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Studies on Multiple Antibiotic Resistance Gene in *Aeromonas*

Narayan Roy Md Belaluddin and M. Shahjahan
Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh

Abstract: Three xylanase-producing *Aeromonas* strains (St_1, So_1 and So_2) were isolated from stagnant water from Rajshahi University campus. The strains were isolated on xylan agar media and screened by β-xylanolysis method. This strains showed drug resistance to cotrimoxazole, ampicilline and amoxycilline when tested by disc diffusion method on nutrient agar or xylan agar plate respectively. This strains also showed sensitive to erythromycin, tetracycline and doxycyclin. The minimum inhibitory concentration (MIC) of erythromycin, tetracycline and doxycyclin was determined by serial dilution technique against three test bacterial strains. The MIC values of the above three drugs against St_1 were 250, 125 and 250 μg ml^{-1}, against So_1 and So_2 were 125, 62.5 and 125 μg ml^{-1} respectively. Plasmid DNA was isolated the multi-drug resistant strain St_1. These drug resistant plasmids were transformed into sensitive *E. coli* LE 392. The transformed LE392 became resistant to cotrimoxazole, ampicilline and amoxycilline and the respective plasmid DNA was detected in the plasmid DNA isolated from transformed *E. coli* cells. The antibiotic resistant in *Aeromonas* was plasmid born.

Key words: *Aeromonas sp.*, Plasmid, Antibiotic resistant, xylanase, minimum inhibitory concentration

Introduction

Bacteria of *Aeromonas* genus are gram-negative, cells are straight, rod-shaped with rounded ends to coccoid ranging from 1.0-4.4 μm in diameter. They are molile by polar flagella, generally monotrichous but some species are non-motile (John, G. R. Krieg and H. Peter, 1994). Most *Aeromonas* strains produce hydrogen sulfide which react with the ferric ammonium-citrate in the medium to produce black centered pink colonies. Bacteria of this genus generally ferment carbohydrates with the production of acid and gas. Typically glucose, fructose, lactose, maltose and cellobiose are fermented. They produce xylanase, deoxyribonuclease, arginine dehydrogenase and phosphatase but can not produce urease. Microorganisms resistant to certain drug may also be resistant to other drugs that share a mechanism of action. Such relationships exist mainly between agents that are closely related chemically or that have a similar mode of binding of action. In certain classes of drugs, the active nucleus of the chemical is so similar among many congeners (e. g. tetracycline) that extensive cross-resistance is to be expected. Most plasmid DNA produces enzymes that modify the antibiotic by the addition of chemical group causing acetylation. Some plasmid can alter cell transpor of tetracycline, cadmium and perhaps other agents.

Xylanases widespread in nature and they have reported to be present in marine and terrestrial bacteria, ruman and reminant bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants. Commercial applications suggested for xylanases involve the conversion of xylan, which is present in wastes from agricultural and food industry, into xylose (Biely, 1985). Similarly, xylanases could be used for the clarification of juices, for the extraction of coffee, plant oils and starch (Wong ***et al.***, 1988) and for the production of fuel and chemicals feedstocks (Linko ***et al.***, 1989). Recently, the use of xylanolytic enzymes in pulp bleaching has been considered as one of the most important new biotechnological applications of these enzymes (Viikari ***et al.***, 1991 and 1994). The environmental impact of wastewaters arising from the pulp and paper industry, especially the formation of toxic organic chlorines, has attracted public attention in the last few years (Coughlan ***et al.***, 1993). The most important enzyme that is used in enzyme-aided bleaching is endoxylanase (Kantelinen ***et al.***, 1988).

While biomedical scientists are discovering newer and more potent anti-microbial drugs, the pathogenic bacteria with their demonstration to survive, are gaining resistance against them in a curious way. In a sensitive bacterial population, there may be a small number of drug resistant bacteria which develop resistance spontaneously as a result of mutation. More frequently resistance is due to the presence of additional gene (s) as extra-chromosomal DNA known as R-factors (plasmids) has reported (Choudhury, 1995).

Aeromonas strain was suspected to have some drug resistance. This report makes a sense of urgency to study the mechanism of acquisition and transfer of xylanase gene as well as the genetic organization of the gene coding for resistance to cotrimoxazole, ampicilline and amoxycillin. Therefore, the aim of this study was to isolate and to characterize the xylanase gene structure which would be helpful to elucidate the mechanism of such multi-drug resistance.

Materials and Methods

Bacterial strains

Aeromonas strain used in this study was collected from stagnent water. *E. coli* 392 strain used in the transformation experiment was supplied by the Department of Biochemistry and Molecular Biology, Yamaguchi University, Japan.

Media and culture conditions

Luria-Beriani (LB) broth was used as a complete medium. Nutrient agar was used as a solid medium throughout the work.

Multi-drug resistant in *Aeromomas*

The multi-drug resistant *Aeromonas* strains were isolated from the selected strains containing of xylanolytic activity using type disc diffusion method of Bauer and Kirby (1966). A 16 h broth cultures of the collected strains when grown at 37° C and was spread on both nutrient agar plate and xylan agar plate using sterilised glass spreader. Then the cotrimoxazole (25 μg $disc^{-1}$) ampicilline (10 μg $disc^{-1}$) and amoxycilline (10 μg $disc^{-1}$) antibiotics were

distributed on plates and kept the plates at 4°C for 4 h, so that the antibiotic can diffuse on the agar media. The plates were then incubated at 37°C for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected strains were resistant to that antibiotic. The drug resistant bacteria tested by disc diffusion method were again confirmed by spreading its culture on the selected antibiotic plates of different concentrations. The plates were then incubated at 37°C and observed on next day. The clear plate indicated that the strains were sensitive to this selective concentration and presence of colonies on the plate indicated that the strains were resistant to that selective concentration.

Determination of minimum inhibitory concentration of erythromycin, tetracycline and doxycyclin

The minimum inhibitory concentration of erythromycin, tetracycline and doxycyclin were determined by serial tube dilution technique (Abdul Hye Khan ***et al.***, 2000) using nutrient broth media.

Plasmid DNA extraction and agarose gel electrophoresis

A single colony of the isolated multidrug resistant ***Aeromonas*** was inoculated into 100 ml LB broth containing 0.25 mg ml^{-1} of antibiotic solution in 250 ml conical flask and incubated at 37°C overnight with constant shaking. This culture was then subjected for the extraction of plasmid DNA according to Holmes and Quigley (1981). The extracted plasmid DNA was then purified with polyethylene glycol (PEG) according to Maniatis ***et al.*** (1989). The purified plasmid DNA was then subjected to electrophoresis by using 0.8% agarose according to Meyers ***et al.*** (1976). Plasmid transfer to a sensitive ***E. coli*** LE 392 strain. Competent cells were prepared by calcium chloride procedure modified from Cohen ***et al.*** (1972). An ***E. coli*** LE 392 strain sensitive to amoxycillin, ampicillin and cotrimoxazole was inoculated in a 20 ml LB broth and grown for 8 hours at 37°C with slow shaking for the experiment. Transformation of the isolated plasmid DNA to the ***E. coli*** LE 392 was carried out according to Cohen ***et al.*** (1972).

Extraction of transformed plasmid DNA from *E. coli* LE 392

After the transformation experiment, plasmid DNA was extracted from the transformed ***E.coli*** LE 392 according to Holmes and Quigley (1981). The extracted plasmid DNA was purified and subjected to agarose gel electrophoresis.

Results

Isolation and characterisation of ***Aeromonas***: The three bacterial sample (St_1, So_1 and So_2) which was collected from stagnant water were identified ***Aeromonas*** as described Roy and Abedin (2002).

Result of multi-drug resistance test

The three drug resistant strains were isolated by disc diffusion method on nutrient agar plates against some commonly used antibiotics to see the resistance pattern of these strains. All

Table 1: Overall drug resistance pattern of isolated three multidrug strains by disc diffusion method on nutrient agar plate

	Name of the drug used						
	TS	AP	A	E	T	DXT	Resistant to
Strain no	Diameter of the clear zone produced (mm)						
St_1	--	--	--	26	24	27	TS,AP,A
So_1	--	--	--	25	23	26	,,
So_2	--	--	--	24	25	25	,,

TS=Cotrimoxazole, AP=Ampicilline and A=Amoxycilline, E=Erythromycin, T=Tetracyclin and DXT=Doxycyclin.

Table 2: Results of antibiotic spread plate method against different selection concentration of antibiotics (cotrimoxazole, ampicilline and amoxycillin)

	No.of resistant colonies appeared on different antibiotic plates															
	TS (conc. μg ml^{-1})					AP (conc. μg ml^{-1})					A (conc. μg ml^{-1})					
sample no	10	20	30	50	100	10	20	30	50	100	10	20	30	50	100	Resistant to
St_1	45	32	21	13	5	36	27	19	12	7	41	32	20	10	6	TS,AP,A
So_1	32	26	13	8	0	31	23	12	6	0	33	21	14	5	0	,,
So_2	41	34	19	9	0	38	32	23	13	0	36	24	12	6	0	,,

strains showed multiple resistance to the antibiotic used. Resistant and sensitive strains were separated according to their diameter of zone of inhibition produced around the antibiotic disc and growth type and No. of colonies on clear zone and comparative study with control strains (sensitive). The result of drug resistance pattern on nutrient agar showed that the three strains were resistant to cotrimoxazole, ampicilline and amoxycillin. The results of overall multi-drug resistant tests on nutrient agar plates have shown in the Table 1.

Results of antibiotic spread plate method against different selection concentration of antibiotics

Multidrug resistant strains through the disc diffusion method were also reisolated and again their drug resistant were confirmed by antibiotic spread plate method with different selective antibiotic concentrations (10, 20, 30, 50, 100 and 120 μg ml^{-1}). The antibiotics used were cotrimoxazole, ampicilline and amoxycilline for confirmation of drug resistance of those strains were isolated from disc diffusion method. Sample St_1 showed growth on the plate at 100 μg ml^{-1} concentration of cotrimoxazole, ampicilline and amoxycilline respectively. This bacterial sample of ***Aeromonas*** was remarked as highly resistant to these three antibiotics. Appearance of drug resistant colonies on these antibiotic plates indicated that all these strains were resistant to these selective concentrations of antibiotic. While no growth at next other concentrations indicated that these strains were sensitive to these selective concentration of antibiotic (Table 2).

Table 3: Minimum inhibitory concentration of erythromycin (MIC) for strains St_1, So_1 and So_2

No of strains	No of tubes	Nutrient broth (ml)	Diluted solution of E (μg ml^{-1})	Inoculum added (μl)	Observation MIC (μg ml^{-1})	MIC (μg ml^{-1})
St_1	1	1	500.00	10	NG	250
	2	1	250.00	10	NG	
	3	1	125.00	10	G	
	4	1	62.50	10	G	
	5	1	31.25	10	G	
	6	1	15.60	10	G	
	7	1	7.80	10	G	
	T_{MS}	1	500.00	0	NG	
	T_{MI}	1	0.00	10	G	
	T_M	1	0.00	0	NG	
So_1	1	1	10.00	10	NG	125
	2	1	10.00	10	NG	
	3	1	10.00	10	NG	
	4	1	10.00	10	G	
	5	1	10.00	10	G	
	6	1	10.00	10	G	
	7	1	7.80	10	G	
	T_{MS}	1	500.00	0	NG	
	T_{MI}	1	0.00	10	G	
	T_M	1	0.00	0	NG	
So_2	1	1	500.00	10	NG	125
	2	1	250.00	10	NG	
	3	1	125.00	10	NG	
	4	1	62.50	10	G	
	5	1	31.25	10	G	
	6	1	15.60	10	G	
	7	1	7.80	10	G	
	T_{MS}	1	500.00	0	NG	
	T_{MI}	1	0.00	1	0G	
	T_M	1	0.00	0	NG	

NG= No growth, G= Growth

Minimum inhibitory concentration (MIC) of erythromycin, tetracyclin and doxycyclin antibiotics against *Aeromonas* strains

The MIC of erythromycin, tetracycline and doxycyclin were determined by serial dilution technique that against three tests organisms. In this method, the sterile nutrient broth media was taken (1ml taken) in a number of autoclaved test tubes. The test sample was added to the first test tube containing nutrient broth media and mixed well. Then 10 μl of the test organisms (about 10^7 cell ml^{-1}) was added to each of the broth media mixed well and incubated at 37°C for 12 to 18 h. For antibiotic erythromycin, the first sign of growth of organism St_1, So_1 and So_2 was observed in the test tube (No.St_1 -3, So_1-4 and So_2-4) containing 125, 62.5 and 62.5 μg ml^{-1} respectively (Table 3). The minimum inhibitory concentrations where no bacterial growth was observed were from 250, 125 and 125 μg ml^{-1} (tube no St_1 -2, So_1-3 and So_2-3) against St_1, So_1 and So_2 strains respectively.

For antibiotic tetracycline, the first sign of growth organism St_1, So_1 and So_2 was observed in the test tubes (No St_1-4 , So_1-5 and So_2-5) containing 62.5, 31.25 and 31.25 μg ml^{-1} respectively. The minimum inhibitory concentrations where no bacterial growth was observed were from 125, 62.5 and 62.5 μg ml^{-1} (tube no St_1-3, So_1-4 and So_2-4) against St_1, So_1 and So_2 strains respectively (data not shown). For antibiotic doxycyclin, the first sign of growth of organism St_1, So_1 and So_2 was observed in the test tubes (No.St_1 -3, So_1-4 and So_2-4) containing 125, 62.5 and 62.5 μg ml^{-1} respectively. The minimum inhibitory concentrations where no bacterial growth was observed were from 250, 125 and 125 μg ml^{-1} (tube no St_1-2, So_1-3 and So_2-3) against St_1, So_1 and So_2 strains respectively (data not shown).

Plasmid profile of transformed *E. coli* LE392 and donor *Aeromonas* strain

An attempt was taken to transfer plasmid DNA from multi-drug resistant ***Aeromonas*** to a sensitive ***E. coli*** LE392. This attempt was totally successful. The plasmid DNA isolated from transformed ***E. coli*** LE392 by boiling method described in materials and methods. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg ml^{-1}) for 20 min. Then the gel was washed with tap water and placed on an UV-transsilluminator and finally photographed by a camera. Plasmid profile showed that plasmid DNA from transformed ***E. coli*** LE392 and plasmid DNA of donor ***Aeromonas*** strain were same size i.e. about 30 kb in size. This plasmid DNA band corresponding to that of original strain is the indication of successful transformation. This transformation study indicated that multiple-drug resistance and xylanase activity in the selected ***Aeromonas*** strain might be due to the presence of 30 kb plasmid DNA.

Discussion

In this study the bacterial strains were isolated from stagnant water, which degraded â-1, 4 xylans and to belong to ***Aeromonas*** genus. It was observed that, the strains were resistant to three antibiotics i.e. cotrimoxazole, ampicilline and amoxycillin and were sensitive to three antibiotics i.e. erythromycin, tetracycline and doxycyclin. The multi-drug resistance in each of these three strains were confirmed by antibiotic spread plate method using cotrimoxazole (0.5 mg ml^{-1}), ampicilline (0.5 mg ml^{-1}) and amoxycillin (0.5 mg ml^{-1}) in 10, 20, 30, 50 and 100 μg ml^{-1} concentration on nutrient agar plates. The minimum inhibitory concentration of erythromycin, tetracycline and doxycyclin was determined by serial dilution technique against three test bacterial strains. The MIC values of the above three drugs against St_1 were 250, 125 and 250 μg ml^{-1} against So_1 and So_2 were 125, 62.5 and 125 μg ml^{-1} respectively.

The xylanase-encoding plasmid DNA from multi-drug resistant ***Aeromonas*** bacteria was isolated and transformed. After transformation experiment, the transformed strains appeared on plates were tested for their xylanolytic activity by their colonical growth as clear zone on xylan agar plate after 48 hours at 37°C. From this experiment it was observed that ***E. coli*** LE392 which was found non-xylan degrading (do not produce clear zone on xylan agar plate) before transformation became xylan degrading (produce clear zone on xylan agar plate) due to xylanase-encoding plasmid acquisition. This xylanase activity study indicated that xylanase gene was transformed from ***Aeromonas*** strain into non-xylanase-producing ***E. coli*** LE392. The transformed strains

appeared on plates were tested for their multi-drug resistance by disc diffusion method on both nutrient agar and xylan agar plates. From this experiment it was observed that *E. coli* LE392 which was sensitive to cotrimoxazole, ampicilline and amoxycillin before transformation became resistant to these antibiotic due to this plasmid acquisition. These strains again tested by spread method using 30, 40 and 60 ìg/ml of cotrimoxazole plates on which 20, 17 and 12 drug resistant colonies were appeared respectively. Similarly, in case of ampicillin and amoxycillin using same concentration plates; 18, 12 and 7 and 19, 13 and 10 drug resistant colonies were appeared on the respective plates respectively. But no drug resistant colonies were appeared on the control plates. This drug resistant study indicated that cotrimoxazole, ampicillin and amoxycillin resistance was transformed from *Aeromonas* strain into *E. coli* LE392. This result indicated that plasmid of 30 kb in size was transferred. Transformation study showed that xylanase activity and multi-drug resistance in *Aeromonas* strain from stragnent water is due to the presence of extrachromosomal DNA or plasmid present in the bacteria. It could be concluded that the xylanase gene was localized in a 30 kb plasmid DNA in *Aeromonas* bacteria. The plasmid also contained at least three drug resistant genes. The three drug resistant genes were expressed with xylanase genes simultaneously or co-operatively. Toshiaki Kudo, Riken Institute, Japan, reported that plasmid pAX1 from *Aeromonas* sp. No 212 was isolated from transformants producing xylanase and the xylanase gene was located in a 6.0 kb HindIII fragment (Kudo Toshiaki ***et al.***, 1985).

Further study is now going on to digest the plasmid DNA with different restriction enzymes, which will help us to establish a complete restriction map of the plasmid. From this restriction map we may characterize the plasmid structure of the xylanase-producing *Aeromonas*.

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