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Research Article

ISOLATION AND CHARACTERIZATION OF NONCOMPLEMENTRY DIPLOIDS BY PROTOPLAST FUSION OF *PSEUDOMONAS AERUGINOSA* AND *E. COLI*

***G. Vaidu**
Department of Biotechnology, Willingdon College, Sangli
**Author for Correspondence*

ABSTRACT

Growing cells of *Ps. aeruginosa* and *E.coli* were quantitatively transform into protoplast by separate treatment of submilimolar EDTA and microgram Lysozyme, resultant transformed protoplast of both cells was fused by calcium phosphate. Fusion product were analyzed and found to be mixture of parental, Ncds (Non complementary diploids) and recombinant genotypic cells. These Ncds carry both parental chromosomes, but only one is expressed. In this study we analyzed these Ncds is not result of genetic transfer among bacteria and lysates or protoplast and lysates or protoplast and protoplast, bacteria and bacteria combinations. Ncds colonies were mixture of unstable Ncds, stable Ncds and recombinants. Among these unstable Ncds was reversible chromosomal inactivated mean its segregates might contain active genotype. In this work we used these instability pattern of segregation to detect unstable Ncds. While stable Ncds was irreversible chromosome inactivated mean its segregate remain persist with inactive genotype for longer period, thus it become difficult to differentiate phenotypically stable Ncds. So in this work we used Calcium phosphate induced self fusion destabilisation to detect and characterise stable Ncds. Late or slow grew colonies of Ncds on both selective medium were consider as recombinants, results of cross over among the diploid genome was recombinants thus recombinants showed both expresses and unexpressed phenotype. In present study recombinants were analyzed by considering slow grew colonies on both selective medium and complex pattern of sugar fermentation and biochemical test then both parents. Over all concluded that how long Ncds persist was unpredictable.

Keywords: *Non Complementary Diploids, Selective Medium, Protoplast Fusion, Dettol Medium, Lactose Bromothymole Blue Medium, EDTA, Lysozyme*

INTRODUCTION

Diploid form of cells is not a basic form of cell but they might become diploid in their life by natural process known as conjugation (Hayes, 1968). Diploid in *E.coli* was first proposed by the Lederberg in 1949 to account for various observation on prototroph's obtained in conjugation experiments with *E.coli k-12* was confirmed by the analysis of colonel pedigrees from isolated single cells (Zelle and Lederberg, 1951). More recently, diploid has been achieved artificially, by polyethylene-glycol, calcium phosphate, sodium nitrate induced protoplast fusion in various bacteria: *Bacillus megaterium* (Fodor and Alfoldi, 1976), *Bacillus subtilis* (Schaeffer *et al.,* 1976), *Providencia alcalifaciens* (Coetzee *et al.,* 1979) and even *Streptomyces* spp. (Hopwood *et al.,* 1977). Hotchkiss and gabori observed that polyethyleneglycol (PEG)-induced protoplast fusion of strains of *Bacillus subtilis* carrying multiple markers produced diploid cells in which one of the *two* parental chromosomes was randomly silenced. These bacteria, called noncomplementing diploids (Ncds), show the phenotype of only one parent (Gabor and Hotchkiss, 1983). Extensive genetic experiments showed that the silent chromosome remained unexpressed until segregation occurred, after which it was reactivated (Gabor, 1983; Sanchez-rivas *et al.,* 1982). 'A' diploid fusion product displaying phenotype 'A', grown in medium selective for parent 'A', typically yielded cells of phenotype B at a frequency of 10^{-2}-10^{-4}. Therefore, Ncds characterized by following the

inheritance of the biparental trait (BP) are unstable (Schaeffer and Hirschbein, 1985). Most importantly, cells recombinant for parental traits A and B were identified in the Ncds progeny, indicating that inactive nucleoids could coexist with active nucleoids in the same cell (Gabor, 1983; Levi-Meyruecis and Sanchez-rivas, 1984). Segregating and recombinant Ncds converted spontaneously to a stable form (Sanchez-rivas *et al.*, 1982; Guillen *et al.*, 1985). Stable Ncds after segregation remain phenotypically one parental, it indicates irreversible silencing of one parental chromosome.

Overall rate of segregation and chromosome silencing might be useful in investigation of gene-product interactions in genetic recombination and biosynthesis. Some of these possibilities, as well as the identification of cells that have fused (Fodor and Alfoldi, 1976; Schaeffer *et al.*, 1976), are based upon the common expectation that in diploids complementation will normally occur between alleles of the same gene. Support for this expectation comes from work with several kinds of partial diploids. For genes governing structure of biosynthetic enzymes it is generally true that the wild-type allele is dominant to ones producing a defective enzyme. Protoplast fusion also used in strain improvement because strain improvement requires the establishment of appropriate vectors for introducing the foreign genes into the desired hosts. The development of genetic engineering however had been hampered in those bacteria that are poorly characterized genetically hence the protoplast fusion technique has been studied extensively as a means of gene transfer or recombination (Jolles, 1969).

In this study we investigated that fusion product of *Ps. aeruginosa* and *E.coli* showed presence of Unstable Ncds, stable Ncds and recombinants. These Ncds were characterized by suger fermentation and biochemical tests.

MATERIALS AND METHODS

Bacterial Isolates

The *Ps. aeruginosa* and *E.coli* isolated from soil and sewage respectively were used throughout these studies. Growth of *Ps. aeruginosa* was possible on Dettol medium but not on lactose bromothymole blue medium, while growth of *E.coli* was in contrast to *Ps. aeruginosa.* Advantage of using these genus *Ps. aeruginosa* and *E. coli* was difference in their colony morphology, sugar fermentation, biochemical test.

Media

Protoplasting medium, hypertonic growth medium and 5% yeast extract supplemented hypertonic growth medium, for solid medium 1% agar (difco) in plates, while 0.5% agar (soft agar) used for overlays. Lactose bromothymole blue Medium (LBBM)-Lactose; 1gm, Magnesium sulphate; 0.02gm, Ammonium dihydrogen phosphate; 0.1gm, Dipotasium phosphate; 0.06gm, pH; 7.2, D/w; 100, Bromothymole blue; 0.008gm, Agar-agar; 2.8gm and Dettol Medium for screening of Ncds. Growth promoting medium-Peptone; 1gm, Meat extract; 0.3gm, NaCl; 0.5gm, pH; 7.2, D/W; 100ml, Agar-agar; 2.5gm.

Preparation of Protoplast

In this study to transform enteric bacterial suspension to protoplast, method employed by small modification in method described by Katalin fodor and Lajos alfoldi for gram positive bacteria. Here bacterial suspension inoculated into Yeast extract supplemented hypertonic media and cultured overnight at 37^0c. One millimeter of this culture was pipette into 100 ml of fresh yeast extract supplemented hypertonic media and incubation was resumed for 6-8 hours at 37^0 c. the viable count was around 2×10^8 cells per ml at the time of harvesting. the culture were centrifuge at 4^0c for 20 minutes at 4000 X g and bacteria were resuspended in 10 ml of protoplasting medium containing EDTA and incubates at 37^0c for 10 minutes and pipette out 1ml and added into the 9 ml of Protoplasting Medium containing Lysozyme (300µg/ml) and incubate for 15 minutes at 37^0 C. After 15 minutes at 37^0 C virtually all bacteria were transformed to protoplast. The suspensions were then centrifuged at 4° for 20 min at 4000 X g, and the protoplasts resuspended in the same volume of protoplasting medium. Protoplast suspensions did form

only 5% colonies from 2×10^8 bacterial cells and Spherical form of protoplast was also observed by microscope, indicate transformation of bacterial cells into protoplast (Fodor and Alfoldi, 1976).

Calcium Phosphate Treatment

The method described by Katalin fodor and Lajos alfoldi for gram positive bacteria *Bacillus megaterium* was used for protoplast fusion. Here The equal volume of two bacterial suspension were taken in protoplasting medium, in first minute of Lysozyme treatment, digest were mixed and centrifuged as before. The pellet was resuspended in its original volume of protoplasting medium. Then 0.05 volume of 0.02M KH_2PO_4 and 1.0M $CaCl_2$ were added in that order to the protoplast suspension addition of $CaCl_2$ produces slightly increases in the turbidity of the suspension. Sample 0.1 ml were then pipetted into 2 ml of molten hypertonic soft agar medium (maintained at 40^0c) immediately poured on the surface of plates with yeast extract supplemented hypertonic medium. The plates were Incubated at 37^0c for 4-5 days. Colonies appeared on these selection plates after 4-5 days of incubation at 37^0C, among these 30 colonies were selected for further study. These colonies suspended in 2ml saline water and concentrate it in 0.1ml by centrifugation. Thus concentrated 0.1ml was plated on growth promoting medium and both selective media (Fodor and Alfoldi, 1976).

Protoplast Lysates Preparation

The protoplast Lysates were made by resuspending a pellet of protoplast in distilled water then the osmolarity was then adjusted to that of the protoplasting medium till the growth promoting Lysis of cell was ensure by microscope (Fodor and Alfoldi, 1976).

Analysis of Fusion Products

Analysis of fusion product reveals unstable Ncds, stable Ncds and recombinants which were derived from Ncds. Ncds were isolated by colonies from growth promoting medium obtained from plating concentrated 0.1ml of 30 colonies suspension were replica on selective medium of one parent then obtained colonies again replica on selective media of Another parent thus common colonies on both selective medium were considered as Ncds. these Ncds were collected and alternatively subculture on selective medium like colonies grew on Dettol medium were replica on lactose bromothymole blue medium those colonies grew on lactose bromothymole blue medium were replica again on Dettol. These biparental phenotypic colonies were not grew on Dettol medium such type of colonies called as unstable Ncds. Colonies grew on lactose bromothymole blue medium was used similar way to isolation of unstable Ncds. Stable Ncds were isolated and characterized by self fusion destabilization was performed similar to calcium phosphate treatment among stable Ncds and lastly Recombinants were identified by their biparental traits and slow growth, these traits make them Ncds to identified as recombinants, here we used sugar fermentation and biochemical tests to characterize unstable Ncds, stable Ncds and recombinant one (Schaeffer *et al.*, 1976).

RESULTS

Preparation of Protoplast

Gram negative bacteria have cell wall and capsule for protection and longer survival. Both cell wall and capsule is made from the peptidoglycan, lipopolysaccharide and polysaccharides or polypeptides respectively (Jolles, 1969). So it is obvious that these may interfere during preparation of protoplast. In present study for preparing the protoplasts lipopolysaccharide, polysaccharides, polypeptides was removed by chelating agent EDTA (Jolles, 1969). While peptidoglycan was removed by using Lysozyme (Jolles, 1969). Treatment of EDTA, Lysozyme and protoplast transformation was given in table 1 and protoplast count was taken in % by plating treated cells on growth medium. Before treatment initial count of *E.coli* and *Ps. aeruginosa* cells was 8×10^8 and 9×10^{10} CFU/ml respectively. After treatment if no colony forming unit (CFU) was observed on growth medium it indicates 100 % efficiency of protoplast transformation (3) or Lysis of all cells and if count was similar to above mentioned considered as 0% protoplast transformation.

Table 1: % Efficiency of protoplast transformation by separate or combined treatment of EDTA and Lysozyme

		Separate treatment								
EDTA in mM		1			2			3		
Lysozyme in µg/ml		100	200	300	100	200	300	100	200	300
% efficiency of protoplast transformation *E.coli* per ml	Exp.1	21	38	43	30	51	71	41	59	79
	Exp.2	18	36	47	34	57	69	45	64	74
% efficiency of protoplast transformation *Ps. aeruginosa* per ml	Exp.1	19	29	41	28	47	64	36	52	70
	Exp.2	19	24	39	32	48	66	40	59	72
Combined treatment										
% efficiency of protoplast transformation *E.coli* per ml	Exp.1	11	32	40	15	38	57	39	49	59
	Exp. 2	19	37	39	16	34	55	42	52	54
% efficiency of protoplast transformation *Ps. aeruginosa* per ml	Exp.1	8	12	34	11	22	45	33	39	48
	Exp.2	10	11	31	9	24	42	31	46	51

Here it is seen that separate treatment of 3 mM EDTA and 300µg/ml Lysozyme transform up 70-80% cells into protoplast. While combine treatment was showed upto 50-60% cells into protoplast.

It is conclude that separate treatment of EDTA and Lysozyme was more effective for to transform cells into protoplast.

Protoplast Combination Tested for Genetic Transfer or Cross Feeding in Ps. Aeruginosa and E.Coli.

When cells of *Ps. aeruginosa* and *E.coli* cells were mixed and plated on Dettol agar then only growth of *Ps. aeruginosa* was observed while when same plate replica on Lactose bromothymole blue agar medium then no growth was observed. The same was true when lysates *Ps. aeruginosa* was given to *E.coli* or vice versa (Table 2). Protoplast of both cells were plated on soft agar will revert normal form (Fodor *et al.*, 1975; Hadlaczky and Fodor, 1976). It was our expectation that cells would grow on both selection medium. Because protoplasts of some bacteria might take up biologically active DNA molecules, we first wanted to know in mixture of protoplasts and intact DNA in crude lysates is taken up by protoplast competent for transformation (Hotchkiss, 1951). Lysates of protoplasts were therefore tested with bacteria and with protoplasts of the other bacteria. No recombinant colonies were ever produced by these procedures

Table 2: Protoplast combination tested for genetic transfer in *Ps. aeruginosa* and *E.coli*. *Dettol*$^+$/ *Dettol; Does or Does not Grew on Dettol respective. LBBM*$^+$/ *LBBM*$^-$*; Does and Does not grew on LBBM*

Cross		No. of CFU/0.1ml			
Ps. aeruginosa	*E.coli*	Dettol to LBBM		LBBM to Dettol	
Dettol$^+$ *LBBM*$^-$	*Dettol*$^-$ *LBBM*$^+$	Dettol	LBBM	LBBM	Dettol
Protoplast	Protoplast	>200	No	>200	No
Lysates	Protoplast	No	No	>200	No
Protoplast	Lysates	>200	No	No	No
Bacteria	Bacteria	>200	No	>200	No
Lysates	Bacteria	No	No	>200	No
Bacteria	Lysates	>200	No	No	No

Many times two bacterial cells showed biparental traits because of Cross feeding (Fodor, K. and Alfoldi, L. 1976). Therefore in present study we used Dettol agar and lactose bromothymole blue agar as a

selection medium, so cross feeding may possible by diffused products from colony growing at few millimeters distance can occur, but only after several days of incubation; it was not observed in short periods of incubation (20-24 hrs).

Isolation of Recombinants and Unstable or Stable Noncomplementry Diploids (Ncds)

The bacterial genus *Ps. aeruginosa* and *E.coli* are distinguished by their nutritional requirements and colony morphology when plated on selective and growth promoting solid medium. In present study we used these differences to find Ncds and recombinants. When bacteria of two parents were converted to protoplast, mixed, centrifuged and plated in soft agar layer on hypertonic yeast extract supplemented media, colonies grew out with over gas production after 4-5 days of incubation at 37^0c. Here fusion was mediated by calcium phosphate treatment resultant 10^{-7} to 10^{-5} colonies was appeared per protoplast plating. Thirty colonies obtained on hypertonic yeast extract supplemented medium were suspended in 2ml saline and concentrate it to 0.1ml by centrifugation. This 0.1 ml when plated on growth promoting medium, instead of two there was three colony morphology was observed. Among these *Ps. aeruginosa* colonies were large, translucent, irregular, blush green pigment. Whereas the *E.coli* colony were small, translucent, regular, Non pigmented. When same plate replica on both Dettol medium and lactose bromothymole blue medium colony morphological difference was observed among these selective medium but not among the colonies on each selective medium, 5-10 common colonies among the both selective medium show presence of Ncds (Valerie *et al.,* 1996) and those not common on both selective medium were parental nature. This Ncds showed totally different colony morphology, these Ncds were resuspended, diluted and again plated on both growth promoting medium and selective medium. Colonies from lactose bromothymole blue medium will continue manifest growth on Dettol medium but when growth on is further tested, the Dettol medium colonies grow only on Dettol medium. In contrast colonies grown on Dettol medium exhibit growth on lactose bromothymole blue medium, while those grown lactose bromothymole blue medium will not exhibit growth on Dettol medium those colonies were unstable Ncds (Valerie *et al.,* 1996). Those remaining colonies is seen only on growth promoting medium but not on selective medium recombinant type of colonies (Valerie *et al.,* 1996). When 0.1ml concentrate when plated on Dettol medium 80-100 same colonies observed plate when replica on growth promoting medium and lactose bromothymole blue medium, here growth promoting medium show two types of colony morphology. When these colonies subculture from Dettol to lactose bromothymole blue medium and bromothymole blue to Dettol 2-3 times, only one type of colony stay remained these colonies were stable Ncds (Valerie *et al.,* 1996). Similar was observed when 0.1ml concentrate plated on lactose bromothymole blue medium.

To estimate rate of generation of Ncds, recombinants and segregation rate require to investigate genetic analysis.

Confirmation of Unstable and Stable Ncds

To confirm Stable Ncds among Ncds was not done phenotypically alone but it's not true with unstable Ncds because segregation pattern of unstable Ncds give confirmation of presence of unstable Ncds. To overcome this problem we used self-fusion of stable noncomplementry diploids, which should lead to the destabilization of both chromosomes and reactivation of the previously unexpressed genes. Table 3 illustrates the results of sugar fermentation test of *Ps. aeruginosa*, *E.coli*, recombinants, stable Ncds and stable Ncds self fusion product. After self-fusion of the Ncds protoplasts and regeneration on hypertonic yeast extract supplemented medium, recombinants and BP colonies were recovered, revealing the diploid nature of the Ncds by sugar fermentation tests. Here stable Ncds A1 showing sugar fermentation tests similar to *Ps. aeruginosa* when self fused then resultant each segregates C1, C2, C3, C4 and C5 showed either *Ps. aeruginosa* or *E.coli* type of sugar fermentation test. Similarly table 2.4.2 illustrates the results of biochemical test of *Ps. aeruginosa*, *E.coli*, recombinants, stable Ncds and stable Ncds self fusion product.

Results suggested that bacterial diploids may form but their diploid nature was how longer exhibit is questionable.

Table 3: Sugar fermentation test of *Ps. aeruginosa* (X), *E.coli* (Y) and their fusion products X x Y; fusion among *Ps. aeruginosa* and *E.coli* and A1, A3 were stable Ncds (X x Y) A1 x (X x Y) A1; self fusion of stable Ncds isolates A1
- ; Negative test
+ ; Positive test

Cross	Sugar fermentation tests						
	Lactose	**Glucose**	**Ribose**	**Arabinose**	**Dextrose**	**Xylose**	**Mannitol**
Ps. aeruginosa (X)	_	_	_	_	_	_	_
E.coli (Y)	+	+	+	+	+	+	+
Recombinants	+	+	_	_	+	_	_
X x Y	+	+	+	+	+	+	+
A2, A3 (stable Ncds isolates)							
X x Y (stable Ncds) A1	_	_	_	_	_	_	_
(X x Y)A1 x (X x Y)A1 C1,C3,C4	+	+	+	+	+	+	+
(X x Y)A1 x (X x Y)A1 C2,C5	_	_	_	_	_	_	_

Table 2: Biochemical test of *Ps. aeruginosa, E.coli* and their fusion products X x Y; fusion among *Ps. aeruginosa* and *E.coli* and A1, A3 were stable Ncds (X x Y) A1 x (X x Y) A1; self fusion of stable Ncds isolates A1
- ; Negative test
+ ; Positive test

Cross	**Biochemical test**						
	Methyl red	**Indole**	**Citrate Utilisation**	**Urea hydrolysis**	**Lysine decarboxylase**	**Nitrate reductase**	**Arginine decarboxylase**
Ps. aeruginosa (X)	_	_	+	+	+	+	+
E.coli (Y)	_	+	+	+	+	_	+
Recombinants	_	+	+	+	_	+	+
X x Y (stable Ncds) A1	_	_	+	+	+	+	+
X x Y (stable Ncds) A3	_	+	+	+	+	_	+
(X x Y)A1 x (X x Y)A1 C1	_	+	+	+	+	_	+
(X x Y)A1 x (X x Y)A1 C2	_	_	+	+	+	+	+

DISCUSSION

Bacterial protoplast might be useful for cultivation of virus, enzyme synthesis, transformation, transfection. Nevertheless, prior only gram-positive bacteria was transformed into protoplast in large scale because of easy susceptibility to Lysozyme (Joshua, 1956). Now it has been found efficient production of protoplasts from enteric bacteria such as *Escherichia coli* and Salmonella *typhimurium, Ps. aeruginosa.* The technique consists exposure of growing cells to submilimolar EDTA and Microgram Lysozyme

(Jolles, 1969). In present study it is seen that separate then combined treatment of EDTA and Lysozyme in sucrose Hypertonic medium was effective to transform intact cells to protoplast because mg+2 responsible for to preserve protoplast which was kept in high concentration in sucrose hypertonic medium. So it obvious that when combine treatment of EDTA and Lysozyme is given, mg+2 get chelated and deprive resultant unstable protoplasts which brought protoplast less in No. when combined treatment of EDTA and Lysozyme is given in mg+2 lacking sucrose hypertonic medium resultant similar results. So it was concluded that separate then combined treatment of EDTA and Lysozyme in sucrose Hypertonic medium was effective to transform intact cells of enteric bacteria to protoplast.

No conjugation like genetic transfer mechanism exist among *Ps. aeruginosa* and *E.coli* but there may possibility of transformation. In this work, we were used medium not support for generation of competent cells likewise we unable to detect transformation among *Ps. aeruginosa* and *E.coli* protoplast; protoplast, protoplast; Lysates, Lysates; protoplast, Bacteria; Bacteria, Bacteria; Lysates. Lysates; Bacteria respectively. Fusion of *Ps. aeruginosa* and *E.coli* protoplast by fusing agent like calcium phosphate results yield was good. It is clear that all fusion products formed primary colonies on a cell wall regenerating medium among these parental haploid, recombinants, Complementary diploids, Non complementary diploids. These all fusion products were isolated and characterised they clearly demonstrated that both segregation of diploid genome and recombination were going with the process of reversion to bacillary form and cell division (Fodor and Alfoldi, 1976).

Fusion products showed one parental phenotype, when it divides or segregate into two cells one of them expressed all phenotype of the other parent. Such type of fusion products were considered Ncds provided neither parents grew on both selective medium and each parent had different phenotype (Valerie *et al.,* 1996). In this work among those Ncds, unstable Ncds as described in results provided Ncds of *Ps. aeruginosa* and *E.coli* contained two distinct genome one of which had been phenotypically unexpressed. These Ncds is not result of crossfeeding because of sub cloning, purification of culture was considered as evidence for Ncds Nature (Rollin and Magda, 1980). Crossfeeding to occur from spots growing millimetres distance it requires several days of incubation (Rollin and Magda, 1980).

Among the fusion product late colonies or those not grew on selective medium were considered to be recombinants (Valerie *et al.,* 1996). In present study recombinants were obtained from Ncds, those Ncds showed late or slow grew on both selective medium and they were persistence in biparental expression because of complex pattern of cross over resultant phenotype of both expressed and unexpressed chromosome (Valerie *et al.,* 1996). It was considered that illegitimate recombination which is RecA independent may responsible for recombinants cells among Ncds (Rollin and Magda, 1980).

Stable Ncds were stable in diploid stage but expression wise only one chromosome was expressed while other remain unexpressed (Valerie *et al.,* 1996). This expression pattern of stable Ncds made difficult to phenotypic differentiation of stable Ncds from unstable Ncds. In this work we characterise stable Ncds by calcium phosphate induced self fusion destabilisation. This results suggest that self fusion among stable Ncds result in destabilisation of diploid chromosome which might be responsible for mixture of different phenotype cells in single colony.

Before knowledge of segregation it was considered that diploid nature of bacteria was contributed by lack of segregation but today it was prove that segregated cells showed diploid nature (Schaeffer *et al.,* 1976; Guillen *et al.,* 1985). In unstable Ncds chromosome silencing is reversible until the chromosome become irreversible stabilized through a process involving the *spoOA* gene (Gabor and Hotchkiss, 1983). Segregation and irreversible silencing genome will not be able to support cell growth in all time, thus Ncds cells with an irreversible stabilised inactive chromosome bearing one parental phenotype and inviable cells (Schaeffer *et al.,* 1976). Thus segregation and the different viability of the segregants may explain why Ncds by repeated Sub culturing and growth in selective medium failed to show evidence for diploid cells.

Summery; Fusion product of *Ps. aeruginosa* and *E.coli* analysed and it was found that colonies formed by fusion products to be mixed populations of individual bacteria. Containing parental, unstable Ncds, stable Ncds and recombinants segregating genotypes colonies.

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