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Effect of Iron-Limiting Conditions on Growth of Clinical Isolates of *Acinetobacter baumannii*

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Different clinical isolates of *Acinetobacter baumannii*, typed by plasmid profile, were able to grow in iron-chelated medium by secreting iron-regulated siderophores. This iron-scavenging phenotype was associated with the production of iron-repressible catechol. Siderophore utilization bioassays showed the presence of 2,3-dihydroxybenzoic acid in the growth medium, and neither enterobactin nor aerobactin was detected in culture supernatants obtained under iron-deficient conditions.

Acinetobacter baumannii, a gram-negative bacterium frequently found in the skin (2) and throats (26) of healthy humans, usually causes severe nosocomially acquired infections in compromised patients (7, 13, 15, 16, 18, 22, 25, 27, 32). However, Anstey et al. (3) recently described the largest series of fatal cases of community-acquired *Acinetobacter* pneumonia in patients affected by different risk factors. The ability of *A. baumannii* to colonize and invade the human host suggests that it can obtain essential nutrients, such as iron, which are normally restricted, for bacterial utilization. Only very few reports showed the ability of clinical isolates of *A. baumannii* to grow and produce siderophore compounds under iron-deficient conditions (21, 33). Recently, we described a high-affinity siderophore-mediated iron uptake system in a clinical strain of *A. baumannii* (14). We show in this work that different *A. baumannii* strains were able to grow under iron-deficient conditions. The bacterial growth was accompanied by the secretion of iron-regulated catechol siderophores, independently of the bacterial plasmid content.

The *A. baumannii* isolates were obtained from sputum specimens, respiratory support equipment, and the hands of personnel at the Oregon Health Sciences University hospitals during a nosocomial outbreak of respiratory tract infections (16). Strains were cultured on Luria agar, Luria broth, and M9 minimal medium (28). Human apo-transferrin (TF) (substantially iron free), 2,2′-bipyridyl (DIP), ethylenediamine-di-(*o*-hydroxyphenyl)acetic acid (EDDA), and nitrilotriacetic acid (Sigma Chemical Co., St. Louis, Mo.) were used as iron chelators to achieve iron-limiting conditions. The MIC for iron chelators was determined in M9 liquid medium (14). Iron-rich conditions were achieved by adding 100 μM $FeCl_3$ dissolved in 0.1 M HCl.

Plasmid DNA was prepared either by the method described by Kado and Liu (17) or by the Triton lytic method (9) modified by the inclusion of a heating step (16). DNA samples were analyzed by gel electrophoresis using 0.7% agarose vertical gels and Tris-borate buffer (10). Plasmids contained in *Escherichia coli* V517 (20) were used as molecular weight standards.

Extracellular siderophores were detected by the method of Schwyn and Neilands (30). The Arnow (4) and Csaky (12) tests were used to detect phenolic and hydroxamate extracellular compounds, respectively. The catechol siderophore enterobactin and the hydroxamate siderophore aerobactin were used as standards in chemical and biological assays. The biosynthesis of aerobactin, enterobactin, and 2,3-dihydroxybenzoic acid was examined by plate assays as previously described (1, 11, 19). Binding of TF to bacterial cells was assayed as described by Schryvers and Morris (29).

The 96 clinical isolates of *A. baumannii* obtained during the outbreak were classified by their plasmid content. The yield of plasmid DNA by the method of Kado and Liu was low, and most of the preparations appeared to be degraded after electrophoresis (data not shown). Conversely, the hot Triton lytic method yielded *Acinetobacter* plasmid DNA of good quality and in amounts suitable for agarose gel electrophoresis. Figure 1 shows the electrophoretic plasmid patterns of representative isolates. This analysis revealed that all the strains harbored plasmids, which varied in copy number, had sizes that ranged from 1.8 to about 100 MDa, and displayed great diversity in their electrophoretic profiles. The bacterial isolates were classified into 33 different types, based upon the plasmid patterns (Table 1). As reported before (16), profile A isolates harbor plasmids of 18, 4.8, 3.2, and 2.3 MDa and profile A-related isolates harbor plasmids of the same size, together with other plasmids. Those designated profile B have 17- and 1.8-MDa plasmids, and profile B-related isolates have these two plasmids together with other plasmids. Isolate K, having plasmids of 18, 17, 4.8, 3.2, 2.3, and 1.8 MDa, was designated AB. The rest of the isolates showing plasmid profiles different from those described above were designated OTHER. This typing method showed that the outbreak of low-tract respiratory infections was caused by different *A. baumannii* strains. In addition, it was found that strains with plasmid profiles A, A related, B, B related, and AB were the isolates most commonly obtained from the hospitalized patients, the hands of the personnel, and the respiratory support equipment (16).

Twelve *A. baumannii* strains representing the most (A, A related, B, B related, or AB) and least (OTHER) prevalent plasmid groups were examined for their ability to grow under conditions of iron limitation. These strains were able to grow on M9 minimal medium plates containing the iron chelator DIP, EDDA, or nitrilotriacetic acid (Table 1). MIC assays showed that these isolates were able to grow in liquid M9 medium containing up to 1,000 μM EDDA or nitrilotriacetic acid or up to 200 μM DIP. In addition, all the strains grew

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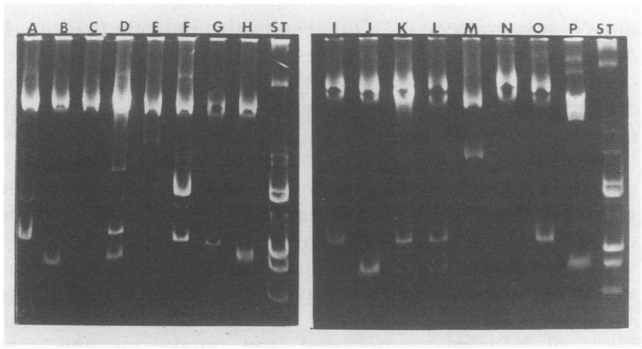

FIG. 1. Agarose gel electrophoresis of plasmid DNAs prepared from various *A. baumannii* strains (designated A through P above the lanes) isolated from hospitalized patients, respiratory equipment, and hands of hospital personnel. ST, molecular weight standard plasmids.

well after overnight incubation at 37°C on M9 agar plates containing 30 μM TF. The possibility of the acquisition of iron from TF by direct interaction with this host iron-binding protein was eliminated, since none of the *A. baumannii* isolates bound horseradish peroxidase-labeled transferrin (data not shown).

The universal chemical assay for siderophores showed that individual colonies of the 12 clinical isolates were surrounded by orange halos after overnight incubation at 37°C on agar plates containing chrome azurol S. Furthermore, the addition of iron-limited, but not iron-rich, culture supernatants of the *A. baumannii* isolates to the chrome azurol S reagent produced a color change of the reagent from blue to orange. These results strongly indicated that these isolates excreted an iron-regulated siderophore into the culture medium. The Arnow (4) and Csaky (12) colorimetric reactions were used to determine the chemical nature of the siderophores secreted by these isolates. The Arnow test revealed the presence of catechol, which was detected only in iron-deficient culture supernatants. Conversely, neither the iron-deficient nor the iron-rich culture supernatants reacted by the Csaky test, indicating that no hydroxamate compounds were present in the culture supernatants. We also investigated biologically the ability of the *A. baumannii* isolates to produce aerobactin and enterobactin. The presence of aerobactin was ruled out, since aerobactin-deficient, receptor-proficient *E. coli* LG1522 (34) was not able to grow under conditions of iron limitation in the presence of cell-free iron-deficient culture supernatants of the *A. baumannii* strains (Table 2). This result agrees with that obtained with the Csaky test. Conversely, the same culture supernatants were able to reverse the EDDA inhibitory effect on the growth of the *Salmonella typhimurium enb-7* mutant (Table

TABLE 1. Plasmid content, ability to grow in iron-deficient medium, and siderophore production of *A. baumannii* isolates obtained from patients, respiratory devices, and hands of hospital personnel

Isolate plasmid profile	Size(s) of plasmid(s) harbored (MDa)	Growth in Fe^{3+} chelated media[a]	Siderophore production[b]
A	18, 4.8, 3.2, 2.3	+	+
B	17, 1.8	+	+
C	18	+	+
D	45, 4.8, 4.2, 3.2, 2.4, 1.9	+	+
E	56, 6.2, 3.6	+	+
F	100, 45, 16, 4.6, 3.4, 2.2	+	+
G	18, 2.2	+	+
H	60, 17, 1.8	+	+
I	56, 18, 4.8, 3.2, 2.3	+	+
J	100, 60, 17, 1.8	+	+
K	18, 17, 4.8, 3.2, 2.3, 1.8	+	+
L	60, 40, 18, 17, 4.8, 3.2, 2.3, 1.8	ND[c]	ND
M	8, 4	ND	ND
N	60, 15	ND	ND
O	90, 60, 18, 4.8, 3.2, 2.3	ND	ND
P	56, 15, 10, 2.8	ND	ND
Q	15, 12, 7, 4	ND	ND
R	15, 7	ND	ND
S	40, 30, 14, 8.6, 5.4	ND	ND
T	30, 25, 8.6, 6.7, 1.2	ND	ND
U	27, 16, 8.6, 5.4, 3.7, 2.5	ND	ND
V	49, 27, 16, 11, 8.6, 5.4, 5.0, 3.6, 2.5, 1.9	ND	ND
W	16, 8.6, 3.6	+	+
X	49, 32, 16, 1.9	ND	ND
Y	49, 32, 7.0, 5.4	ND	ND
Z	49, 27, 1.9, 1.0	ND	ND
AA	32, 19, 9.6, 3.6, 2.6	ND	ND
AB	20, 8.2, 2.6	ND	ND
AC	45, 5	ND	ND
AD	39, 7.6	ND	ND
AE	35, 7.2, 2.6	ND	ND
AF	38, 16, 6.8, 4.8	ND	ND
AG	42, 14, 6.8, 3.3	ND	ND

[a] M9 minimal medium containing 50 μM DIP, EDDA, or nitrilotriacetic acid or 30 μM TF.
[b] Assayed by the chrome azurol S universal test for siderophore production. All culture supernatants were catechol positive and hydroxamate negative.
[c] ND, not determined.

TABLE 2. Results of siderophore utilization bioassays[a]

A. baumannii plasmid profile	Growth of[b]:		
	E. coli LG1522	*S. typhimurium*	
		enb-1 mutant	*enb-7* mutant
A	−	−	+
B	−	−	+
C	−	−	+
D	−	−	+
E	−	−	+
F	−	−	+
G	−	−	+
H	−	−	+
I	−	−	+
J	−	−	+
K	−	−	+
W	−	−	+

[a] Isolated *A. baumannii* colonies were toothpicked on M9 minimal medium agar plates containing 200 μM DIP and seeded either with *E. coli* LG1522 cells or with cells of the *S. typhimurium enb-1* or *enb-7* mutant.

[b] Growth halos were recorded after overnight incubation at 37°C. −, no growth; +, growth present.

2), indicating the presence in the culture supernatants of 2,3-dihydroxybenzoic acid, which can be utilized by this *S. typhimurium* mutant as a precursor to produce enterobactin (24). However, none of the *A. baumannii* culture supernatants were able to promote the growth of the *S. typhimurium enb-1* mutant (Table 2), an iron uptake mutant that requires enterobactin for growth under conditions of iron limitation (24). Thus, 2,3-dihydroxybenzoic acid, but not enterobactin, is secreted by all of the 12 *A. baumannii* strains when cultured under iron-limiting conditions.

The increased number of outbreaks of infection due to *A. baumannii*, also known as *A. calcoaceticus* subsp. *anitratus* (5), revealed the role of this opportunistic pathogen in affecting hospitalized as well as nonhospitalized patients. However, the bacterial virulence factors involved in these devastating infections are still ill defined. It was proposed that the production of slime (23) and lipases (33) could contribute to the virulence of *A. baumannii*. Another potential virulence factor could be the expression of high-affinity iron uptake systems, as was demonstrated for other human and animal bacterial pathogens (8). Only a few previous reports have described the production of siderophore compounds in clinical isolates of *A. baumannii* growing under iron-limiting conditions (21, 31, 33). Recently, we reported that a clinical isolate of *A. baumannii* expresses a high-affinity iron uptake system mediated by an extracellular siderophore (14). However, the iron uptake proficiency, the mechanisms of iron acquisition, and the incidence of siderophore production in different clinical isolates of *A. baumannii* are actually not known.

The results described here demonstrate that 12 different clinical *A. baumannii* strains can grow in the presence of high concentrations of different iron chelators. Furthermore, the fact that these strains grew in the presence of 30 μM TF strongly suggests that all of them must express a high-affinity iron uptake system, allowing them to scavenge iron from the iron-chelating host proteins. This iron-scavenging system is expressed independently of the plasmid content and does not require direct interaction between the cells and the iron-TF complexes, as was described for other human pathogens such as *Haemophilus influenzae*, *Neisseria meningitidis*, and *N. gonorrhoeae* (8). Our results also indicate that the *A. baumannii* clinical strains only produce iron-regulated catechol compounds with siderophore activity. This finding confirms the results previously reported by Martinez et al. (21), although only the production of aerobactin was investigated in that work. Clinical isolate 8399, which belongs to plasmid group H (B related), was included in this work. The growth behavior of this strain, previously used for the characterization of a high-affinity iron uptake system (14), was identical to that of the other strains classified into the various plasmid groups. It is thus highly possible that all the *A. baumannii* clinical isolates have either similar or even identical siderophore-mediated iron uptake systems that involve the synthesis of a catechol siderophore that is at least different from enterobactin as well as from 2,3-dihydroxybenzoic acid. It is also possible that *A. baumannii* can produce more than one catechol compound with siderophore activity, an important matter that is currently under investigation.

The expression of this type of iron uptake system, involving the secretion of a soluble siderophore, represents a benefit for the invading bacterium by allowing it to compete with host binding proteins for essential iron. The secretion of siderophores may also represent a detrimental factor for the host. Recently, it has been reported that *Pseudomonas aeruginosa*, another important respiratory pathogen, can cause tissue damage by the synergistic interaction between pyocyanin and ferripyochelin (6). This interaction occurs close to the endothelial cells and leads to the formation of highly toxic hydroxyl radicals that produce local tissue damage at the site of infection. This virulence mechanism may also explain the pathogenesis of the respiratory infections caused by *A. baumannii*. Although the catechol siderophore detected in iron-deficient culture supernatants could be one of the bacterial factors involved in this mechanism, the existence and nature of synergistic extracellular bacterial products and the formation of the hydroxyl radicals remain to be elucidated.

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