Adaptative response and degradation of quaternary ammonium compounds by *Pseudomonas putida* A ATCC 12633

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Quaternary ammonium compounds (QACs) are commercially available chemicals commonly used in a variety of products such as cosmetics, antiseptic solutions, textile finishes, and fabric softeners. Released into the environment, the biodegradability of QACs may be limited by their antimicrobial activity. However some species of bacteria, notably strains of *Pseudomonas*, have high resistance to QACs and are thought to be responsible for biodegradation in activated sludge. Here, we show how tetradecyltrimethylammonium bromide (TTAB), a representative of QACs, might be degraded by *P. putida* A ATCC 12633. We also establish that the response to TTAB involves changes in the membrane phospholipids composition.

P. putida A ATCC 12633 uses TTAB as a sole carbon, nitrogen, and energy source. The TTAB degradation is initiated by N-dealkylation catalyzed by a monooxygenase activity resulting in the formation of tetradecylalkanal and trimethylamine (TMA). The TMA produced is used by *P. putida* A ATCC 12633 as a nitrogen source through trimethylamine monooxygenase activity and is also accumulated inside the cell, decreasing the bacterial growth an effect counteracted by the addition of $AlCl_3$. In the presence of 0.1 mM $AlCl_3$, the intracellular concentration of free TMA is decreased by the formation of an Al^{3+}:TMA complex, and the TTAB is fully consumed without accumulation of undesirable compounds.

On the other hand, in *P. putida* A ATCC 12633, the adaptative response and resistance to the bactericidal activity of TTAB involves an initial mechanism, producing changes at membrane levels involving specific variations in the content of phosphatidic acid, phosphatidylglycerol, and cardiolipin. These modifications indicate that these phospholipids are involved in cellular responses to QACs, utilizing principally phosphatidic acid to neutralize the high positive charge density given for the ammonium quaternary moiety from TTAB. Also, when *P. putida* are grown with TTAB in the presence of $AlCl_3$, phosphatidylcholine increased. This indicate that this particular phospholipid may be involved in the bacteria response to binding Al^{3+} and allowing us to formulate a physiological role of phosphatidylcholine as a temporary reservoir of available Al^{3+}.

Keywords: *Pseudomonas putida*; cationic surfactant; aluminum.

1. Introduction

Surfactants are usually amphiphilic organic compounds commonly classified into four categories according to the formal charge present in their hydrophilic head: anionic, cationic, nonionic and amphoteric. For each classification, it is possible to further sub-classify them according to the functional group of their hydrophilic head. For the household industry, the most common cationic surfactants are those with quaternary ammonium groups [1]. Quaternary ammonium compounds (QACs) are molecules with at least one hydrophobic long alkyl chain attached to a positively-charged nitrogen atom. Among the QACs, the generic term Cetrimide relates to mixtures of n-alkyltrimethyl ammonium bromides where the n-alkyl group is between eight and 18 carbons long, whereas Cetrimide USP is tetradecyltrimethylammonium bromide (TTAB). Benzalkonium chlorides are always mixtures of n-alkyldimethylbenzylammonium chlorides, where the n-alkyl groups can be of variable length within a specified range. QACs have a wide-range of commercial and consumer uses such as detergents, antistatics, wetting and softening agents, biocides, germicides, deodorizers and emulsifiers. In quantitative terms, this utilization translates into mostly fabric softeners (66%), coated clays (16%), and biocides (8%) [2].After use, the residual product is discharged to sewage treatment plants or surface waters and finally to coastal waters. Released into the environment, the biodegradability of QACs is limited by their antimicrobial activity [3, 4].

As biocides, QACs are active against Gram-negative and Gram-positive bacteria and some viruses, fungi and protozoa [5, 6]. When bacteria are exposed to QACs, the following sequence of events has been proposed to occur: (i) adsorption of the cationic compound into the bacterial cell surface; (ii) diffusion through the cell wall; (iii) binding to the cytoplasmic membrane; (iv) disorganization of the cytoplasmic membrane; (v) release of cytoplasmic constituents; and (vi) death of the bacterial cell [6]. Thus, the bactericidal action of QACs is centred upon physical disruption and partial solubilization of the cell wall and membrane. However, several reports have described intrinsic and acquired resistance to these compounds, especially among Gram-negative species [7-9]. Some microorganisms are able to demonstrate intrinsic resistance through inactivation of the biocide, and this will assist in the removal of such agents from the environment. Consequently, some strains of *Pseudomonas* from the environment, including *Pseudomonas* sp. and *Pseudomonas fluorescens*, have high resistance to QACs and are thought to be responsible for biodegradation of QACs in activated sludge [10, 11].

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In this context, we investigated *Pseudomonas* strains for their adaptive response and resistance to the bactericidal activity of a representative QAC, TTAB. Because the resistance to antimicrobial agents can be due to enzymes transforming the biocide to a non-toxic form and this phenomenon is usually investigated from the biodegradation point of view, we also analyzed if TTAB might be degraded by a pure culture of different species from the *Pseudomonas* genus and whether factors in the medium influenced its biodegradability. Because the different *Pseudomonas* strains, which can be fluorescent or not, and plant or animal pathogens or saprophytes [12], *Pseudomonas putida* A ATCC 12633 which utilizes TTAB as its sole carbon, nitrogen and energy source when screened on a basal salt liquid medium (HPi-BSM) [13], was selected for the present study.

2. Adaptative response and resistance to the bactericidal activity of TTAB

2.1. Effects of TTAB on the growth of *P. putida* A ATCC 12633

The activity of antimicrobial agents is often quantified as the minimum concentration that is required to inhibit the growth of the target organisms (minimum inhibitory concentration or MIC) or as the concentration that leaves no detectable survivors after a specified contact time (minimum bactericidal concentration (MBC), generally taken as > 99.9% killing) [14]. The MIC detected for TTAB in *P. putida* A ATCC 12633, defined as the lowest concentration of the biocide that prevented an increase in turbidity at 660 nm after 48-72 h incubation at 30ºC [15], was 150 mg L^{-1}. Even though the intrinsic susceptibility of an organism to a biocide, documented from an MIC or MBC determination by standard methods, is not a fixed value, the MIC value detected for TTAB in *P. putida* A ATCC 12633 was similar to those reported for other QACs, such as benzalkonium chloride or dodecyl dimethylammonium chloride against other *Pseudomonas* [9, 16]. The viability of *P. putida* A ATCC 12633, tested at 100 mg L^{-1} of TTAB, indicated that the number of viable cells (cfu mL^{-1}) decreased 5 to 6 orders of magnitude, with respect to the initial number of bacteria. With 50 mg L^{-1} TTAB, the bacterial survival was more than 99% (Figure 1) and this concentration was used for further study.

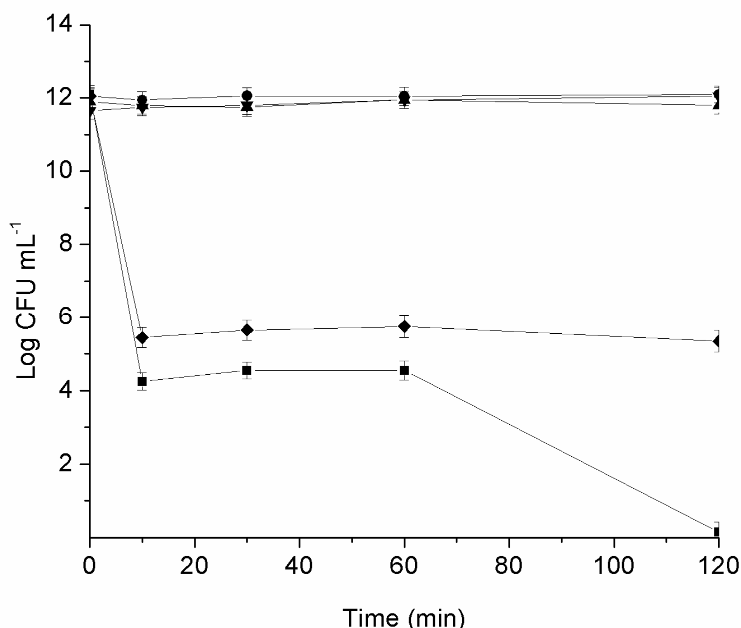

Fig. 1 Survival of *P. putida* A ATCC 12633 that was grown in HPi-BSM with 20 mmol L^{-1} glucose and 18.7 mmol L^{-1} NH_4Cl until the culture reached an OD_{660} of 1. At this point the culture was divided, and the following TTAB concentrations were added: (●) 0, (▲) 10, (▼) 50, (♦) 100 and (■) 150 mg L^{-1}. The number of viable cells (cfu mL^{-1}) were determined, at the indicated times, by plating the serially-diluted cell suspension on LB plates. Values are the means ± SD of three independent experiments.

P. putida A ATCC 12633 also exhibited tolerance to the highest concentration of TTAB (280 mg L^{-1}) after successive subculturing in the presence of progressively higher concentrations of TTAB. For adaptation to growth in the presence of TTAB, the first range of concentrations used was 10-30 mg L^{-1}. Bacteria showing growth after 24-72 h in the presence of this concentration were used to inoculate the next culture series (increasing by 10 mg L^{-1} for each step). The same adapted bacteria lost the increased QAC resistance after two sub-cultures without TTAB, followed by a higher growth rate; the MIC remained unchanged (150 mg L^{-1}) [17]. This reversible adaptive response of *P. putida* A ATCC 12633 to the bactericidal activity of TTAB, which is dependent on the presence of the biocide, seems to be a characteristic of bacterial adaptation [18].

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2.2. Growth of *P. putida* A ATCC 12633 with TTAB as the sole carbon and nitrogen source

Because some microorganisms are able to demonstrate intrinsic resistance through inactivation of a biocide and this will assist in the removal of such agents from the environment, we tested the growth of *P. putida* in the presence of TTAB as the sole carbon (C) and nitrogen (N) source. *P. putida* A ATCC 12633 was grown with 50 mg L^{-1} of TTAB, and after 48 h, the cells entered in a stationary phase and growth ceased. To gain definitive evidence of the metabolism of TTAB, the correlation between growth yield and QAC consumption was determined. TTAB consumption was analyzed by a colorimetric method based on the reaction of TTAB found in the culture supernatants with bromothymol blue [19], and the growth was expressed by the protein content determined as previously described [20]. The growth yield for the biodegradation process was 0.22 mg protein mg $TTAB^{-1}$. The net increase in cell protein when the culture reached the stationary phase was 6.27 mg L^{-1}, and the TTAB consumption was 28.5 mg L^{-1}. Because the average N content of proteins is about 15%, the N incorporated into the cell biomass was 0.94 mg L^{-1}. This represented about 79 % of the initial N-TTAB incorporated into the cell biomass. The rest of the initial N (21% of the total) was not incorporated in protein synthesis and was found as trimethylamine (TMA), which accumulated with time [21].

2.3. Experimental approach to combat a stress situation

The maximal growth of *P. putida* A ATCC 12633 is clearly not optimal when TTAB is available as the sole carbon and nitrogen source, and the low biomass obtained was attributed to an accumulation of intracellular TMA in the cell after initial TTAB oxidation. Considering that TMA is a Lewis base, we hypothesized that the addition of a Lewis acid, such as $AlCl_3$, should diminish the intracellular TMA concentration through the following reaction: $Al^{3+} + \mathbf{:}N(CH_3)_3 \rightarrow Al^{3+}\mathbf{:}N(CH_3)_3$. As shown in Figure 2, the addition of 0.1 mM $AlCl_3$ to the *P. putida* A ATCC 12633 culture after 48 h of growth with TTAB, increased both the bacterial growth and TTAB consumption, and the free-TMA intracellular concentration decreased to approximately zero by the formation of a soluble Al^{3+}:TMA complex [21]. For these experiments, the intracellular TMA concentration was determined by fluorescence using the fluorochrome 2´,3,4´,5,7-pentahydroxyflavone (morin reagent) with the addition of $AlCl_3$. Morin reacts with Al^{3+} to form a fluorescent complex, Al^{3+}:morin [22], and in the presence of compounds that sequester Al^{3+}, Al^{3+}:morin is not formed [23, 24]. We determined that in the presence of TMA, the fluorescence intensity of the Al^{3+}:morin complex decreased linearly with increased TMA concentrations, providing evidence for the formation of an Al^{3+}:TMA complex. Consequently, the concentration of TMA was calculated by calibration graphs constructed by plotting the fluorescence intensity of the Al^{3+}:morin complex versus TMA concentration, and the intracellular concentration of free TMA in the cells was calculated by taking the difference between the total TMA detected by GC-MS in the cellular extracts, and the TMA present in the Al^{3+}:TMA complex [21].

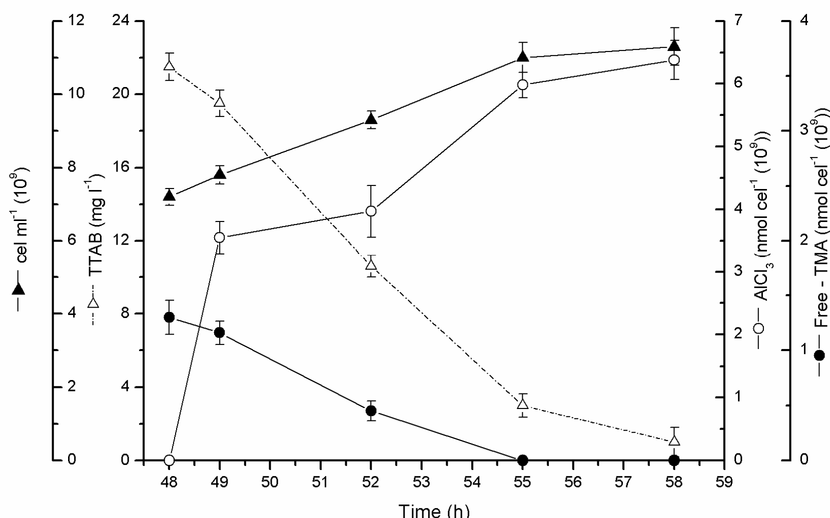

Fig. 2 Growth (▲) and TTAB consumption (Δ) of *P. putida* A ATCC 12633 in HPi-BSM with 50 mg L^{-1} TTAB as the carbon and nitrogen source**.** After 48 h, the culture was divided, and growth and consumption continued in the presence of $AlCl_3$ 0.1 mmol L^{-1}. $AlCl_3$ (○) and free-TMA (●) were measured in resuspended cells. Values are the means ± SD of three independent experiments.

Thus, the utilization of Al_3Cl was a good experimental approach to combat a stressful growth situation and allows the bacteria to better and more efficiently utilization TTAB. It is also possible to postulate that the intracellular increase of any substituted or non-substituted ammonium ion (e.g. alkylammonium ions, amino sugars, amino acids, phosphatidylethanolamine (PE), etc.) may be useful bacterial strategies to survive by sequestering ionic species of Al^{3+}. Although Al^{3+} is known to interfere with the normal functioning of most cellular systems, numerous organisms have

elaborate strategies to combat metal stress, such as active efflux, intracellular sequestration, volatilization, and biotransformation [25, 26]. Hence, the formation of an Al^{3+}:TMA complex can also enable bacteria to overcome the possible damage caused by aluminium [21].

2.4. Changes in phospholipid composition in *P. putida* A ATCC 12633 induced by TTAB

The mode of action of QAC against bacterial cells is thought to involve a general perturbation of lipid bilayer membranes that leads to a generalized and progressive leakage of cytoplasmic materials to the environment. Low concentrations of QAC bind firmly to anionic sites found on the membrane surface, causing cells both to lose osmoregulatory capability and to leak potassium ions and protons [27]. Intermediate levels perturb membrane-located physiologies such as respiration, solute transport, and cell wall biosynthesis [28]. The high concentrations used in many biocidal formulations however, kill cells by solubilization of the membranes to release all of the cells contents, hence their designation as biological detergents [29]. It has been demonstrated in different microorganisms that the adaptation to grow in high concentrations of QAC is followed by changes in fatty acid composition [8, 15, 30, 31], changes in the acidic phospholipid content of the membrane [32] or with the acquisition or hyperexpression of multi-drug efflux pumps [31, 33, 34].

The reversible adaptive response and the resistance of *P. putida* A ATCC 12633 to the bactericidal activity of TTAB involve an initial mechanism producing changes in the membrane involving the phospholipid composition. In Pseudomonas there are three main phospholipids: phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) [35]. Although their ratio is influenced by the growth phase, PE is the most abundant of the total phospholipid content; PG is most abundant in cells in the logarithmic phase, whereas CL accumulates in stationary-phase cells [36, 37]. In the absence of TTAB, the phospholipid composition of *P. putida* A ATCC 12633 was similar to that described for other fluorescent *Pseudomonas* cultivated in non-stressed conditions, such as *P. aeruginosa* [38]; *P. putida* DOT-T1 [36]; *P. putida* Idaho (solvent-tolerant) and *P. putida* MW1200 (solvent-sensitive) [39]. In the long-term response of *P. putida* A ATCC 12633 to TTAB and in TTAB-adapted bacteria, phosphatidic acid (PA) and PG increased and CL decreased. The changes in adapted cells reverted after two subcultures without the biocide (Figure 3). Since in the presence of the biocide PG and PA showed the greatest changes with respect to non-treated cells, it is possible that these phospholipids, by their anionic characteristics, were initially and consecutively damaged by TTAB and need to be replaced more rapidly. Although at present how these changes are interrelated has not been studied because the amount of CL decreases, it is possible that the turnover of CL might be an efficient method to replenish PG and PA pools, through the action of a phospholipase-hydrolyzing CL, such as the phospholipase D enzyme present in Gram-negative bacteria, such as *E. coli*, *Pr. vulgaris*, and *P. aeruginosa* [40]. Alterations in the membrane could activate this enzyme, resulting in the release of PA and PG, which would maintain the cell envelope integrity. Independent of the phopholipases acting on different phospholipids species, it is clear that the major bacterial response in TTAB-containing media is to produce a highly negatively-charged membrane, exemplified here as an increase in the anionic phospholipids, PA and PG, principally to neutralize the high positive charge density given for the ammonium quaternary moiety from TTAB [17, 41].

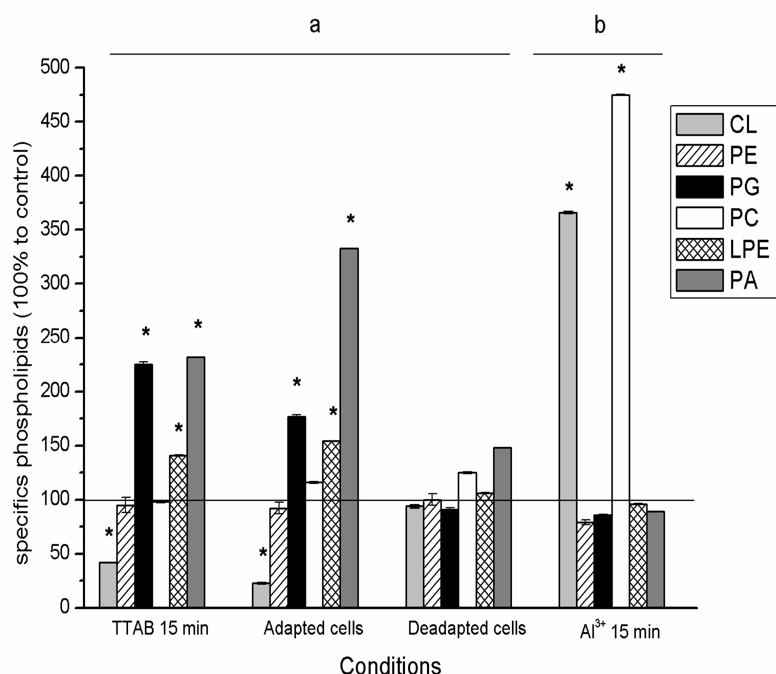

Fig. 3 Effect of TTAB and Al^{3+} on phospholipid composition of *P. putida* ATCC 12633. TTAB 15 min: Cells grown with glucose and NH_4Cl and exposed to TTAB for 15 min. Adapted cells: Cultures grown with glucose and NH_4Cl containing gradually increasing concentrations of TTAB (280 mg l^{-1}). De-adapted cells: Adapted cells were subcultivated twice without TTAB. Al^{3+} 15 min: Cultures grown with TTAB and exposed to $AlCl_3$ for 15 min. Results are expressed as percentages of the specific PL compared to 100% content of the PL in control: a) cells grown with glucose/NH_4Cl and b) cells grown with TTAB. Values are the means ± SD of three independent experiments.* Significantly different from the values obtained in control conditions ($p<0.005$).

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2.5. Physiological role of phosphatidylcholine in the *P. putida* A ATCC 12633 response to TTAB and Al^{3+}

The Al^{3+} in the membrane can be visualized using morin, which becomes fluorescent when it reacts with aluminium. In *P. putida* A ATCC 12633 exposed to Al^{3+}, a high level of fluorescence intensity was visualized in the cell membranes, consistent with the presence of more Al^{3+} content [41]. In cells grown with TTAB and exposed to Al^{3+}, an increase of CL and phosphatidylcholine (PC) (3-4 fold) was detected (Figure 3) with respect to cells grown without Al^{3+}, but the molar ratio of PC/PE, which suggests a transitory change in the membrane fluidity [42, 43], was similar for both conditions. Consequently, cells adapt to external stimuli by altering their lipid composition, such that the bilayer fluidity remains relatively constant and maintains cellular homeostasis.

Because the PC was always modified in the presence of Al^{3+} and Al^{3+} binds to the phosphate group of PC in multilamellar vesicles [44, 45], we propose a physiological role for PC as a temporary reservoir of available Al^{3+} through the formation of Al^{3+}:PC complexes. Consequently, these complexes are utilized as a reservoir of Al^{3+} in the membrane, and when *P. putida* cells grown with TTAB in the presence of Al^{3+}, the bacteria can obtain the ion to reduce the TMA accumulated inside the cell, through the formation of the Al^{3+}:TMA complex [21], in concordance with the total degradation of TTAB [41].

3. Biodegradation of TTAB by *P. putida* ATCC 12633

It has been shown that QAC biodegradation may occur through different pathways [4, 10], among others, one of these pathways is N-dealkylation, which involves monooxygenase activity with the production of TMA and an alkyl residue [10, 46]. When *P. putida* A ATCC 12633 grown with TTAB induces NADH or NAD(P)H-dependent TTAB monooxygenase activity, TMA and tetradecanoic acid is produced and is responsible for the first step in the degradation of this QAC [21]. Assays of monooxygenase activity are performed using a GC-MS method [10, 21, 46], and, although this method is appropriate, it is time-consuming and tedious, particularly in studies where numerous samples need to be tested. To overcome these limitations, we developed a simple fluorescent method to determine TTAB monooxygenase activity by measuring the formation of TMA with the use of the morin reagent and the addition of Al^{3+}. The method is based on the fluorescence quenching of the Al^{3+}:morin complex by the formed TMA [47]. The activity of the enzyme and its kinetic behaviour, as determined by these two quantitative methods, GC-MS and the fluorescence assay, showed that TTAB monooxygenase has an activity of 4.9 nmol min^{-1} mg $prot^{-1}$ and an allosteric behaviour with $K_{0.5}$ and napp (apparent Hill coefficient) values of 4.41 x 10^{-4} M and 2.35, respectively. Also, the enzyme was inhibited by the production of TMA, but this inhibition was avoided by the presence of $AlCl_3$ [47].

As shown the Figure 4, in *P. putida* A ATCC 12633 the *N*-dealkylation of TTAB catalyzed by TTAB monooxygenase activity resulted in the formation of tetradecylalkanal and TMA. Tetradecylalkanal is oxidized to tetradecanoic acid and metabolized by β-oxidation, a metabolic pathway present in *P. putida* [48]. The second product, TMA, is, in part, accumulated inside the cell [47] or metabolized to NH_3 through oxidation and demethylation [49]. In the latter case, TMA is oxidized to TMA *N*-oxide through TMA monooxygenase activity, NADH or NADPH-dependent, with an activity of 0.084 nmol min^{-1} mg $prot^{-1}$ (measured with the same extracts as for determination of TTAB monooxygenase activity). The product, TMA *N*-oxide, is demethyled to dimethylamine and methanal by a TMA *N*-oxide demethylase, whose activity is also NADPH-dependent. Dimethylamine monooxygenase oxidizes dimethylamine producing monomethylamine and methanal, and monomethylamine monooxygenase then produces methanal and ammonia [50].

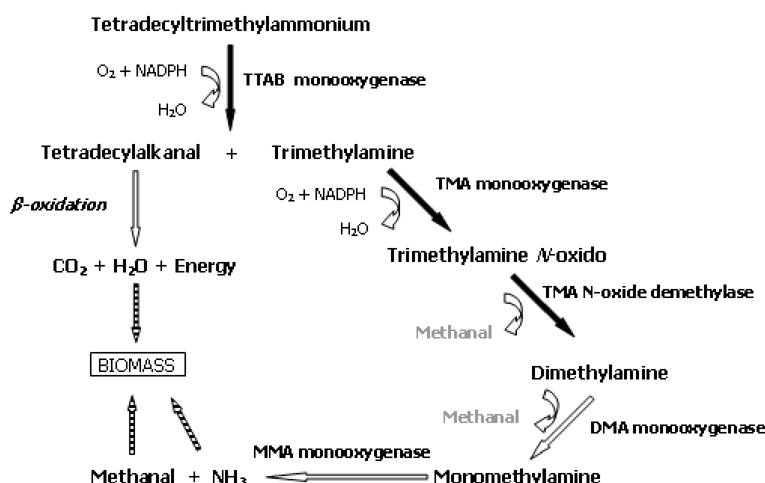

Fig. 4 Complete pathway of mineralization of TTAB by *P. putida* A ATCC 12633.

On the other hand, based on these results, we consider that the different catalytic activities for TMA monooxygenase and TTAB monooxygenase are responsible of the accumulation of TMA inside the cells when *P. putida* is grown with TTAB [49]. In this sense, the low catalytic activities of TMA monooxygenase (0.084 nmol min^{-1} mg $prot^{-1}$) were not sufficient to completely metabolize the TMA produced in the cell by TTAB monooxygenase (4.9 nmol min^{-1} mg $prot^{-1}$) and, therefore, TMA is accumulated in the cell in sufficient quantities to inhibit TTAB monooxygenase and, consequently, the growth. This effect can be avoided by the addition of Al^{3+}, with the consequential formation of the Al^{3+}:TMA complexes [49]. Thus, *P. putida* A ATCC 12633*,* is an organism capable of completely mineralizing TTAB in the presence of Al^{3+} and offers promising opportunities for the efficient biological removal of this or similar quaternary ammoniums.

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