CURRENT MICROBIOLOGY Vol. 47 (2003), pp. 32–39
DOI: 10.1007/s00284002-3944-x

Current Microbiology
An International Journal

The Metabolism of Aluminum Citrate and Biosynthesis of Oxalic Acid in *Pseudomonas fluorescens*

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Received: 13 August 2002 / Accepted: 4 September 2002

Abstract. ^{13}CNMR and ^{1}HNMR studies revealed that aluminum citrate (Al-citrate) was metabolized intracellularly and that oxalic acid was an important product in the Al-stressed cells. This dicarboxylic acid was produced via the oxidation of glyoxylate, a precursor generated through the cleavage of isocitrate. In the control cells, citrate was biotransformed essentially with the aid of regular tricarboxylic cycle (TCA) enzymes. However, these control cells were able neither to uptake nor to metabolize Al-citrate. Al-stressed cells obtained at 38–40 h of growth showed maximal Al-citrate uptake and biotransforming activities. At least a fourfold increase in the activity of the enzyme isocitrate lyase (ICL, E. C. 4.1.3.1) has been observed in the Al-stressed cells compared with the control cells. The transport of Al-citrate was sensitive to *p*-dinitrophenol and sodium azide, but not to dicyclohexylcarbodiimide. Experiments with the dye 9-aminoacridine revealed that the translocation of Al-citrate led to an increase in intracellular pH. Thus, it appears that after the uptake of Al-citrate, this complex is metabolized intracellularly.

Although aluminum is the most abundant metal on the earth crust, there has as yet been no demonstrable utilization of this trivalent metal in any biological functions [11]. It appears that living organisms may have evolved by obviating the need to incorporate this element in any essential biochemical reactions, most probably as a consequence of its toxic properties. Indeed, aluminum has been found to be toxic to a wide variety of organisms. This problem has been further compounded owing to an increased bioavailability of aluminum triggered by industrialization and acid rain [20].

The presence of organic acids such as citrate, succinate, malate, and humic acids in the environment may also mobilize the metal and allow its easy access to biotic systems [7, 10]. Our laboratory has been investigating the interaction of the soil microbe, *Pseudomonas fluorescens*, and Al-citrate as the sole carbon source. The trivalent metal is apparently processed intracellularly and is eventually deposited as a residue in association with phosphatidylethanolamine (PE) and oxalic acid [2, 3, 13]. The transport of numerous metals in various cellular systems is a well-studied subject and appears to necessitate the participation of a wide variety of proteins that facilitate the passage of the metallic elements across the membrane barriers [22]. Metals are usually translocated either as metal ions or as organic complexes. Iron (III), for instance, enters numerous organisms associated with siderophores. A ferric siderophore receptor in the outer membrane delivers the substrate in an energy-dependent manner to the periplasm that eventually crosses the cytoplasmic membrane. Following reduction or degradation of the siderophore-metal complex, the iron is released [8]. Aluminum, a toxic metal that is known to mimic iron (III), has been shown to gain access into cellular systems via hydroxamates. These Al complexes enter the cell with the aid of the siderophore-receptor uptake system [15]. In our study, as citrate is the only source of carbon, the organism is faced with the following possibilities if it is to survive: a) metabolize the Al-citrate intracellularly; b) degrade the Al-citrate extracellularly; and/or c) liberate the citrate from the aluminum prior to the entry of the tricarboxylic acid into the cells. It appears that the intracellular decomposition of the Al-citrate is the preferred strategy invoked in this instance [3]. In this report, we describe the degradation

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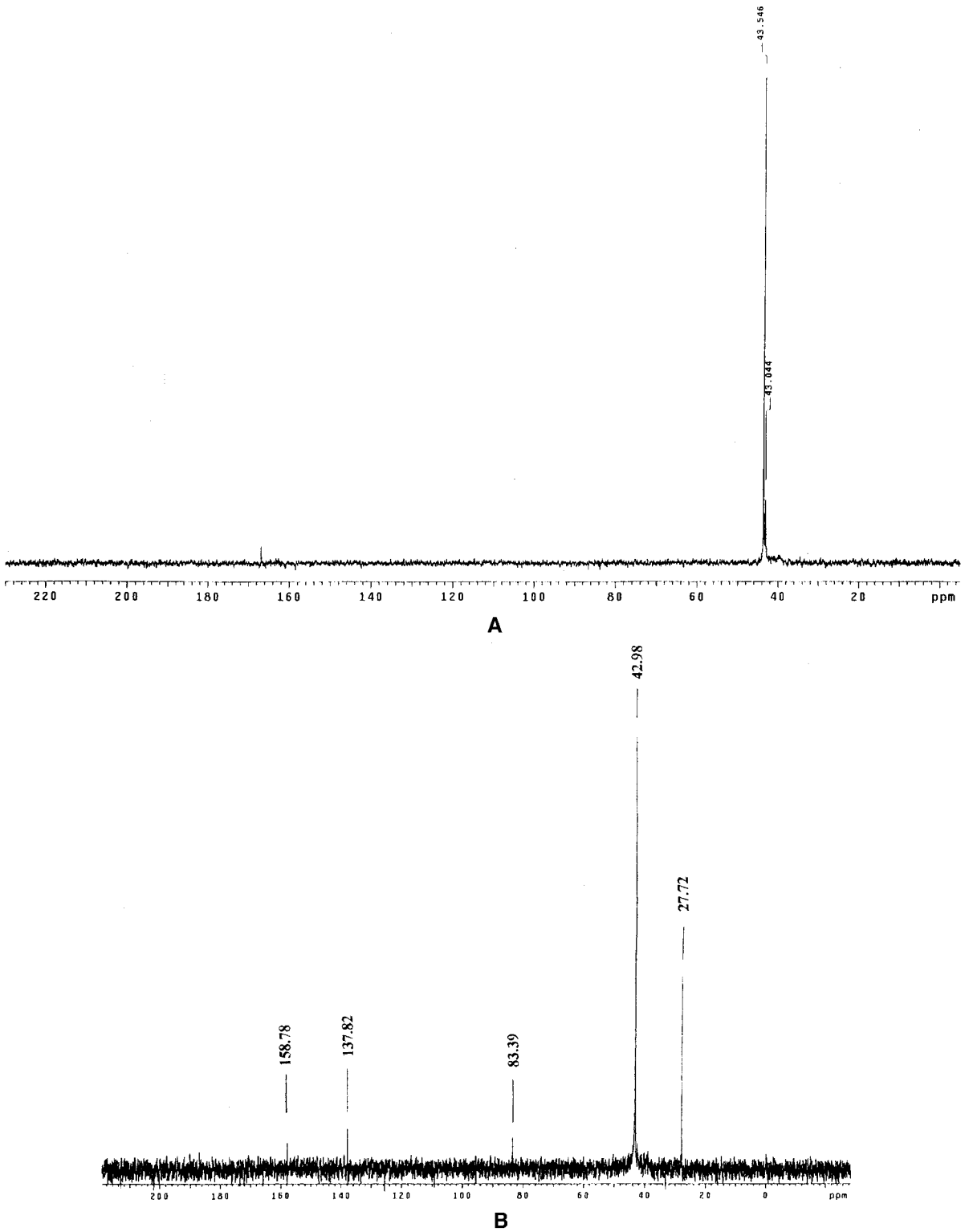

Fig. 1. ^{13}CNMR spectra obtained from *Pseudomonas fluorescens* grown under various conditions: (A) Control cells incubated with Al-citrate (2,4 $^{13}C_2$) for 30 min. (B) Al-stressed cells incubated with Al-citrate (2,4 $^{13}C_2$) for 10 min. Spectrum of the soluble fraction was obtained after sonication. (Note the presence of the citrate peak at 43 ppm.)

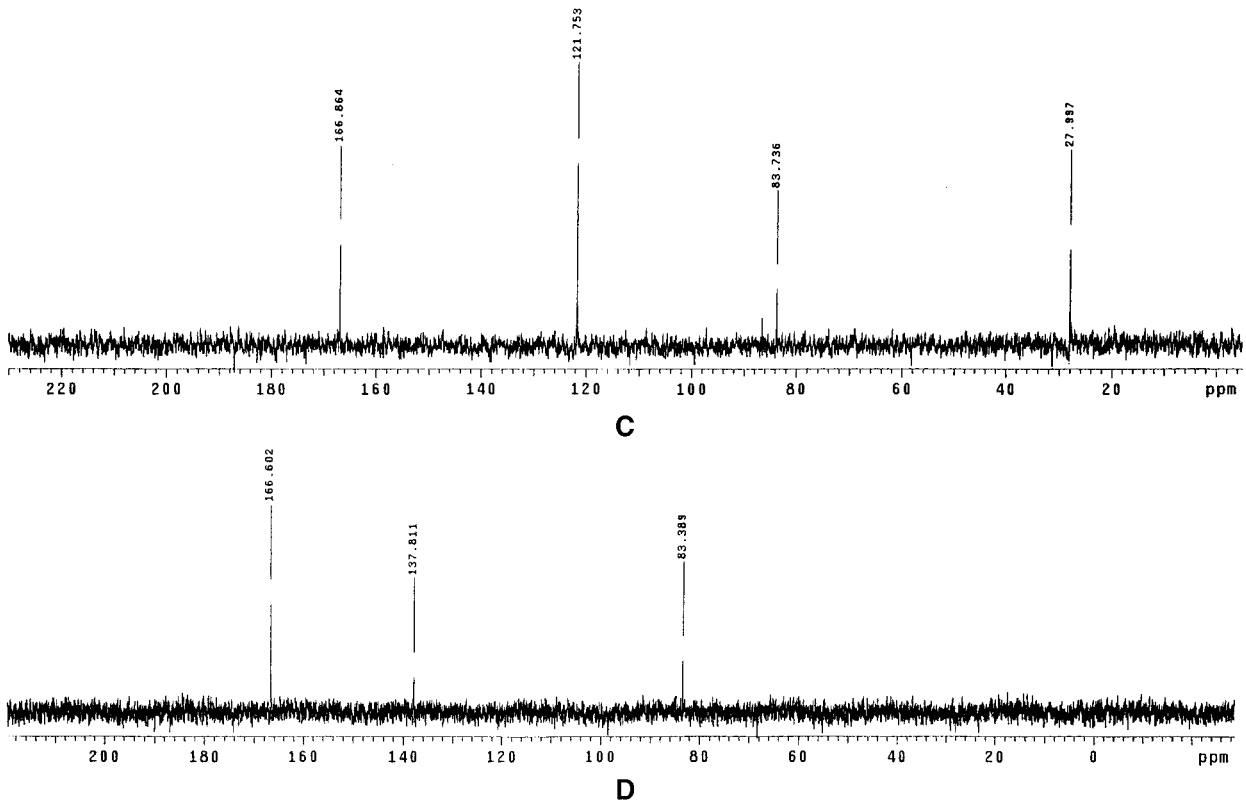

Fig. 1. Continued. (C) Al-stressed cells incubated with Al-citrate (2,4 $^{13}C_2$) for 60 min. Spectrum of the sonicated cells. (Note the complete disappearance of the citrate peak at 43ppm.) (D) Al-stressed cells incubated with Cr-citrate (2,4 $^{13}C_2$) for 60 min. Spectrum of the sonicated cells.

of this substrate, preferentially to oxalate, and the transport system effecting the translocation of Al-citrate is also discussed. The control cells are unable to either uptake or metabolize Al-citrate.

Materials and Methods

Bacterial strain and cultivation. *Pseudomonas fluorescens* ATCC 13525 was obtained from the American Type Culture Collection and grown in a mineral medium containing Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NH_4Cl (0.8 g), $MgSO_4$. $7H_2O$ (0.2 g), and citric acid (4.0 g) per liter of deionized distilled water. Trace elements were present in concentrations as previously described [1]. Aluminum chloride was complexed to citric acid in a 1:1.26 ratio, i.e., 15 mM aluminum to 19 mM citric acid. The pH was adjusted to 6.8 with dilute NaOH, and the media were dispensed in 200-mL amounts in 500-mL Erlenmeyer flasks. This was then inoculated with 1 mL of stationary-phase cells grown in a medium unamended with the test metal and aerated on a gyratory waterbath shaker, model 76 (New Brunswick Scientific) at 26°C at 140 rpm. The Bradford protein assay was performed in order to determine the amount of cells in the culture by measuring solubilized cellular proteins, and bovine serum albumin was used as the standard [6]. The cells were treated with 1 M NaOH in order to afford the solubilized proteins.

Determination of maximal Al-citrate uptake by intact Al-stressed cells. Cells obtained at various incubation periods from the mineral medium with 15 mM Al were harvested, washed twice with 0.85% NaCl, and resuspended in a Tris buffer (25 mM) pH 6.8. Whole cells (3.0 mg of protein equivalent) were used for the transport assays. Typically, the cells were incubated in a starvation buffer consisting of 25 mM Tris buffer with 0.8 mM $MgCl_2$ for 30 min at 26°C, and the uptake was initiated by the introduction of Al-citrate (1 mM Al:1.26 mM citrate and/or Al 1:1 citrate). The cells were separated from the uptake buffer after 1–30 min of incubation by centrifugation and washed with EDTA (1 mM) to eliminate any fortuitously bound Al (this treatment assured the removal of most of the loosely bound Al without affecting the transport process). Citrate in the supernatant was monitored enzymatically [19]. Following nitric acid digestion, Al was measured by the aluminum assay [14] and by an atomic absorption spectrophotometer model 703 from Perkin-Elmer. All experiments were run in triplicate, and as the cells obtained at 40 h of growth did show maximal Al-citrate translocation profile, they were subsequently subjected to further investigation. Cells harvested in the same citrate medium without added Al at various growth periods were used as controls, as were heat-inactivated (50°C) Al-stressed cells.

Characterization of Al-citrate transport system. The effects of pH on the transport of Al-citrate were investigated in *Pseudomonas fluorescens* isolated from the Al-stressed medium. The pH of the phosphate buffer (25 mM) ranged from 5.8 to 8.2. The cells were also subjected to different temperatures and were heated to 50°C or cooled to 4°C for 30 min, and their ability to transport Al-citrate was evaluated. The cells were initially incubated in the presence of such inhibitors as sodium azide, NaN_3 (2 mM), chloramphenicol (1 mM), dicyclohexylcarbodiimide (DCCD) (1 mM), *p*-dinitrophenol (DNP) (2 mM), and $HgCl_2$ (0.1

mM) for 30 min respectively prior to the evaluation of their Al-citrate uptake ability. The change in intracellular pH was probed with the aid of 9-aminoacridine, a membrane-permeable dye that is known to have different fluorescent characteristics at various pHs. The cells were treated with 9-aminoacridine (4–8 nM) for 30 min, and uptake experiments were initiated by the addition of Al-citrate. Cells that were subjected to the fluorescent dye and incubated in the absence of Al-citrate served as controls. Fluorescence was measured (emission wavelength 455 nm) with the luminescence spectrometer LS from Perkin-Elmer. The influence of other citrate-metal complexes such as Fe^{3+}, Cr^{3+}, Ni^{2+}, and Cu^{2+} was also evaluated. A 1:1 metal-citrate complex was used. Isocitrate lyase activity was measured with 2,4-dinitrophenylhydrazine (DNPH) as described in [12].

Al-citrate metabolism: ^{13}C and ^{1}HNMR studies. Cells (200 μg soluble protein equivalent) obtained at late logarithmic phase (40 h in Al-citrate and 24 h in citrate media, as these times correspond to similar growth phases [4]), were suspended in a fresh Al or Cr-^{13}C-labeled citrate (2, 4-$^{13}C_2$) medium devoid of any source of nitrogen. After incubation for 10–60 min, the cells were harvested, sonicated, and ^{13}CNMR spectra were obtained on a Varian Gemini 200 MHz spectrometer operating at 50.38 MHz for ^{13}C. Chemical shifts were referenced to shifts of standard compounds obtained under the same conditions. ^{1}HNMR spectra were also obtained from the soluble fractions of the Al-stressed and control cells incubated with Al-citrate for 10 min. The ICL activity was studied as follows: the soluble fraction (200 μg) (control and Al-stressed cells) was incubated with isocitrate (2 mM), and the formation of succinate and glyoxylate was monitored. The reaction mixture was freeze dried and solubilized in 10% D_2O. The use of the homodecoupler allowed for the suppression of the H_2O peak. The parameters were adjusted as follows: continuous modulator, delay 0, pulse 2, acquisition time 1, pulse width 90°, decoupler low power to 2000, and the number of transients to 5, which was subsequently changed to 200, following the suppression of H_2O.

Results

Al-citrate was readily degraded by Al-stressed cells, but not by cells not a priori exposed to this toxicant. Intact cells appeared to produce oxalic acid as an important constituent. At 10 min of incubation, the soluble fraction revealed a major peak at 43 ppm indicative of the unreacted Al-citrate. The peaks at 158.8 ppm and 83.4 ppm have been attributed to free oxalate and glyoxalate, respectively. The signal at 137.8 ppm has not been characterized yet. However, after 1 h of incubation, the citrate peak disappeared. A peak at 166 ppm indicative of Al-oxalate was observed, as were resonances at 27 ppm and 83 ppm that were assigned to the (CH_2) of succinate and (CHO) of glyoxylate, respectively. Succinate, glyoxylate, and oxalate were further confirmed by enzymatic assays [9, 25, 26]. The control cells did not metabolize Al-citrate when intact cells were incubated with 5 mM of this substrate. In the presence of this carbon source, only a 43-ppm peak, indicative of the unreacted ^{13}C-labeled citrate (2,4-$^{13}C_2$), was observed in the supernatant (Fig. 1A–C). The ^{1}HNMR spectrum revealed a peak indicative of citrate and its degraded products in the soluble fraction of Al-stressed cells, while no peak was observed in the soluble fraction of the control cells exposed to Al-citrate. Indeed, the Al-citrate was localized in the supernatant (Fig. 2). These findings pointed to the intracellular decomposition of Al-citrate and led us to probe other metal complexes. Cr bound to citrate (1:1) was also metabolized by the cells. Peaks at 83 ppm, 137 ppm, and 166 ppm were observed. No citrate peak was observed as this moiety was rapidly degraded (Fig. 1D). As succinate and glyoxylate were key products of this metabolism, the different cellular fractions were incubated with isocitrate in order to localize and monitor the activity of isocitrate lyase. This enzyme was restricted to the cytoplasmic compartment and had a fourfold higher activity than the cells obtained in the control cultures (cells in late logarithmic phases were utilized in both cases). The control cells were unable to deal with Al-citrate. ^{1}HNMR spectroscopy was utilized to monitor the degradation of isocitrate. The peak at 2.0 ppm, indicative of succinate, was readily discerned in the soluble fraction

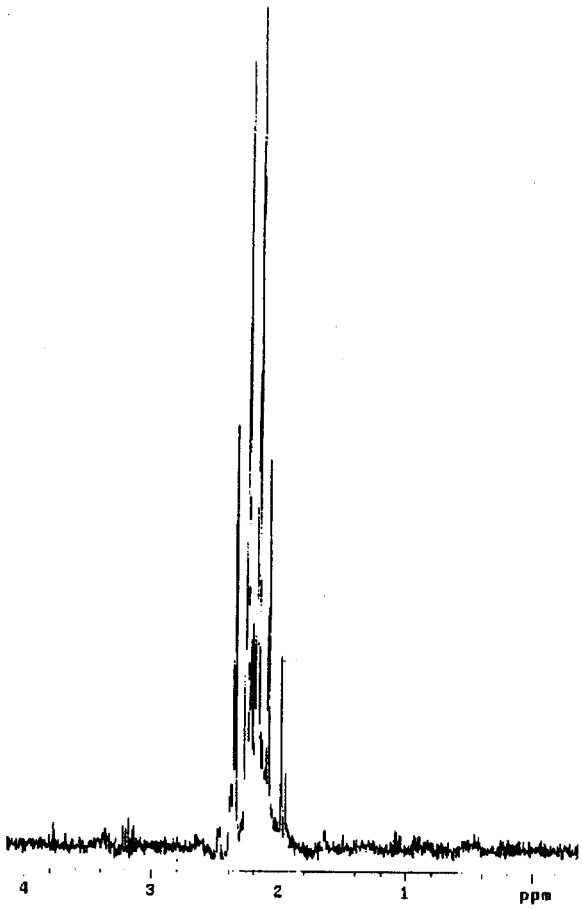

Fig. 2. ^{1}HNMR spectrum of the soluble fraction obtained from Al-stressed cells exposed to Al-citrate for 10 min. (Note: no peak was discerned in the soluble fraction from the control cells.)

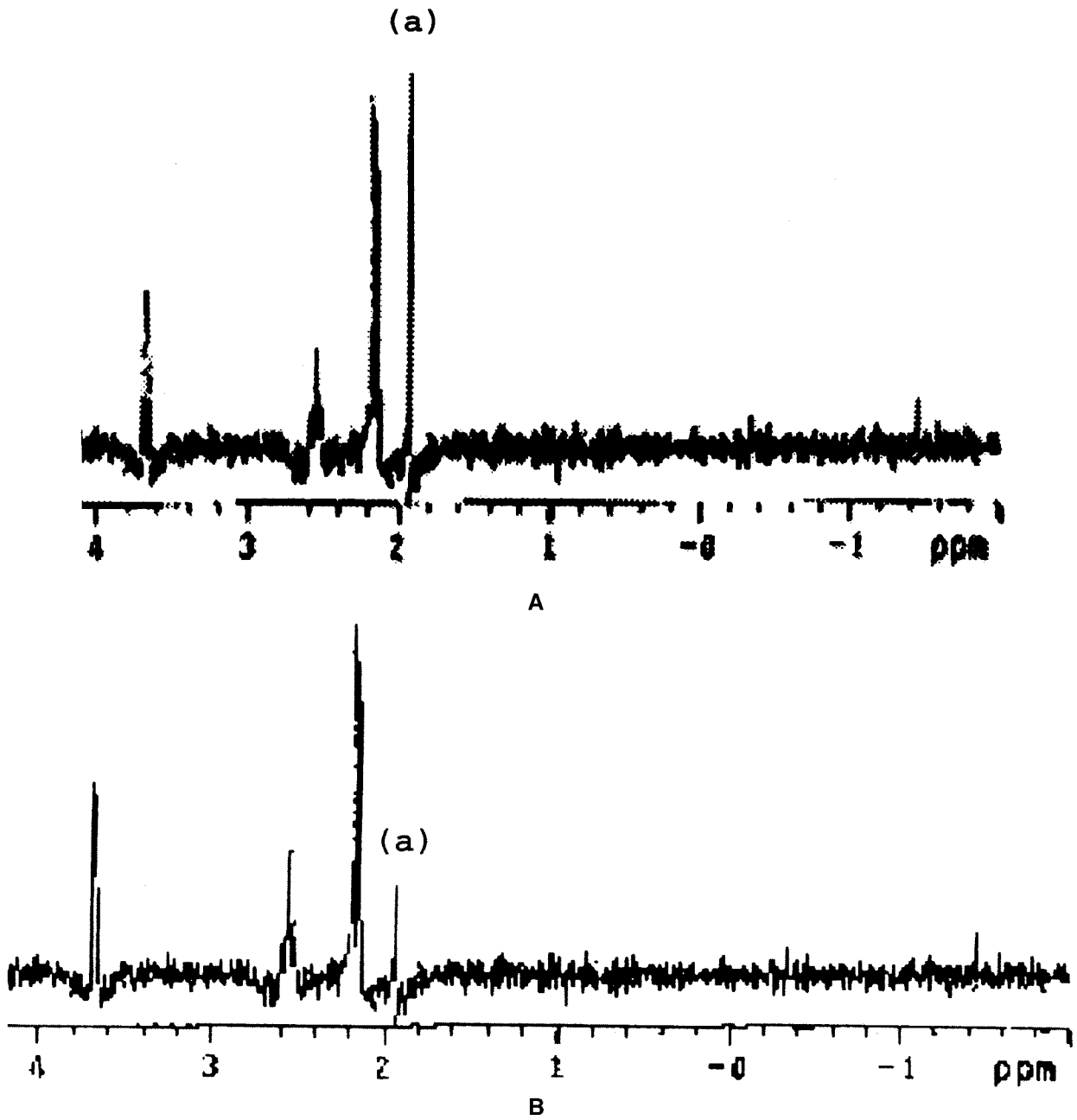

Fig. 3. ^{1}HNMR spectra of soluble fraction from control and Al-stressed cells incubated with 2 mM isocitrate. (A) Al-stressed soluble fraction. The appearance of a 2.0-ppm peak (a) is indicative of succinate formation. (B) Control soluble fraction [succinate (a) formation is slow].

from the Al-stressed cells. This reaction was slow in control cells. The glyoxylate reasonance was not evident as the D_2O peak was visible in the same region (Fig. 3).

The maximal entry of Al-citrate occurred within 5 min of incubation and was optimal in cells obtained after 40 h of growth (Fig. 4). This process was pH dependent, showing an optimal uptake at pH 7.2 and a fourfold diminution at pH 5.8. Its sensitivity to temperature was demonstrated by marked reduction in activity at both 4°C and 60°C. The transport system that facilitated the entry of Al-citrate in *Pseudomonas fluorescens* was also markedly affected by DNP and NaN_3. The former moiety is known to dissipate the proton-motive force, while the latter impedes energy production. DCCD, an inhibitor of ATP synthesis, did not appear to have any appreciable impact on the Al-citrate-translocating machinery of this

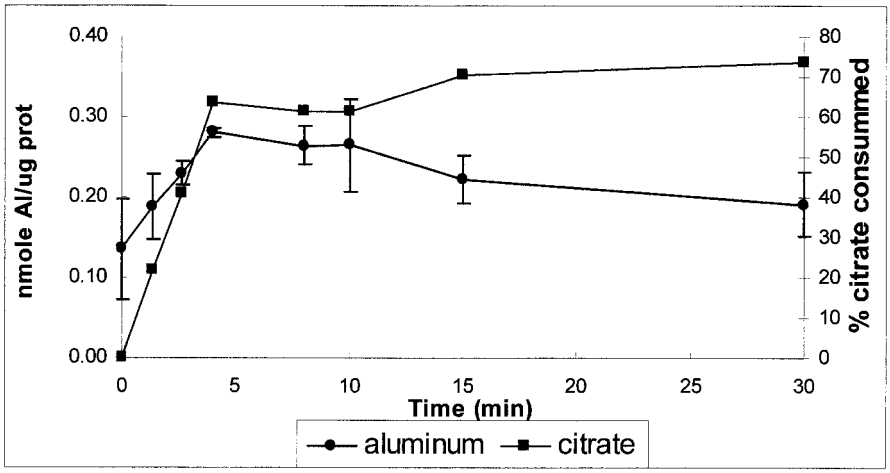

Fig. 4. Al uptake and citrate utilization in cells obtained at 40 h of growth in an Al-supplemented medium.

Table 1. Effects of inhibitors on Al-citrate transport in *Pseudomonas fluorescens*

Inhibitors	Activity (%)
Control (no inhibitors)	100
DNP	38.8
$HgCl_2$	75.7
NaN_3	55.2
DCCD	100
Chloramphenicol	100

Standard deviations of four different experiments ranged from 4% to 7%. (100% represents 0.27 μmol Al mg^{-1} protein equivalent of cells.)

microbe (Table 1). While a 25% reduction of Al-citrate uptake was observed in the presence of $HgCl_2$, no inhibition was recorded when chloramphenicol was included in the assay mixture. 9-Aminoacridine, a membrane-permeable dye that fluoresces in an alkaline environment, was utilized to observe the change in intracellular pH. Following the entry of Al-citrate, an increase in pH was observed, as evidenced by elevated fluorescent activity of the dye. The fluorometric studies with 9-aminoacridine revealed internal alkalinization after the entry of Al-citrate. The Al-stressed cells were ineffective in dealing with citrate bound to divalent metals like Cu and Ni. In this instance, only trace amounts of Ni or Cu were detected (Fig. 5).

Discussion

The foregoing data suggest that *Pseudomonas fluorescens* degrades Al-citrate intracellularly. The resting Al-stressed cells readily metabolized the metal-citrate complex, while control cells were not responsive to this carbon source. When exposed to Al-citrate, the microbe appeared to produce glyoxylate, succinate, and oxalate, as evidenced by the respective labeled ^{13}CNMR resonances of these compounds. The control cells did not respond to Al-citrate, as only a peak at 43 ppm (the unreacted Al-citrate) was observed in the supernatant only. The intracellular localization of the citrate peak at 10 min of incubation and its disappearance at 60 min would indicate that Al-citrate was degraded after its uptake. Thus, it is quite evident that only the cells exposed to Al-citrate were able to metabolize this carbon source. The formation of oxalate allows for the sequestration of Al and its eventual exocellular deposition. Oxalate has been shown to detoxify Al by rendering the trivalent metal biologically unavailable [17]. However, its de novo production, triggered by Al, has not been reported. In this study, it appears that glyoxylate and oxalate are two important metabolites resulting from the metabolism of Al-citrate. The production of glyoxylate is mediated by ICL. A fourfold increase that has been observed [12] was easily followed by ^{1}HNMR by the appearance of the succinate peak. Thus, the adaptation to Al-citrate appeared to be aided by an increase in activity of ICL and a glyoxylate-oxidizing enzyme [12, 13].

Since Al-citrate was being processed intracellularly, it was important to elucidate how the cell was acquiring its sole carbon source. It is evident that the control cells, i.e., cells that have not seen Al-citrate, could neither translocate nor metabolize this substrate. Hence, the microbe subjected to Al-citrate appeared to be acclimatized to this compound. Indeed, the Al-stressed cells did translocate Al-citrate. As this process was sensitive to DNP, it is not unlikely that this transport system is being fueled by a proton-motive force and not by the hydrolysis of ATP. Its insensitivity to DCCD, an inhibitor of ATP synthesis, would indicate that the translocation of Al-citrate was not dependent on ATP hydrolysis. Further-

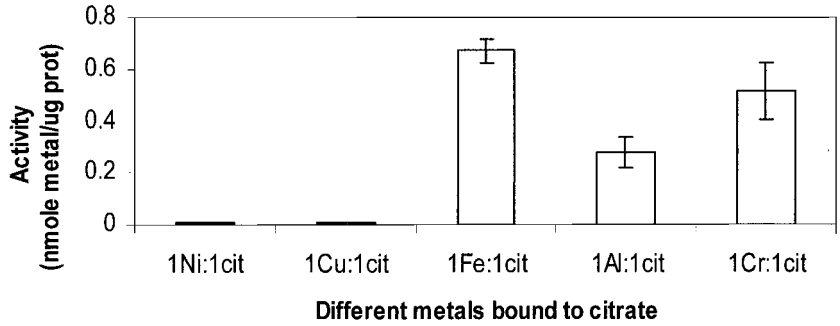

Fig. 5. Uptake of citrate-metal complexes in Al-stressed *P. fluorescens*. (Note: no citrate-metal complex transport was observed in the control cells.)

more, the inability of chloramphenicol to inhibit this transport process would indicate that de novo protein synthesis in the 40-h cells is not an important modulator of this uptake phenomenon, i.e., the cells have all the proteins needed to mediate this process. The entry of Fe (III)-citrate in various cellular systems involves the participation of such proteins as Fec A, Ton B, Exb B, Exb D that are energized by a proton-motive force. The metal-citrate complex then traverses the inner membrane with the aid of an ATP-binding cassette transporter [8]. The transport of the free citrate in some cells is driven by the proton-motive force, while in other organisms citrate uptake is coupled to the entry of Na^+ or with the efflux of lactate [16, 23, 24]. In some instances, a citrate/succinate antiporter helps shuttle the tricarboxylic acid in the cells, and the positive role played by metals like Mg^{2+} in the transport of citrate has also been documented [5, 21]. Siderophore-mediated iron uptake does also invoke the function of a specific receptor on the outer membrane and an electrochemical potential [8]. In this study, it is important to note that this uptake system appears to be operative only in the Al-stressed cells, as the control cells did not effect the translocation of Al-citrate. This may be a crucial adaptive feature that allows the organism to acquire its source of energy. In the absence of any extracellular citrate-lyase-like activity or the diminution of pH that may enable the liberation of citrate from the metal toxicant, the microbe is compelled to resort to this Al-citrate import machinery [13]. The presence of a citrate peak in the soluble cellular fraction would indicate such a possibility. It is unlikely that the same set of proteins is involved in the uptake and/or processing of citrate and Al-citrate, since the cells not subjected to aluminum stress did not accumulate the trivalent metal. Furthermore, Al-stressed cells readily metabolized Fe-citrate (1:1) and Cr-citrate (1:1), while citrate complexes of Cu and Ni respectively were not biodegradable. This may point to the preference of this system to acquire and metabolize trivalent metal complexes.

An initial intracellular increase of aluminum followed by a subsequent diminution may be necessary owing to the toxicity of the metal. It is not likely that, as aluminum reaches a critical toxic concentration and owing to space limitation in this cellular model, the metal is expelled. Indeed, when cells containing aluminum were incubated with citrate, aluminum was released with the concomitant utilization of the tricarboxylic acid (personal observation). Thus, the degradation of citrate appears to effect the efflux of Al from the cells. The inhibition observed in the $HgCl^{-}_{2}$-treated cells may be indicative of a pivotal role for sulfhydryl groups in this Al-citrate uptake process, while the negative impact of NaN_3 confirms the necessity of a source of energy for the entry of this complex into the cells. The decrease in the transport of Al-citrate at lower pH may be due to the fact that the speciation between aluminum and citrate may be pH-dependent, thus preventing its access to the cells. The other possibility, that moieties affecting the transport process may be sensitive to acidic pH, may also be operative. Indeed, the speciation of Al-citrate complexes is known to vary with pH [18].

The data from this study demonstrate that the adaptation of *Pseudomonas fluorescens* to Al-citrate is mediated by its ability to translocate and degrade this moiety. The uptake of Al-citrate appears to be energized by a proton-motive force, while its metabolism results in the production of oxalate, a key ingredient in the detoxification of the trivalent metal. The generation of glyoxylate and succinate, through isocitrate cleavage, provides the necessary precursor(s) to sustain this process. Work on the enzyme(s) participating in this metabolic route(s) is currently in progress.

ACKNOWLEDGMENTS

This work was supported by funding from Industry Canada and Human Resources Development Canada.

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