrea lurida* and *Crassostrea gigas*). J Fish Res Board Can 24: 2155–2159.
- Beaumont AR (1994) The application and relevance of genetics in aquaculture. In: Beaumont AR (ed) Genetics and Evolution of Aquatic Organisms. Chapman & Hall, London, pp. 467–486.
- Brewster F and Nicholson BL (1979) *In vitro* maintenance of amoebocytes from the American oyster *Crassostrea virginica*. J Fish Res Board Can 36: 461–467.
- Colombo L, Bortolussi M., Belvedere P and Battaglia B (1990) Genetic improvement in aquaculture. In: Hummel H (ed) The Genetics of Marine and Estuarine Organisms. DIHO, Yerseke NDL, pp. 32–33.
- Cornet M (1993) A short-term culture method for chromosome preparation from somatic tissues of adult mussel (*Mytilus edulis*). Experientia 49: 87–90.
- Cornet M (1995) A study of some factors affecting the efficiency of *in vitro* mussel tissue culture for chromosome preparation. Cytobios 84: 7–12.
- Domart-Coulon I, Doumenc D, Auzoux-Bordenave S and Le Fichan Y (1994) Identification of media supplements that improve the viability of primary cell cultures of *Crassostrea gigas* oysters. Cytotechnology 16: 109–120.
- Griffiths JB (1990) Scale-up of suspension and anchorage-dependent animal cells. In: Pollard JW and Walker JM (eds) Methods in Molecular Biology, vol. 5, Animal Cell Culture. Humana Press, Clifton NJ, pp. 49–63.
- Hummel H and Patarnello T (1994) Genetic effects of pollutants on marine and estuarine invertebrates. In: Beaumont AR (ed) Genetics and Evolution of Aquatic Organisms. Chapman & Hall, London, pp. 425–434.
- Ieyama H and Inaba I (1974) Chromosome numbers of ten species in four families of Pteriomorpha (Bivalvia). Venus 33: 129–137.
- Ieyama H, Takubo M and Moribe S (1979) Explant culture of the oyster, *Crassostrea gigas*. Mem Ehime Univ Sci, B, 8: 1–4.
- Jayme D, Watanabe T and Shimada T (1997) Basal medium development for serum-free culture: a historical perspective. Cytotechnology 23: 95–101.
- Jones BM (1966) Invertebrate tissue and organ culture in cell research. In: Willmer EN (ed) Cells and tissues in culture. Methods, biology and physiology, vol. 3. Academic Press, London, pp. 397–457.
- Kadouri A and Spier RE (1997) Some myths and messages concerning the batch and continuous culture of animal cells. Cytotechnology 24: 89–98.
- Kleinschuster SJ and Swink SL (1992) *In vitro* culture of presumptive nervous tissue of *Crassostrea virginica* (Gmelin, 1791). J Shellfish Res 11: 349–361.
- Le Deuff RM, Lipart C and Renault T (1994) Primary culture of Pacific oyster, *Crassostrea gigas*, heart cells. J Tissue Cult Meth 16: 67–72.
- Li MF, Stewart JE and Drinnan RE (1966) *In vitro* cultivation of cells of the oyster *Crassostrea virginica*. J Fish Res Board Can 23: 595–599.
- Macgregor HC and Varley JM (1983) Working with Animal Chromosomes. John Wiley & Sons, New York.
- Moore MN, Lowe DM, Livingstone DR and Dixon DR (1986) Molecular and cellular indices of pollutant effects and their use in environmental impact assessment. Wat Sci Tech 18: 223–232.
- Ozturk SS (1996) Engineering challenges in high density cell culture systems. Cytotechnology 22: 3–16.
- Palomares LA and Ramirez OT (1996) The effect of dissolved oxygen tension and the utility of oxygen uptake rate in insect cell culture. Cytotechnology 22: 225–237.
- Perkins FA and Menzel RW (1964) Maintenance of oyster cells *in vitro*. Nature 204: 1106–1107.
- Penso G and Balducci D (1963) Tissue culture in biological research. Elsevier, Amsterdam.
- Renault T, Flaujac G and Le Deuff RM (1995) Isolation and culture of heart cells from the European flat oyster, *Ostrea edulis*. Meth Cell Sci 17: 199–205.
- Sami S, Ahmed I and Faisal M (1991) *In vitro* cultures of oyster *Crassostrea virginica* cells: stimulation by mitogens. Chesaapeake Res Cons Pub 137: 403–407.
- Sokal RR and Rohlf FJ (1981) Biometry. Freeman WH, San Francisco.
- Spier RE (1997) Factors limiting the commercial application of animal cells in culture. Cytotechnology 23: 113–117.
- Thiriot-Quievreux C and Ayraud N (1982) Les caryotypes de quelques espèces de Bivalves et de Gastéropodes marins. Mar Biol 70: 165–172.
- Thiriot-Quievreux C (1984) Analyse comparée des caryotypes d'Ostreidae (Bivalvia). Cah Biol Mar 25: 407–418.
- Thiriot-Quievreux C, Noel T, Bougrier S and Dallot S (1988) Relationships between aneuploidy and growth rate in pair mating of the oyster *Crassostrea gigas*. Aquaculture 75: 89–96.
- Thiriot-Quievreux C and Insua A (1992) Nucleolar organiser region variation in the chromosomes of three oyster species. J Exp Mar Biol Ecol 157: 33–40.
- Tripp MR, Bisignani LA and Kenny MT (1966) Oyster amoebocytes *in vitro*. J Invert Pathol 8: 137–140.
- Vago C and Chastang S (1960) Culture de tissus d'huître. C R Acad Sci Paris 250: 2751–2753.
- Wallis AM (1972) A new technique for the preparation of primary cultures of oyster tissue. Lab Pract 21: 32.
- Wen CM, Kou GH and Chen SN (1993) Establishment of cell lines from the Pacific oyster. In Vitro Cell. Dev Biol 29A: 901–903.