Oligotrophic Bacteria: A General Introduction

Moderate terrestrial environment (environments with near neutral pH, temperature between 20-40 °C, air pressure 1 atm ; and sufficient water with solutes comprising of nutrients and inorganic salts) is considered most favorable to sustain life except the extreme habitats. However, a variety of microbes survive and grow well in other unusual habitats too, such as deserts and the ocean beds, acidic or hot springs, saline and/or alkaline lakes, glaciers, and very low nutrient milieu (Satyanarayana *et al.*, 2005). Tolerance of these microbes to extreme environments is an intrinsic property that helps to survive; for example, acidophiles show optimum growth at or below pH 3, alkaliphiles demonstrates an optimum growth at pH 9 or above, endolith can live inside rocks, thermophiles can thrive at 60 °C or above, halophiles requires at least 2M NaCl for their growth, psychrophiles grow optimally at 4 °C or below, piezophiles grow in high hydrostatic pressure (very common in deep sea surfaces), xerophiles prefers to grow in dry condition like deserts, oligophiles/ or oligotrophs grow in very low nutrient condition etc. Except in rare instances, nature does not offer bacteria such a verdant life like that when grown under laboratory condition. In the natural environment, there exists sharp competition among the competing bacteria for retrieval of nutrient from available sources. Therefore, the growth of pure cultures under laboratory conditions does not accurately mimic the situation found in nature. Bacteria in less nutrient environment struggles to adapt the fluctuating nutrient availability and thus compromises to modify its growth and tends to remain viable for a longer period of time rather than to perish (Poindexter, 1981; Mortia, 1988). Bacteria termed as 'oligotroph' [term "oligotroph" was introduced in by Weber (1907)] employ novel strategies to cope with circumstances of poor nutrient availability in their milieu and modify their growth according to nutrient conditions. Oligotrophic bacteria can therefore be defined as the "bacteria that grow and multiply in poor nutrient environments using low concentration of organic substrates". The studies of oligotrophic bacteria dates back to1920s; where survival and persistence of bacteria in absence of carbon, energy or other essential growth nutrients was reported by Winslow and Falk (1923). Later on many authors have studied the physiology of oligotrophic bacteria (Postgate and Hunter, 1962; Akagi *et al.*, 1977; Poindexter, 1981; Roszak and Clewell, 1987; Button, 1991; Button, 1993; Mortia, 1997). Earlier studies have described that an oligotrophic bacterium dominating under low nutrient condition possess mechanisms to assimilate substances present in the low-nutrient environment. Such bacteria are ubiquitous in nature and have been reported from diverse sources including clinical samples (Akagi *et al.*, 1977; Mallory *et al.*, 1977; Kuznetsov *et al.*, 1979; Tada *et al.*, 1995; Watve *et al.*, 2000; Nagarkar *et al.*, 2001; Miyake *et al.*, 2003; Pramanik *et al.*, 2003; Katsunori and Masafumi, 2006; Hu *et al.*, 2007; Ishii *et al.*, 2011). Experimentally, the ability of aquatic bacteria to develop at minimal concentration of organic matter was shown by Zobell and Grant (1943), and Jannasch (1967). Nonetheless, it was also found that many bacteria isolated on nutrient-poor media from environmental samples possess the ability either spontaneously or by adaptation, to grow on rich media. The possible explanation of the failure of obligate oligotrophic bacteria to grow on nutrient-rich media is still obscure. One possible reason could be because of their inability to cope up with the toxic products of metabolism e.g hydrogen peroxide. The growth of *Leptothrix pseudoochraceae*, *Siderocapsa eusphaera* and *Metallogenium personatum* was found inhibited due to accumulation of hydrogen peroxide when cultured on nutrient-rich media; when catalase was supplemented to the rich medium it enabled growth of those bacteria (Kuznetsov *et al.*, 1979). In *Pseudomonas oxalaticus* for example, it was found that enzymes involved in active transport of substrate into the cell or respiration) are inhibited in presence of high nutrient concentrations (Dijkhuzen and Harder, 1975; Kuznetsov *et al.*, 1979). Studies have also

shown that the cells grown in low-nutrient medium take up leucine more efficiently at low concentrations than the cells grown under high-nutrient conditions (Yoshinaga, 1990; Yoshinaga and Ishida, 1992). To investigate this physiological adaptation to low-organic nutrient conditions, Maeda *et al.* (2000) examined the protein composition of the bacterial cells by growing them into different organic nutrient concentrations. The induction of certain specific proteins designated as OlgA, -B and –C, were observed under low- organic nutrient conditions. The most significant ecological factor responsible for the development of oligotrophic bacteria is the concentration of dissolved organic substances which is indicator for the heterotrophic bacteria to assess the amount of energy substrates available for its sustenance (Al-Talhi, 2000). The effective substrate uptake system which renders the ability to acquire nutrients from very low nutrient environment is an important characteristic of the oligotrophic bacteria. Thus, oligotrophic bacteria are expected to be equipped with large surface area to volume ratio, high-affinity uptake systems with broad substrate specificities, and an inherent resistance to environmental stresses that provide them to grow, sustain and maintain their structure in low-nutrient environments. Due to potential biotechnological, medical and environmental importance, oligotrophic bacteria merit attention.

I. Nutrient availability for bacterial growth in oligotrophic environment

Growth is integrated phenomena of every live cell to increase in number and biomass; for which energy has to be derived from substrates present in its niche (Morita, 1988; Tranvik, 1988). Besides energy substrates, various elements like C, H, O, P, K, N, S, Ca, Na, Mg and Fe (macro-elements) and the trace elements; Mn, Zn, Mo, Cu, Co, Ni, V, B, Cl, Se, Si, W as well as others growth factors for example vitamins are essential for growth. Minimally these elements are acquired from the environment where bacteria sustain. Lack of one or more of these essential nutrients may wipe out a bacterial species from that environment. Hence, growth of an organism in an environment is self-explanatory that all nutrients were initially present, even if analytical techniques fails to detect the presence of any such component (because of rapid utilization of the component in extremely diluted conditions) (Al-Talhi, 2000). Life of bacteria is often difficult due to scarcity of substrates (which are vital for cellular function) to allow copious growth without any interruptions. Oligotrophic (low nutrient) environments are often created in (i) soil; (ii) marine water; and (iii) fresh water.

Soil

Nature of soil present elsewhere in the earth is not at all uniform and immensely diverse where the organic and inorganic components, that sustains bacterial life in it, are often present in extremely low concentrations (Ohta and Hattori, 1983). Much of the organic matter enters into the soil are utilized by higher living systems. Even in locations around roots of higher plants where there is regular supply of energy yielding substrate, input is often found inadequate to maintain an enormously active microbial community (Barber and Lynch, 1977). Water extract from soils usually contain < 4.2 µg/L of carbo-hydrate (glucose equivalents) and 1.9 µg/L amino acids (Ko and Lockwood, 1967). Hence, soil indisputably is a poor medium in comparison to conventional media, like nutrient agar which contains approximate 4000 mg/L carbon, used for routine bacterial culture. So we may conclude that bacteria growing in nature are more prone to oligotrophic condition than copiotrophic laboratory culture conditions.

Marine Water:

Natural seawater is characterized by extremely low nutrient concentrations, generally around 0.5 mg of carbon per liter that is, far less than conventional bacterial culture media, which range from 1,000-20,000 mg of carbon per liter (Maeda *et al.*, 2000). Oceanic volume, under 200-300 m in depth, the dissolved organic carbon concentration ranges from 0.35 to 70 mg/L and the particulate organic

carbon from 0.3-1.0 mg/L (Martin and Macleod, 1984). Bacteria that thrive in low-nutrient conditions also have mechanism to survive in starved condition (Morita, 1982; Kjelleberg *et al.*, 1987). During starved conditions, physiological and morphological changes occur that induce several characteristic proteins that helps bacteria to adapt in the low nutrient environment (Amy and Morita, 1983; Jouper-Jaan*et al.*, 1986; Morton and Oliver, 1994). It was found that even in the presence of viable bacteria there was no significant variation in the concentration of dissolved organic carbon in marine water over an incubation period of two months (Barber, 1968).

Fresh Water

Fresh water samples from diverse sources and locations like fast flowing rivers or comparatively static lacustrine system or pristine water bodies (both static and dynamic) or distilled water storage systems display a wide spectrum of low nutrient conditions. It was found that the concentration of organic matter from numerous American lakes ranged from 1 to 26 mg of carbon per liter, and averages of 1.36 mg of carbon for suspended organic substance and 15.24 mg of carbon for dissolved organic substance per liter (Birge and Juday, 1927; Birge and Juday, 1934); while Canadian lakes, the lake Opinikon contained approximately 5-10 µg of sucrose and 2-5 µg of glucose per liter (Vallentyne, 1954). Similar ranges of sugars and glucose were also found in oligotrophic lakes, Rudnlake and hypolimnion (Kuznetsov *et al.*, 1979).

II. Oligotroph: Definition

The Bacteria that have an ability to grow in very low nutrient concentration are labeled as oligotrophs, but there is no generally accepted definition of oligotrophic bacteria. Yanagita *et al.* (1978) define oligotrophs, organisms that able to grow at nutrient concentration of 5 mg C/L but not at concentration of 7.5 g C/L. The bacteria from the environmental sample which are able to form colonies when directly plated on media containing organic matter approximate 1-15 mg carbon per liter are termed as oligotroph; in spite of their ability to grow on rich-media at subsequent re-cultivations (Kuznetsov *et al.*, 1979). Other authors have recommended the use of 10 mg or 1 mg C/L in the media for the cultivation criterion specific for oligotrophic bacteria (Akagi and Taga, 1980a; Ishida and Kadota, 1981). However, Poindexter (1981) is of view that oligotrophy is a phenomenon demonstrated by bacteria to survive in ecological niche having a nutrient flux from near zero to a fraction of milligram of carbon per liter per day. Some of the definitions proposed by different authors are given in Table I.I.

Table I.I: Definitions of oligotrophic bacteria by different authors (Schut *et al.*, 1997)

- Oligotrophic micro-organisms are prokaryotic and eukaryotic organisms that are evolutionarily adapted to exploit ecological niches characterized by low substrate concentrations and low energy fluxes. Oligotrophs may develop in rich as well as in poor environments.
- Oligotrophic bacteria are heterotrophic bacteria capable of growth in the presence of organic nutrients equivalent to 16.8 mg/L.
- Bacteria capable of growth on un-amended BWA (agar-solidified Chesapeake Bay water).
- Bacteria which can grow at substrate concentration of less than1 mg C/L.
- Oligocarbophilic bacteria are capable of to grow on media containing only minerals, and they meet their carbon and energy requirements from trace amounts of organic substance found in the air.
- A trophic group of bacteria that can grow only in the presence of minor amount of nutrilites and not in the presence of a large amount.
- Those bacteria that develop at the first cultivation on the media with the minimal content of organic matter of about 1-15 mg C/L and that grow on such media on subsequent re-cultivation through they can grow on richer media.

Table I.I: continue....

- Organism that grow in media containing organic matter at a concentration of 1 mg C/L. Obligate oligotrophs may decrease in number or disappear with the onset of man-made eutrophication, facultative oligotrophs can tolerate or rapidly adapt to the higher concentration of organic substances.
- Oligotrophic bacteria can be conceived of as those whose survival in nature depends on their ability to multiply in habitats of low nutrient fluxes (approaching zero to a fraction of mg C per liter per day.
- Bacteria that can be isolated on a low nutrient medium (unsupplemented Bushnell Haas agar) and that are restricted to growth at low nutrient concentrations.
- Oligotrophs are defined as those organisms known to be able not only to survive but particularly to multiply under conditions of extremely low and often discontinuous supply of nutrients. In other words, organism adapted to low and irregular fluxes of substrates.
- Obligately oligotrophic bacteria are capable of growth in $SF10^{-1}$ (0.2 mg C/L) but not in $SF10^{-1}$(200 mg C/L).
- Obligate oligotroph as an organism which does not grow in rich (200 mg C/L) media, and the facultative oligotroph as an organism grow in not only in poor (0.2 mg C/L) but also in rich media.
- Oligotrophic isolates are defined as bacteria capable of growth on OEMS agar (0.4 mg C/L).
- Oligotrophic bacteria can broadly be defined as organisms that grow on low concentrations of organic substrates. Obligate oligotrophs cannot grow at substrate concentrations above 6 g C/L.

III. Classification of oligotrophic bacteria

Oligotrophic bacteria can be classified broadly in two classes- obligate and facultative. Ishida *et al.* (1986) differentiated facultative oligotrophs from obligate oligotrophs that fails to grow in substrate concentration above 0.3 g C/L. Obligate oligotrophs grow only at low nutrient condition and fail to grow on a richer media while facultative oligotrophs have the flexibility to grow in low as well as on the nutrient-rich media and can enjoy life in both environments. Horowitz *et al.* (1983) used the term 'euryheterotroph' for facultative oligotroph, later on Baxter and Sieburth (1984) replaced the term 'facultative oligotroph' by eurytroph. Kuznetsov *et al.* (1979) divided oligotrophs in four categories (Table I.II). The 16S rDNA probe-based hybridizations with environmental DNA revealed multitude of uncultured organism (Pace, 1997; Hugenholtz *et al.*, 1998). These unculturable bacteria constitute fourth class of non-cultivable organisms (Kuznetsov *et al.*, 1979). The fact that these bacteria could not be cultured may be because of the following reasons: (i) the deficiency of knowledge-base for designing suitable media; (ii) non-availability of knowledge of existing consortia of different kinds of bacterial communities that aid symbiotic association to each other; (iii) existence of bacteria in the form of inert cell or spore with an ability to revert to vegetative form by switching infrequently for short periods to the growing state in a particular ecological niche (Koch, 2001). These uncultivable bacteria may also include the "obligate oligotrophs". The prototypic organisms generally used for studying oligotrophs are *Caulobacter crescentus* and *Arthrobacter* spp. (Poindexter, 1981); but the fact remains that they are 'facultative oligotroph' and not as "obligate oligotroph" as they could grow both in nutrient-poor and nutrient-rich medium. Hattori's (1976) and other workers observed that frequency of obtaining colonies from soil and environmental samples is more in diluted organic nutrient agar medium than the usual rich nutrient medium.

IV. Predicted properties of oligotrophs

Proposition of a model oligotroph with possible attributes on the basis of nutrient uptake and utilization was one of the outcomes of the Dajhelm conference (Hirsch *et al.*, 1979). The predicted properties that a bacterium should possess to be branded as an oligotroph are as follows: (a) having a shape pertaining to high surface per volume ratio (also expected to be small or bear prostheca); (b) possessing an intrinsic preference to utilize metabolic energy for uptake of nutrients during periods of stagnancy of growth; (c) having ability for nutrient uptake which are expressed constitutively; (d)

existence of high affinity, low-specific transport system to facilitate simultaneous uptake of mixed substrate; and (e) having mechanisms of storing nutrients after the uptake.

Table I.II: Categories of oligotrophic Bacteria

Category	Characteristic	Species	References
1.	Demonstrates formation of colonies on sterile-water agar medium but fails to grow when sub-cultured.	Bacteria of unusual morphology.	(Kuznetsov *et al.*, 1979)
2.	Initially could be grown on nutrient-poor media and do not readily grow in rich media, but can be adapted slowly to grow in nutrient-rich media.	Some species from *Pseudomonas, Agrobacterium, Photobacterium, Vibrio, Aeromonas, Flavobacterium, Micrococcus roseus, M. luteus, M. rajahs, Staphylococcus sapro. phyticus, Corynebacterium, Arthrobacter.*	-do-
3.	Isolated and subsequently re-cultivated in special nutrient-poor media.	Species *of Hyphomicrobium, Caulobacter, Microcyclus, Leptothtix, Ochrobium, Metallogenium, Pasteuria* etc.	-do-
4.	Detected in natural water reservoirs, only under electron microscope; could not be cultivated under laboratory conditions.	Prosthecate bacteria; a number of bacteria with gaseous vacuoles.	-do-

The small size of cells was predicted to provide a distinct advantage in terms of grazer dodging (Ostrowski, 2006) and increased efficiency of nutrient uptake. It is also expected that broader specificity in nutrient uptake would be present in oligotrophic bacteria. Button (1998) observed that the ability of organisms to compete for substrates at low concentrations is influenced by the quantities and types of transport proteins and enzymes like different permeases as opposed to conditions when bacteria are exposed to high nutrient growth environments where metabolic cost will not be borne by the cells in expressing enzymes that are not required when nutrients are in abundance. The regulation of biosynthetic routes operating in an oligotroph would be in line with nutrient uptake rates (Poindexter, 1979). Oligotrophs were also expected to have certain savings and therefore predicted to have the ability to store diverse nutrients in reserves (Hirsch *et al.*, 1979). Physiological studies on oligotrophs are still meager to support and validate the above-mentioned predictions.

V. Oligotroph versus Copiotroph

Oligotrophic bacteria have the ability to grow and multiply in extremely nutrient poor environments; often defined to have the ability to grow when carbon flux is 1 to 15 mg soluble carbon per liter while copiotrophic bacteria grow in carbon-rich environments that provide about 1000 mg soluble carbon per liter (Paul and Clark, 1996). In concept, the oligotrophic bacteria survive in a perennially meager environment. Copiotrophs, or eutrophs, are coupled with richer environments and are usually adapted to utilize resources quickly when available. Habitats with continually low levels of nutrients (oligotrophic), of course, are a foremost and important extreme environment. Button (1991) demonstrated that copiotrophic bacteria have high V_{max} and K_m values and adapted to high nutrient environments, while oligotrophic bacteria have high substrate (carbon) affinity as evidenced by the low K_m values of their transport systems, therefore, oligotrophic bacteria have competitive advantages over copiotrophs when substrate is low. To sustain life in a nutritionally robed environment must involve the expression of genes that have evolved over long periods of good and bad environmental conditions. The way of life of oligotrophs, especially facultative oligotrophs are very important to understand the microbial evolution. Although both oligotrophs

and copiotrophs can survive in a low nutrient environment, but only oligotrophs can persist in chronic starvation conditions and, conversely, may not be able to persist for long periods in richer environments. Study of Hu *et al.* (1999) illustrated an inverse relation between soil copiotrophs and oligotrophs in response to carbon availability. It was shown that high carbon availability inhibited oligotrophs in natural soils. Earlier workers (Akagi *et al.*, 1980b; Fry, 1990) have reported that high carbon concentrations were detrimental to the oligotrophs on agar or liquid media. Akagi *et al.* (1980b) showed that the colony number of marine bacteria was maximal at 400 mg peptone-carbon per litre of medium and then decreased significantly as carbon concentration increased. There are no environments that are really exhibit constant nutrient concentration except for a perfect chemostat or turbidostat culture. In nature there are reasonably upswings and downswings in the nutritional state in the immediate environment hence microorganism must have to develop mechanisms to resist, capitalize on, and exploit such bonuses and survive deficits. The term 'oligotroph' is approximately equivalent to the term 'autochthonous' which was introduced by Winogradsky in 1924, it's because of many bacteria inhabiting soil environment grow in sparse and difficultly metabolizable substrates (Koch, 2001). Thus according to the terminology these autochthonous bacteria can be placed in oligotrophic group, and can be differentiated from "copiotrophs", a term introduced by Poindexter (Poindexter, 1978; Poindexter, 1981).

Table I.III: Some possible reasons that, why oligotrophs failed to grow on rich nutrient media and copiotrophs on poor nutrient media.

Possible reasons for oligotrophs to succumb during challenges by too high nutrition*	**Possible reasons for copiotrophs to succumb during low nutrition or starvation***
➢ **OSMOTIC CHALLENGES** **Transport Raises the Internal Osmotic Pressure** • Cell dies by osmotic swelling because solute pumping is too rapid for consumption • Cell, inappropriately, pumps a substrate inward that the cell cannot use. • Collective import of many substances makes the osmotic pressure too high. • Cell pumps a specific substance that harms the cell because growth is blocked by absence of other growth factors • Wall growth is blocked, but not import of nutrients • (Example-Cell forms bulges in presence of low levels of penicillin and ruptures at division site) ➢ **GROWTH IMBALANCE** **Energy depletion of ATP and/or proton motive force** • Too many transportable non-metabolic substances suddenly available **Rigid and slowly-enlarging wall may rupture due to cytoplasm accretion** • Wall enlargement rate may not be adequate to prevent wall rupture (Counter example- *S. mutans* in presence of penicillin type inhibitors) **Cell insertion of inappropriate membrane proteins** • The cytoplasmic membrane has too high protein to lipid ratio not permitting growth • Too many integral membrane protein may weaken membrane and block growth ➢ **TOXIC SUBSTANCE** **Lactose Death** (Example-Galactoside excess is caused in an unknown way) **Killing due to free radicals** • Higher rate of free radicals formation generated from nutrients in the presence of oxidants, such as oxygen, generation **Inappropriate SOS-like response** • DNA synthesis blocked, but cell never recovers	➢ **LACK OF ADEQUATE REGULATORY SYSTEMS** • Cells have lost, or inactivated, their starvation mechanisms • Cells never did evolve starvation protection mechanisms and have no other strategies • Stripped-down cells that have jettisoned protection Mechanisms ➢ **LACK OF ADEQUATE TIME TO ADAPT TO POOR NUTRITION** • Regulatory mechanisms may need adequate time to make protective proteins ➢ **HOLDUP DUE TO LACK OF REPAIR OF DNA DAMAGE DUE TO CELL'S INADEQUATE SUPPLIES OF NUCLEOTIDES** ➢ **SOS SYSTEM FAILS TO FUNCTION AND FAILS TO BLOCK CHROMOSOME REPLICATION AND CELLS DIVISION AFTER DNA DAMAGE HAS TAKEN PLACE** ➢ **UNDER ENERGY STARVATION, SYNTHETIC REACTIONS ACT IN THE REVERSE DIRECTION** ➢ Enzymes (and Protein Synthesis Machinery) Have to Catalyze Reversible Processes and Unless Specifically Blocked, Hydrolysis or Leakage May Occur ➢ **METABOLIC PRODUCTION OR NON-UTILIZATION OF A SUBSTANCE POISONS CELL** • (Example-Galactose-1-phosphate killing in both humans and bacteria is due to metablic imbalance) • Cell depletes a necessary consumable cofactor and cannot grow

Table I.III: Continue.....

Unbalanced syntheses • Blockade of DNA synthesis because failure of chromosome initiation Blockade of cell division because failure of constriction or septum formation **Cell depletes a necessary cofactor for protein synthesis and cannot grow or recover** • (Example-This is one possible explanation of how streptomycin kills, suggested by Robert Harvey, unpublished) **Generation of inactive cells** • (Example-Extension of quiescent cells formation as in low-dilution rate chemostats) **Possible role of a VBNC** of a syndrome or a shut-down cell phenomenon***** • Cell regulatory systems turn down metabolism, but • inappropriately and not easily reversed *That is, either no growth, slow growth, or dying. **Variable but not cultivable. ***Cell limited to motility in its metabolic role.	➢ **COMPETITION WITH OLIGOTROPHS** • In nature, the concentration of certain nutrients may be maintained at quite small levels because the oligotrophs deplete the resource and compete with any copiotroph entering the habitat ➢ **MAINTENANCE COSTS ARE TOO BIG** • Energy for maintenance is needed to repair and possible resynthesize of 'worn out' cellular components. Oligotrophs may have adapted to need little energy for such purposes, while copiotrophs have not. *No growth, slow growth, or dying

[adapted without any modification from Koch A.L. 2001. Oligotrophs versus copiotrophs. *BioEssays* 23: 657-661]

Oligotrophs are characterized both by the ability to grow in low concentrations of substrates, and inability to grow and prosper in environments with high levels of nutrients. Some doable rationales for the oligotrophic and copiotrophic life strategies pointed by Koch (2001) are presented in the table I.III.

VI. Methods for studying oligotrophic bacteria

The effect of low nutrient habitats on bacteria is studied either by *in-situ* detection (direct observation of live bacteria in the environmental samples) or by cultivating environmental bacterial samples in media devoid of any energy source or by cultivating environmental bacterial samples in the sterile media containing the same constituents from where the samples were isolated [like sterile soil (for cultivating the soil bacteria); filtered sea water (for cultivating marine bacteria); and filtered river waters (for cultivating riverine bacteria)]. Due to troublesome operations, there are only few reports of the direct observation of bacteria in soils and rocks (Balkwill *et al.*, 1997). The difficulty in isolating oligotrophs from the environment has been pointed by several authors (Schut *et al.*, 1997a). The difficulties, faced by the experimenter in isolating oligotrophs and subsequently adapt them to the laboratory culture conditions for in-depth studies, are due to several factors like intolerance to high concentrations of nutrient levels in the conventional culture media; or inappropriate growth substrates used for enrichment; or the lack of appropriate growth factors/cofactors like specific microelements and or vitamins; or presence of undetected inhibitory growth substrates or other additives; or incompatibility to grow in close proximity to other cells (in colonies on agar plates); or inability to resist oxidative respiratory burst upon upshift and outgrowth in the sudden nutrient replenishment conditions when fresh nutrients are added during cultural practices; or because of the enrichment of the phage population that perishes the population. However, efforts to cultivate oligotrophs using different methodologies did not fade away and several trouble-shootings were innovated.

Isolation of oligotrophic bacteria by plating method

Oligotrophs mostly have been isolated on several fold diluted traditional media or on agar plates without any organic nutrients. Nutrient-poor media which contained 10 mg of polypeptone and either deionized water or aged sea water was used by Yanagita *et al.* (1978) to isolate oligotrophs. Brain heart infusion esturian salts agar (brain heart infusion 3.7g/L, mineral salts 15g/L) was used

by MacDonell and Hood (1982) to isolate oligotrophic aquatic bacteria. Bushell Haas agar which containing exclusively mineral salts was used by Horowitz *et al.* (1983). Some workers used agar amended with water from natural reservoir supplemented with minimal quantities of mineral nitrogen and phosphorus for cultivating oligotrophic bacteria (Kuznetsov *et al.*, 1979). Diluted (10^{-3} and 10^{-4}) Nutrient broth amended with agar was used by Tada *et al.* (1995) to isolate oligotrophic bacteria from clinical samples. Watve *et al.* (2000) and Nagarkar *et al.* (2001) used Ravan medium (glucose, 5 mg/L; peptone, 5 mg/L; sodium acetate, 5 mg/L; sodium citrate, 5 mg/L; yeast extract, 5 mg/L; sodium pyruvate, 2 mg/L; agarose, 10 g/L) . Reasoner and Gerldreich (1985) introduced R2A medium for cultivating environmental bacteria (oligotrophic) from potable water. Massa *et al.* (1998) compared results obtained from plate count agar (rich nutrient medium) and R2A medium for enumeration of heterotrophic bacteria; and found that bacterial counts on R2A agar were 34.3% greater than the bacterial counts on plate count agar. These results indicate that R2A medium was better than the plate counts agar for cultivating bacteria from natural mineral water. Some workers used 100- 10,000 fold diluted nutrient agar for isolating and cultivating oligotrophic bacteria from different sources (Tada *et al.*, 1995; Ishii *et al.*, 2011). Recently diluted Luria Bertani (LB) borth and diluted Luria broth was used to isolate oligotrophic bacteria (Oh *et al.*, 2009; Kumar *et al.*, 2010, 2011). Hu *et al.* (2007) in their study demonstrated that application of diluted LB produced more colonies representing diverse bacterial communities including the novel ones. Also, some workers used comparatively rich media to isolate facultative oligotroph. F5 agar (1 g/L polypeptone, 0.1 g/L yeast extract) was used for isolating facultative oligotrophic bacteria (Ishida and Kodota, 1981). However, growth on nutrient rich medium do not exclude the oligotrophic organism since facultative oligotrophs have the capability to grow on nutrient-rich as well as on nutrient-poor media. To differentiate colonies of oligotrophs from copiotrophs, replica plating on nutrient-rich and nutrient-poor plates is preferred. The facultative oligotrophic bacterial colonies will appear on the imprints of both nutrient-poor and nutrient-rich agar medium. The colonies of obligate oligotrophs on nutrient-poor master plates will show growth only on nutrient-poor replica plates. The bacterial colonies on nutrient-rich master plates which would show growth only on nutrient-rich replica plates will be differentiated as copiotroph.

Isolation of oligotrophic bacteria using glass fiber filter

A novel method using an indecisive utilizable organic material, devoid of agar but retained a solid surface in form of glass fibers was designed and used for isolation of oligotrophic bacteria (Akagi *et al.*, 1977). In this method annealed plates of pressed glass in sterile Petri dish (9 cm in diameter) filled with 15 mL of nutrient-poor media devoid of agar were placed at the bottom of dish. On the plates sterile membrane filters were placed through which volumes of sea water (0.5- 500 mL) was passed. The system which was devoid of agar (with an uncertain complement of utilizable organic material) retained the advantages of offering a solid surface. The dilute nutrient medium used contained 16.8 mg carbon per liter. The composition of the medium (in charcoal treated sea water) polypeptone,10 mg/L; protease; peptone, 5 mg/L; bacto soytone, 5 mg/L; yeast extract, 5 mg/L; sodium glycolate, 5 mg/L; sodium malate, 5 mg/L; D-mannitol; 5 mg/L; sucrose, 5 mg/L; and ferric citrate, 0.5 mg/L.

Isolation of oligotrophic bacteria using liquid media

Some of the workers used liquid media for the isolating oligotrophic bacteria by diluting bacterial suspension in low nutrient liquid medium followed by incubation at 20 °C for 15-30 days. But the original technique turned to be problematic for recording growth in dilute media. Ishida and Kadota (1981) resolved this problem by labeling substrate with ^{14}C in the medium; and growth was assessed by $^{14}CO_2$ evolution using a scintillation counter. Baxter and Sieburth (1984) detected growth by

epiflourescence microscopy after growing in a series of diluted inorganically supplemented sea water with 0.01 and 0.1 mg/L of glucose at 17 °C for 2 days. Sub culturing onto agar was done from higher dilutions to obtain isolated colonies. HuiXia *et al.* (2007) isolated an oligotrophic bacterium SGB-5 from biological soil crust using KH_2PO_4 0.2 g/L; NaCl 0.2 g/L; $MgSO_4$ 0.1 g/L; $CaCO_3$ 3 g/L; C 10 mg/L (quite glucose 0.025 g); neutral pH.

Isolation of oligotrophic bacteria by enrichment method

VenderKooj and Hijnen (1985) used batch culture enrichment technique to isolate facultative oligotrophs from drinking water. They incubated tap water with added substrates at 15 °C. *Flavobacterium* spp. was isolated from medium with the added starch, while strain 166 and strain S12 were isolated from medium containing 10-20 µg/L and 100 µg/L starch respectively. These isolates grew well on low concentrations of one or more of the substrates used in the enrichment medium. Hence this method may be a good alternative for isolating oligotrophs with specific nutrient requirements.

Isolation of oligotrophic bacteria by extinction dilution method

Extinction dilution method is the most successful isolation technique. The method was often used to obtain strains of *S. alaskensis* (Button *et al.*, 1993; Schut *et al.*, 1993) and *Cycloclasticus oligotrophus* (Button *et al.*, 1993, Wang *et al.*, 1996). In this method the samples (from where it originated, for example seawater) were diluted in the filtered sterile diluents (made from source) until only few bacteria remained in each dilution tube (Button *et al.*, 1993). The lack of additional substrate prevents the possibility of substrate toxicity and removed competition for substrates by less abundant indigenous copiotrophs allowing for long term incubation of potentially pure cultures in the highest dilutions. Long term incubation of these cultures (6-12 months) in the dark and at 5 °C initiated an unknown mechanism that enabled the cells to grow on a rich nutrient medium, i.e a transition from an obligate to a facultatively oligotrophic state (Schut *et al.*, 1993). This changing behavior from one state to another is still blurred, in an experiment conducted with an isolate, RB2256, which demonstrated that the time of exposure and dose of concentration of external nutrient is an imperative factor to determine whether an oligotrophic bacterium would survive on nutrient rich medium or not (Schut *et al.*, 1997b). The transition phase (from oligotrophic to copiotrophic) may involve gradual changes in cellular reaction or cellular composition that provide resistance to cell to protect from osmotic stress induced by the initial uptake of nutrients and/ or the initial oxidative respiratory burst (Ostrowski, 2006). Isolation of previously uncultivated members of the SAR11 clade, as well as novel Gammaproteobacteria representatives from coastal and ocean environments (Rappé *et al.*, 2002; Cho *et al.*, 2004) are some fruitful outcome of this technique.

Isolation of oligotrophic bacteria by micro-encapsulation method

This technique combines encapsulation of cells in gel micro-droplets for massively parallel microbial cultivation under low nutrient flux conditions, followed by flow-cytometry to detect micro droplets containing micro colonies (Zengler *et al.*, 2002). In this method filtered seawater for diluting seawater sample and PBS buffer (pH 7.2) were used for diluting soil samples. The diluted samples (10^7cells/mL) were mixed with preheated agarose (at 40°C) and added into Cell Mix emulsion matrix and emulsified at room temperature. On cooling, the oil-bacterial suspension resulted in the formation of 10^7 gel micro-droplets (GMDs). Microscopic monitoring ensures encapsulation of single cell. Incubation of GMDs (at least 3 h-5 weeks) into respective medium resulted into development of micro colonies that were either sorted individually or by flow cytometry.

Isolation of oligotrophic bacteria by filtration–acclimatization method (FAM)

Hahn *et al.* (2004) developed this technique for isolation and cultivation of bacteria which were unable to cultivate by standard methods. The original method involved two steps; first step was filtration which removed most of the readily cultivable bacteria that overgrow than the slow growers; and second step was acclimatization that provided a slow transition from the low to the high nutrient concentration. In this method sudden exposure to high nutrient concentration is avoided to prevent substrate shock there-by increasing the viability of the oligotrophs (bacteria adapted to low substrate concentration). Neither rich nutrient media nor solid media were used for culturing the isolates. In FAM, bacteria were slowly acclimatized to higher substrate concentrations. By using FAM they isolated many previously uncultured bacteria which belonged to the group *Actinobacteria, Alpha-, Betaproteobacteria, Bacteroidetes,* and *Spirochaeta.*

VII. Ubiquity of oligotrophic bacteria

Several oligotrophic bacterial species from varied genera and sources have been isolated. Most of them were Gram negative bacteria. In few instances oligotrophic isolates were obligate oligotrophic, non-motile, Gram negative and oxidase positive rods (Ishida and Kadota 1981). Characterization of 162 oligotrophic bacteria isolated on low nutrient media from estuarine environment showed that 90% bacteria belonged to the genera, *Alcaligenes, Corynebacterium, Hyphomicrobium, Hyphomonas, Listeria, Nocardia, Pedomicrobium, Planococcus, Sphaerotilus, Streptothrix,* and *Streptomyces* (Mallory *et al.*, 1977). The remaining 10% were unidentified sheathed bacteria. In one thesis available on the internet, there was a mentioning of a work by West and Fry (1989) describing 421 isolates; of them, 94.8% were facultative and 1.9% obligates oligotrophs; the facultative oligotrophic bacteria mostly reported were from the genera *Acinetobacter, Bacillus, Brevibacterium, Corynebacterium, Escherichia, Klebsiella, Salmonella, Acidovorax, Comamonas, Serratia, Providencia, Enterobacter, Micrococcus, Proteus* and *Pseudomonas* (Al-Talhi, 2000). Katsunori and Masafumi (2006) isolated 538 oligotrophic bacteria from hospital tap water, of which 23.6% (108) were *Methylobacterium* followed by *Pseudomonas* (13.2%) and 60% bacteria could not be identified. A number of oligotrophic species, were isolated from various sources, have been compiled as follows: *Spirillum* sp. (freshwater pond), *Pseudomonas* sp.486 (coastal water), *Pseudomonas* sp. R.P 303 (coastal water), *Arthrobacter* spp. (soil), *Caulobacter cresentus* (freshwater, marine water), *Asticcacaulis biproshtecum* (unknown), *Aeromonas* sp. No.6 (Lake Biwa), *Flavobacterium* sp. M1 (Lake Mergozzo, Italy), *Pseudomonas flourescens* (drinking water), *Flabobacterium* sp. S12 (Tap water), *Spirillum* sp. NOX (slow sand filter), *Hypomicrobium* sp T37 (fresh water), *Acinetobacter* sp GO1 (sea water), *Agromonas* sp. (soil, rice roots), *Corynebacterium* sp. MC2 (canal water, UK), *Curtobacterium* sp. CF2 (taff feeder canal, UK), *Pseudomonas flourescens* (spring water, UK), *Bacillus pumilis* WF01 (Lianishen reservoir, UK), *Pseudomonas* sp. WO01 (Lianishen reservoir, UK) [Fry, 1990]. Oligotrophic strains like *Acintobacter johnsonii* MB52, and *Klebsiella pneumoniae* strain MB45 were reported from river water (Kumar *et al.*, 2010, and 2011). A new genus *Agromonas* with oligotrophic species *A. oligotrophica* was established by Ohta and Hattori (1983). The above said acetylene-reducing oligotrophic strain, *A. oligotrophica* was isolated from paddy soil. Similarly a halo- and organo-sensitive oligotrophic bacterium *Sphingomonas oligophenolica* was also isolated from paddy soil near Sendai in Japan (Ohta *et al.*, 2004). Two novel gram-negative oligotrophic strains, *Xanthobacter xylophilus and Ancylobacter abiegnus* were isolated from dystrophic humified waters formed by xylotrophic fungi grown on decaying spruce wood (Zaichikova *et al.*, 2010a, 2010b). Han *et al.* (2012) isolated 200 oligotrophic bacterial strains from rhizospheres of various soil samples in Korea, of them two oligotrophic bacterial strains, *Pseudomonas monteilii* B001 and *Bacillus cereus* C003 were found to provide a broad spectrum of induced systemic resistance to the plants.

Recently a gram-positive facultative oligotrophic stain, *Brevibacterium siliguriense* was isolated from river water sample (Kumar *et al.*, 2012).

VIII. Antibiotic resistance in oligotrophic bacteria

The age of antibiotics is usually traced back to 1928, the year of penicillin discovery by Alexander Fleming. Since then a vast number of antibiotics have been introduced in market for treatment of several fatal and non-fatal diseases. Besides fundamental application in human health, antibiotics (antimicrobials in broad) have also been used for preventing and treating animals and plants infections as well as for promoting growth in cattle farming (McManus *et al.*, 2002; Smith *et al.*, 2002; Singer *et al.*, 2003; Cabello, 2006). It is important to state that several antibiotics are produced by environmental microorganisms (Waksman and Woodruff, 1940) that exerts selective pressure in natural environment for bacteria growing in that habitat (Baya *et al.*, 1986). On the other hand, getting antibiotic resistance genes via Horizontal Gene Transfer (HGT) is another cause of spread of antibiotic-resistance in environmental and clinical bacteria (Davies, 1997). Antibiotic-resistance genes can also evolve under strong antibiotic selective pressure through natural selection (Martinez and Baquero, 2000; Martinez *et al.*, 2007). Additionally, mobile genetic elements like such as integrons, transposons, and plasmids contribute to the spread antibiotic resistance among the environmental bacteria (Recchia and Hall, 1995; Carattoli, 2001; Rowe-Magnus and Mazel, 2002). However, spent antibiotic residues in environment also contribute to a different type of pollution enriching the population of intrinsically resistant bacteria, and consequently leading to the reduction of the susceptible bacterial population. There are reports which illustrates that antibiotics themselves can promote targeted mutations (Cirz *et al.*, 2005; Kaufmann and Hung, 2010; Kohanski *et al.*, 2010; Thi *et al.*, 2011). Recently Li *et al.* (2010) performed a comparative study on impact of antibiotics in bacterial resistance. They collected samples from river surface water from 5 km upstream and 20 km downstream from the discharge point receiving effluent (waste water) from an oxytetracycline production plant. They found that almost all bacteria (97%) from the immediately discharged waste water samples, and downstream water samples, were multidrug-resistant while in upstream water samples, these were less frequent (28%). Evidence suggests that non-pathogenic environmental bacteria constitute the reservoir of antibiotic-resistance genes with potential to be transferred to pathogens (Riesenfeld *et al.*, 2004; Allen *et al.*, 2009; Donato *et al.*, 2010). Whole genome sequence analysis of an oligotrophic, water disinfection resistant bacterium, *Minibacterium massiliensis*, revealed the presence of a unique genomic island that encoded resistance to several antibiotics and heavy-metal ions and metalloids (Audic *et al.*, 2007).

Majority of studies on antibiotic-resistance, knowingly or unknowingly, were concentrated on copiotrophic bacteria. In comparison to copiotroph, little is known about antibiotic resistance in oligotrophic bacteria (Nikitin *et al.*, 1988; Zlatkin *et al.*, 1991; Kimura *et al.*, 1995; Oh *et al.*, 1995; Tada *et al.*, 1995; Riesenfeld *et al.*, 2004; Kumar *et al.*, 2011). Miyake *et al.* (2003) isolated bacteria on diluted nutrient broth agar medium from the sediment of oligotrophic Lake Biwa, where predominance of sulfamethoxazole resistant bacteria was noted. A recent study conducted on oligotrophic bacteria of a pristine cave, Lechuguilla Cave in New Mexico, has revealed that most of the bacteria were resistant to different antibiotics used in their study; even some were multiple-antibiotic-resistant strains resisting up to 14 different antibiotics (Bhullar *et al.*, 2012). Two large plasmids, pREV1 and pREV2 (about 150 and 250 kb, respectively) isolated from an oligotrophic bacterium, *Ancylobacter vacuolatus* , carrying resistance genes for chloramphenicol and trimethoprim in addition to genes coding functions related to oligotrophy have been reported very recently (Zlatkin *et al.*, 2012). Oligotrophic bacteria therefore can be a "potential reservoir of antibiotic resistance genes that can be acquired by pathogens through plasmid transfer".

IX. Recent advances in studies related to oligotrophic bacteria

Several oligotrophic bacteria isolated in recent times were previously unidentified and uncultured. Recent developments in the molecular methods, without the need of cultivation of bacteria enabled the breakthrough in the discovery of specific prokaryotic taxa which were not cultivated or cultured previously (Spring *et al.*, 2000; Handelsman, 2004; Lee *et al.*, 2007; Vaz-Moreira *et al.*, 2011). Epifluorescence microscopy (Porter *et al.*, 1980) and direct viable count (Kogure *et al.*, 1979) had shown that only 0.01 to 0.1% of all the microbial cells from marine environments formed colonies on standard agar plates (Ferguson *et al.*, 1984). Culture independent measurement of microbial diversity which is based on 16S rRNA gene sequencing explained that there is great discrepancy in the data of direct count and plate count methods (Giovannoni *et al.*, 1990; DeLong, 1992; Suzuki *et al.*, 1997). Deininger and Lee (2001) developed an ATP assay for rapid determination of bacteria in potable water. This test was fast and could determine the total bacterial populations in a very short time. The ATP bioluminescence assay allows an estimation of bacterial populations within minutes and it can be applied on-site. The present concern is that many microbial groups are still uncultivated and there is a need to cultivate these uncultivated groups to make possible genome-enabled physiology. This culturing of uncultured bacteria will possibly require new approaches other than standard plating methods (Cho and Giovannoni 2004). Recently a number of novel approaches were applied to cultivate the ones which were previously uncultured microorganisms, for example, high-throughput culturing (HTC) using dilution-to-extinction (Connon *et al.*, 2002), cultivation with a diffusion growth chamber (Kaeberlein *et al.*, 2002), encapsulation of cells in gel microdroplets (Zengler *et al.*, 2002), and modified plating methods (Eilers *et al.*, 2001; Janssen *et al.*, 2002) have enabled to reveal the reality. One significant achievement was the cultivation of members of the SAR11 clade, previously branded as unculturable (Rappé *et al.*, 2002). In addition to SAR11 clade, numerous novel strains belonging to *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia* were also identified and cultivated in the laboratory. One reason for this success is thought to be the use of growth conditions which closely mimic the chemical composition of natural environments (Connon *et al.*, 2002; Kaeberlein *et al.*, 2002; Zengler *et al.*, 2002). Some of the strains obtained by high-throughput culturing have already been taxonomically classified as novel genera in a novel order or family (Cho and Giovannoni 2004).

The definitions of the oligotroph is polemic and profoundly confusing from several decades (Schut *et al.*, 1997a), it is widely accepted that a general characteristic of oligotrophic bacteria is the ability to grow in low-nutrient (0.5 to 15 mg of C/L) media, irrespective of whether they grow in rich nutrient media or not. Cho and Giovannoni (2004) observed that twelve representative isolates in the oligotrophic marine *Gamma-proteobacteria* (OMG) group grew well in carbon-unamended natural seawater medium and addition of mixed carbons (176 mg of C/L^1) significantly reduced specific growth rates. No isolates grew in the medium containing more than 351 mg of dissolved organic C/L. It implies by definition that obligately oligotrophic bacteria cannot grow at substrate concentrations above 0.3 g of C/L (Ishida *et al.*, 1986) or 6 g of C/L (Fry, 1990). The fact that failure of microorganism to grow in nutrient-rich media upon first cultivation from natural habitats (Button *et al.*, 1993; Schut *et al.*,1993; Schut *et al.*, 1997b) supports obligate oligotrophic character of the isolates obtained from high-throughput culture collection in the OMG group (Cho and Giovannoni, 2004). *Sphingopyxis alaskensis* strain $RB2256^T$, isolated from Resurrection Bay, Alaska (Button *et al.*, 1993; Schut *et al.*, 1993), is defined as a model oligotrophic marine ultra-micro-bacterium because of following reasons: (i) ability to grow in very low nutrient media (<1 mg of C/L) but not in 5 mg of dissolved organic C/L; (ii) ultramicrosize of <0.1 μm^3; (iii) relatively low growth rate (μ = <0.2 h^{-1}); (iv) and high-affinity substrate uptake systems (Button *et al.*, 1993; Schut *et al.*, 1993; Eguchi *et al.*, 1996; Schut *et al.*, 1997; Fegatella *et al.*, 1998; Fegatella and Cavicchioli, 2000; Eguchi *et al.*, 2001;

Ostrowski *et al.*, 2001). However prolong storage at 4 to 5 °C and under laboratory cultural conditions, the strains RB2256^T and AFO1 turned to be facultative oligotrophs (Schut *et al.*, 1993; Schut *et al.*, 1997b; Eguchi *et al.*, 2001). Antibodies for PstS (a periplasmic binding protein required for high affinity uptake of phosphate by marine *Synechoccus* and *Prochlorococcus* during phosphate) were used to demonstrate the seasonal phosphate stress in *Synechoccus* and *Prochlorococcus* populations (Scanlan *et al.*, 1997; Fuller *et al.*, 2005). Though number of recent techniques enabled the scientists to cultivate and understand diverse physiology of the oligotrophic bacteria, still large gap remains to understand completely the nature of the oligotrophic bacteria. Hence there is need of innovating new media and cultivation methodologies to cultivate the uncultivated bacterial species; and to understand their physiologies in detail.

X. Aim of this study

The proposed research was undertaken to excavate data based on phenotypic and genotypic analysis of the oligotrophic bacterial population of a city-waste polluted river of northern West Bengal, India, with special emphasis on the genomics of integrons. As integrons are the dynamic platform for acquiring and disseminating gene cassettes in an ecological niche, predicting structure and functions of the putative ORFs borne by the cassettes will throw possible light of adaptive gene-acquisition phenomenon.

XI. Significance of study

The present study of integrons in oligotrophic bacteria is significant in terms of novelty because such study has never been reported elsewhere (as far as the available literature). Profiling of antibiotic resistance and identification of genes harbored in integrons in oligotrophic bacterial genome will help to reveal the actual reservoirs of resistance-gene pool in the environment. The present study (like previous studies) demonstrated that oligotrophic bacteria required very low nutrient for their survival and could be cultured. Once culturable strains are available, the physiology and gene-expression studies are also possible. Since these bacteria can survive in very low nutrient and can sustain for long periods with extremely stressed conditions in the hospital settings and also on surgical instruments, the study implying the mechanisms to survive are very important to devise novel therapeutic measures. The oligotrophic bacteria are good source of industrially useful enzymes and other substances. As this study has revealed several genes captured within integrons, it has expanded the horizon to study novel ORFs besides those that encode antibiotic resistance genes. The Department of Biotechnology, Government of India, has been acknowledged in all occasions of publications that have resulted from this study.

XII. Objectives undertaken in this study

1. To provide detailed descriptive information about the nature of antibiotic resistance in culturable oligotrophic bacteria from the water samples of river Mahananda
2. To study the diversity of the oligotrophic bacteria of Mahananda river
3. To apply molecular systematics in ascertaining taxonomic status of the isolates
4. To explore the incidence of resistance integrons in oligotrophic bacterial population
5. To explore the molecular diversity of the antibiotic resistance gene cassettes

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