OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

This document has been produced without formal United Nations editing. The designations employed and the presentation of the material in this document do not imply the expression of any opinion whatsoever on the part of the Secretariat of the United Nations Industrial Development Organization (UNIDO) concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries, or its economic system or degree of development. Designations such as "developed", "industrialized" and "developing" are intended for statistical convenience and do not necessarily express a judgment about the stage reached by a particular country or area in the development process. Mention of firm names or commercial products does not constitute an endorsement by UNIDO.

FAIR USE POLICY

Any part of this publication may be quoted and referenced for educational and research purposes without additional permission from UNIDO. However, those who make use of quoting and referencing this publication are requested to follow the Fair Use Policy of giving due credit to UNIDO.

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

15830

APPLICATION OF BIOTECHNOLOGY IN ALGAL MARICULTURE

Madeline Wu

Department of Biological Sciences

University of Maryland, Baltimore County

Catonsville, MD 21228 USA

Abstract

Seaweeds have been used as food, fodder or fertilizer since ancient times. Recently, unique and valuable polysaccharides have been isolated from several species of seaweed. These phycocolloids are used in a wide range of industries. Raw material shortages have stimulated seaweed mariculture in the Orient and Indo-Pacific. Some species are farmed as a source of food while other species are cultivated for their phycocolloids. If genetically improved stocks are used in maricultures, it should be possible to produce commercially-valuable products at reduced cost and increased dependability. Aimed at this long term goal, a unicellular green algae *Chlamydomonas reinhardtii* was used as a model system to test whether genetic engineering of an algal genome is feasible. An algal chloroplast DNA replication origin was mapped, cloned and characterized. The cloned replication origin was used for vector construction. A chloramphenicol resistance gene was successfully introduced into the algal genome.

Introduction

The abundance and diversity of seaweeds have made them prime material for human use. Many of the old ways of using seaweeds, e.g. as food, fodder, and fertilizer, are still practiced while new ways of using them

are constantly being research and developed. Seaweeds have been utilized more or less systematically by coastal dwellers for feeding purposes all over the world far back into history. In the Mediterranean area the Romans made some use of seaweed for feeding horses. Along the coasts of France, Scotland, Iceland, and Norway seaweed formed a valuable supplement to the rations for sheep, cows, and pigs through centuries. Today a significant quantity of seaweed is used for the preparation of seaweed meal as a feed additive in animal husbandry. Over 50,000 tons of seaweed meal are produced annually in Canada, France, Great Britain, Ireland, Norway, South Africa, and the United States of America. Seaweed meal is obviously a good source of iodine. Iodine deficiency is becoming more common with higher-yielding animals. An investigation carried out in 1963 in Great Britain by Alderman and Strank (1967) showed that 15 out of 18 dairy herds at pasture were iodine deficient.

Seaweeds have been used as human food since ancient times; the earliest record being 600-800 B.C. in China. Analyses of certain edible seaweeds show that many contain significant amounts of proteins, vitamins, and minerals essential for human nutrition (Abbott and Cheney, 1982). For example, *Porphyra*, a popular delicatessen in Japan and China, has a high content (20-25% of its dry weight) of digestible protein, as well as a high vitamin C content similar to that of lemons (Levring *et al*, 1969). It is estimated that approximately 168 species of marine algae serve as commercially important food sources worldwide, with an estimated market value of well over one billion dollars annually (Abbott and Cheney, 1982). Most of this total comes from cultivation and consumption of *Porphyra* (nori), *Laminaria* (kombu) and *Undaria* (wakame) in the countries of Japan, China, and Korea. The *Porphyra*, or nori,

industry in Japan alone is estimated to involve over 60,000 hectares in cultivation area and to be worth over $730 million annually.

In the western hemisphere, seaweeds are principally utilized today as a source of the phycocolloids agar, carageenan and alginate. These polysaccharides are produced in the cell walls of certain red (for agar and carrageenan) and brown (for alginate) algae. These constituents of seaweeds are unique to these plants and for which no other sources have been found. Commercial exploitation of these useful and unique seaweed chemical has become the mainstay of the seaweed industry in the 20th century. Carrageenan and alginate are primarily used as gelling or stabilizing agents in the food industry. Their applications include uses in: stabilizers, emulsifiers, cosmetics and soaps, dental technology, bakery and candy products, dairy products, and fish, meat, and beverage processing. They are also used in a wide range of industrial products, e.g. dyes, paints, textile production, pesticides, lubricants and ceremics (Levring *et al* 1969, Abbott, and Cheney, 1982). Agar, in addition to being used in the food industry, is uniquely important to microbiology as a culture medium. Agarose, a neutrally-charged fraction of agar, is widely used in biotechnology for immunodiffusion and electrophoretic separations.

Future uses of marine algae will undoubtedly be far more diverse. Some of the potential uses that have been suggested for algal polysaccharides include applications in enhanced oil recovery, enzyme immobilization technology, and the modification of viscosity or interfacial properties of aqueous systems (Whitesides and Elliott, 1984). Marine algae may also serve as rich sources of valuable pharmaceuticals

and specialty chemicals in the future (Colwell, 1983). For example, diatomaceous deposits are used to recover high yields of silica. A number of laboratories are currently conducting extensive screening of seaweeds for biologically-active compounds (B. Fenical, Scripps Oceanographic Institution, per. commun.). Perhaps an indication of the potential for new seaweed products is the recently developed and fast growing use of phycobiliprotein fluorescent dyes in labeling monoclonal antibodies and other probes. These dyes are isolated from the pigments produced by red algae and can substitute for many of the radioactive substances now widely used for cell or protein identification. They are currently being used clinically to detect Acquired Immune Deficiency Syndrome (AIDS) virus in blood samples.

Seaweed utilization in the past has been limited by the availability of suitable raw material (Moss, 1978; Cheney, 1984). Unregulated field harvests have caused reductions in wild populations of many desired species. Already in some areas of Japan, the present harvest approaches the estimated potential harvest. Raw material shortages have stimulated seaweed mariculture. altogether, there are approximately 11 genera of seaweeds being cultivated to a significant commercial extent worldwide (Tseng, 1981). Most seaweed cultivation occurs in the Orient and Indo-Pacific and includes, for example, the large-scale farming of *Porphyra* in Japan, *Laminaria* and *Undaria* farming in China, *Gracilaria* pond culture in Taiwan and *Eucheuma* farming in the Philippines (Tseng, 1981). *Porphyra*, *Laminaria* and *Undaria* are cultivated as a source of food, while *Eucheuma* and *Gracilaria* are primarily farmed for their phycocolloid, carrageenan and agar, respectively. If genetically improved strains can be cultivated, it should be able to produce new and

improved products that are not available in existing wild plants. It should also be possible to produce commercially-valuable, existing products at reduced costs and increased dependability.

While the commercial cultivation of seaweeds is quite different from land agronomy in many respects, it shares the same basic need for genetically improved stocks. In the past decade, major improvements of land crop plants have been achieved by classical breeding techniques. However, these methods are slowly reaching a plateau. Recent intensive research efforts in plant molecular biology strongly suggest that the improvements of many agronomical traits of crop plants, such as increases in resistance to environmental stresses and pathogens, improvements of nutritional quality and decreases in fertilization requirements, can be accomplished by genetic engineering. These same techniques can and should be applied to the genetic improvement of seaweed.

In my laboratory, a well-studied unicellular green algae, *Chlamydomonas* has been used as a model system to test whether genetic engineering of an algal genome is feasible. At this stage we have limited our study to the important and well-studied chloroplast genome. *Chlamydomonas* is a fresh water algae, however, it is closely related to a salt water flagellate, *Dunaliella*. In the life cycles of all multicellular seaweeds, single cell stages, such as spores or gametes, have been detected. The single cell stage resembles its closely related unicellular algae in many aspects. Some single cells which can be cultivated to generate the multicellular plant will be a prime target for genetic engineering. Our experimental approach is using a functional algal DNA replication origin for vector construction. Therefore, some

fundamental study about cloning and characterization of algal DNA replication origin will be included.

Initiation of Chloroplast DNA Replication in *Chlamydomonas reinhardtii*.

The chloroplast (Chl) is a self-duplicating extrachromosomal organelle with its own hereditary apparatus. Chl DNAs from higher plants and green algae have been isolated as covalently closed circular molecules with lengths in the range of 110-215 kb. Chloroplasts contain multiple copies of the circular genome (Bedbrook and Kolodner, 1979). Chl DNA can be purified on the basis of either its location within the organelle or its distinct base composition. In *Chlamydomonas reinhardtii*, Chl DNA has been isolated from a satellite component in a CsCl gradient (Sager and Ishida, 1963). The replication of Chl DNA was studied by Chiang and Sueoka (1967) using density labeling experiments. They demonstrated that during the vegetative growth of this unicellular green algae, Chl DNA and chromosomal DNA replicated at different times in the division cycle. Recently, several approaches have been used to isolate the algal DNA sequences which could promote autonomous replication. Rochaix *et al* (1984) isolated plasmids that could replicate autonomously in *C. reinhardtii* (ARC) by inserting random algal DNA fragment into the yeast plasmid pYe arg4. Plasmid pYe arg4 can replicate autonomously in yeast but not in *Chlamydomonas*. After transforming a strain of *C. reinhardtii* lacking arginiosuccinate lyase with recombinant plasmids constructed from pYe arg4 and algal DNA, arginine prototrophy was used to select ARC plasmids. DNA inserts of several ARC plasmids were localized on the physical map of the Chl genome. Using a yeast transformation system, several algal DNA sequences which could promote

autonomous replication in yeast and which approximate the yeast ARS core sequence have also been mapped on the Chl genome of *C. reinhardtii* (Loppes and Denis, 1983; Vallet *et al*, 1984). However, the ARC sequences do not coincide with the ARS sequences.

Using electron microscope, we have mapped 2 putative replication origins on the physical map of the Chl DNA of *C. reinhardtii*. Chl DNA isolated from a synchronized culture at the time when Chl DNA was replicating was used for this study. The purifed Chl DNA was digested with either EcoR1 restriction enzyme or BamH1 restriction enzyme, the restriction sites for both enzymes have been mapped by Rochaix (1978). Restriction fragments containing displacement loop (D-loop) were photographed and measured. Examples of the D-loop containing Eco R1 fragment are shown in Fig. 1. The length of the restriction fragment and the position of the D-loop in relation to a particular restriction site was determined and plotted. Statistical analyses indicated that D-loop initiated at specific sites on the physical map of Chl DNA. One D-loop site was mapped at about 10 kb upstream of the 5' end of a 16s rRNA gene. The second D-loop site was spaced 6.5 kb apart and was about 16.5 kb upstream of the same 16s rRNA gene (Wang *et al*, 1984). In Fig. 2, ori A and ori B designate the map positions of these 2 D-loop sites.

The following evidence suggests that a D-loop is probably a transient structure formed after initiation of the replication process. D-loops with various sizes of displaced regions were observed. In a D-loop with the displaced region shorter than 0.5 Kb, one strand of the loop was observed to contain complete single-stranded region while the other strand was complete double stranded (Fig. 1). Formation of these types

of D-loop was resulting from the synthesis of one daughter strand. Large D-loops with the displaced region longer than 0.5 kb have the structural feature of a replication loop (Fig. 3a). Both strands of the loop were observed to contain complete double stranded regions or predominant double stranded regions with single stranded gaps (Fig. 3b). The single stranded regions were frequently located at positions that were trans with respect to each other at both replication forks (Fig. 3c). To deterimine whether the replication loops grew bidirectionally, or unidirectionally, the distance between one particular restriction site and the center of the nearest replication loop was measured. If the loop grew bidirectionally at the same rate, this distance should remain constant. If the loop grew unidirectionally, the same measurement should either increase or decrease proportionally with the loop size. Those observations imply that the very early replication of Chl DNA is unidirectional in forming a D-loop. After the D-loop expanded to the size corresponding to the synthesis of 350 ± 130 bp of one daughter strand, synthesis of the opposite strand initiated. DNA synthesis then proceeded in both directions.

Mapping and Cloning of Functionally Important DNA Sequences Adjacent to One Replication Origin.

The EcoRl fragment, R-13, which contains the D-loop site designated as Ori A was cloned into the EcoRl site of plasmid pBR325 to yield clone CR-13 (Wang *et al*, 1984). Functionally important sequences within this fragment were delimited based upon the assumption that these sequences would be conserved between closely related yet distinguishable species. We have isolated a new species of *Chlamydomonas*, *C. sp. strain WXM.* The

EcoR1, BamH1 and BglII restriction patterns of purified Chl DNA isolated from *C. sp. strain WXM* are markedly different from those of *C. reinhardii cc-125* (Fig. 4). Fig. 4 also shows the hybridization pattern obtained when ^{32}P-labeled CR-13 insert was used as the probe in a Southern blotting analysis. The strongest hybridizing band in the EcoR1 digest of *C. sp. strain WXM* Chl DNA was denoted as I-10. EcoR1 fragment I-10 was estimated to be 9.5 kb in length and was longer than the EcoR1 insert of CR-13 which was 5.5 kb in length. A partial library of Chl DNA EcoR1 fragments of *C. sp. strain WXM* was constructed. The clone containing I-10 (CI-10) was selected and verified. CR-13 and CI-10 were digested with EcoR1 to excise the inserts from the plasmid vectors. Heteroduplex between the 2 inserts was prepared and observed in the EM. Three homologous regions between R-13 and I-10 were detected (Fig. 5a) when the DNA sample was spread under high stringency conditions: 60% formamide in the spreading solution and 30% formamide in the hypophase. The 2 EcoR1 sites were respectively located within the 2 conserved regions between Chl DNAs derived from the 2 species. The respective assignment of each region are shown in the explanatory tracings and the length measurements are summarized in Fig. 5c. Two additional homologous regions were detected (Fig. 5b, c) when DNA samples were spread under sequentially lower stringency conditions: 50% and 40% formamide in the spreading solutions, 20% and 10% formamide in the hypophase respectively. The homologous regions between R-13 and I-10 are identififed as regions A, B, C, D, and E in the explanatory tracings.

The restriction endonuclease map of the cloned R-13 fragment was generated with single, double, and partial restriction digests with various restriction endonucleases. The result is shown in Fig. 6a.

Precise alignment of this restriction map with the EM heteroduplex pattern described above was achieved by using certain restriction fragments of R-13 for EM heteroduplexing studies. The mapping results are summarized and shown in Fig. 6c. In *C. reinhardtii cc-125*, the D-loop region in R-13 fragment was mapped at 0.5 ± .2kb away from the single BamH1 site (Waddell *et al*, 1984). After incorporating this result in Fig. 5b, we drew the conclusion that homologous region C between R-13 and I-10 may include the D-loop sequences which initiate the Chl DNA replication in *C. reinhardtii cc-125*. In fragment I-10, homologous region C was calculated to be 4.1 ± 0.2kb away from the nearest EcoR1 site. According to the EM study results (Wang *et al*, 1984), we can also identify this region as the one containing the D-loop sequences of *C. sp. strain WXM*. Fig. 6a also shows that the suitable restriction sites to subclone homologous region C are ClaI and BamH1. To construct a subclone containing the D-loop region, the 1.05 kb restriction fragment generated by BamH1 and ClaI digestions of R-13 was cloned into pBR322 which was similarly restricted. This subclone was designated as SC3-1.

Test for Origin Function.

In the absence of an effective chloroplast transformation method, *in vitro* DNA replication system using algal proteins has become the method of choice to test the origin function of cloned DNA fragment. The algal proteins were isolated from exponentially growing cells of *C. reinhardtii cc125*. Thylakoid membrane fraction was isolated and purified by a floatation procedure according to Chua *et al* (1973) with minor modification. One mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5mM Σ-amino-n-caproic acid were added to the lysis buffer and floatation

buffer to inhibit protease activitites. High salt extract of the membrane fraction was obtained according to the extraction procedure of Orozco Jr. *et al* (1985). The extract can initiate transcription of Cp DNA but is not sufficient to support *in vitro* DNA synthesis. In addition to the high salt extract, a soluble protein fraction is essential for DNA replication. For the preparation of a soluble protein fraction, the disrupted algal cell suspension was centrifuged at 10K in a sorvall SS34 rotor for 7 min. at 2°C. The supernatant was collected and mixed with ammonium sulfate to 20% saturation. The precipitate was discarded and the supernatant was precipitated again by the addition of solid ammonium sulfate to 45% saturation. Precipitates were collected by centrifugation, resuspended in a minimal volume of extraction buffer, dialyzed and stored at -80°C until use. The standard reaction mixture analogous to Fuller *et al* (1981) was used for DNA replication assay. It contained the following components: Hepes (pH7.6), 40mM, ATP, 2mM; GTP, CTP, and UTP, each 500 uM; bovine serum albumin, 50 ug/ml; creatine phosphate, 21.6 mM; dATP, dGTP, dCTP, and dTTP, each 100 uM, with -^{32}p dTTP added at 3400 cpm per pmol of total deoxynucleotide; magnesium acetate, 11mM; creatine kinase, 100 ug/ml; and supercoiled DNA, 4ug/ml, PEG 6000, was added to the final concentration of 5% (wt/vol). The mixtures were assembled at 0°C. The reactions were initiated by addition of 4 ug proteins extracted from thylakoid membrane isolated from 7x10^{6} cells and 40 ug soluble protein to 25 ul reaction mixture and incubated at 30°C. The reaction was stopped by addition of EDTA and total nucleotide incorporation was measured by determining radioactivity after trichloroacetic acid precipitation. Some characteristics of the *in vitro* system are shown in Table I. The system is ATP-dependent and the extent

of incorporation is template-specific. Both R-13 and SC3-1 are effective templates in this *in vitro* system, but higher incorporation of labeled dNTP into R-13 was consistently observed. Fragment R-13 contains ARC1 and ARS-8 in addition to the D-loop region cloned in SC3-1. This is the only region on the Chl genome that 3 replication-related sequences are closely clustered. Relative position of these functional regions, some useful restriction sites, and the 5 conserved regions, shared between *C. reinhardtii* and *C. sp strain WXM*, mapped previously by EM heteroduplex analysis are shown in Fig. 7. The length and location of these conserved regions are indicated by black bars A to E in Fig. 7. Vallet and Rochaix (1985) mapped the ARC1 element within the 0.35-kb conserved region B and indicated the ARS08 sequence could overlap with conserved region E (Fig. 9). The higher incorporation of labeled dNTP into R-13 could either be due to the presence of multiple initiation site or due to more effective usage of one initiation site. The initiation site of DNA replication in this *vitro* system was investigated by the ddCTP method, ddCTP blocks chain elongation at an early phase of replication (Conrad *et al*, 1979). Replicative intermediates of decreasing extents of replication were generated by using an increasing ratio of the chain terminator ddCTP to dCTP in a series of reactions. The replication products were analyzed by restriction digest, agarose gel eletrophoresis and autoradiography. When R-13 was used as the template, labeling of the 1.65 kb fragment generated by EcoR1 and ClaI digest persisted up to the highest concentration of ddCTP (Wu *et al*, 1986). As shown in Fig. 7, oriA and the D-loop region are located within this fragment; thus they are the first to be replicated. ARC1 is located in the 2.50 kb fragment, ARS08 is located in the 1.35 kb fragment. Labeling of both fragments are decreasing in the

presence of increasing concentration of ddCTP. Therefore, oriA is probably a functional replication origin. the adjacent ARC1 and ARS8 might play some important auxillary roles.

Gene transfer using the cloned replication origin.

Recent work has demonstrated DNA transformation of plant cells, but these experiments are routine only for *Agrobacterium tumefaciens* Ti-plasmid-mediated DNA transfer (Herrera-Estrella *et al*, 1983; Bevan *et al*, 1983). Unfortunately, *A. tumefaciens* DNA transfer has been limited to dicots and a few non-cereal monocots. Techniques are needed to extend gene transfer to other plant species. The transformation of tobacco protoplasts by treatment with DNA and polyethylene glycol has been reported (Krens *et al*, 1982; Paszkowski *et al*, 1984). However, polyethtylene glycol is often detrimental to protoplast viability (Kartha, 1974). Successful nuclear transformation of *Chlamydomonas reinhardtii* by treatment with a plasmid carrying a yeast replication origin in the presence of polyethylene glycol or poly-L-orinithine has been reported. However, the transformation yield is low and it has been very difficult to optimize the transformation procedure (Rochaix and Dillewijn, 1982). Electrical impulses of high field strength reversibly permeabilize biomembranes (Zimmermann, 1982) and thus have had two important applications: the introduction of macromolecules into cells (Neumann *et al*, 1982; Potter *et al*, 1984) and the induction of cell fusions (Zimmermann and Scheurich, 1984; Bates *et al*, 1983). Conditions required to electrically transfer DNA into mouse cells have been reported and a simple apparatus was used to transfer DNA into mouse and human

cells by electroporation (Potter *et al*, 1984). Recently electroporation of DNA into plant protoplasts has been reported (Fromm *et al*, 1985).

To initiate electroporation experiments using algal cells, we have designed and built and electronic device which can deliver short and sharp electrical impulses of high field strength. Exponentially growing algal cells of *C. reinhardtii* were suspended at the concentration of 10^7 cells/ml in the shock medium and placed in a sterilized shock chamber which holds 400 µl of cell suspension. Electric shock of voltages ranging from 1.5KV to 10KV/cm was applied. The shocked algal cells were held on ice for 10 min. before diluting into growth medium. The viabilities of the treated cells were estimated by plating out a fraction of the treated cell on non-selective growth medium. A killing curve was established. Algal cells can sustain the eletroporation condition.

A recombinant plasmid containing the functional Chl DNA replication origin and a selective marker was constructed. Chloramphenicol resistant gene coding for chloramphenicol acetyl transferase (CAT), was used as a selective marker. Chloramphenicol specifically inhibits the protein synthetic activity of chloroplast ribosomes. CAT catalyses the formation of acetylated derivatives of chloramphenicol which are inactive as inhibitors of protein synthesis. The CAT gene used in this construction was subcloned from bacterial transposable genetic element Tn*9* using the Pst I site in IS1 region (Alton and Vapnek, 1979). It has the *E. coli* promoter and can only be expressed in chloroplast. Our testing result shows that for the wild type algal cells, the killing concentration of chloramphenicol is 250 µg/ml. The rate of spontaneous mutation to acquire chloramphenicol resistance is very low (less than 1 out of 10^9

cells). Fig. 8 shows the strategy for the construction of recombinant plasmid pCMori A. Plasmid R-13-43 contains a functional replication origin cloned in a suitable orientation in *E. coli* plasmid pBR325. It contains 2 PstI sites flanking a dispensable region. The larger PstI fragment from R-13-43 and the smaller PstI fragment from M13pCmA were gel purified and ligated by uising the enzyme ligase. The ligated mixture was used to transform *E. coli,* the recombinant plasmid pCMoriA was recovered. Plasmid pCMOriA contains a functional chloroplast DNA replication origin and a bacterial CAT gene. DNA molecules of pCMOriA was electroporated into autolysin-treated algal cells. Surviving cells were plated out in algal growth medium containing 300 ug/ml chloramphenicol to screen for potential transformants. DNA from potential transformants was isolated and dotted on nitrocellulose filter for southern blot analyses. Nick translated CAT gene was used as the probe in the dot-blot analysis. There was no CAT homologous sequence detected in the DNA isolated from wild type algal cells when this particular hybridization condition was used. Potential transformants which show clear positive signals in the dot-blot analysis were counted as real transformants. The transformation rate obtained in several electroporation was in the range of 1 transformant per 3 to 8 x 10^4 surviving cells. When various CAT-containing plasmids which do not contain the cloned replication origin were used in electroporation, the transformation rate were less than 1 transformant per 10^7 surviving cells. This transformation rate is considerably higher than previously reported. It should be noted that gene transfer has been reported for *Chlamydomonas*, using plasmids (Rochaix and van Dillewijn 1982, Hasnaim *et al* 1985) and somatic hybridization (Matagne *et al*, 1979) techniques.

Conclusion, Perspective and the Role of Developing Countries.

Genetic engineering of a unicellular green algae, *Chlamydomonas* has been successful in several laboratories. Recent studies have improved the rate of transformation. In the life cycle of most multicellular algae, single cell stage such as gametes or spores have been detected. These single cells resemble unicellular algae in many respects and could be suitable for genetic modification. In the near future, it should be feasible to transfer specific desirable genes from various sources into a specific algal genome to generate suitable stocks for mariculture. Mariculture as well as the screening and field test of the potential transformant are labor-intense and only involve labor of low scale training. They should be carried out in a developing country which has the natural resources. Scientists interested in marine algae in the developing country should start to collect, characterize, and study the life cycle of different strains of algae which yield useful biopolymers. A rich germplasm collection will provide the source of desirable genes. Complete knowledge of algal life cycle will facilitate the recovery and cultivation of potential transformants. Scientists in the developed country will carry out the cloning of the specific gene, construction of the recombinant DNA, and the initial transformation process. The potential transformants will be shipped to developing countries for screening, cultivation and field test.

Bibliography

Abbott, I.A., and D.P. Cheney. (1982) Commercial uses of algal products: introduction and bibliography. *In*: J. Rosowski and B. Parker, Eds., Selected Papers in Phycology, Vol. II, Phycological Soc. Amer., Lawrence, KA, pp. 779-787.

Alderman, G., and M.H. Stranks. (1967) F. Sci. Fd. Agric. 18: 151.

Alton, N.K., and D. Vapnek. (1979) Nature 282: 864-869.

Bedbrook, J.R. and R.D. Kolodner. (1979) The structure of chloroplast DNA. Ann. Rev. Plant Physiology 30: 593-620.

Bevan, M.W., R.B. Flavell, and M. Chilton. (1983) Nature (London) 304: 184-187.

Cheney, D. (1984) Genetic modification in seaweeds: application to commercial utilization and cultivation. *In*: R. Colwell, E. Pariser, and A. Sinskey (Eds.), Biotechnology in the Marine Sciences, Wiley-Interscience, NY, pp. 161-175.

Chiang, K.S. and N. Sueoka. (1967) Replication of chloroplast DNA in *Chlamydomonas reinhardii* during the vegetative cell cycle: its mode and regulation, Proc. Natl. Acad. Sci. USA. 57: 1506-1513.

Chua, N.H., G. Blobel, P. Siekevitz, and G.E. Palade. (1973) Attachment of chloroplast polysomes to thylakoid membranes in *Chlamydomonas reinhardtii*, Proc. Natl. Acad. Sci. USA. 70: 1554-1558.

Colwell, R. (1983) Science 222: 19-24.

Conrad, S.E., M. Wold and J.L. Campbell. (1979) Origin and direction of DNA replication of plasmic RSF 1030, Proc. Natl. Acad. Sci. USA. 76:736-740.

Fromm, M., L.A. Taylor, and V. Walbot. (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. U.S.A. 82: 5824-5828.

Fuller, R.S., J.M. Kaguni, and A. Kornberg. (1981) Enzymatic replication of the origin of the *Escherichia coli* chromosome, Proc. Natl. Acad. Sci. USA 78: 7370-7374.

Hasnain, S., E. Manavathu, and W. Leung. (1985) Mol. Cell. Biol. 5: 3647-3650.

Herra-Estrella, L., A. Depicker, M. Van Montagu, and J. Schell. (1983) Nature (London) 303: 209-213.

Kartha, K.K., O.L. Gamborg, E.F. Consstabel, and N. Kao. (1974) Can. J. Bot. 52: 2435-2436.

Krens, F.C., L. Molendijk, G.J. Wullems, and R.A. Schilperoort. (1982) Nature (London). 296: 72-74.

Levring, T., H.A. Hoppe, and O.J. Schmid. (1969) Marine Algae: A Survey of Research and Utilization, Cram, DeGruyter and Co., Hamburg.

Loppes, R., and C. Denis. (1983) Chloroplast and Nuclear Fragments from *Chlamydomonas* Promoting High Frequency Transformation of Yeast. Current Genetics 7: 473-480.

Matagne, R., D. Deltour, and L. Ledoux. (1979) Nature (London), 278: 344-346.

Moss, J.R. (1978) Essential considerations for establishing seaweed extraction factories. *In*: The Marine Plant Biomass of the Pacific Northwest Coast, ed. Krauss R.W. pp. 301-314. Oregon State University Press, Corvallis.

Neumann, E., M. Schaefer-Ridder, Y. Wang, and P.H. Hofschneider. (1982) EMBO J. 1: 841-845.

Orozco, Jr., E.M., J.E. Mullet, and N.J. Chua. (1985) An *in vitro* system for accurate transcription initiation of chloroplast protein genes, Nuc. Ac. Res. 13: 1283-1302.

Paszkowski, J., R.D. Shillito, M. Saul, V. Mandak, T. Hohn, B. Hohn, and I. Potrykus. (1984) EMBO J. 3: 2717-2722.

Potter, H., L. Weir, and P. Leder. (1984) Proc. Natl. Acad. Sci., USA 81: 7161-7165.

Rochaix, J.D. (1978) Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardii*, J. Mol. Biol. 126: 567-617.

Rochaix, J. and J. Van Dillewijn. (1982) Nature 296: 70-72.

Rochaix, J.D., J. Van Dillewijn, and M. Rahire. (1984) Construction and characterization of autonomously replicating plasmids in the green unicellular alga *Chlamydomonas reinhardii*, Cell 36: 925-931.

Sager, R., and M.A. Ishida. (1963) Chloroplast DNA in *Chlamydomonas*, Proc. Natl. Acad. Sci. USA 50: 725.

Tseng, C.K. (1981) In the Biology of Seaweeds, Ed. Lobban and Wyunne. University of California Press.

Vallet, J.-M., M. Rahire, and J.-D. Rochaix. (1984) Localization and sequence analysis of chloroplast DNA sequences of *Chlamydomonas reinhardii* that promote autonomous replication in yeast. EMBO J. 3: 415-421.

Vallet, J.-M., and J.-D. Rochaix. (1985) Chloroplast origins of DNA replication are distinct from chloroplast ARS sequences in two green algae. Current Genetics 9: 321-324.

Waddell, J., W. Wang, and M. Wu. (1984) Electron microscopic localization of the chloroplast DNA replicative origins in *Chlamydomonas reinhardii*, Nuc. Ac. Res. 12: 3843-3856.

Wang, W., C.H. Chang, J. Waddell, and M. Wu. (1984) Cloning and delimiting one chloroplast DNA replicative origin of *Chlamydomonas*, Nuc. Ac. Res. 12: 3857-3872.

Wu, M., J.K. Lou, D.Y. Chang, C.H. Chang, and Z.Q. Nie. (1986) Structure and function of a chloroplast DNA repliciation origin of *Chlamydomonas reinhardtii*, Proc. Natl. Acad. Sci. USA (in press).

Zimmermann, U. and J. Vienken. (1982) J. Mebr. Biol. 67: 165-182.

Figure Legends

Fig. 1. Electron micrographs of Chl DNA EcoRl fragments containing D-loops

Fig. 2. The positions of D-loops on Rochaix's restriction endonuclease map of the *C. reinhardtii* Chl DNA.

Fig. 3. a) Histogram shows the size distribution of replication loops. Open bars represent D-loops with 1 complete single strand. Black bars represent replication loops containing double stranded region on both strands of the loop. b) Micrograph of a large replication loop, both strands containing predominant double stranded regions with single stranded gaps. c) Explanatory tracing of the molecule. Solid line represents double stranded region, dotted line represents single stranded region. Bar represents 1 kb.

Fig. 4. A) and B) show the hybridization pattern of ^{32}P-labeled R-13 to the EcoRI digest (lane E), the BamHl digest (lane Bm) and the BglII digest (lane B_g) of the Chl DNA isolated from *C. reinhardii cc 125*, (A) shows the ethidium bromide staining pattern. (B) shows the hybridization pattern after autoradiography. HindIII digest of λ DNA (lane M) is the size marker, triangles are placed next to the hybridizing fragments in (A). (C) and (D) show the Hybridization pattern of ^{32}P-labeled R-13 to the BglII (lane Bg), the BamHl (lane Bm) and the EcoRI (lane E) digests of Chl DNA isolated from *C. sp strain WXM*, HindIII digest of λ DNA (lane M) is the size marker, (C) shows

the ethidium bromide staining pattern, (D) shows the hybridization pattern after autoradiography. Triangle is placed next to the strong hybridizing EcoRl fragment.

Fig. 5. Electron micrographs and explanatory tracings of heteroduplexes prepared from the EcoRl inserts of clones CR-13 and CI-10. a) The spreading solution was 60% formamide in 0.1M Tris (pH8.5), 0.01M Na_3EDTA. b) The spreading solution was 50% formamide, the hypophase was 20% formamide. c) The spreading solution was 40% formamide, the hypophase was 10% formamide. The homologous regions between those 2 EcoRl fragments are labeled with A, B, C, D, E consecutively in the explanatory tracings. Bar in each micrograph represents the length of lkb double stranded DNA.

Fig. 6. Restriction endonuclease map of R-13 fragment. b) A diagram shows the relative position of D-loop region in R-13 fragment c) Diagram of a heteroduplex molecule between R-13 and I-10, the homologous regions are labeled in the same order as in Fig. 5. Numeral in each region represents the average length in kb measured from more than 10 molecules each, standard deviations were less than 8%.

Fig. 7. Physical and functional map of clone R-13. Restriction sites of EcoRl and Clal are shown. The Kb lengths of each restriction fragment generated by EcoRl and Clal double digest are indicated in parantheses. Dotted line represents pBR325 sequence. Waved line represents sequence cloned in SC3-1. The 5 conserved regions between *C. reinhardtii* and *C. sp. strain WXM* mapped by Wang et al (1984) are indicated by black bars labeled A to E.

ARC1 and ARS 08 regions were mapped by Vallet and Rochaix (1985). The replication origin of pBR325 and ChlDNA (OriA) are also indicated.

Fig. 8. Cloning strategy to construct a recombinant plasmid containing the functional replication origin and the chloramphenicol resistant gene.

Table I Requirements for DNA synthesis and the Effects of Inhibitor in vitro

Condition	DNA Synthesis activity(%)
Complete	100%
Without DNA	<5%
Without ATP, creatine phosphate, creatin kinase	25%
Without rCTP, rGTP and rUTP	73%
Without dCTP	56%
Without Mg^{++}	31%
Without soluble proteins	18%
Without membrane extracts	57%
Supplemented with 2 μg/ml Ethidium Bromide	24%
Supplemented with 10 μg/ml Ethidium Bromide	0

DNA synthesis activity was measured for 60 min. at $30^{\circ}C$ in the standard reaction assay as described in the text. 100% equaled 9.6 pmole dNTP incorporated.

Fig. 1

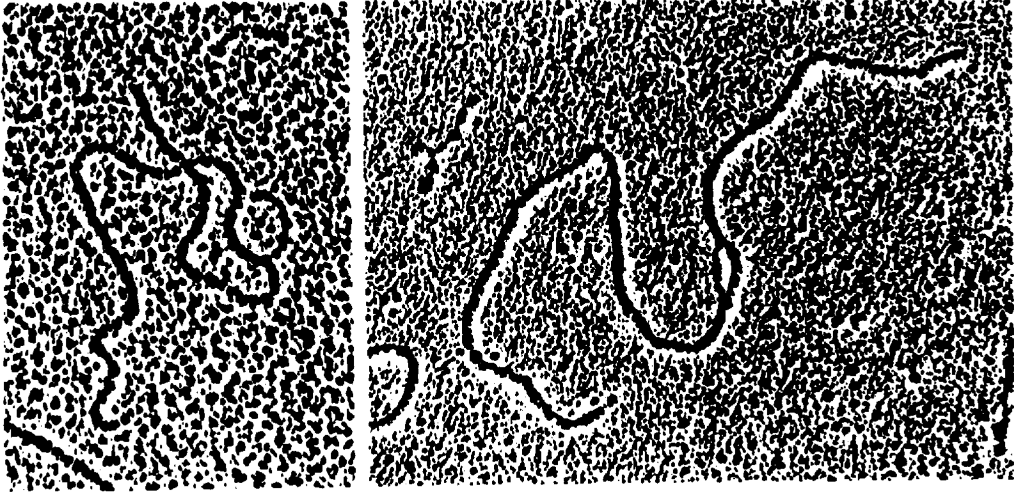


Fig.2

Ba1 Ba4
5S 23S 16S

R07
R24 R19 R04 R2
R16 (R11,R6a,R5,R3)
R12
(R7,R8)
R15
(R18,R02)
R4
(R23,R9)
R25
R26
R01
R24'
(R20,R1,R03,R05)
R10
R17
R13
R22
R21
R6b R14 R24 R07

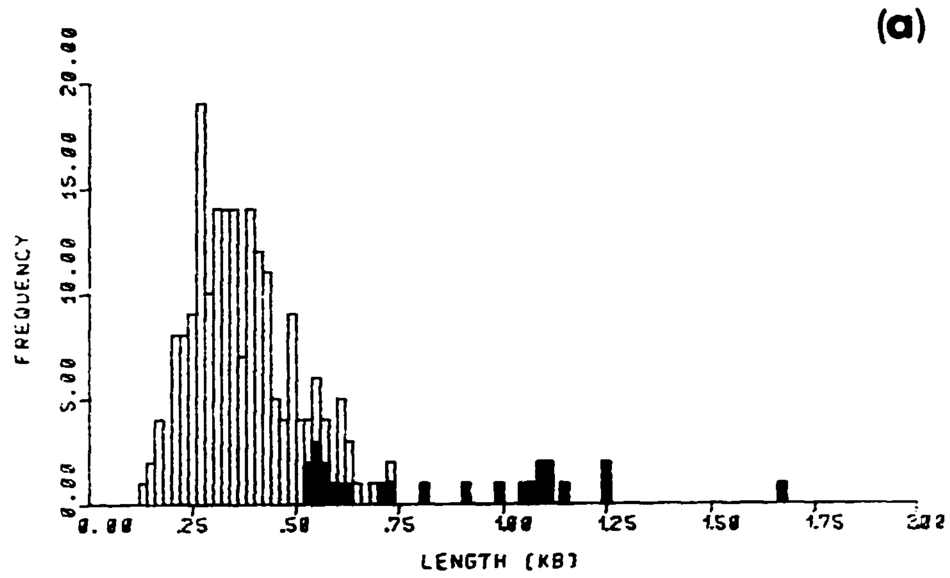
Ba1 Ba4 Ba5
Ba14
Ba7 Ba01
Ba15
Ba13
Ba9
Ba10
Ba11
Ba3
Ba02
Ba6
Ba12 Ba1 Ba4

Bg13
Bg12
Bg10
Bg5
Bg2 Bg1
Bg3
Bg11
Bg14
Bg8
Bg4 Bg6

Ori A
Ori B

Ba1 Ba4
5S 23S 16S

Fig.3



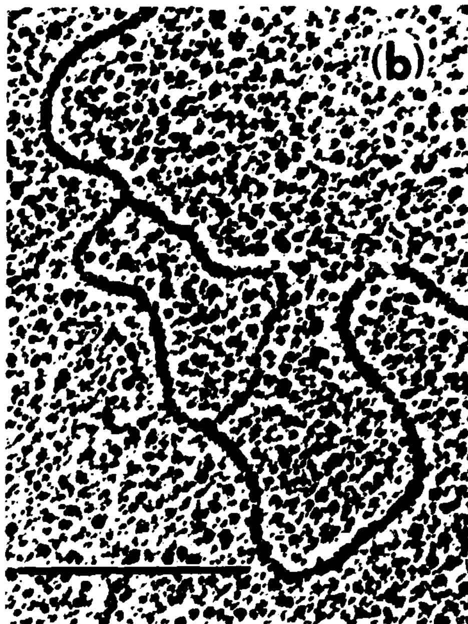
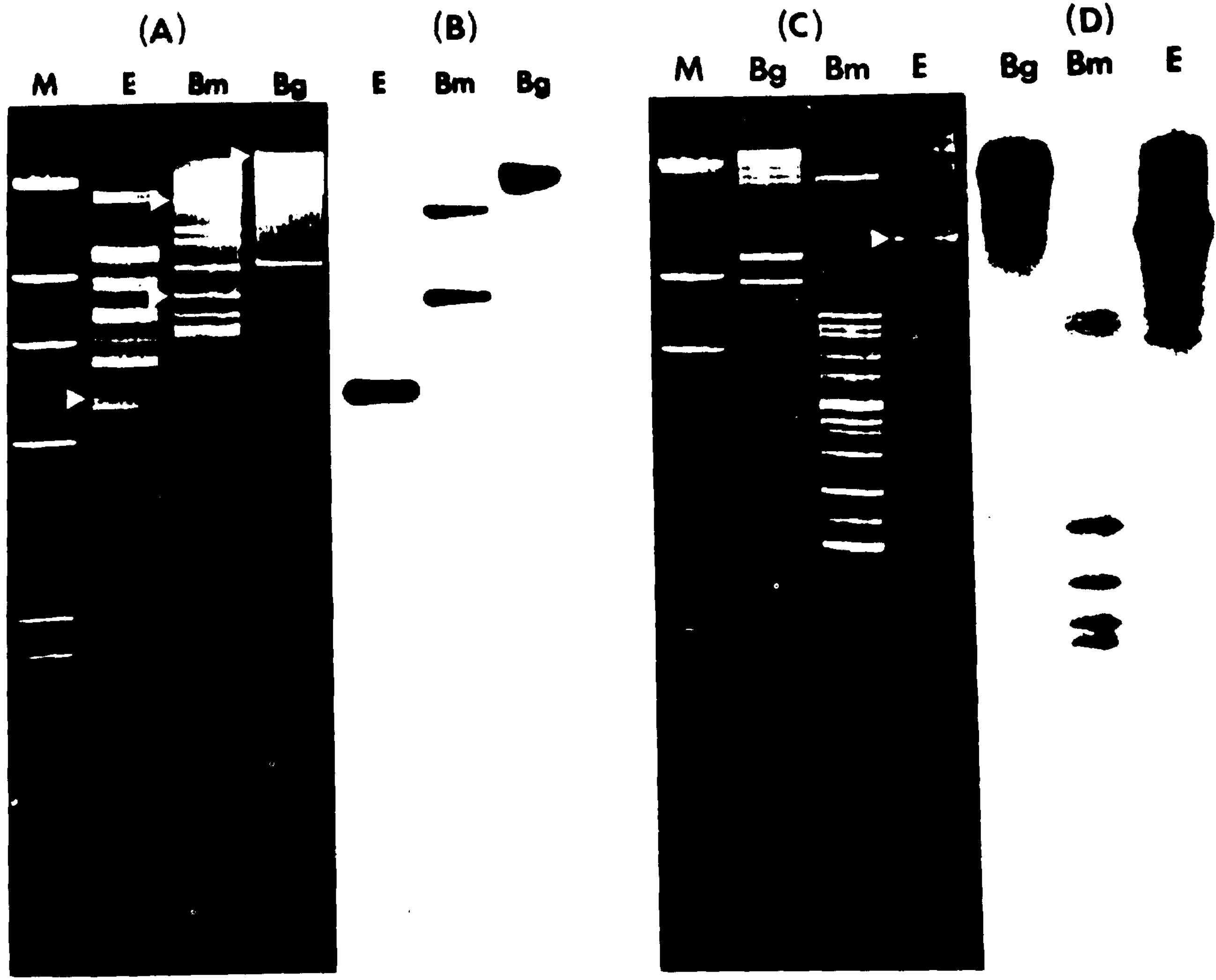
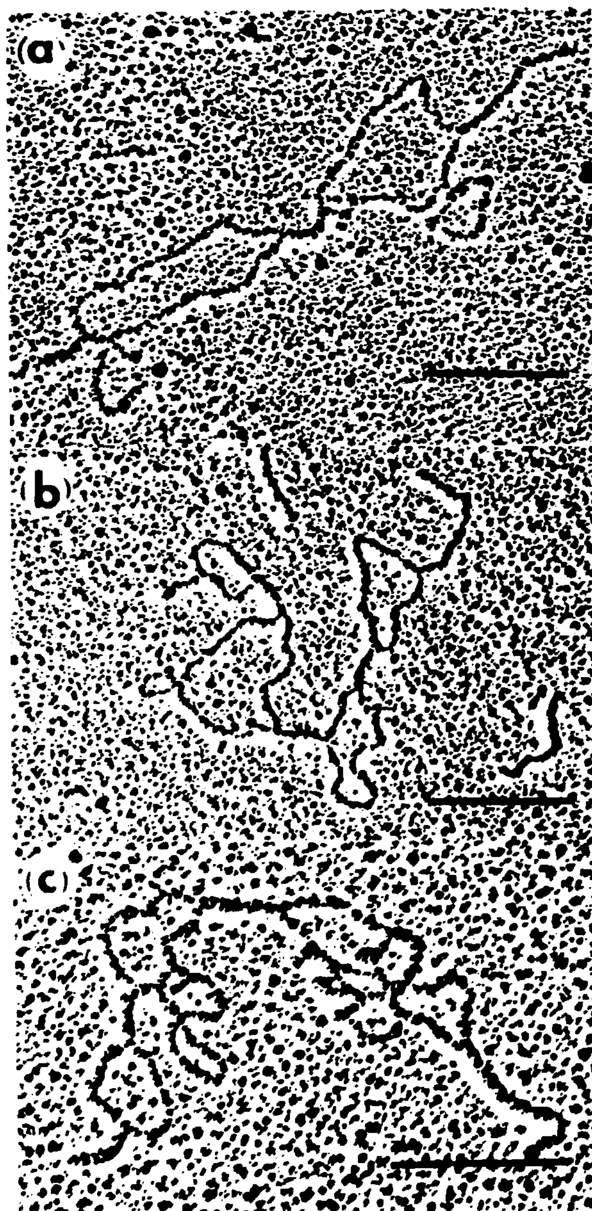
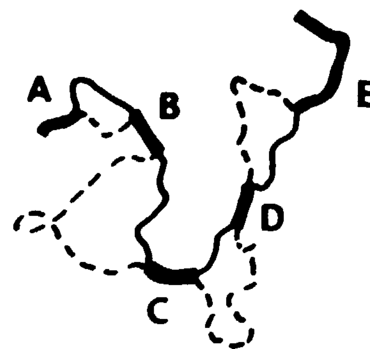


Fig.4




(a)
(b)
(c)

E
C
A


A
B
E
D
C

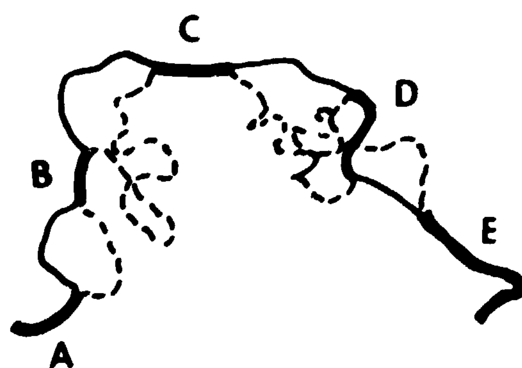
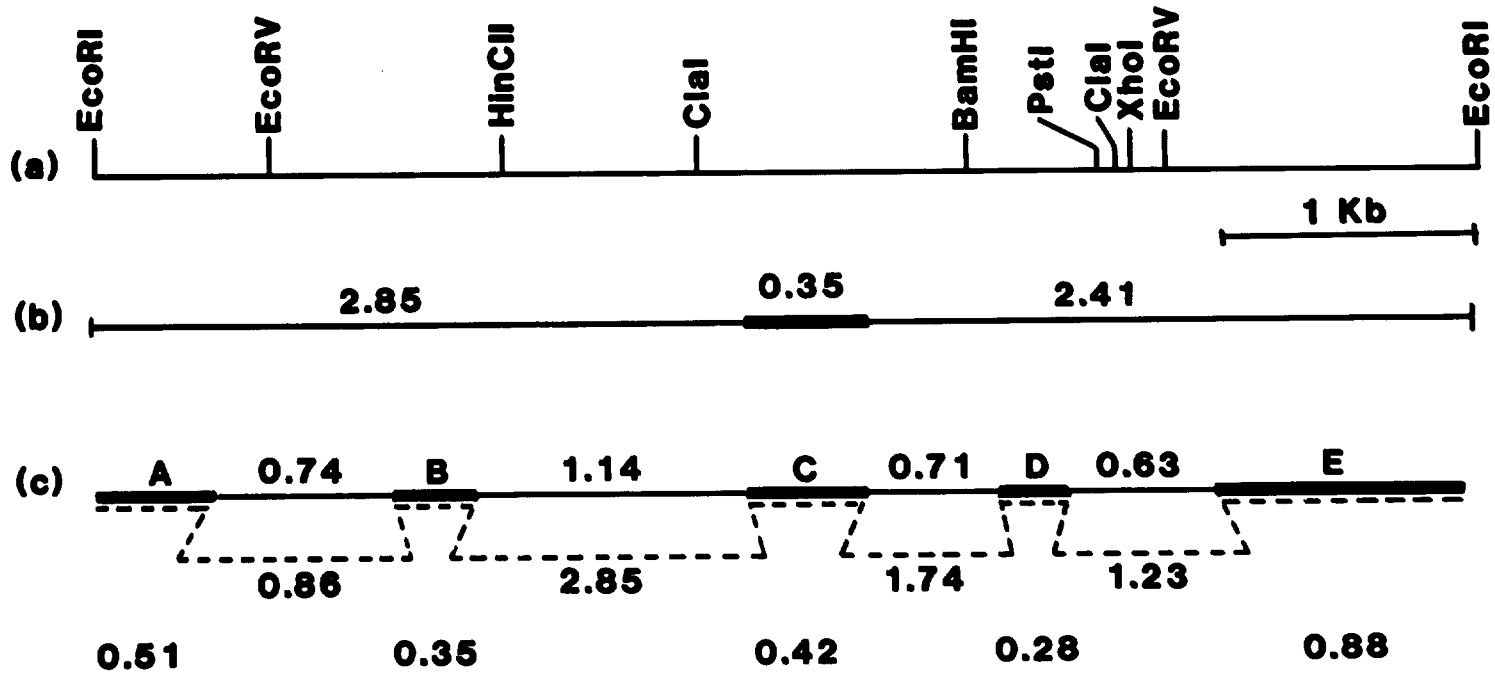

C
D
B
E
A

Fig.6



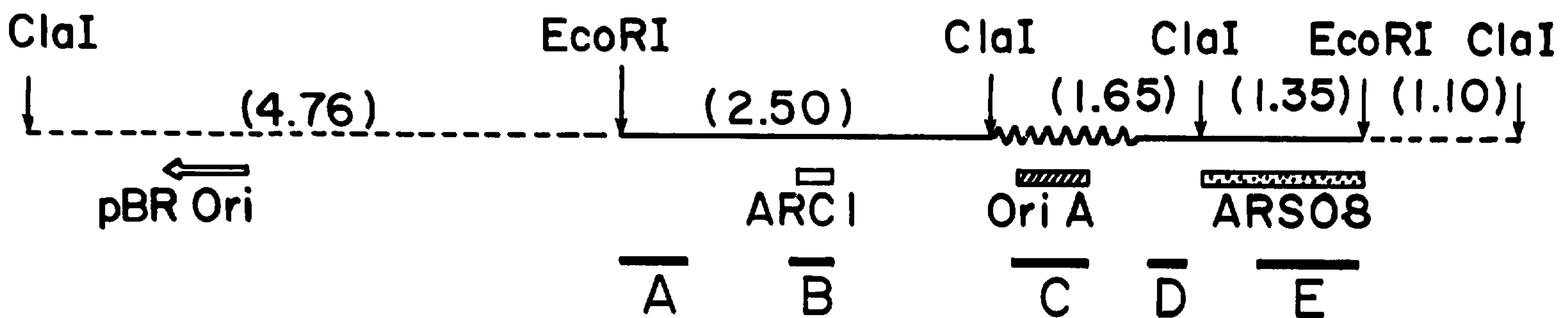


Fig.7

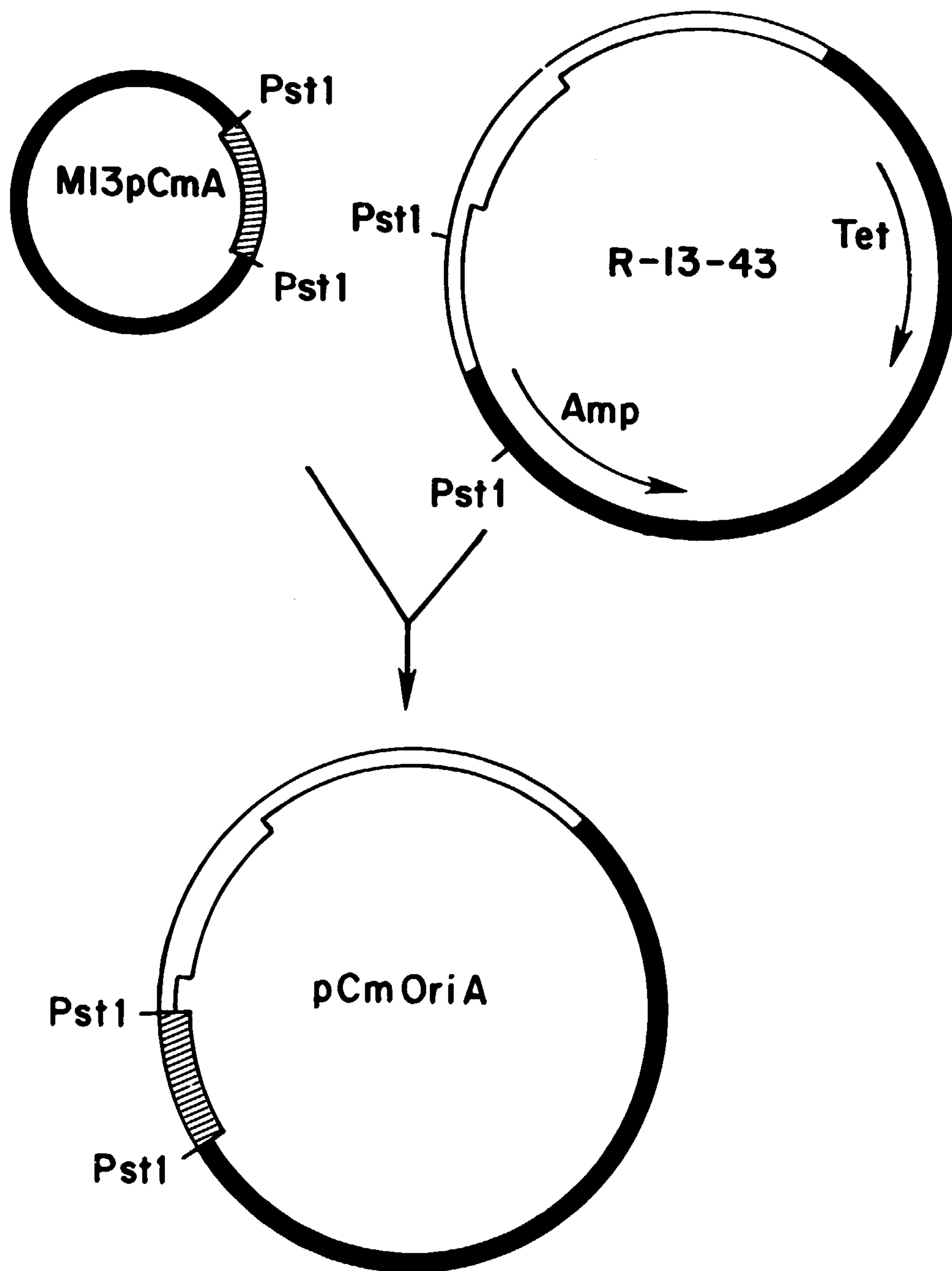


Fig. 8