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Respiratory enzyme activities in the oxygen-deficient waters of the Arabian Sea

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Denitrification in the oxygen-poor waters at intermediate depths in the Arabian Sea is very intense. Depth profiles of nitrite and the activities of the electron transport system (ETS) and dissimilatory enzymes such as nitrate reductase and nitrite reductase within the nitrite-bearing waters, display depth-mismatched maxima. There are indications of contribution by both dissimilatory nitrate reduction and nitrification to the nitrite enrichment of the secondary nitrite maximum (SNM) between 150 and 350 m. The waters below the SNM, not bearing nitrite but within the oxygen minimum zone, are also potentially denitrifying.

Analyses of the relationships between various parameters indicate the existence of a layered ecosystem in which nitrification and denitrification activities are present in several closely-spaced layers. This is probably caused by the inflow of different water masses into this hypoxic region, as suggested by the strong maxima and minima in enzyme activities at equivalent depths.

A thick layer of low-oxygen water exists between ~ 150 and 1100 m in the Arabian Sea. It is associated in its upper part with large nitrite accumulation and nitrate deficit, denoting intense denitrification. The secondary nitrite maximum (SNM) layer around 150–600 m is characterized by high metabolic activities[1] and maximum concentrations of both suspended particles and particulate protein[2]. The reasons for the acute oxygen depletion are unclear. So also the carbon source sustaining the intense denitrification remains to be demystified. Recent studies on transparent exopolymer particles (TEF)[3,4] provide a clue as to how the carbon demand could be met. The bacterial activities associated with these waters have yet to be fully identified, although respiration measured as the electron transport system (ETS) activity in suboxic waters containing > 0.2 μM NO_2^- has been assumed to be predominantly due to denitrification[1,5,6]. However, nitrifiers are also known to be intimately involved in the nitrogen metabolism of such regimes[7]. And, the numerical model of Anderson *et al.*[8] incorporating oxidation of ammonium and nitrite by nitrifiers at the boundaries of the oxygen-deficient waters has been largely substantiated by the work of Lipschultz *et al.*[9] in the eastern tropical south Pacific. Similar analyses are lacking for the Arabian Sea.

The carbon and nitrogen cycles operating in the oxygen minimum zone (OMZ) of the Arabian Sea have global implication, since these waters serve as strong sinks for fixed nitrogen on a global scale[10] and a significant source of N_2O (which is produced by both nitrification and denitrification) to the atmosphere[11,12].

The objective of this study was to look at the fine details of distribution of metabolic activities within the OMZ of the Arabian Sea, specifically around the SNM, in order to produce some significant ecological insights

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into the nature of microbial processes operating therein. This has been done by relating overall microbial respiration (ETS activity) to dissimilatory respiration/ denitrification, viz. activities of nitrate reductase (NR) and nitrite reductase (NiR).

The study was conducted aboard FORV *Sagar Sampada* (cruise 150) during 20 November–3 December 1996, at five stations (Figure 1). A SeaBird Electronics conductivity–temperature–depth (CTD) profiler (SBE-9) equipped with an oxygen probe (SBE-13-02), a Sea-Tech 25-cm beam transmissometer (129D) on a General Oceanics bottle rosette with 10 l Go-Flo bottles, was employed. Dissolved oxygen values as read from the oxygen probe, were corrected with reference to the titrimetric Winkler concentrations determined soon after sampling. Apparent oxygen utilization (AOU) was calculated as the difference between the oxygen solubility obtained from Benson and Krause[13] and the observed oxygen concentrations. A Skalar analyser was used to analyse nutrients by standard methods[14].

Samples for ETS, NR and NiR activity measurements were collected from the same 10 l sampler from depths defined by the subsurface particle maximum taken as the reference. Samples for ETS (5 l) and NR plus NiR (5 l) were vacuum-filtered (< 0.5 atm) through Whatman GF/F glass-fibre filters[15] in a cold room (8–12°C) within 2.5 h of collection. ETS activity was measured by the tetrazolium reduction technique[16] and converted into respiration rates following Naqvi and Shailaja[1]. NR activity was determined by the method of Packard *et al.*[15]. The crude homogenate, prepared immediately after filtration by grinding the filter at 0–4°C for 2 min in 3 ml of 0.2 M phosphate buffer (pH 7.9) containing 9 mg polyvinyl pyrrolidone and 0.5 mg dithiothreitol, was used for both NR and NiR assays. Incubations of the crude extract with substrate for NR assay were not done anaerobically, since the reduction in enzyme activity after half an hour of exposure to air has been found to be < 20% (ref. 15). The reaction mixture for NiR assay was as described by Eppley and Rogers[17], except that NADH was substituted for methyl viologen. Briefly, the assay was as follows: 1 ml of enzyme extract was reacted with substrate mixture consisting of 1.7 ml of phosphate buffer (0.2 M, pH 7.9), 0.1 ml of NADH (2 mM), 0.1 ml of $NaNO_2$ (9 mM in glass-distilled water) and 0.1 ml of freshly prepared sodium dithionite-bicarbonate solution (60 mg each of sodium dithionite and sodium bicarbonate in 2.5 ml glass-distilled water). Incubations were carried out for 30 min at 24°C in an atmosphere of helium, as there was no information available on the effect of oxygen on the activity of the enzyme. The reaction was stopped with 0.1 ml zinc acetate and 1.9 ml of 95% ethanol. The mixture was clarified by centrification and the supernatant taken for NO_2^- analysis. Inhibition studies were conducted in the presence of 50 and 100 μM NO_3^- added to the substrate buffer.

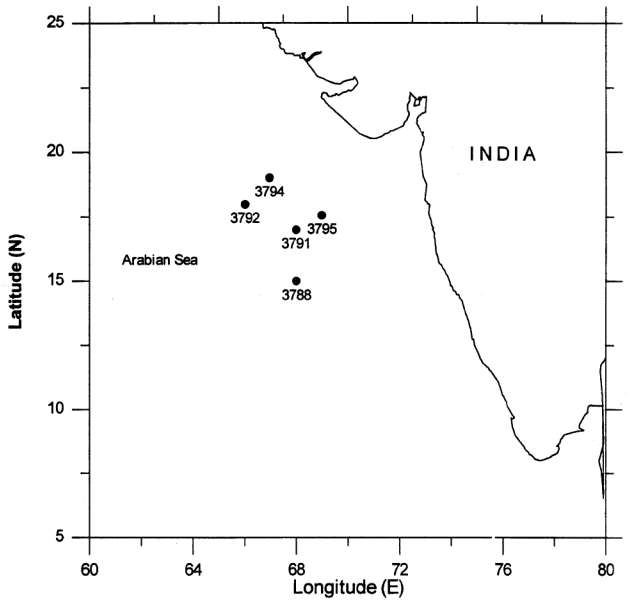

Figure 1. Station positions.

Concentrations of NO_2^-, NO_3^-, O_2, AOU and suspended particles represented as beam attenuation coefficient (BAT) measured at different depths at 5 stations (Figure 1), together with activities of ETS, NR and NiR at corresponding depths are presented in Table 1. The Winkler technique for oxygen measurements is not very accurate at low O_2 concentrations. A comparison of O_2 values obtained by this method and those measured colorimetrically (S.W.A. Naqvi, unpublished) at location 19°N, 67°E showed the former to be 3.5 to 7.8 times higher. Due to practical constraints, replicate analyses of enzyme activities were not done routinely. However, values obtained in duplicate analyses of the activities at one station (19°N, 67°E) at 250 m depth were found to vary by 0.98% (ETS), 4.1% (NR) and 2.1% (NiR).

The various enzyme activity maxima (Table 1) were often vertically separated. At four of the five stations studied, NO_2^- and particle maxima occurred at the same depth. Interestingly, NR and NiR activities were observed even at depths below the nitrite-bearing waters within the oxygen minimum layer.

Nitrite accumulation in the oceanic OMZs has been interpreted as the consequence of partial denitrification and undersaturation of N_2O in similar regimes as implying complete denitrification[18]. The presence of both the signals at overlapping depths, as seen in the eastern tropical south Pacific (ETSP)[19] as well as in the Arabian Sea[12] would, therefore, signify an imbalance in the reduction sequence. Ward[20] attributes such a situation to the presence of an excess of bacteria that are capable of reducing nitrate to nitrite and not further and/or, to reac-

Table 1. Depth-wise distribution of NO_2^-, NO_3^-, O_2, AOU, BAT and activities of the ETS, NR and NiR at different stations

Station No. (location)	Depth (m)	ETS (μeq l^{-1} h^{-1})	NR (nmol NO_2^- l^{-1} h^{-1})	NiR (μmol NO_2^- l^{-1} h^{-1})	NO_2^- (μmol l^{-1})	NO_3^- (μmol l^{-1})	O_2 (μmol l^{-1})	AOU (μmol l^{-1})	BAT (m^{-1})
3788	80	0.0611	12.70	0.1153	0.10	21.3	24.4299	197.5289	0.353188
(15°N, 68°E)	130	0.0788	0.68	0.0833	3.09	16.2	4.2462	236.0209	0.383470
	169	0.0174	8.19	0.2194	4.13	16.4	3.3098	246.7839	0.392286
	210	0.0594	23.00	0.0394	3.40	18.1	2.4775	252.9737	0.370722
	300	0.0523	1.36	0	0	30.3	2.9457	263.2276	0.357576
	400	0.0660	8.60	0.0613	0	32.3	2.3735	269.6049	0.348836
3791	100	0.1452	1.87	0.2682	0.21	22.4	18.8117	207.6131	0.348836
(17°N, 68°E)	170	0.1146	2.76	0.2366	3.20	15.7	4.7664	239.9689	0.387876
	220	0.0673	2.76	0.4288	3.48	15.6	2.4775	247.1680	0.375113
	350	0.1093	2.41	0.2570	2.63	18.5	5.5987	260.1237	0.353203
	500	0.0485	4.24	0.1953	0	29.5	1.8533	270.1230	0.353203
3792	100	0.1564	1.80	0.1282	0.08	19.6	12.8814	217.5632	0.344437
(18°N, 66°E)	150	0.0993	4.61	0.2751	3.87	18.2	2.9457	232.4125	0.357576
	200	0.1685	10.1	0.0670	4.07	16.9	2.7896	241.9428	0.392286
	350	0.1166	0	0.1674	3.63	18.5	2.0613	257.8572	0.375113
	600	0.0443	2.68	0	0	29.7	1.6972	270.2793	0.357576
3794	100	0.0594	8.3	0.0767	0.09	17.7	76.3977	145.1121	0.383496
(19°N, 67°E)	150	0.0653	15.5	0.3733	0.69	20.3	4.8184	235.4493	0.396702
	250	0.0925	11.9	0.0825	3.30	16.7	2.3214	252.6834	0.413968
	350	0.0951	14.9	0.0206	2.63	20.8	2.0093	263.7148	0.396702
	500	0.1299	3.9	0.2474	0.03	27.9	1.9573	270.0203	0.387876
3795	66	0.0285	0	0.4489	0.14	10.8	96.9975	110.2241	0.427345
(17.56°N,	112	0.0486	1.9	0.6275	1.16	12.8	4.6103	226.2807	0.401122
69°E)	155	0.2938	0.5	0.5165	3.08	18.2	2.7896	247.3020	0.436214
	300	0.0313	0	0.3498	3.03	20.0	2.0613	258.3061	0.413968
	500	0.2080	1.2	0.6593	0	28.1	1.8533	270.1230	0.405547

tions subsequent to nitrite formation being limiting to the overall rate of formation of molecular nitrogen. But these deductions do not seem to hold for the Arabian Sea, as observed in the present study. Firstly, within the nitrite-bearing waters, NR activity which could be deemed as a labile chemical/biological index of the process of active nitrate reduction[15], was found to be both poorly and negatively correlated with NO_2^- concentrations. Besides, relatively high NO_2^- levels were observed in the presence of low NR activity at 3 out of 5 stations, similar to the results of Packard *et al.*[15] in the ETSP. These observations strongly suggest the presence of another source of NO_2^- to the SNM, apart from nitrate reduction. The additional NO_2^- could have come from ammonium oxidation (nitrification), as discussed in a later section, and masked the relationship between NO_2^- and NR activity. However, there are no data on the nitrifying activity in the Arabian Sea to confirm it. On the other hand, Lipschultz *et al.*[9] do not consider nitrification to be a significant source of NO_2^-, as they failed to detect significant $^{15}NO_2^-$ formation from $^{15}NH_4^+$ in the ETSP. It is suspected that because of the oxygen limitation in the ETSP, nitrification is not a likely source of much NO_2^- (B. B. Ward, pers. commun.). It need not be so in the Arabian Sea, where several modes of oxygen replenishment of the oxygen-deficient zone exist[21,22].

Secondly, NiR activity was not limiting within the SNM at any station; on the contrary, it was generally much higher than NR activity at most sampled depths. The mean activities of the two enzymes were: NR 7.22 ± 6.67 nmol NO_2^- l^{-1} h^{-1} and NiR 250.8 ± 170.6 nmol NO_2^- reduced l^{-1} h^{-1}. As noted from culture experiments[23], the activity of NiR and all subsequent reductases in the denitrifying sequence remains suppressed until concentrations of NO_3^- become very low. Evidently, this does not happen in the Arabian Sea, since the ambient nitrate concentrations are quite high in these waters. Besides, inhibition experiments carried out concurrently demonstrated that the extent of *in vitro* inhibition of NiR activity by nitrate is ca 80%. It is likely, therefore, that rather than the concentration of NO_3^- alone, the relative proportions of both NO_2^- and NO_3^- are important in triggering the denitrification reaction sequence. The excess NO_2^- contributed by another source (e.g. ammonium oxidation) would help desuppress the denitrifying enzymes, by raising the NO_2^- to NO_3^- ratio.

Observed chemical concentrations were not highly correlated with the measured enzyme activities such as ETS, NR and NiR, probably because of the presence of several simultaneously-active bacterial processes. In general, denitrification was found to occur throughout the SNM, as shown by the negative relationship between NiR and NO_2^- ($P < 0.1$) in the combined data set from all stations and depths with NO_2^- > 0.2 μmol l^{-1}. ETS correlated poorly with both NR and NiR activities, while NO_2^- levels were associated positively with ETS activity but not with NR activity, indicating the probable presence of other bacterial processes, besides nitrate reduction, forming NO_2^-.

The occurrence of depth-mismatched maxima and minima in ETS, NR and NiR activities (Table 1) is a probable indication of different bacterial activities in discrete layers within the nitrite-rich waters. Although the enzyme activities as measured are potential activities, in the case of NR and NiR the potential activities could very well be *in situ* activities, since both are inducible enzymes. The respiration rate of an organism being a rough reflection of its growth efficiency, an examination of the interrelationships among the enzyme activities, NO_2^- and NO_3^- concentrations at some distinct depths (corresponding to various maxima) was able to yield information on the different bacterial processes predominating at those depths. Significant correlations existing between different parameters that show a strong probability of a causal relationship are discussed below.

Maximum ETS activity occurred at or just below the NO_2^- peak and it was not uncommon to find a second activity peak in the lower part of the SNM, as seen at stations 3788 and 3791 (Table 1). At the depths corresponding to the ETS activity maxima, the major bacterial activity was significantly related ($P < 0.05$) to denitrification. In the core of the SNM, which was generally the depth of the particle maximum as well, ETS activity was not related to either NR or NiR activity but correlated well with NO_3^- concentration ($P = 0.05$), signifying nitrite oxidation as the major bacterial activity.

NR activity was detected almost throughout the SNM. It usually peaked close to the NO_2^- maximum and showed an increasing trend again, further down, extending below the nitrite-bearing layer. At all stations, around 250–350 m a minimum in NR activity, going down to zero at stations 3792 and 3794, was seen in waters with AOU corresponding to 259.8786 ± 1.797. The observed density (~ 26.8) corresponded to σ_t of the Persian Gulf water. Interestingly, nitrite was present at these depths, although NR activity was close to zero. There was no correlation between the ETS and NR activities at any depth, including the depth where the reductase activity was maximum, which meant that nitrate reduction was not the major bacterial activity within the SNM. Also, at the depth corresponding to the NR peak, NR activity did not correlate with NO_2^- but AOU did ($P < 0.1$). A positive but not highly significant relationship was also noted between ETS activity and NO_2^- concentration. Thus, it could be inferred that in the upper part of the SNM, NO_2^- was not formed by nitrate reduction alone. Nitrification might also produce NO_2^- there, as seen from the AOU and NO_2^- correlation which, though significant, is subject to the imprecision in the Winkler oxygen values at low concentrations. In contrast to this, in the lower part of the SNM associated with decreasing oxygen concentrations, NR activity showed a positive correlation ($P < 0.1$) with NO_2^- concentrations, thus being a major contributor of NO_2^-. And, with ETS and NiR activities correlating well ($P < 0.05$), denitrification could be said to be the predominant bacterial activity at these depths.

A maximum in NiR activity usually occurred in waters with higher dissolved oxygen concentrations compared to NR (station 3971 at 100 m and station 3792 at 150 m, Table 1), which is understandable, since NiR can tolerate higher oxygen levels than NR[24,25]. Possibly for the same reason, NiR activity persisted although NR activity tapered to vanishingly low levels at ca 300 m, affected by the intrusion of the Persian Gulf waters with a higher oxygen content. Yet, at the depth where NiR activity reached its peak, the prevalence of a highly dynamic biological situation was apparent from the poor correlations seen between ETS activity and all other variables.

Immediately below the SNM, but within the oxygen minimum layer, ETS, NR as well as NiR activities showed an increasing trend with depth, even in waters with no measurable NO_2^-. Also, ETS activity was highly correlated with NiR activity ($P < 0.01$) at those depths.

Clearly, there was no accumulation of NO_2^- because of its complete consumption during denitrification. Although the sampling protocol did not extend deep enough into the oxyen minimum region to reveal further maxima in enzyme activities, it is interesting to note that isotopic measurements of N_2O have indicated its production through denitrification even below the SNM[26].

From the data discussed above, a layered ecosystem could be visualized for the OMZ of the Arabian Sea. In the upper part of the SNM, active nitrite formation by both ammonium oxidizers and dissimilatory nitrate reducers (and/or nitrate respirers) is indicated. Within this layer nitrate- and nitrite-reducing (denitrifying) activities maximize at closely-located depths, possibly depending on their oxygen sensitivities. This is followed, depth-wise, by a predominance of nitrite oxidizers, and, to a small extent, nitrite reducers occurring in the core of the SNM (which often houses the particle maximum as well), and denitrifiers in the layer below, which incorporates a steep gradient in nitrite concentration. Embedded in this layer is a minimum (often going down to

zero) in NR activity. Below the SNM, in waters with low oxygen and no obvious accumulation of nitrite, > 85% of the variation in bacterial activity is attributable to the denitrifiers. It is emphasized, though, that none of the layers described above was absolutely 'pure' in the sense that all of them consisted of more than one kind of bacterial activity. A closer vertifical sampling might have yielded better samples in this respect. Still, it is amply evident that the denitrifying activity is concentrated in more than one layer within and below the secondary nitrite maximum and is interleaved with a layer predominated by another bacterial activity, presumably nitrification. The significance of a nitrification–denitrification coupling is that it could lead to greater amounts of fixed nitrogen loss than is reflected in the net nitrate deficit[20]. However, the implications of a layered ecosystem for denitrification and carbon flow in the Arabian Sea are not easily discerned, given the paucity of data on nitrification.

Despite the small size of the data set, it is clearly evident from Figure 2, depicting the density characteristics of the waters bearing maximum NR and NiR activities, that the different water masses forming the intermediate layers of the northern Arabian Sea notably, the Persian Gulf water and the Red Sea water, play a role in the concentration of bacteria at certain (optimum) depths. For instance, nitrite reduction was maximum in waters having σ_t values between 25.0 and 25.8 corresponding to the depth range 80–150 m, while nitrate reduction predominated between σ_t values 26.2 and 26.5 corresponding to the depth range 170–220 m. These waters form the mixing zone of the Arabian Sea high salinity water and the Persian Gulf water[27]. Further, the minimum in NR activity was associated with the core of the Persian Gulf water (σ_t 26.6–26.8) in the depth range of 250–350 m. Below the SNM, increasing activities of both NR and NiR were recorded in waters at 500–600 m depth, bearing no nitrite and whose density corresponded to Red Sea water (σ_t 27.0–27.2).

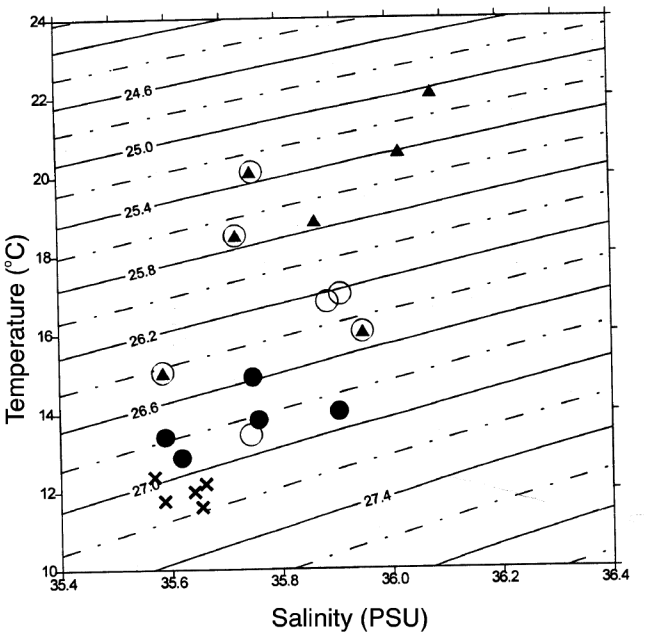

Figure 2. Association of NR maximum (○) and minimum (●), NiR maximum (▲) and increasing activities of both NR and NiR (×) with different density levels in the Arabian Sea.

From the close association obtaining between ammonium oxidizers and nitrate reducers, it is surmised that the former aid the activity of the enzyme NR of the latter, by lowering the oxygen levels. Also, in addition to the transparent exopolymer particles (of phytoplankton origin)[3,4], it would be attractive to ponder upon chemolithotrophy of nitrifiers as a (part) source of the carbon supply that sustains the observed intense denitrification[28,29]. Incidentally, exopolymeric substances are also known to be elaborated by ammonia-oxidizing, *Nitrosomonas*-type organisms[30]. A similar benefit is likely to be derived by the nitrite-reducing organisms from the nitrite-oxidizing ones, because there were indications that these two activities also co-occur at certain depths.

Other than oxygen, the NR (but not NiR) activity is sensitive also to the availability of organic carbon[8]. Confirming this, ETS and NR activities revealed excellent correlation during the SW monsoon (Shailaja, unpublished), a period when the organic carbon flux to the deep sea is the highest[31]. In other words, an increase in the availability of organic carbon can enhance dissimilatory NR activity. This implies seasonal variations in the layered structure of the denitrifying enzyme activity.

The interrelationships between the activities of the respiratory ETS and dissimilatory enzymes such as NR and NiR within the denitrifying waters of the Arabian Sea indicate the operation of a layered ecosystem having the following features: (i) nitrification and denitrification activities are present in several closely-spaced layers within the SNM layer, perhaps as a result of the intrusion of oxygenated water masses, viz. subantarctic mode water, Persian Gulf water and Red Sea water at various depths within the oxygen-poor zone; (ii) both dissimilatory nitrate reduction and ammonia oxidation (nitrification) are likely to contribute to the nitrite enrichment of the SNM; and (iii) waters below the SNM not bearing nitrite, but within the oxygen minimum layer, are also potentially denitrifying.

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Differential expression of a unique protein by intracellular *Mycobacterium tuberculosis* complex

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We have investigated the changes in the protein synthesis pattern in guinea pig peritoneal macrophages following infection with virulent *Mycobacterium tuberculosis* H37Rv *in vitro*. By ^{35}S methionine labelling of the newly synthesized proteins followed by ultracentrifugation, SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and autoradiography, the protein synthesis pattern of the control uninfected macrophages and the infected macrophages *in vitro* were compared. By adding cycloheximide to the macrophage cultures, the protein synthesis of macrophages was inhibited and the protein synthesis pattern of *M. tuberculosis* has been analysed. We have identified a mycobacterial protein of molecular weight 17 kDa which was expressed exclusively in the cytosolic fraction of *M. tuberculosis*-infected guinea pig macrophages *in vitro*.

TUBERCULOSIS is a major cause of morbidity and mortality worldwide. There is an enormous increase in the incidence of tuberculosis due to HIV epidemic and rise in multi-drug resistance. *Mycobacterium tuberculosis* is one of the successful intracellular pathogens infecting over one-third of the world's population and causing 8 million new cases of active tuberculosis annually[1]. The resurgence of tuberculosis has highlighted the need for new strategies to combat *M. tuberculosis*. To develop such strategies, one needs to learn more about the pathogenesis of *M. tuberculosis* infection.

M. tuberculosis is an intracellular pathogen. The macrophage fails to eliminate this pathogen in spite of the powerful array of antimicrobial defences it puts forth, along with other components of the immune system. This shows that *M. tuberculosis* adopts itself to the intracellular environment by mounting a response appropriate to ensure its survival. The resistance by *M. tuberculosis* would involve a number of genes. The protein products of these genes could not only be potential virulent determinants but also important in cell-mediated and protective immune response to *M. tuberculosis*, because they are processed and presented by infected macrophages. So it becomes imperative to identify and characterize *M. tuberculosis* proteins which would help us to understand the pathogenesis of tuberculosis and in turn to develop novel drug targets and attenuated vaccines.

To understand these mechanisms, the bacterial gene products that are specifically required at each stage of the infection process have to be identified. It has been demonstrated by Buchmeir and Heffron[2] that virulent Salmonella strains upon phagocytosis by a macrophage cell line express at least 30 different proteins. Monahan

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