On Further Study of Extracellular Accumulation of DNA by Hydrocarbon-utilizing *Pseudomonas* Species

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Received July 30, 1973

Extracellular accumulation of high molecular weight DNA was further studied using *Pseudomonas* species. More efficient production was obtained by the use of glucose-grown seed culture and by controlling the broth-pH at around 6.0 for first 24 hr and then around 8.0 during the fermentation. The maximum yield was 5 to 6 g per liter of the broth culture, which corresponded to 10-fold of that reported in the previous work.

Purified DNA (4×10^6 daltons) was obtained successfully by applying an aqueous biphasic system of dextran-polyethyleneglycol and dextranase.

Significant release of DNA occurred only with cell lysis of *n*-paraffin-grown bacteria. The primary cause of rapid lysis was explained by the exhaustion of cellular glucose pool. Relation of DNA accumulation to the effect of rhamnolipids on cell membrane was also investigated.

In our previous paper,¹⁾ we reported that some hydrocarbon-utilizing *Pseudomonad* and *Arthrobacter* extracellularly accumulated high molecular weight DNA. The primary cause of DNA accumulation by *Pseudomonad* was explained by a rapid cell lysis occurred with the exhaustion of *n*-paraffin.

The present investigation was undertaken, on the one hand, to find the culture condition for the production of larger quantity of DNA and to isolate high molecular weight DNA more efficiently, on the other hand, to provide further evidence for the accumulation mechanism relating to the cell lysis.

This paper deals with the effect of carbon source in the seed culture and the pH of the culture medium, and with large scale-application of an aqueous biphasic extraction of DNA. Relation of the cell lysis to internal glucose pool in *n*-paraffin-grown cells is also described.

MATERIALS AND METHODS

Microorganisms. *Pseudomonas* strains were from our laboratory stock. Among them, *Pseudomonas fluorescens* KY4032 was studied extensively.

Culture methods. The composition of media for seed culture and fermentation was described previous-

ly.¹⁾ The bacteria were grown in 250 ml Erlenmeyer flasks or jar fermentors depending on the experiments at 30°C. All the experiments in flasks were carried out with shaking. The jar fermentor was operated under the same condition as described previously.¹⁾

Assays. Cell growth was estimated by absorbancy at 660 nm of appropriately diluted broth culture using a spectrophotometer. Assay of high molecular weight DNA was performed as follows; two times volume of ethanol was added to an aliquot of the broth supernatant and the resulted fibrous precipitates were subjected to the DNA assay of Dische²⁾ after dissolving in a given amount of saline. Total amount of DNA was also determined by the same colorimetric method with 75% ethanol insoluble precipitates. RNA was assayed with 75% ethanol insoluble precipitates according to the method of Zamenhof.³⁾ Dextran and glucose were determined respectively with Anthrone reagent⁴⁾ and by the method of Somogyi.⁵⁾

Chemicals. *n*-Paraffin mainly containing C-12 to C-14 fractions was obtained from the Nikko Petrochemical Co. (Japan). Dextran T-500 was the product of Pharmacia (Sweden). Polyethyleneglycol, "Carbowax 6000" was purchased from Union Carbide Chemical Co. (U.S.A.). Rhamnolipid was prepared by the method indicated previously.⁶⁾ Dextranase was prepared from the broth supernatant of *Aspergillus candidus* by precipitation with ammonium sulfate (0.8 sat.) followed by acetone precipitation. The specific activity of the enzyme was found to be 2600 mg

of glucose liberated from the substrate/hr/mg. All other chemical compounds were the best grade reagents.

RESULTS AND DISCUSSION

Effect of carbon sources in the seed culture

Since most of hydrocarbon-utilizing bacteria, belonging to *Corynebacterium* and its closely related group, utilize glucose poorly as the carbon source, sugar alcohols such as sorbitol have been generally used in the seed medium for their *n*-paraffin fermentation. *Pseudomonad* can utilize glucose effectively, thus sorbitol in the seed medium was replaced by glucose and its effect on accumulation of DNA was examined. As shown in Fig. 1, the growth rate in the fermentation medium was increased by the use of glucose-grown seed culture. In addition, the lysis of cells was also enhanced, thus it led to the accumulation of considerably great amount of DNA.

Effect of pH levels during the fermentation

In preliminary experiments with flasks, we observed that the pH of the medium affected greatly on the growth rate and on the ac-

cumulation of DNA. Therefore detailed examination was attempted about the effect of the pH on the fermentation. When the pH was controlled at around 6 for the first 24 hr and then at around 8, the accumulation of high molecular weight DNA increased considerably and about 5 g per liter were obtained as the result of cell lysis. When the pH was controlled at around 7, little DNA was recovered as high molecular weight polymer as shown in Table I and Fig. 2. However the total amount of DNA (low and high molecular weight) were not different significantly between the cultivations controlled at pH 7 and pH 8. From the above finding, the presence of active DNase was suspected in the broth culture controlled at pH 7.

Isolation of DNA from the broth culture

Since a fairly large amount of the broth culture must be treated in this work, we chose the method of Rudin *et al.*⁷⁾ because of mildness of the procedure. The method involves multistep liquid-liquid extraction with an aqueous polymer mixture of dextran and polyethyleneglycol. The original application of this method was made to the purifica-

FIG. 1. Effect of Carbon Sources of Seed Medium on DNA Accumulation.

Incubation was carried out in 5 liter jar fermentors as described in the methods. The inoculum size for fermentation was 5% in volume.

(A) sorbitol-grown seed culture, (B) glucose-grown seed culture.

—, pH; ▲—▲, growth; ●—●, DNA.

TABLE I. EFFECT OF pH CONTROL ON DNA PRODUCTION

Seed cultures were performed with glucose medium. All measurements were made as indicated in the text.

pH	Time (hr)	High molecular weight DNA mg/ml	Total DNA mg/ml	RNA mg/ml
6	0	0	0.31	0.24
	48	0.02	0.65	0.26
	60	1.0	2.2	1.5
	72	3.0	4.1	2.2
7	0	0	0.28	0.26
	48	0.09	0.64	0.17
	60	0	2.8	1.1
	72	0.02	4.6	1.4
6→8	0	0	0.32	0.28
	48	0.6	1.3	0.41
	60	4.2	5.5	3.1
	72	3.8	4.8	2.5

FIG. 2. Effect of pH on DNA Production.

Experiments were carried out in 5 liter jar fermentors. The open symbols indicate the amount of DNA accumulated and the closed symbols indicate the growth of bacterium (A_{660}).

■—■, controlled at pH 6; ▲—▲, pH 7; ●—●, controlled at pH 6 for the first 24 hr and then at pH 8 during fermentation.

tion of DNA from cell lysates of *E. coli* or *B. subtilis* in the definite composition of salts. In order to isolate high molecular weight DNA from the broth culture, this method was principally followed, although salts concentration was different from that as indicated by Rudin *et al.* Prior to the extraction, the supernatant fluid obtained by centrifugation of the broth culture was shaken with an equal volume of the mixture of chloroform and isoamylalcohol (24:1, v/v). After centrifugation at 7000 rpm for 15 min, the upper layer containing nucleic acids was pipetted out. This procedure was repeated three times with lower layer. To the aqueous solution thus collected, NaCl (4 M at the final concentration) was added to salt-out residual protein and to adjust the ionic strength for the subsequent procedure. The supernatant solution was subjected to the subsequent extraction with dextran-polyethyleneglycol (0.2-7.3%). As shown in Fig. 3, protein and low molecular weight RNA were removed by repeating this extraction. High molecular weight DNA was obtained in upper layer of successive eight extraction with dextran-polyethyleneglycol (0.4-5.9%) containing NaH_2PO_4 (5 mM),

FIG. 3. Extraction Profile of Two Phase System.

Procedures for extraction were described in the text. A, protein and low molecular weight RNA; B, DNA; C, high molecular weight RNA.

Na_2HPO_4 (2 mM) and sodium citrate (1 mM). Minor fraction was obtained separately from DNA fraction by further extraction with dextran-polyethyleneglycol (0.4-5.9%) containing Na_2HPO_4 (5 mM) and Na_3PO_4 (5 mM) and characterized as high molecular weight DNA.

As mentioned above, the method of Rudin

Broth culture

| centrifuged

Supernatant fluid

| mixed with CHCl_3 + isoamylalcohol

| centrifuged repeated the above procedure three times added 4 M NaCl

Supernatant fluid

| mixed with dextran (6%), polyethyleneglycol (12%) and 4 M NaCl

Lower layer

| mixed with dextran (0.2%), polyethyleneglycol (7.3%) and 4 M NaCl

Lower layer

| repeated the above procedure four times mixed with dextran (0.4%), polyethyleneglycol (5.9%), 5 mM NaH_2PO_4 , 5 mM Na_2HPO_4 and 1 mM sodium citrate

Lower layer

| repeated the above procedure eight times

Upper layer (DNA fractions)

| added 0.1 vol. polyethyleneglycol (40%) and 0.05 vol. of 0.4 M Na_2HPO_4

Upper layer

| mixed with 1 vol. of CHCl_3

Upper layer

| added dextranase, dialysed and deproteinized

Supernatant fluid

| added ethanol

DNA (purified)

Final yield 13%

FIG. 4. Isolation Procedure of DNA by Phase System.

FIG. 5. Zone Sedimentation of DNA.

Samples were layered on the top of the gradient (53 ml) of sucrose (5 to 20%) in SSC (0.15 M NaCl + 0.015 M sodium citrate, pH 7.2). After centrifugation, fractionation of the tube contents was carried out through a needle introduced in the bottom of the tube. Centrifugation was conducted with a Hitach RPS25-2 rotar at 20,000 rpm for 20 hr. (A) without dextranase, (B) with dextranase treatment.

et al. proved to be applicable for the broth culture without modification. Figure 4 presented a typical extraction flow for the large scale preparation. As shown in Fig. 4, DNA fraction thus obtained was mixed with 0.1 vol. of polyethyleneglycol (40% w/w) and 0.05 vol. of Na_2HPO_4 (0.4 M), and centrifuged at 3000 rpm for 5 min. Upper layer was extracted again with chloroform to remove concomitted polyethyleneglycol. Small amount of dextran contaminated in the aqueous layer thus obtained. Therefore dextranase treatment was employed in 0.2 M citrate buffer (pH 5.0) for 30 min at 30°C. Under this condition, 5 to 100 μg of dextranase per ml was proved to be available without any degradation of DNA, by the observation of sedimentation pattern in sucrose density gradient. From the solution treated with dextranase, purified fibrous DNA was obtained by ethanol precipitation. Molecular weight of the isolated DNA was calculated to be 4×10^6 daltons from the result of zone sedimentation as shown in Fig. 5.

Some possible factors responsible for DNA accumulation

Rhamnolipids. In our previous papers^{6,8)} it was reported that many strains of *Pseudomonas aeruginosa*, when grown on *n*-paraffin as the sole source of carbon, produced a fairly large amounts of two sorts of rhamnolipids, either of which was made as an important factor for *n*-paraffin utilization by the bacteria. A possible role of rhamnolipids in *n*-paraffin utilization was realized in their activity as a surface active agent which is responsible for dispersing *n*-paraffin in the broth culture.

TABLE II. PRODUCTION OF DNA BY VARIOUS *Pseudomonas*

Incubation was carried out in 250 ml flasks with shaking. All measurements were performed after 3 days of fermentation.

Strain		DNA	Rhamno	Growth
		mg/ml	lipids	
<i>P. fluorescens</i>	KY3965	0.5	0	+
	KY3974	0.6	0	—
	KY3975	1.3	1.5	+
	KY4032	3.1	5.7	++
	KY4033	0.5	0	+
<i>P. syncyanea</i>	KY4018	0.5	0	±
<i>P. boreopolis</i>	KY3967	0.4	0	±
<i>P. chlorophis</i>	KY4000	0.6	0	+
<i>P. convexea</i>	KY4014	0.4	0	+
<i>P. cruciviae</i>	KY3960	0.2	0	+
<i>P. dacunhae</i>	KY3952	0.4	0	+
<i>P. fairmontensia</i>	KY4022	0.2	0	—
<i>P. marginalis</i>	KY4013	0.3	0	±
<i>P. melanogenum</i>	KY3986	0.6	0	±
<i>P. oleovorans</i>	KY3981	0.2	0	—
<i>P. polycolor</i>	KY4015	2.1	5.2	+++
<i>P. robescens</i>	KY3993	0.5	0	+
<i>P. aeruginosa</i>	KY3951	0.4	0	±
	KY3955	2.8	6.5	+++
	KY3976	0.3	0	±
	KY3977	1.2	0.8	+
	KY3996	3.0	5.9	+
	KY3997	2.0	0.8	+
	KY4020	1.5	0.8	+
	KY4025	3.5	6.3	+++
	KY4027	3.8	3.2	+
	KY4276	3.0	0.9	+
	KY8508	3.1	3.4	++
	KY8509	3.7	4.5	++
	KY8520	3.6	10.0	+

However it was obscure how these lipids functioned on the cell surface when *n*-paraffin was exhausted at the end of the incubation even though they functioned as a growth stimulator during the growing phase. Considering that a primary cause of DNA release was the cell lysis which occurred with the exhaustion of *n*-paraffin, if rhamnolipid played also a part in the cell lysis of *n*-paraffin-grown cells, some correlations must be found between DNA accumulation and rhamnolipids. On the basis of the above assumption, various *Pseudomonas* species were compared of their growth on *n*-paraffin and productions of rhamnolipids and DNA. As summarized in Table II, almost all strains capable of producing rhamnolipids accumulated a fairly large amount of DNA. These results suggest a correlation of DNA accumulation and production of rhamnolipids.

In order to provide further evidence for the role of rhamnolipids in DNA release, the function of rhamnolipids as a surface activating agent was examined using spheroplasts. Spheroplasts of *Pseudomonas fluorescens* KY 4032 was prepared by lysozyme-EDTA method⁹: bacterial cells grown on glucose or *n*-paraffin were collected respectively by centrifugation. Each preparation was then suspended (O.D.₆₆₀=4.0) in Tris-buffer (10 mM, pH 7.4) containing 4 mM EDTA, 0.5 M sucrose and lysozyme (0.2 mg/ml) and incubated at 30°C for 15 min. The cells of both cultures proved microscopically to be converted to spheroplasts to the same extent. Spheroplasts thus obtained were used for subsequent experiments. To the surfactants dissolved in Tris-buffer (10 mM, pH 7.4) containing 0.5 M sucrose, one eighth volume of spheroplasts suspension was added and the mixture was followed by the measurement of optical density at 660 nm by a spectrophotometer. As shown in Fig. 6, the rhamnolipid (2-O- α -L-rhamnopyranosyl- α -L-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate, R-1) bursted the spheroplasts of glucose-grown cells at the concentration of 50 μ g/ml which was far less amount compared with Tween 20 or

FIG. 6. Bursting of Spheroplasts by Rhamnolipid, R-1.

Spheroplasts were prepared from glucose-grown cells except those shown in open circles, which were prepared from *n*-paraffin-grown cells.

▲—▲, Tween 20; ■—■, SDS; ●—●, ○—○, R-1.

sodium dodecyl sulfate. Comparing with spheroplasts prepared from hydrocarbon-grown cells, there was a little difference in the susceptibility of those from glucose-grown cells towards the rhamnolipid (Fig. 6). As shown previously,⁶ either lysis or release of DNA was not observed in the glucose-medium even though 0.7 mg/ml of rhamnolipids were present. Thus it is difficult to explain the distinct difference in DNA production between glucose- and hydrocarbon-medium, from the difference in the susceptibility of spheroplasts towards rhamnolipids.

Subsequently the susceptibility of intact cells

FIG. 7. Effect of Phamnlipid, R-1 to Intact cells.

●—●, R-1; ▲—▲, SDS.

towards R-1 was also examined. Glucose or hydrocarbon-grown cells were respectively collected by centrifugation and washed once with Tris-buffer (10 mM, pH 7.4). Washed cells suspended in the same buffer were contacted with R-1. As shown in Fig. 7, incubation for 30 min at 30°C did not cause the bursting of cells even at the concentration of 5 mg of R-1 per ml, which corresponded to five fold amount produced in the glucose medium.⁶⁾ In other experiment, R-1 (1.0 to 10 mg/ml) was added to cells incubated overnight in glucose medium, but it did not also result in detectable lysis even by longer incubation. Accordingly it seems likely that rhamnolipids play only a minor role in lysis of cells and release of DNA, although the triggering action of rhamnolipids in autolysis was not resolved yet.

Glucose. We have shown in the previous paper¹⁾ that *Pseudomonad* cells grown on glucose were resistant to lysis and did not release DNA. Therefore effect of glucose was examined again. Comparing with *n*-paraffin medium, glucose medium gave a less growth and no release of DNA (Fig. 8). When the concentration of glucose was reduced to 2.5%, it resulted in the release of DNA associating with cell lysis after glucose was

FIG. 8. Effect of Concentrations of *n*-Paraffin and Glucose on DNA Accumulation.

Incubation was carried out in 250 ml flasks with shaking at 30°C.

(A) *n*-paraffin, (B) glucose.

—, 10%; ----, 5%; - · - · -, 2.5%.

TABLE III. EFFECT OF CARBON SOURCES ON DNA PRODUCTION

Incubation was carried out in 250 ml flasks with shaking for 3 days. Carbon sources were added at the concentration indicated in the Table.

Carbon source	DNA produced (mg/ml)		
	2.5%	5.0%	10%
<i>n</i> -Paraffin	1.06	2.24	2.74
Glucose	1.02	0.76	0.66
Sucrose	1.18	1.17	1.26
None	1.18	—	—

consumed completely (Table III). The above finding suggests that starvation of the carbon source could be the primary cause of cell lysis.

Other possible factors. Although it is likely that the exhaustion of glucose from cellular pool was the primary cause of the cell lysis, the factor responsible directly for the lysis may remain to be found. In attempt to find it, the examination was focussed on the broth culture of hydrocarbon medium. The supernatant fluid obtained from the broth culture after 48 hr incubation was passed through a membrane filter (Millipore filter, pore size 0.3 μ) to remove living cells and used as the conditioned medium for subsequent experiments. Glucose-grown cells harvested at 48 hr of incubation were incubated further in the conditioned medium supplemented with glucose or R-1. As indicated in Fig. 9, cells grew well in the conditioned medium without any supplement and lysed rapidly. Growth was stimulated extensively with supplementation of glucose, which was distinct from the observation in Fig. 8 where no lysis of cells were observed. This might indicate that the conditioned medium contained a factor stimulating growth. Residual glucose in the media was measured and found that in the conditioned medium glucose was almost completely consumed (Table IV). The above results suggested that exhaustion of carbon source might be the primary cause of rapid lysis. The presence of a factor stimulating autolysis was not plausible because cell lysis was not observed immediately after change of the medium.

TABLE IV. RESIDUAL GLUCOSE AFTER EXCHANGE OF THE MEDIA

Incubation was carried out for 3 days after exchange of the media.

Additives	Residual glucose (mg/ml) Conditioned medium	Fresh medium
None	1.0	0.3
Glucose 5%	0.9	2.2
Rhamnolipid, R-1 5 mg/ml	0.8	0.6
Glucose 5%+R-1 5 mg/ml	0.9	2.0

FIG. 9. Lysis of Cells in Conditioned or Fresh Medium. Experimental procedures were described in the text.

(A) conditioned medium, (B) fresh medium.

○—○, none; ●—●, glucose, 5%;

■—■, rhamnolipid, R-1, 5 mg/ml;

▲—▲, glucose, 5% plus rhamnolipid, 5 mg/ml.

Thus the rapid lysis in hydrocarbon-medium might be explained as follows; the intracellular pool of glucose, steadily synthesized from

hydrocarbon, reduced rapidly after the substrate was exhausted and it consequently led to the cell lysis.

It has been shown that phospholipase C production parallels with the production of rhamnolipid¹⁰⁾ and that phospholipase C might cause the triggering of lysis in *Bacillus subtilis*.¹¹⁾ In our case also phospholipases might cause the triggering action after exhaustion of carbon sources in cellular pool. The investigation from the aspects of enzymic action are in progress in our laboratory.

REFERENCES

- 1) F. Tomita and T. Suzuki, *Agr. Biol. Chem.*, **36**, 133 (1972).
- 2) Z. Dische, *Mikrochemie*, **8**, 4 (1930). Cited by Chargaff and Davidson, "The Nucleic Acid," Vol. 1, 1955, p. 287.
- 3) "Methods in Enzymology," Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N.Y., 1957, p. 696.
- 4) T. A. Scott, Jr. and E. H. Melvin, *Anal. Chem.*, **25**, 1656 (1953).
- 5) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).
- 6) S. Itoh, H. Honda, F. Tomita and T. Suzuki, *J. Antibiotics*, **24**, 855 (1971).
- 7) L. Rudin and P. A. Albertsson, *Biochim. Biophys. Acta*, **134**, 37 (1967).
- 8) S. Itoh and T. Suzuki, *Agr. Biol. Chem.*, **36**, 2233 (1972).
- 9) H. C. Neu and L. A. Heppel, *J. Biol. Chem.*, **239**, 3893 (1964).
- 10) S. Kurioka and P. V. Liu, *J. Bacteriol.*, **93**, 670 (1967).
- 11) Y. Koga and I. Kusaka, *Europ. J. Biochem.*, **16**, 407 (1970).