The effect of p-nitrophenylglycerol on swarming and the production of some virulence factors in *Proteus vulgaris*

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Abstract: *Proteus vulgaris* is an opportunistic pathogen, commonly responsible for urinary and septic infections; often nosocomial. *Proteus vulagris* has a number of putative virulence factors, including the secreted hemolytic, which has been suggested to contribute to host cell invasion and cytotoxicity, an inducible urease which, by generating ammonia, causes precipitation of bladder and kidney stones, fimbriae which promote bacterial adherence to the uroepithelium, a secreted protease able to digest immunoglobulins. In this study we have verified the ability of p-nitrophenylglycerol (anti-swarming agent of *Proteus mirabilis*) to inhibit *Proteus vulgaris* swarming and the expression of some virulence factor (haemolysin and urease).Swarming inhibition was determined on Luria Bertani agar with PNPG and then bacteria was harvested to assay cell length and the production of haemolysin and urease. P-nitrophenylglycerol significantly inhibited swarming and virulence factor expression but its effect on growth rate was not significant.

[Mohammed Ghaidaa, Wang Yanchang, and Hindi Abdallah. **The effect of p-nitrophenylglycerol on swarming and the production of some virulence factors in *Proteus vulgaris.*** *N Y Sci J* 2013;6(9):8-14]. (ISSN: 1554-0200).
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Keywords: P-nitrophenylglycerol, *P.vulgaris*, Swarming,virulence factors

Introduction

Proteus vulgaris is a rod-shaped Gram-negative chemoheterotroph bacterium. The size of individual cells varies from 0.4~0.6μm by 1.2~2.5μm. *P. vulgaris* possesses peritrichous flagella, making it actively motile. It inhabits the soil, polluted water, raw meat, gastrointestinal tracts of animals, and dust. In humans, *Proteus* species most frequently cause urinary tract infections, but can also produce severe abscesses; *P. mirabilis* produces 90 percent of cases, and is encountered in the community, but *P. vulgaris* is associated with nosocomial infection (O'Hara *et al*.2000 and Struble 2009).

The invader *P. vulgaris* has numerous factors including fimbriae, flagella, outer membrane proteins, lipopolysaccharide, capsule antigen, urease, immunoglobulin A proteases, hemolysins, amino acid deaminases, and, finally, the most characteristic attribute of *Proteus*, swarming growth, enabling them to colonize and survive in higher organisms (Rozalski *et al*.1997 and Emody *et al*.2000).

P-nitrophenylglycerol or 1-(4-Nitrophenyl-β-D-glucuronicacid) (PNPG) is a chromogenic β-glucuronidase substrate (Sartory and Watkins, 1999). It has been found to has anti-swarm properties by adding it to a solid culture medium.So, it used as one of methods which have been reported to prevent *Proteus* species from swarming over the surface of a solid culture medium(Hernandez *et al*.1999).The antiswarming activity of p-nitrophenylglycerol (PNPG) has been found invaluable for the recognition and isolation of pathogenic bacteria from specimens contaminated with swarming strains of *Proteus spp*(Williams 1973).In addition, PNPG has little effect on the results of a variety of identification tests performed directly on colonies from media containing PNPG(Lai 1994 and Jun *et al.* 2004). It is relatively cheap, nontoxic and doesn't affect red blood cells; even fastidious pathogens will grow well and with characteristic colony morphology in its presence (Liaw *et al*. 2000).Its heat stability and long 'shelf – life' make it convenient to use in the preparation of media(Liaw *et al*. 2000).

According to the ability of *Proteus spp* to express virulence factors and invade human urethelial cells is correlated with its swarming activity (Liaw *et al*. 2001 and 2004). So, the purpose of the present study was to investigate the activity of PNPG against uropathogenic *P. vulgaris* swarming and it's producing of some virulence factors (haemolysin and urease) which are correlated with its ability to invade uroepithelial cells.

Materials and methods

Chemicals: PNPG (Sigma) was sterilized by filtration through a 0.22um pore membrane.

Bacteria and growth conditions

Proteus vulgaris was isolated from a patient with UTI and identified by biochemical tests (MacFaddin 2000). *P. vulgaris* isolate was cultured overnight at 37˚C in Luria Bertani (LB) broth and then used to inoculate LB broth and LB agar contained different concentration of PNPG (0, 10, 50, 100, 150 and

200µg/ml) to assay its growth rate, swarming activity and ability to express virulence factors (haemolysin and urease) in the presence of PNPG.

Swarming behavior assay

The swarming migration distance assay was performed as described previously (Liaw *et al*. 2000 and 2004, Wang *et al*. 2006, and Al-Dulaimi 2009**)**. Briefly, an overnight bacterial culture (5 µl) was inoculated centrally onto the surface of dry LB swarming agar (1.5 % w/v) plates with or without PNPG, which were then incubated at 37˚C. The swarming migration distance was assayed by following swarm fronts of the bacterial cells and recording progress at 1h intervals.

Bacterial growth assay

P.vulgaris was cultured overnight at 37C in Luria broth (LB) diluted 1:100 in LB containing various concentrations of PNPG (0, 10, 50,100,150 and 200µg/ml) and the growth rate was monitored at 1h intervals (Liaw *et al*. 2000 and 2004, Wang *et al*. 2006, Echeverrigaray *et al*. 2008, Vanessa and Zunino, 2009).

Measurement of cell length, haemolysin and urease activities

Sections of agar containing vegetative cells (colony center) and swarming cells (colony edge) have been taken from plates with and without appropriate concentration of PNPG. Bacteria were washed from agar segments with 5ml of phosphate buffered saline (PBS) after that the cell length, haemolysin and urease production was assayed (Liaw *et al*. 2000 and 2004, Wang *et al*. 2006, and Echeverrigaray *et al*.2008).

Measurement of cell length

Measurement of cell length was performed as described by (Liaw *et al*. 2000 and Echeverrigaray *et al*.2008]. Briefly, 150µl of stationary-phase LB cultures were spread onto LB agar plates without or with appropriate PNPG concentration and incubated at 37°C. After incubation, Cells from the entire surface of agar plates were harvested by washing into 5 ml of PBS. Bacterial cells were fixed and subjected to gram stain (Ward's Science, USA), examined by light microscopy (Carl Zeiss, Germany) at a magnification of 100x, and digitalized using a digital camera. The lengths of 100 cells in each sample were determined, and the average was calculated.

Haemolysin Production assay

Haemolysin production was carried out by inoculating a blood agar medium containing 2%washed horse erythrocytes with bacterial cells taken after suspended in 5ml of PBS then incubated at 37◦C for 24h.The appearance of a clear zone around the colonies referred to a complete hemolysis (β-hemolysis). The appearance of greenish zone around the colonies referred to a partial hemolysis (α-hemolysis), whereas no change of zone referred to non-hemolysis (γ- hemolysis) (Buller 2004, Ray *et al*. 2004, and Al-Dulaimi 2009).

Urease production assay

Preparation of cells for urease assay was performed as described previously (Liaw *et al*. 2000 and Echeverrigaray *et al*.2008).In this test we inoculated the urea slant from bacterial suspension by streaking the entire slant surface, incubated the tubes with loosened caps at 37^{o}C then color change of medium was examined after 16h incubation. Urease production was indicated by changed medium color into pink (Winn *et al*. 2006 and Al-Dulaimi 2009).

Statistical Analysis

All the results represent the average of three independent experiments. The data were presented as mean and analyzed by one-way analysis of variance with $P < 0.05$ being significant, calculated using the GraphPad Prism 5 statistical software.

Results

Inhibition of *P. vulgaris* swarming by PNPG

In this study, we found that PNPG had the ability to block the swarming migration of *P.vulgaris* in a dose-dependent manner (Fig. 1a). The swarming behavior was significantly inhibited at concentrations 50µg/ml and was blocked completely at 150 and 200µg/ml (Fig. 1a, b). To test the inhibitory effect of PNPG on bacterial growth in addition to swarming, an overnight culture of *P.vulgaris* was inoculated into LB containing various concentrations of PNPG and the growth rate of bacteria was monitored as shown in Figure1c.The growth rate of *P. vulgaris* wasn't inhibited by PNPG. At 16 h post-inoculation, the bacteria grew approximately to similar densities, regardless of the presence of PNPG. We concluded that the inhibitory effect of PNPG on swarming was unlikely to be due to inhibit of cell growth.

Inhibition of swarming differentiation and virulence factor expression of *P. vulgaris* by PNPG

Cell morphology was mointered after inoculation of an overnight culture of *P.vulgaris* onto LB swarming plates containing various concentrations of PNPG. As shown in Figure 2a, in the absence of PNPG, the swarming cells were longer than the bacterial cells in the presence of PNPG at the concentration 200µg/ml, suggesting that swarming differentiation was inhibited. The inhibition of differentiation started to be observed at PNPG concentration of 50µg/ml. Very few elongated swarming cells were observed at PNPG concentration of 100µg/ml. As PNPG concentration was increased to 150 and 200µg/ml, only short vegetative cells were observed. These results indicate that swarming differentiation of *P.vulgaris* was indeed inhibited by high concentrations of PNPG.

To investigate whether the production of virulence factors (haemolysin and urease) was also influenced by PNPG, the production of haemolysin and urease in *P. vulgaris* which taken from LB agar plates containing different concentrations of PNPG was determined. As shown in Figure 3, the production of virulence factors was not affected significantly at PNPG concentrations (0-100µg/ml) for urease and (0-50 µg/ml) for haemolysin but was inhibited significantly in the presence of increasing concentrations (150 and 200µg/ml).

Figures

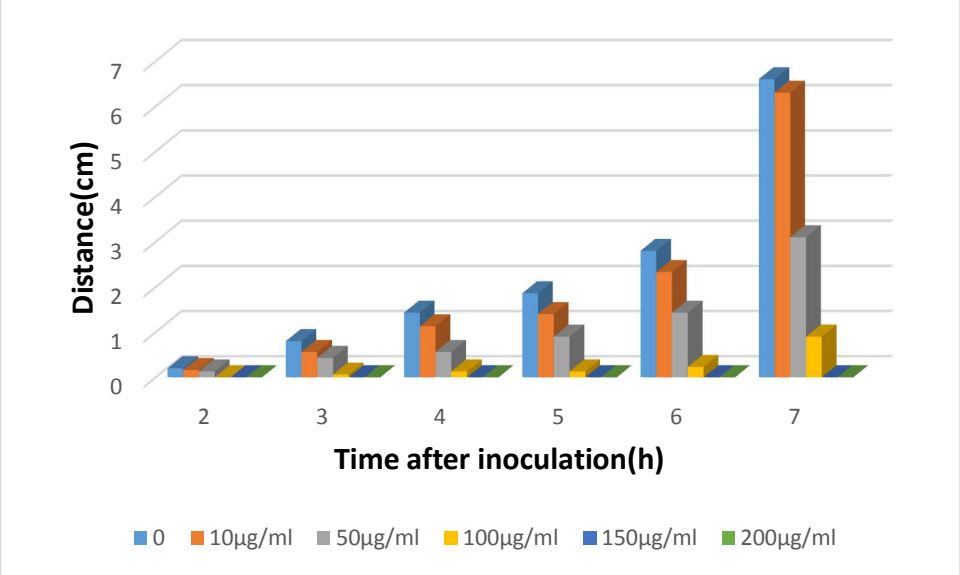

Fig.1.(a). Effect of PNPG on the swarming of *P.vulgaris*. The histogram shows the migration distance of *P.vulgaris* in the presence of various concentrations of PNPG (0, 10, 50, 100, 150, and 200µg/ml). The data represent the mean of three independent experiments and the differences are significant (P value <0.05).

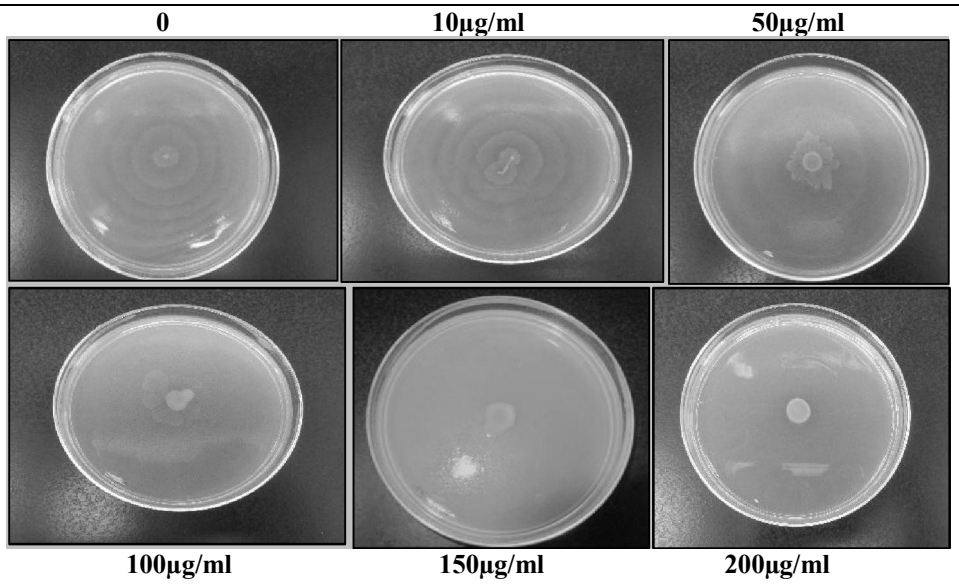

Fig.1.(b).Halo images of swarming plates containing different concentrations of PNPG (0, 10, 50, 100, 150, and 200 µg/ml) at 7h after inoculation.

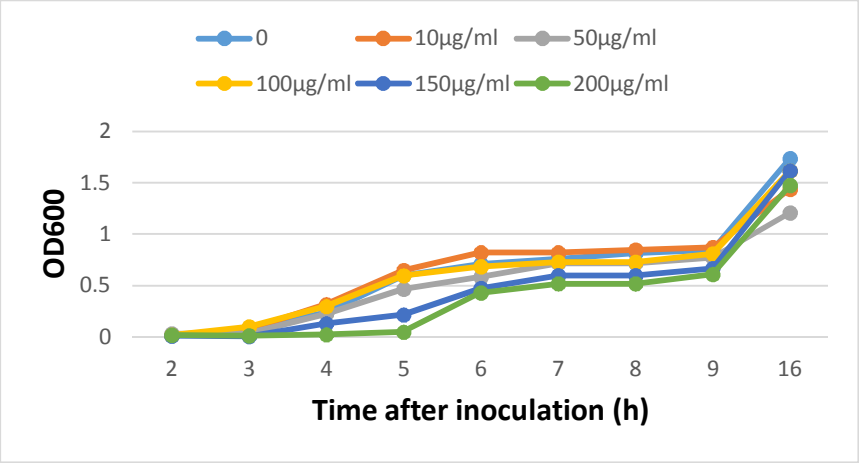

Fig.1. (c). Effect of PNPG on the growth of *P.vulgaris*.OD_{600} was measured overtime in the presence of various concentrations of PNPG (0, 10, 50, 100, 150, and 200µg/ml). The data represent the mean of three independent experiments, there is no significant difference between concentrations (P value >0.05).

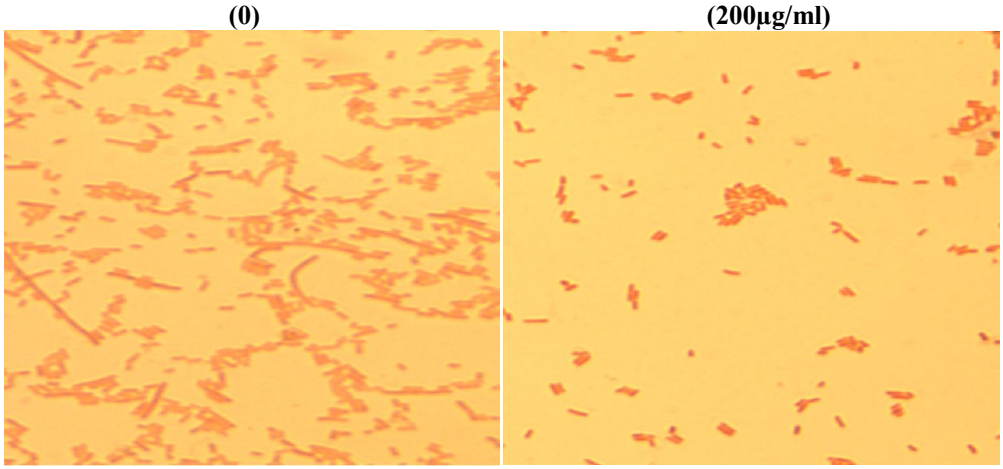

Fig.2 (a).Microscopic observation of *P.vulgaris* isolated from the LB plates without PNPG (0) and with (200 µg/ml) of PNPG.

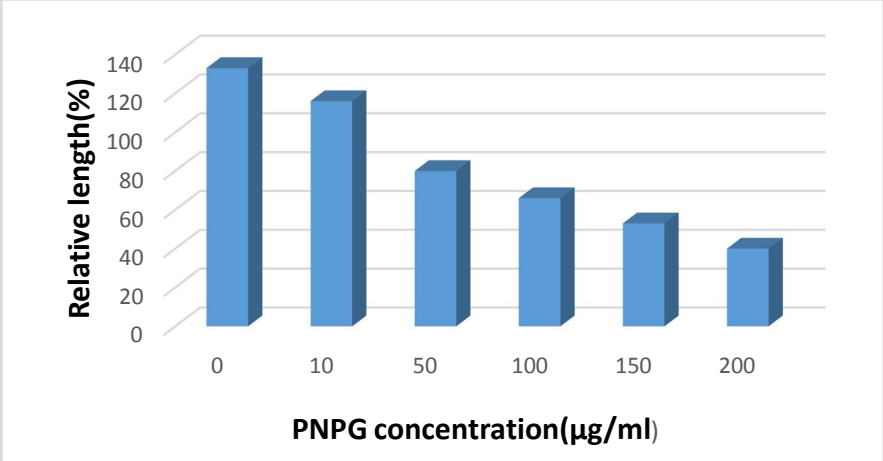

Fig.2.(b). Effect of PNPG on the cell length of *P.vulgaris*. The histogram shows the cell length of *P.vulgaris* in the presence of various concentrations of PNPG (0, 10, 50, 100, 150, and 200µg/ml). The lengths of 100 cells in each sample were determined, and the average was calculated. The difference between concentrations is statistically significant (P value <0.05).

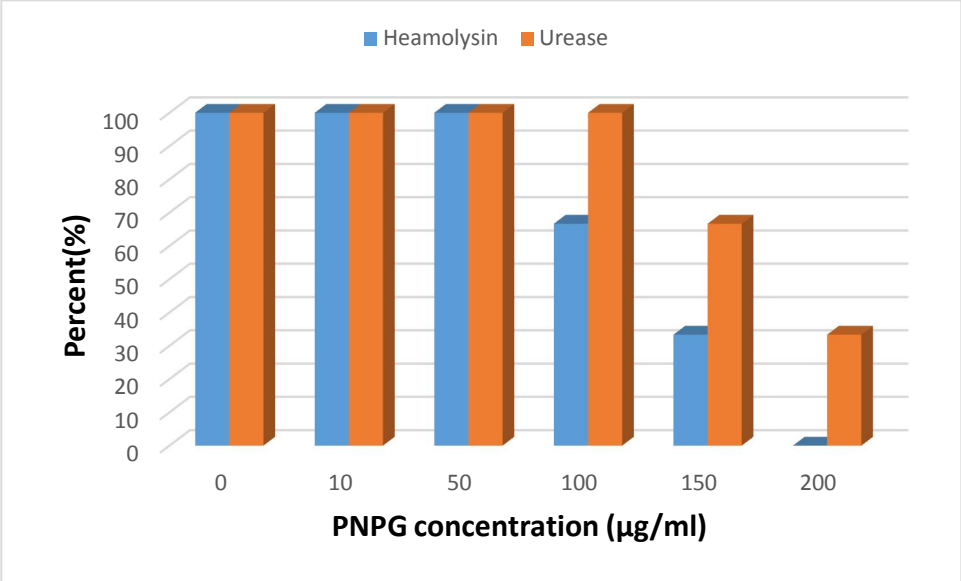

Fig.(3).The influence of PNPG on the expression of virulence factors in *P.vulgaris*. The histogram shows the production of haemolysin and urease at different concentrations of PNPG (0, 10, 50, 100, 150, and 200µg/ml). The data represent the mean of three independent experiments. The difference is statistically significant (P value<0.05).

Discussion

It has been well demonstrated that swarming motility and virulence factor expression are coordinately regulated in several pathogens including *Pr. mirabilis* and *Ps. aeruginosa* which are often implicated in persistent UTI (Ronald 2002, Wang 2006 and Nashikkar *et al*.2010).

P-Nitrophenylglycerol (PNPG) effectively inhibits swarming and virulence factor production of the enterobacterium *Proteus mirabilis* (Liaw *et al*. 2000 and 2001). The underlying mechanism of inhibition is unclear. We have now found that PNPG also inhibits swarming and virulence factor production in another enterobacterium, *Proteus vulgaris*.

In this study, we found that PNPG has the ability to inhibit *P. vulgaris* swarming significantly at a concentration as low as 50 µg/ ml and inhibited swarming completely at 150 and 200µg/ml (Fig.1a and b). Also, it had the ability to suppress the production of virulence factor; hamolysin suppression was at concentrations of (100,150 and 200 µg/ml) whereas urease suppressed at (150 and 200 µg/ml) (Fig.3). But it did not significantly affect the growth of the bacteria even at high concentrations (200 µg/ml) (Fig. 1c). This means PNPG have the ability to inhibit swarming and virulence factor production without significant inhibition of *P. vulgaris* growth. Based on this finding we concluded that the swarming ability of *P.vulgaris* is correlated with its ability to express virulence factors. So, this result corresponds with results recorded by (Liaw *et al.* 2000, Ronald 2002, Wang 2006 and Nashikkar *et al*.2010).

It is now well known that many bacterial functions including swarming, biofilm formation, and secretion of virulence factors important in successful and recurrent establishment bacterial infections are related to cell density-mediated gene expression which is termed 'quorum sensing' (QS). Quorum sensing controls the virulence determinants in most proteobacteria. In this process bacteria communicate with each other within their vicinity using chemical signaling molecules which are known as autoinducers. Quorum sensing (QS) relies on these autoinducers to control gene expression in response to changes in bacterial cell density (Schauder *et al*.2001). Several signaling molecules have been identified and the most common type in most Gram-negative bacteria is the *N*-acylhomoserine lactones (AHLs) (Adonizio *et al*. 2008 and Yin *et al*.2012).

QS regulates diverse bacterial functions including antibiotic formation, virulence factor expression, luminescence, biofilm formation, motility and pigment production (Fuqua and Greenberg, 2003, Whitehead 2001 and Krishnan *et al*. 2012). Thus, inhibiting QS or anti-QS is an important anti-infective measure that does not rely on antibiotics(Vattem *et al*.2007). Anti-QS agents will inhibit the QS mechanism and attenuate the virulence determinants and are unlikely to cause drug-resistance problems (Adonizio *et al*.2008).

Antibiotics are commonly used for the treatment of microbial infections with the widespread appearance of multi antibiotic-resistant bacteria; it is becoming increasingly more difficult to treat bacterial

infections with conventional antibiotics. Thus, there is an increasing need for new strategies to cope with infectious diseases. The discovery that many pathogenic bacteria employ quorum sensing (QS) to regulate their pathogenicity and virulence factor production makes the QS system an attractive target for antimicrobial therapy. It has been suggested that inactivating the QS system of a pathogen can result in a significant decrease in virulence factor production (Schauder and Bassler, 2001, Lyon and Muir,2003, Mihalik *et al.*2008). So, the possible mechanism by which PNPG could inhibit *P. vulgaris* swarming and virulence factor expression may be due to its acting as an inhibitor compound for bacterial quorum sensing (QS). Our results from this study indicate that PNPG has the potential to be an antimicrobial agent against *P.vulgaris* infection.

Acknowledgments

We are grateful to Biomedical Science Department of the Medicine College at Florida State University especially the laboratory of Dr. Wang. We are so grateful to Dr. Wang Yanchang, Dr. Fengshan Liang, Dr. Fengzhi Jin and Kelly McKnight for their assistance.

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Appendices

Appendix (1): Diagnostic features of *Proteus vulgaris*

Number	Test	Result
1.	Growth on MacConkey agar	+
2.	Lactose fermentative	-
3.	Gelatin hydrolysis	+
4.	Catalase	+
5.	Oxidase	-
6.	Gram stain	Gram negative
7.	Shape of bacteria	Short rod
8.	Growth on kligler's Iron agar	Black precipitate
9.	H2S production	+
10.	Iodole test	+
11.	Methyl Red	+
12.	Vogus Proskauer	+
13.	Citrate test	+
14.	Urease test	+
15.	Motility	Swarming
16.	Odor	+ (fishy odor)