

Siderophore-Mediated Utilization of Transferrin- and Lactoferrin-Bound Iron by *Acinetobacter baumannii*

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Acinetobacter baumannii produces the siderophore called acinetobactin (AB) in response to iron starvation. In this study, *in vitro* growth experiments were conducted to evaluate the ability of AB to sequester iron bound to human transferrin and lactoferrin and mediate bacterial utilization of the iron. Strain ATCC 19606 producing AB was able to grow in the presence of either 30% iron-saturated human transferrin (30% Fe-TF) or 15% iron-saturated human lactoferrin (15% Fe-LF) as a sole source of iron even when they were separated by a dialysis membrane. The radiolabel after equilibrium dialysis between ^{55}Fe -TF and AB was accumulated by cells grown under iron-deficient conditions, but not by those grown under iron-sufficient conditions. Addition of AB to the medium caused great enhancement in the growth of a poor producer strain of AB in the presence of 30% TF or 15% LF. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane protein fractions revealed the presence of 77- and 81-kDa proteins only in the cells grown under iron-deficient conditions, suggesting that either of them may function as the receptor for Fe^{3+} -AB complex. No strain tested was able to utilize hemin and hemoglobin as a sole source of iron. These results indicate that *A. baumannii* can utilize iron bound to TF and LF as host iron sources through the action of AB. This system is probably involved in survival and proliferation in the host.

Key words — siderophore, iron acquisition, *Acinetobacter baumannii*, transferrin, lactoferrin

INTRODUCTION

Members of the genus *Acinetobacter* are reported to be involved in a variety of nosocomial infections including bacteremia, urinary tract infection, pneumonia and secondary meningitis, with increasing frequency.¹⁾ Such infections are often extremely difficult to treat because of the widespread resistance of these bacteria to the major groups of antibiotics.¹⁻³⁾ Although the taxonomy of the genus has undergone many changes in the last few years and is still confusing to some extent, the classification established by Bouvet and Grimont⁴⁾ is generally accepted, which is composed of 12 DNA groups or genospecies. Among these species encountered frequently in nosocomial infections are *A. baumannii* and the genotypically closely related *Acinetobacter* genospecies 3 (unnamed).¹⁾

The acquisition of essential nutrients to facil-

itate growth in the host is a problem common for all bacterial pathogens, and such growth is critical to the establishment of infection and depends in part on the ability of the pathogen to scavenge nutrients such as iron. However, the availability of free iron is very low in the host, as most iron is complexed with host proteins such as hemoglobin, transferrin (TF) and lactoferrin (LF). The latter two glycoproteins hold iron tightly with association constants for Fe^{3+} of 10^{-24} M^{-1} and physiologically are partially saturated with iron.^{5,6)} Thus, to overcome this non-specific defense mechanism, successful pathogens have developed efficient iron uptake mechanisms that allow them to scavenge iron from these host proteins, either by synthesis and secretion of high-affinity extracellular iron chelators, known as siderophores, concomitant with expression of cell surface receptor proteins specific for each Fe^{3+} -siderophore complex,⁷⁾ or by direct interaction with these host proteins.⁵⁾

Some reports have shown the ability of *Acinetobacter* species to produce siderophore-like compounds under iron-deficient conditions, but information about their structures was lack-

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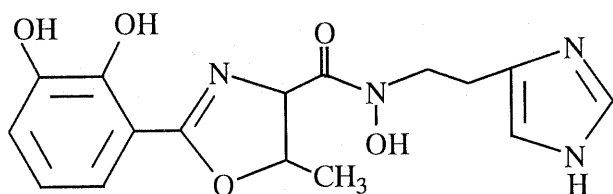


Fig. 1. Structure of Acinetobactin (AB)

ing.⁸⁻¹⁰) Previously, we reported that *A. baumannii* produces a novel siderophore, called acinetobactin (AB) having the structure shown in Fig. 1, in response to iron depletion.¹¹ The current study was undertaken to elucidate whether AB can assimilate iron bound to human TF and LF *in vitro* and facilitate the growth of *A. baumannii*. Some other *A. baumannii* clinical isolates were also examined for their ability to produce AB.

MATERIALS AND METHODS

Chemicals — Thirty percent iron-saturated human TF (30% Fe-TF, physiological saturation level of human TF) was prepared from apo-TF (Sigma Chemicals, St. Louis, MO, U.S.A.) according to the procedure of Morton and Williams,¹² and percent saturation was checked with a diagnostic kit (Fe-Test Wako, Wako, Osaka, Japan) according to the manufacturer's protocol. For preparation of ⁵⁵Fe-labeled 30% Fe-TF (30% ⁵⁵Fe-TF), ⁵⁵FeCl₃ (NEN, specific activity: 2368 kBq/ μ M) was used instead of ferric nitrate. The resulting radiolabeled 30% Fe-TF was dialyzed against 10 mM HEPES (pH 7.4) containing 150 mM NaCl, 10 mM NaHCO₃ (buffer A) until extraneous radioactivity approached background level. Human milk LF was purchased from Sigma Chemicals, whose iron-saturation level was determined to be 15% with the same kit (termed 15% Fe-LF). Prior to use, 30% Fe-TF and 15% Fe-LF dissolved in buffer A at higher concentrations were dialyzed for 24 h against and diluted with buffer A to the concentration equivalent to 100 μ M Fe³⁺. Each diluted solution was sterilized by filtration through a 0.22- μ m cellulose acetate filter. All glassware was filled with 6 M HCl overnight and rinsed several times with distilled water. All reagent solutions and media were prepared with distilled water.

Bacterial Strains — Type strains of *Acinetobacter* species including *A. baumannii* ATCC 19606 were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Strain ATCC 19606

produced AB at a concentration of 17.0 μ M in culture supernatant when grown in Tris-buffered synthetic (TBS) medium (pH 7.4)⁹ at 30°C for 28 h. Eighteen clinical strains including H54Ic isolated from patients with complicated urinary tract infections were provided by Dr. Y. Hirai of Okayama University School of Medicine, which had been defined as *A. calcoaceticus* subsp. *anitratius* (presently designated *A. baumannii*⁴) by the API 20NE technique (API System S.A., Montalieu-Vercieu, France). Strain H54Ic produced AB at *ca.* 0.5 μ M under the same growth conditions as for ATCC 19606, and therefore was used as an AB poor-producer.

Growth Assay — Erlenmeyer flasks (100 ml) with a side-arm were used. Late-log phase cells of strains ATCC 19606 and H54Ic precultured in TBS medium containing 0.15 μ M of added FeCl₃ were each added at an optical density at 660 nm (OD₆₆₀) of 0.02 to 20 ml of TBS medium containing either 30% Fe-TF or 15% Fe-LF at a concentration equivalent to 1 μ M Fe³⁺. In some experiments, to prevent cells from contacting the protein, a sterile dialysis bag (cellulose dialyzer tubing VT802, Nacalai Tesque, molecular weight exclusion limit, *ca.* 8000) containing 2 ml of either 30% Fe-TF or 15% Fe-LF solution (equivalent to 10 μ M Fe³⁺) were placed in 20 ml of TBS medium. In the experiments using strain H54Ic, a 70% methanolic solution of purified AB was added to TBS medium at a final concentration of 5 μ M. Cultures were shaken at 175 rpm at 30°C, and cell density (OD₆₆₀) was monitored at various intervals. Prior to use, the dialysis membrane and closures (Spectrum Medical Industries, Inc., Los Angeles, CA, U.S.A.) were washed with 1 mM EDTA, rinsed with distilled water and autoclaved. Each experiment was repeated three times.

Uptake by Cells of the Radiolabel after Equilibrium Dialysis of 30% ⁵⁵Fe-TF in the Presence of AB

For this experiment, radiolabeled 30% ⁵⁵Fe-TF with a specific activity of 680 cpm/pmol was used. A dialysis bag filled with 2 ml of the labeled protein solution (equivalent to 10 μ M Fe³⁺) was placed in 20 ml of TBS medium in the absence or presence of AB at a concentration of 10 μ M, and incubated overnight at room temperature in a dark room. The resulting TBS medium was then supplemented with late-log phase cells at an OD₆₆₀ of 0.5, and the cell suspension was incubated at 30°C for 30 min. The late-log phase cells were previously prepared by growing in TBS medium containing 0.15 μ M (iron-deficient conditions) or 20 μ M (iron-sufficient conditions) of added FeCl₃, followed by several washings with 100 mM Tris-HCl

(pH 7.5) containing 0.5% NaCl and 10 mM MgCl₂ (washing buffer). An aliquot (1 ml) of the incubated cell suspension was filtered onto a membrane filter (Millipore HAWP, 0.45 μ m) and washed twice with 5 ml of washing buffer. The filter was dried, and the amount of ⁵⁵Fe retained on the filter was measured by liquid scintillation counting (tritium channel). Non-specific adsorption of the radiolabel to cells was corrected for by subtracting the radioactivity of the zero time sample from each value.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Outer Membrane Protein Fractions—Outer membrane protein (OMP) fractions were prepared from cells grown in TBS medium under iron-deficient or iron-sufficient conditions. Cells were cultivated and sonicated in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM benzamidine.¹³⁾ Cell debris was removed by centrifugation at 4000 $\times g$ for 30 min, and then total membranes were collected by centrifugation at 100000 $\times g$ for 1 h. Inner membranes were removed by the procedure of Filip *et al.*¹⁴⁾ using 1% Sarcosyl (Sigma), and OMPs were pelleted by centrifugation at 100000 $\times g$ for 1 h. The pellet was resuspended and washed twice with 50 mM Tris-HCl buffer described above. Protein contents were estimated by the method of Lowry¹⁵⁾ with bovine serum albumin as a standard.

SDS-PAGE was performed using 10% (w/v) acrylamide in the running gel and omitting 2-mercaptoethanol from the sample loading buffer according to the method of Laemmli.¹⁶⁾ Prior to sample application, heat denaturing was carried out at 100°C for 5 min. Proteins were stained with Coomassie blue.

Determination of AB—Type strains of *Acinetobacter* species and clinical isolates were grown at 30°C for 28 h in 50 ml of TBS medium at 0.15 μ M FeCl₃, and each whole supernatant was processed for HPLC analysis as previously described.¹¹⁾ The calibration curve was made up by adding known amounts of purified AB to TBS medium. Recovery rates of AB added to the culture samples were >85% in the range of 0.5–50 μ M AB, and detection limit was *ca.* 2 μ g of AB.

RESULTS AND DISCUSSION

Utilization of 30% TF and 15% LF by *A. baumannii*

The results of growth experiments conduct-

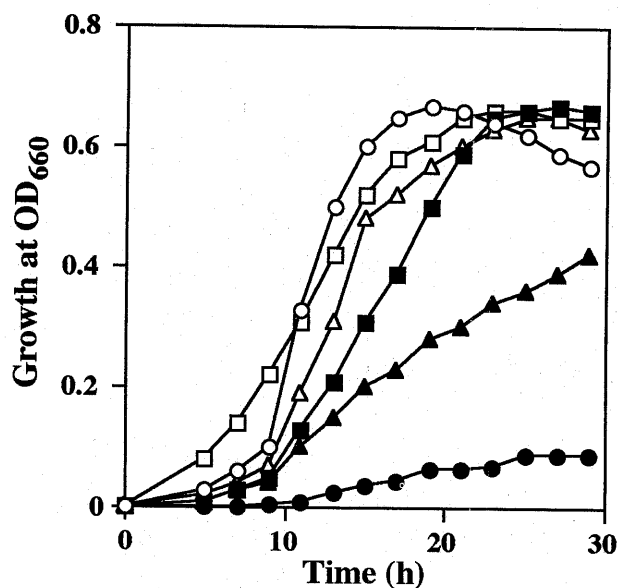


Fig. 2. Growth of *A. baumannii* ATCC 19606 in Iron-Deficient TBS Medium Containing Either 30% Fe-TF or 15% Fe-LF as a Sole Source of Iron

The medium was supplemented with the following iron sources: ●, no supplement (negative control); ○, 1 μ M FeCl₃ (positive control); ■, 30% Fe-TF equivalent to 1 μ M Fe³⁺; □, 30% Fe-TF inside a dialysis bag; ▲, 15% Fe-LF equivalent to 1 μ M Fe³⁺; △, 15% Fe-LF in a dialysis bag. The average of triplicate experiments is shown.

ed for strain ATCC 19606 producing AB were shown in Fig. 2. Growth curves obtained in the absence and presence of added FeCl₃ (1 μ M) were included as negative and positive controls, respectively. The strain was able to grow in the presence of either 30% Fe-TF or 15% Fe-LF equivalent to 1 μ M Fe³⁺; but the latter protein supported the growth to a lesser extent. When placed inside a dialysis bag to prevent their direct contact with the bacterial cells, both iron-containing proteins facilitated the growth, at least showing that AB can remove iron from TF and LF and pass through the dialysis membrane. The possibility of the spontaneous release of iron from these proteins was excluded, since the pH of TBS medium only changed slightly (from pH 7.4 to 7.0) during cultivation and since TBS medium supported no growth even if it had been incubated overnight at 30°C with 30% Fe-TF or 15% Fe-LF inside a dialysis bag without bacterial inoculation. A notable prolongation in the lag phase and reduction in the growth by the presence of TF and LF, respectively, free in TBS medium may be due to their antibacterial activity through damage to the bacterial cell surface.¹⁷⁾ These results did not completely exclude the possibility that this bacterium can utilize iron

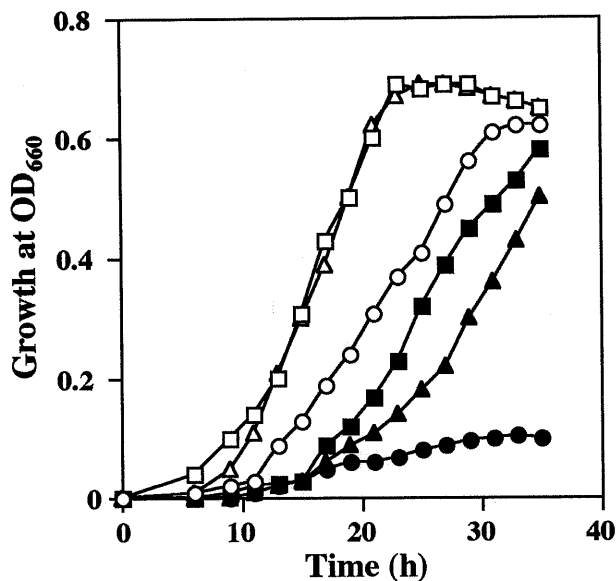


Fig. 3. Growth Enhancement by the Addition of AB of *A. baumannii* H54Ic, a Poor Producer of AB, in the Presence of Either 30% Fe-TF or 15% Fe-LF as a Sole Source of Iron

The iron-deficient TBS medium was supplemented with the following iron sources: ●, no supplement (negative control); ○, 1 μM FeCl_3 (positive control); ■, 30% Fe-TF equivalent to 1 μM Fe^{3+} ; □, 30% Fe-TF plus 5 μM AB; ▲, 15% Fe-LF equivalent to 1 μM Fe^{3+} ; △, 15% Fe-LF plus 5 μM AB. The average of triplicate experiments is shown.

bound to these proteins at least in part through direct interaction. However, there is evidence that this bacterium has no capability to directly bind TF on its cell surface,¹⁰ being indicative of no direct assimilation of TF-bound iron.

Consistent with the above results, the addition of purified AB (5 μM) to TBS medium containing 30% Fe-TF or 15% Fe-LF caused enhancement in the growth and shortening in the lag phase for strain H54Ic, a poor producer of AB (Fig. 3). Less sufficient growth of the strain at 1 μM added Fe^{3+} compared with strain ATCC 19606 suggested that AB was probably functioning as the siderophore in transporting free iron ion present at relatively low concentrations (1 μM Fe^{3+}). It is well known that by its presence in exocrine secretions and on mucosal surfaces, LF can prevent microbial growth at strategic sites of entry.^{6,18,19} In this regard, it is noteworthy that AB has an affinity for Fe^{3+} high enough to remove iron from unsaturated LF.

As an alternative mechanism of iron acquisition, utilization of heme compounds is also important for a number of pathogenic bacteria.^{20,21} However, neither hemin nor hemoglobin facilitated the growth of strain ATCC 19606 as a

Table 1. Uptake by Iron-Deficient Cells of the Radiolabel after Dialysis of 30% ^{55}Fe -TF in a Dialysis Bag against TBS Medium Containing AB^{a)}

Incubation temperature ^{b)}	Radioactivity (cpm) in	
	Iron-deficient cells ^{c)}	Iron-sufficient cells ^{c)}
0°C	790 ^{d)}	805
30°C	20150	1020

a) Conditions for dialysis, see Materials and Methods; b) Cells were suspended in 20 ml of TBS medium (dialysate) at OD_{660} of 0.5 and incubated for 30 min at indicated temperatures; c) Iron-deficient and iron-sufficient cells were prepared by growing at 0.15 μM and 20 μM FeCl_3 , respectively; d) Each value is the mean of triplicate experiments.

sole source of iron (data not shown).

AB-Mediated Uptake of Iron Bound to 30% TF

Strain ATCC 19606 grew well without a lag phase in TBS medium where 30% Fe-TF or 15% Fe-LF inside a dialysis bag had been preincubated in the presence of 5 μM AB for 24 h at room temperature (data not shown). In contrast, preincubation in the absence of AB did not support the growth, further confirming that contact between these proteins and bacteria is not essential for this iron mobilization activity. In order to confirm that iron derived from 30% Fe-TF is, in fact, taken up by cells, radiolabeled 30% ^{55}Fe -TF equivalent to 1 μM Fe^{3+} was used in place of unlabeled one and the resulting TBS medium was incubated at 0°C and 30°C for 30 min with either iron-deficient or iron-sufficient cells. As shown in Table 1, the radioactivity was accumulated by iron-deficient cells at 30°C but not 0°C, suggestive of the involvement of a specific uptake system. Very low uptake was observed for the iron-sufficient cells. Overall, these results indicated that iron bound to TF was incorporated *via* chelation with AB. A similar experiment could not be conducted for LF because apo-LF was unavailable.

Effect of Iron Limitation on OMP Profiles

Bacteria producing siderophores express cognate cell surface receptors which bind respective ferric siderophore complexes as the first step in the iron uptake pathway.⁷⁾ SDS-PAGE of Sarcosyl-insoluble OMP fractions from strains ATCC 19606 and H54Ic cultured under iron-deficient conditions revealed the presence of two prominent proteins with apparent molecular masses of *ca.* 77 and 81 kDa, which were absent in

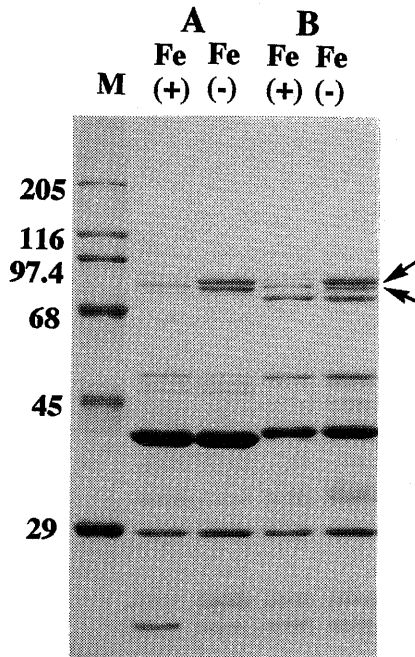


Fig. 4. SDS-PAGE of OMPs Prepared from *A. baumannii* ATCC 19606 (A) and H541c (B) Grown under Iron-Sufficient (+) and Iron-Deficient (-) Conditions

The gel was loaded with 60 μg of sample protein per lane. The apparent molecular masses (in kDa) of the standard proteins (M) are indicated in the left margin. Arrows indicate the prominent OMPs expressed under iron-deficient conditions.

cells cultured under iron-sufficient conditions (Fig. 4). Recently, two iron-repressible OMPs with similar molecular masses (75 and 80 kDa) were reported for *A. baumannii*.²²⁾ It seems likely that either of these iron-repressible proteins can function as a receptor for Fe^{3+} -AB complex.

Distribution of AB in *Acinetobacter* Species

The results described above suggested that the iron acquisition system mediated by AB might be important for establishment of infection by *A. baumannii*. Type strains of other *Acinetobacter* species as well as eighteen *A. baumannii* clinical isolates were thus examined for AB productivity. It was of interest that AB was also detected in *Acinetobacter* genospecies 3 ATCC 19004 (33.6 μM), since this species has been isolated with relatively high frequency from clinical specimens.¹⁾ AB was undetectable in other type strains such as *A. calcoaceticus* ATCC 23055, *A. lwoffii* ATCC 15309, *A. johnsonii* ATCC 17909 and *A. junii* ATCC 17908. However, it remains to be clarified whether these species can produce siderophores other than AB. We previously reported that *A. haemolyticus* ATCC 17906 also produces AB at 1.3 μM , in addition to acinetofe-

rin.^{11,23)} All of the clinical isolates tested produced AB but with a large strain-to-strain variation in AB concentration (0.5–48.8 μM). These strains grew in the presence of either 30% Fe-TF or 15% Fe-LF as a sole source of iron (data not shown).

Most of the iron in the human body is intracellular. A small amount of iron is withheld by iron-binding or transport proteins, TF in the serum, and LF in mucosal secretions and specific granules of neutrophils.¹⁴⁾ These proteins have important roles in host defense by depriving invading pathogens of iron,¹⁷⁾ and their iron-unsaturated forms contribute at least in part to the bacteriostatic and bacteriocidal activity of serum and secretions.¹⁷⁾ Therefore, the ability to acquire iron from these proteins is essential for bacteria to maintain infections in the host. However, not all the siderophores isolated so far can remove iron from both TF and LF. For instance, pyoverdine and pyochelin, the siderophores produced by *Pseudomonas aeruginosa*, can release iron from TF, but not from LF.²⁴⁾ Our data indicated that AB was one of the effective siderophores in terms of acquiring iron from LF.

In conclusion, we demonstrated that *A. baumannii* could utilize iron bound to 30% Fe-TF and 15% Fe-LF through the action of AB. In the species, this iron acquisition system may have important functions for survival and further growth in the host. Determination of the receptor for Fe^{3+} -AB complex and the precise role *in vivo* of this host iron utilization system in relation to pathogenesis of *A. baumannii* are currently under investigation.

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