

Research article

## Monoclonal antibodies against the iron regulated outer membrane Proteins of *Acinetobacter baumannii* are bactericidal

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### Abstract

**Background:** Iron is an important nutrient required by all forms of life. In the case of human hosts, the free iron availability is  $10^{-18}$  M, which is far less than what is needed for the survival of the invading bacterial pathogen. To survive in such conditions, bacteria express new proteins in their outer membrane and also secrete iron chelators called siderophores.

**Results/ Discussion:** *Acinetobacter baumannii* ATCC 19606, a nosocomial pathogen which grows under iron restricted conditions, expresses four new outer membrane proteins, with molecular weight ranging from 77 kDa to 88 kDa, that are called Iron Regulated Outer Membrane Proteins (IROMPs). We studied the functional and immunological properties of IROMPs expressed by *A. baumannii* ATCC 19606. The bands corresponding to IROMPs were eluted from SDS-PAGE and were used to immunize BALB/c mice for the production of monoclonal antibodies. Hybridomas secreting specific antibodies against these IROMPs were selected after screening by ELISA and their reactivity was confirmed by Western Blot. The antibodies then generated belonged to IgM isotype and showed bactericidal and opsonising activities against *A. baumannii* *in vitro*. These antibodies also blocked siderophore mediated iron uptake via IROMPs in bacteria.

**Conclusion:** This proves that iron uptake via IROMPs, which is mediated through siderophores, may have an important role in the survival of *A. baumannii* inside the host, and helps establishing the infection.

### Introduction

Iron is one of the essential nutrients required by the bacteria to be able to multiply and invade a vertebrate host. However as a defense mechanism, availability of iron for the bacteria is limited in the host as most of it complexed to ferritin, transferrin, hemoglobin, heme and hemosiderin [1–4]. For pathogenic bacteria to be able to establish an infection, it must compete with and obtain iron from the host's iron binding protein [5]. The bacteria

have evolved a number of diverse mechanisms utilize the host iron. One of the methods of achieving this, is by secretion of low molecular weight high affinity iron chelators termed siderophores and their specific cell surface receptor, Iron Regulated Outer Membrane Proteins (IROMPs). These receptors are expressed under iron restricted conditions [6–9]. The role of IROMPs in iron uptake have been reported for other bacteria like *Escherichia coli*, *Pseudomonas aeruginosa* and *Neisse-*

*ria gonorrhoeae*. Antibodies directed against these proteins associated with iron uptake exert a bacteriostatic or bactericidal effect, by blocking siderophore mediated iron uptake pathways [10–14].

*A. baumannii* is widely recognized as an emerging nosocomial pathogen and is of particular concern due to the spread of multi-drug resistant strain [15,16]. *A. baumannii* has also been shown to produce siderophores and expresses IROMPs under iron restricted conditions [17,18]. Smith and Alpar had shown the specific antibody response to IROMPs in patients suffering from *A. calcoaceticus* septicemia in the convalescent sera [19].

We carried out the present study to characterize the IROMPs *A. baumannii* ATCC 19606 by producing monoclonal antibodies against them. Western blot analysis was performed to study their specificity. These monoclonal antibodies were tested for their *in-vitro* bactericidal activity and opsonizing activity. Apart from this their role in blocking the siderophore mediated iron uptake system was also studied.

## Results

### Expression of IROMPs and effect of Iron on growth

OMP profiles of *A. baumannii* grown in CDM-Fe and CDM+Fe were compared on SDS-PAGE and it was seen that in absence of iron, bacteria developed 4 new outer membrane proteins which were absent in iron replete conditions. These new proteins are in the range of 77 kDa to 88 kDa and were the IROMPs (Fig. 1).

### Monoclonal antibody production

Five best reactive monoclonal antibodies were selected out of 88 clones after 2 fusions based upon their reactivity to IROMPs by ELISA. These monoclonal antibodies were named as 3D<sub>5</sub>ABIR, 1D<sub>11</sub>ABIR, 2G<sub>9</sub>ABIR, 1F<sub>7</sub>ABIR, and 5D<sub>6</sub>ABIR. All of the five monoclonal antibodies were of IgM isotypes (Table 1). These monoclonal antibodies

were tested in Western blots. Fig. 2 shows the results of western immunoblots of OMPs. All the monoclonal antibodies reacted with one or more OMP bands corresponding to the range of IROMPs but none reacted with the OMPs of bacteria grown in CDM+Fe media. Two antibodies (5D<sub>6</sub>ABIR and 1F<sub>7</sub>ABIR) did not show any reactivity on Western blot this may be due to conformational changes of epitope of proteins. All of the antibodies were tested against *E.coli* and *Pseudomonas aeruginosa* and none of them were cross reactive with IROMPs of these two bacteria on ELISA.

### Characterization of monoclonal antibodies

#### Bactericidal activity

All these monoclonal antibodies were tested for their bactericidal activity against *A. baumannii* ATCC 19606 *in-vitro*. It was found that all the five monoclonal antibodies are bactericidal and they specifically kill the bacteria grown in CDM-Fe media. The % of reduction or killing was 80–90%, and various controls gave 10–20% reduction in bacterial colony (Table 2).

#### Opsonising activity

It was found that human PMNs opsonize bacteria more readily when bacteria is preincubated with monoclonal antibodies as is evident by counting the bacteria within the PMNs (Table 2) as compared to controls. The opsonization activity of PMNs was increased 6–8 folds by prior incubation with monoclonal antibodies.

#### Uptake of Iron through siderophore

The siderophore was secreted by *Acinetobacter baumannii* ATCC 19606 was catecholate type as determined by Arnow's method. The uptake of radiolabeled iron siderophore complex by the bacterial whole cells in CDM-Fe was significantly more than that in case of bacterial cells obtained from CDM+Fe medium (Fig. 3). The control tubes incubated only with <sup>55</sup>Fe without siderophore showed negligible iron uptake. Total radioac-

**Table 1: Characterisation of monoclonal antibodies. The antibodies were tested by ELISA using OMPs from both CDM-Fe and CDM+Fe grown bacteria. The antibody which shows 1.0 or more than 1.0 OD value with CDM-Fe OMPs were selected as an antibody against IROMPs of *A. baumannii* and used for subsequent experiments.**

Monoclonal Antibodies	ELISA		Ig Isotype	Titre in ELISA
	Fe-	Fe+		
2G <sub>9</sub> ABIR	2.762	.141	IgM	1:100
3D <sub>5</sub> ABIR	2.722	.135	IgM	1:3200
5D <sub>6</sub> ABIR	3.093	.562	IgM	1:100
1D <sub>11</sub> ABIR	2.925	.615	IgM	1:8000
1F <sub>7</sub> ABIR	2.975	.412	IgM	1:800
Preimmune sera	.452	.458	-	1:200

**Table 2: Bactericidal and Opsonophagocytic Activity of monoclonal antibodies raised against IROMPs of *A. baumannii*. The antibodies were tested for both type of bacteria i.e. bacteria grown in CDM-Fe and CDM+Fe medium. Results are the mean of three independent experiments in both the cases.  $\pm$  represents the standard deviation.**

Antibodies/ Various Controls	Bactericidal Activity in CDM-Fe medium(%)	Bactericidal Activity in CDM+Fe medium(%)	No. of Bacteria grown in CDM-Fe medium per PMN	No. of Bacteria grown in CDM+Fe medium per PMN
2G <sub>9</sub> ABIR	98.0% $\pm$ 8.0%	15.0% $\pm$ 4%	9.0 $\pm$ 2.0	2.0 $\pm$ 1.0
3D <sub>5</sub> ABIR	97.0% $\pm$ 12.0%	22.0% $\pm$ 9%	11.0 $\pm$ 3.5	2.0 $\pm$ 0.5
5D <sub>6</sub> ABIR	90.0% $\pm$ 11.0%	9.00% $\pm$ 4%	10.5 $\pm$ 2.5	1.0 $\pm$ 0.5
1D <sub>11</sub> ABIR	97.0% $\pm$ 17%	19.00% $\pm$ 6%	9.0 $\pm$ 1.5	3.0 $\pm$ 1.0
1F <sub>7</sub> ABIR+	93.0% $\pm$ 5%	26.0% $\pm$ 12%	10.0 $\pm$ 1.5	3.0 $\pm$ 1.8
Preimmune Sera	12.0% $\pm$ 2%	16.0% $\pm$ 6%	1.5 $\pm$ 0.5	2.5 $\pm$ 1.0
Complement only	12.0% $\pm$ 5%	8.50% $\pm$ 1%	-	-
Tissue culture supernatant only	24.0% $\pm$ 7%	21.0% $\pm$ 4%	2.0 $\pm$ 0.5	1.5 $\pm$ 0.2
Polyclonal Sera	68.0% $\pm$ 13%	39.0% $\pm$ 7%	7.0 $\pm$ 1.0	4.5 $\pm$ 1.0
Only PBS	1.0% $\pm$ 1%	2.0% $\pm$ 1.5%	1 $\pm$ 0.2	1 $\pm$ 0.5

tivity bound to the filter in case of iron deficient bacteria with radioactive siderophore complex was about 85% out of the 100% radioactivity taken of the neat <sup>55</sup>Fe. In control as well as with iron rich bacteria it was not more than 15–25%. This suggests that the iron uptake is mediated through siderophore. The data was analyzed by nonparametric Willcoxon Rank Sum Test for two groups. It was found that iron uptake by iron deficient cells was significantly more as compared to iron rich cells (p value < 0.05). Similarly there was a significant difference in CPM reading of iron deficient cells for uptake through siderophore (p value < 0.05) as compared to the control cells in which no siderophore was added.

#### Binding of <sup>59</sup>Fe-Siderophore complex to OMP

The outer membrane proteins prepared from cells grown in the absence of iron bound significantly higher amount of radioactive iron siderophore complex as compared to that of the OMP prepared from cells grown in iron rich conditions (p value < 0.05). Similarly there was no binding of radiolabeled <sup>59</sup>Fe alone to the filters in absence of siderophore which was put up as control. This strongly indicates that OMP of CDM-Fe grown bacteria contains the IROMPs and that they bind to the iron siderophore complex, hence they may be the receptor for siderophore iron complex. (Fig. 4). That binding receptor, a protein was proved by preincubating the OMPs with proteinase K that abolished all the binding activity of the iron siderophore complex (Fig. 4).

#### Role of monoclonal antibody in Iron pathway

The iron uptake by *A. baumannii* ATCC 19606 when grown in CDM-Fe was inhibited more than 90% by monoclonal antibodies directed against IROMPs of *A. bau-*

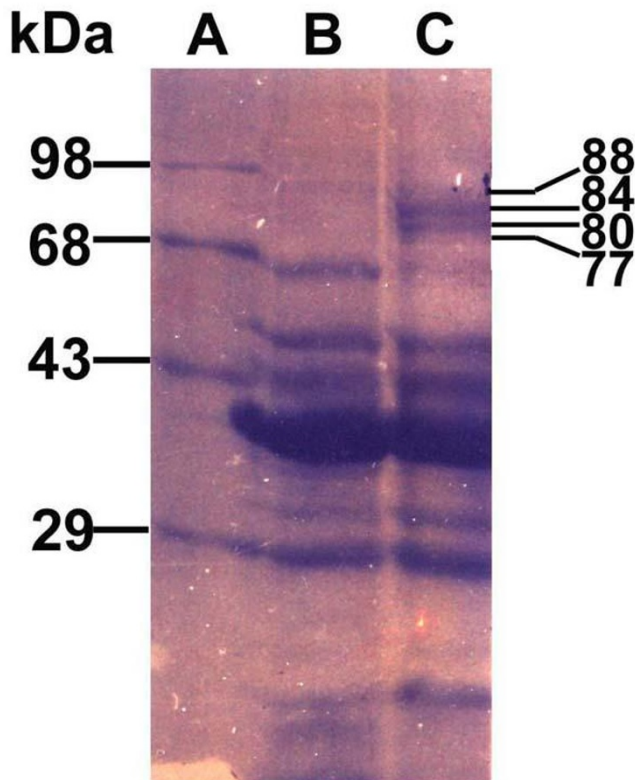
*mannii*, as compared to bacteria grown in CDM+Fe (Fig. 5, p value < 0.05). Similarly, monoclonal antibodies resulted in almost complete inhibition of binding of <sup>55</sup>Fe siderophore complex to its receptor IROMPs, which are the component of OMPs (p value < 0.05). But there was no effect on binding and uptake of iron siderophore complex in absence of monoclonal antibodies or with control sera (Fig. 5).

#### Discussion

Iron is an important nutritional requirement of living cells. Iron is not readily available to the microbes, which invades the human hosts [20] as a part of host defense mechanism against bacterial infections. In iron starved conditions, bacterial growth rate is reduced and morphological changes such as filament formation takes place which suggests an inhibition of DNA synthesis or cell division [21,22]. Besides these the concentration of the proteins whose synthesis is regulated by iron are also observed to get changed [23].

To acquire the iron essential for their growth and metabolism, the microbes have evolved the ability to compete with host iron binding factors. This is achieved by producing powerful iron chelators known as siderophores [24–27]. Siderophores are secreted in the external milieu where they compete with host iron binding proteins to capture iron by forming iron-siderophore complex. This gets internalized through the specific outer membrane protein receptors, termed as Iron Regulated Outer Membrane Proteins (IROMPs).

We found that when *A. baumannii* was grown in iron deficient medium, IROMPs were expressed on their outer



**Figure 1**  
SDS-PAGE profile of Outer Membrane protein of *Acinetobacter baumannii* ATCC 19606. Lane **A** is OMP from bacteria grown in presence of iron and Lane **B** is OMP from bacteria grown in absence of iron. Molecular weights are shown in the left most lane.

membranes with molecular weight of 88, 84, 80 and 77 kDa. We also found that *A. baumannii* secreted catechol type of siderophore in the external milieu. Daniel et al. [28] also reported the production of IROMPs and siderophore during different phase of growth cycle in iron starved medium by *A. baumannii*. The molecular weight of these proteins described by them were in between 70–80 kDa of these 75 and 80 kDa are expressed more strongly in iron repressed bacteria. They have also cloned and characterized the *fur* regulator gene of the IROMPs.

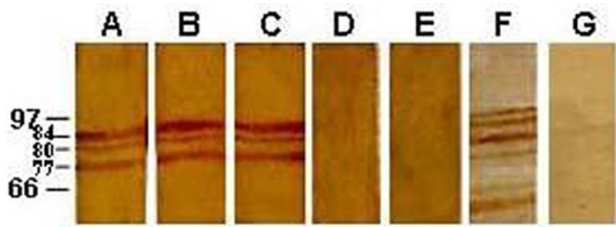
Apart from these, some other high molecular weight IROMPs in *Acinetobacter* have also been identified by Echenique et al. on SDS-PAGE [29]. Smith and Alpar also demonstrated the reactivity by immunoblotting against the IROMPs using the sera of convalescent patients recovering from *Acinetobacter* infection sera by Smith and Alpar [18].

In order to further characterize the biological properties of IROMPs and role of the antibodies produced against them, we raised monoclonal antibodies to the IROMPs by hybridoma technology. The five best reactive and specific clones, which were selected after subcloning, belonged to IgM class. On western blot however only 3 clones reacted with the bands corresponding to IROMPs. The reason for none of the clones reacting, could be due to the fact that monoclonal antibodies recognize only an epitope whose tertiary structure might be changed during denaturation conditions of SDS-PAGE.

The anti IROMPs antibodies showed significant *in-vitro* bactericidal activity against *A. baumannii* grown in absence of iron. Also being IgM subtype, these were efficient in complement mediated lysis of bacteria. They also enhanced the phagocytosis of *A. baumannii* 6–8 folds more as compared to preimmune sera of mice in invitro experiments. These results showed that the IROMPs are surfaced exposed immunodominant antigens, which play significant role in virulence. Earlier also the antibodies against 70 kDa IROMPs of *Neisseria meningitidis* have been shown to be bactericidal [9]. These antibodies may have a role as protective antibodies in the course of infection inside a human host. The antisera against Tbp1 and Tbp2 killed the homologous as well as majority of heterologous strains examined, with varying efficiency [30]. Sokol and Woods [31] have pointed out that the antibodies against pyoverdinin and pyochelin receptor proteins in *Pseudomonas aeruginosa*, enhances the opsonization process by polymorphonucleocytes. They have also shown that these antibodies were protective in a burn mouse model system.

We observed that these antibodies significantly reduced the uptake of iron by *A. baumannii* cells. Meyer et al. [11] and Pintor et al. [13,14] have shown the specific block of iron uptake via siderophore and transferrin using polyclonal and monoclonal antibodies against IROMPs of *Pseudomonas sp.* and *Neisseria sp.* respectively. We also observed that the antibodies raised against the IROMPs of *A. baumannii* also inhibited the binding of iron siderophore complex to purified OMPs. The blocking in the uptake of iron resulted ultimately in the growth inhibition of the bacterium.

This mechanism of iron uptake may constitute an important virulence factor, which can help in establishing infection in the host [26]. The study of this virulence system may help in the better understanding of the bacterial pathogenesis especially in an opportunistic pathogen like *Acinetobacter sp.*



**Figure 2**  
Immunoblot of monoclonal antibodies against IROMPs of *A. baumannii* ATCC 19606 Immunoblot of Monoclonal antibodies with OMP of *A. baumannii* grown in CDM-Fe medium. Molecular Weight markers were marked in kDa. Lane **A.** 2G<sub>2</sub>ABIR; **B.** 3D<sub>5</sub>ABIR **C.** 1D<sub>11</sub>ABIR **D.** 5D<sub>6</sub>ABIR **E.** 1F<sub>7</sub>ABIR **F.** +ve control **G.** -ve control

**Materials and Methods**

**Bacterial strains and growth conditions**

The *Acinetobacter baumannii* ATCC 19606 strain was procured from American Type Cell Culture, USA. It was grown in Iron depleted chemically defined media (CDM-Fe) and in iron rich (CDM+Fe) media. Outer membrane proteins were prepared as described by Goel et.al. [32], and the protein concentration was measured by Bradford assay [33]. The OMPs were resolved on SDSPAGE according to Lugtenberg's protocol [34].

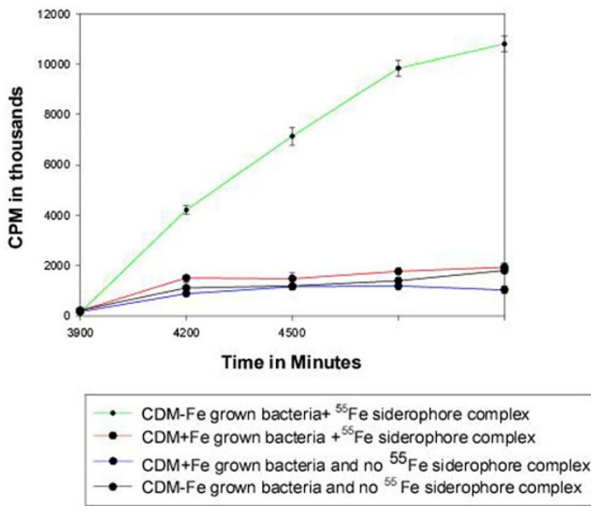
**Production of monoclonal antibodies**

For immunization of Balb/c mice, the area of the SDS-PAGE gel corresponding to the bands representing the IROMPs were cut and used as an antigen [35]. First dose was given Intraperitoneally mixed with 250 µl of complete Freund's adjuvant followed by three doses in Freund's Incomplete Adjuvant at an interval of one week. Final booster was given in normal saline, intraperitoneally, just four days before fusion. The spleen cells were fused with mouse myeloma cell line Sp2O 14/Ag as described previously by using PEG (polyethylene glycol 1450) [36,37]. Screening of hybridomas was done by testing culture supernatants in ELISA using two different antigens.

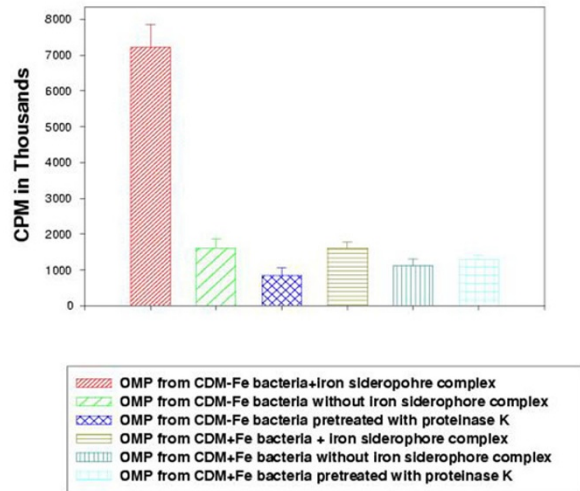
(A) OMPs expressed by bacteria grown in CDM-Fe medium i.e. antigen A

(B) OMPs expressed by bacteria grown in CDM+Fe medium i.e. antigen B

Hybridoma supernatants reacting with antigen A alone without any signal with antigen B were selected and subcloned thrice by limiting dilution. Clones of interest were expanded and cultured in the peritoneal cavities of pristane primed Balb/c mice to obtain ascitic fluid.

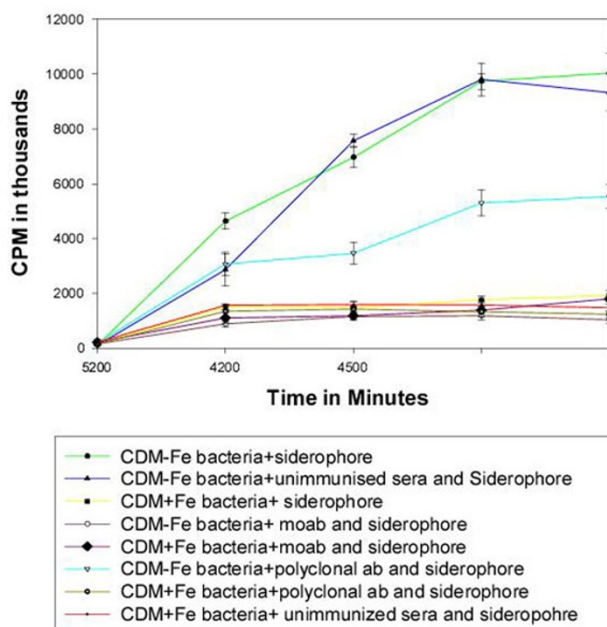


**Figure 3**  
**Invitro iron Uptake by *Acinetobacter baumannii* cells using purified siderophore radiolabelled with <sup>55</sup>Fe.** Each experiment was carried out three independent times and mean values are shown here. The bar represents the standard deviation



**Figure 4**  
Binding of <sup>55</sup>Fe siderophore complex to purified outer membrane proteins of *Acinetobacter baumannii* before and after treatment with proteinase K(10 mg/ml). Each experiment was carried out three independent times and mean values are shown here. The bar represents the standard deviation





**Figure 5**  
Effect of monoclonal antibody on iron Uptake by *Acinetobacter baumannii* ATCC 19606 cells using purified siderophore radiolabelled with  $^{55}\text{Fe}$ . Each experiment was carried out three independent times and mean values are shown here. The bar represents the standard deviation

### ELISA

Antigen A and B were diluted to final concentration of 2.5  $\mu\text{g}/\text{ml}$  in PBS (pH 7.2). 100  $\mu\text{l}$  of this antigen was added to 96 well ELISA plate (High binding ELISA plates, Costar, USA) and were allowed to adsorb for 16–18 hrs at 4°C. The plates were washed thrice with PBS containing 0.05% Tween 20 (Sigma Co. USA). The nonspecific binding sites on the plate were blocked by 2% nonfat milk powder at 37°C for 3 hrs. 100  $\mu\text{l}$  of the antibody was added to the wells and the plates were further incubated for 3 hrs at 37°C. The rabbit antimouse (Dakopats Switzerland) peroxidase conjugated immunoglobulin was used at working dilution of 1:3000. O-Phenylenediamine (OPD, Sigma Inc. USA) and  $\text{H}_2\text{O}_2$  was used as chromogen and substrate, respectively. The optical density were taken by ELISA reader at 492 nm. Cross reactivity of the monoclonal antibodies was checked against *E. coli* and *Pseudomonas aeruginosa* IROMPs in ELISA as described above.

### Determination of immunoglobulin isotype

The isotyping of monoclonal antibodies was done using commercially available isotyping kit based on ELISA (Sigma Inc., USA) as described by manufacturer.

### Immunoblotting

OMPs from SDS-PAGE were transferred electrophoretically to Nitrocellulose paper (0.45  $\mu\text{m}$  Bio-Rad, USA) using semidry blotter (Bio-Rad, USA) as described by manufacturer by a modification of Towbin's [38] procedure. The NC paper was washed twice with PBS pH 7.2. One part of the NC paper was stained with Ponceau S (Merck.Germany) for staining the proteins to check the transfer efficiency and for marking the position of molecular weights. The other part of NC paper was used for immunoblotting. Nonspecific binding sites were blocked by incubating the NC paper in 2% bovine serum albumin and 3% nonfat milk powder at 37°C for 3 hours. NC paper was washed thrice with PBS containing 0.05% Tween 20. The NC paper was incubated at 4°C for overnight with either monoclonal or polyclonal antibodies. The rabbit antimouse peroxidase conjugated immunoglobulin (Dakopats, Switzerland) was used at a dilution of 1:1000 and incubated at 37°C for 3 h. The blots were developed using DAB (diaminobenzimidine) and  $\text{H}_2\text{O}_2$  as chromogen and substrate, respectively.

### Bactericidal assay

Bactericidal assay of monoclonal antibodies was carried out as described by Poolman et.al. [19]. Briefly,  $10^7$  cells of *A. baumannii* ATCC 19606 (grown in iron deficient medium) in 100  $\mu\text{l}$  volume were taken in an eppendorf, 100  $\mu\text{l}$  of monoclonal antibodies culture supernatant (undiluted) was added to it and incubated at 37°C for 1 hour. The human complement from immunoglobulin deficient patient was added (Courtesy of Medical Oncology Unit, AIIMS New Delhi India) to a final concentration of 10% with 10 mM  $\text{MgCl}_2$  and  $\text{CaCl}_2$ , incubated further for 1 hour at 37°C. The reaction mixture were appropriately diluted, plated on to nutrient agar plate and incubated at 37°C for overnight. Bacterial colonies were counted the next day to look for percentage reduction. The percentage reduction was calculated as follows

Control-Test X100/Control

### Opsonophagocytic assay

The assay described by Speert et al. [39] was used to measure opsonophagocytic activity of monoclonal antibodies. The polymorphonuclear leucocytes (PMN) were isolated from pooled normal healthy human blood by Ficoll Hypaque density gradient centrifugation. *A. baumannii* ATCC 19606 ( $10^7$  bacterial cells/100  $\mu\text{l}$ ) grown in CDM-Fe medium were incubated with 100  $\mu\text{l}$  of monoclonal antibody culture supernatant for 30 min at 37°C. To this  $10^6$  PMN in 100  $\mu\text{l}$  volume was added and kept in orbital shaker (100 rpm) at 37°C for 90 min. A duplicate set of assay was incubated at 4°C to measure the nonspecific attachment of bacteria. After incubation the tubes were centrifuged at 300 g and cell pellet was washed gen-

tly in Hank's balanced salt solution (HBSS) at least thrice. The pellet was resuspended in 0.1 ml of HBSS and one drop of it was placed on glass slide, air-dried, heat fixed and stained with Gram Stain. The slide was seen under oil immersion field of light microscope. The number of bacteria within the cytoplasm of 50 PMNs were counted. The mean number of bacteria per PMN obtained from 4°C assay was subtracted from the number obtained from 37°C assay to get the final results. Same set of experiments was repeated with bacteria grown in CDM+Fe medium.

#### **Electron microscopy**

For electron microscopy modified procedure of Root et al. [40] was followed. Mixture of  $10^7$  bacteria and  $10^6$  PMNs were combined and processed for opsonization as mentioned above except the last wash, which was in 0.1 M sodium cacodylate buffer pH 7.2. To the pellet 1 ml of 2.5% glutaraldehyde (made in 0.1 M sodium cacodylate buffer) was added and centrifuged at 1150 g for 10 min, supernatant was decanted. The cell pellet was fixed by adding 2.5% glutaraldehyde for 4 h and the pellet was washed in sodium cacodylate buffer. The pellet was post fixed in 1% osmium tetroxide, embedded in 1% agar. The embedded sample was dehydrated in graded series of alcohol and finally embedded in Araldite resin. It was sectioned, poststained with uranyl acetate and lead and samples were viewed with a Philips 400 electron microscope to look for phagocytosed bacteria.

#### **Blocking of iron uptake by bacteria**

For studying the role of monoclonal antibodies in blocking of iron uptake pathway, we purified the siderophore by a modified method of Smith [17]. Briefly the culture supernatant of bacteria grown in CDM-Fe medium was extracted twice with ethyl acetate. The ethyl acetate was reduced to 0.5 ml by rotary evaporation (Buchi rotary evaporator) at 30°C under reduced pressure. This sample was loaded on Whatman 3 mm chromatography paper and developed using solvent system of water:Acetic Acid:Acetone in ratio of 90:10:1. One portion of the chromatogram was stained with 0.1 M  $\text{FeCl}_3$  in 0.1 M HCl. The corresponding band was excised and siderophore was eluted in ethyl acetate for 48 hrs at room temperature. After evaporation of ethyl acetate, purified siderophore was re-suspended in distilled water and stored at -80°C. The type of siderophore was determined by Arnov's [41] and Csaky's method [42]. The concentration of siderophore was determined using 2–3 dihydroxybenzoic acid as a standard by Universal Chemical assay as described by Schwyn's [43]. 2 mM of 50  $\mu\text{l}$  purified siderophore was incubated with either 10  $\mu\text{M}$   $^{55}\text{FeCl}_3$  (specific activity 2.068 Mci/g of iron, Bhabha Atomic Research Center Trombay, India) or  $^{59}\text{FeCl}_3$  (specific activity 250.49 GBq/g of iron, Bhabha Atomic

Research Center Trombay, India). The mixture was incubated at room temperature for 30 minutes. At the end the unbound radiolabelled iron was removed by chromatography using sephadex G-10 as described by Rosenberg [44]. The uptake of radiolabelled iron siderophore was studied by the method described by Rosenberg [44]. Briefly *A. baumannii* was grown in CDM-Fe medium also in CDM+Fe medium to a mid exponential phase and was washed twice with uptake medium (30 mM  $\text{Na}_2\text{HPO}_4$ , 10 mM  $\text{K}_2\text{HPO}_4$ , 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1 mM  $\text{MgCl}_2$  and 40  $\mu\text{M}$   $\text{CaCl}_2$  pH 6.9) at 4°C and re-suspended in the same medium at a density of  $A_{470} = 0.4$ . The washed cells were incubated at 37°C for 15 minutes. The uptake was initiated by adding equal volume of radiolabelled  $^{55}\text{Fe}$  siderophore to the bacterial suspension and shaken at 300 rpm. Aliquots of 500  $\mu\text{l}$  were withdrawn after every 5 minutes interval and filtered immediately on glass microfiber filter and washed with 2 ml of uptake medium. The membranes were dried at 37°C and measured as counts per minutes on  $\beta$ -scintillation counter (Pharmacia) on the  $^3\text{H}$  channel. In parallel, bacterial cells were also incubated with radiolabelled iron alone and unlabelled siderophore as a control.

To look for the role of monoclonal antibodies against IROMPs in blocking the iron uptake the bacterial cells were pre-incubated with 100  $\mu\text{l}$  of undiluted monoclonal antibody culture supernatant for 1 h at 37°C before addition of radiolabelled iron siderophore complex. After incubation with antibodies the bacterial cells were washed twice with uptake buffer to remove unbound antibodies. Similarly as like in uptake, radioactivity retained on the filter was counted on  $\beta$ -scintillation counter on  $^3\text{H}$  channel.

#### **Binding of radiolabelled iron siderophore complex to OMPs**

Binding of radiolabelled  $^{55}\text{Fe}$  siderophore complex to OMPs was carried out as described by Meyer et al. [11]. 100  $\mu\text{g}$  of OMPs in 0.125 M Tris HCl pH 6.8 was mixed with 0.1 ml of siderophore labeled with  $^{55}\text{Fe}$  (as described above). The total volume of the mixture was adjusted to 1 ml with uptake medium and the reaction mixture were incubated for 10 minutes at 37°C. This was filtered through 0.22  $\mu\text{m}$  filter and washed twice with 2 ml of uptake medium. The radiolabeled iron retained on the filter was counted as counts per minutes on  $\beta$ -scintillation counter on  $^3\text{H}$  channel. To determine the nature of the binding receptor, the OMP preparation was subjected to digestion with proteinase K (10 mg/ml) for 1 hr at 37°C prior to the incubation with iron siderophore complex.

To test the blocking of iron siderophore to OMPs, it was preincubated with monoclonal antibodies for 1 h at 37°C as described in uptake experiment and then binding assay was performed

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