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# Oxalate Decarboxylase from *Agrobacterium tumefaciens* C58 is Translocated by a Twin Arginine Translocation System

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**Oxalate decarboxylases (OXDCs) (E.C. 4.1.1.2) are enzymes catalyzing the conversion of oxalate to formate and $CO_2$. The OXDCs found in fungi and bacteria belong to a functionally diverse protein superfamily known as the cupins. Fungi-originated OXDCs are secretory enzymes. However, most bacterial OXDCs are localized in the cytosol, and may be involved in energy metabolism. In *Agrobacterium tumefaciens* C58, a locus for a putative oxalate decarboxylase is present. In the study reported here, an enzyme was overexpressed in *Escherichia coli* and showed oxalate decarboxylase activity. Computational analysis revealed the *A. tumefaciens* C58 OXDC contains a signal peptide mediating translocation of the enzyme into the periplasm that was supported by expression of signal-peptideless and full-length versions of the enzyme in *A. tumefaciens* C58. Further site-directed mutagenesis experiment demonstrated that the *A. tumefaciens* C58 OXDC is most likely translocated by a twin-arginine translocation (TAT) system.**

**Keywords:** Oxalate decarboxylase, *Agrobacterium tumfaciens*, prokaryotic expression, enzymatic activity assay, signal peptide, twin-arginine translocation system

Oxalate decarboxylases (OXDCs) (E.C. 4.1.1.2) are enzymes catalyzing the conversion of oxalate to formate and $CO_2$ [13]. These enzymes, which have been found in fungi and bacteria [13, 16, 23, 30], belong to a functionally diverse protein superfamily known as the cupin [10, 11] that are characterized by their conserved motifs, most recently defined as $GX_5HXHX_{3\text{-}4}EX_6G$ and $GX_5PXGX_2HX_3N$ [18] constituting a conserved six-stranded β-barrel fold. Owing to the presence of a duplication of this domain, OXDCs are members of the bicupin subclass and are thus thought to contain two β-barrels, each comprising six β-strands [18].

The best-characterized OXDCs are enzymes that have a wood-rotting fungal origin, such as *Flammulina velutipes* [17, 23], *Postia placenta* [24], *Aspergillus niger* [12, 26], and the bacterium *Bacillus subtilis* [30, 31]. The fungal OXDCs are secreted enzymes, and a secretion signal has been found in the *Flammulina velutipes* oxalate decarboxylase that can mediate the secretion of heterologous proteins into the medium and periplasmic space in *Schizosaccharomyces pombe* [3]. It is believed that oxalate synthesized by fungi contribute to lignin degradation, nutrient availability, pathogenesis, and competition. The oxalate-degrading enzymes secreted by these organisms are likely to be involved in pH regulation, and to help reduce toxicity caused by excess accumulations of oxalic acid in the microenvironment. In contrast, most bacterial OXDCs are localized in the cytosol [30, 31]. A decarboxylative phosphorylation mechanism has been described in the Gram-negative bacterium *Oxalobacter formigenes*, in which the antiporting of oxalate and formate are coupled to oxalate decarboxylation by oxalyl-CoA decarboxylase, thereby generating a proton-motive gradient that drives ATP synthesis [1, 22]. It is possible that the bacterial cytosolic OXDCs have a similar function in energy metabolism as oxalyl-CoA decarboxylase in *O. formigenes*. Recently, *B. subtilis* OxdC and OxdD were found to be targeted to the interior layer of the spore coat and found to be spore-associated proteins [2, 7, 19], suggesting a further novel role for this type of enzyme.

In *A. tumefaciens* C58, a locus for a putative oxalate decarboxylase (here denoted as AtuOXDC) is present [15]. In this study, the enzyme was overexpressed in *E. coli*, and its oxalate decarboxylase activity was determined. Further computational analysis and experimental data indicated that the AtuOXDC is a secreted enzyme that is most likely translocated by a twin-arginine translocation system.

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## Materials and Methods

### Strains, Culture Conditions, and Vectors

*E. coli* DH5α (Invitrogen, USA) was used as the cloning host for plasmids pBluescript II SK (+) (Stratagene, U.S.A.), pTrc200 [27], and pET-30b (+) (Novagen, Germany) and was also used in site-directed mutagenesis experiments. *E. coli* BL21 (DE3) (Novagen, Germany) was used as the prokaryotic expression host. The vector pBluescript II SK (+) was used for cloning the *AtuOXDC* gene and for carrying out the site-directed mutagenesis experiments. Vectors pET-30b (+) and pTrc200 were used as expression vectors in *E. coli* BL21 (DE3) and *A. tumefaciens* C58, respectively. *E. coli* was cultured for cloning procedures at 37°C in LB medium, whereas *A. tumefaciens* C58 was grown at 28°C in LB liquid medium or in petri dishes containing LB medium with 1.5% agar. For plasmid propagation, media were supplemented with streptomycin (50 μg/ml for *E. coli* and 100 μg/ml for *A. tumefaciens*), spectinomycin (50 μg/ml for *E. coli* and 300 μg/ml for *A. tumefaciens*), kanamycin (50 μg/ml for *E. coli*), and ampicillin (100 μg/ml for *E. coli*).

### Preparation of the *AtuOXDC* Gene

Standard methods were used for DNA preparation, modification, and cloning [25], with enzymes purchased from MBI Fermentas (Canada) and New England Biolabs (NEB, U.S.A.). The polymerase chain reaction was used to amplify the *AtuOXDC* gene from total DNA of *A. tumefaciens* strain C58 with a high-fidelity Pfu DNA polymerase system (Sangon, China). The oligonucleotide primers used for amplification were 5'-CTCGCCATGAACAAGGAGTT-3' and 5'-GCCGGTCAATAGAGGAACAA-3'. The purified PCR products were inserted into the pBluescript II SK (+) vector at the SmaI site and confirmed by sequencing. The resulting plasmid containing the DNA fragment, hereafter referred to as pBSK-AtuOXDC, was subsequently used as a template for PCR amplification of DNAs designed for constructing the expression vector.

### Expression of AtuOXDC in *E. coli*

To generate the construct for expression in *E. coli*, a truncated *AtuODXC* gene lacking the sequence encoding for the putative signal peptide at the N-terminal was amplified using 5'-GCGGATCCATGAATGC-CAATGCCGCA-3' and 5'-GCGAGCTCTCAGCCCGGCATGAT-3' primers. The PCR products were inserted into the SmaI site of pBluescript II SK (+) for sequencing. After digestion with a combination of BamHI and SacI, the required DNA fragment was ligated into pET-30b (+) digested with the same enzymes. The resulting plasmid, pET-AtuOXDC, was introduced into the expression host *E. coli* BL21 (DE3) and transformants were screened on LB agar plates supplemented with kanamycin. Standard methods were used for subsequent expression and pellets collection [9]. The final concentrations of IPTG (Promega, U.S.A.) and $MnCl_2$ were 1 mmol/l and 2.5 mmol/l, respectively. The collected pellets were washed with extract buffer (50 mmol/l sodium phosphate, 300 mmol/l sodium chloride, pH 8.0) and then stored at −80°C until used.

### Purification of Truncated AtuOXDC Expressed in *E. coli*

Truncated AtuOXDC protein expressed in *E. coli* was purified using Chelating Sepharose Fast Flow (Amersham, Sweden) charged with nickel ions (hereafter Ni-NTA resin), as described by the manufacturer's manual. The recombinant protein was eluted with extract buffer containing 300 mmol/l imidazole and its purity was determined by SDS-PAGE. For enzymatic activity assays, the purified protein was dialyzed overnight against 100 mmol/l phosphate buffer (pH 5.0), changing the buffer three times. The protein concentration was determined with a Bio-Rad Protein Assay Kit (Bio-Rad, U.S.A.).

### Oxalate Decarboxylase Activity Assays

The oxalate decarboxylase activity assays were performed as described by Magro *et al.* [21] with some modification. Briefly, a reaction mixture containing 50 μl of enzyme and 50 μl of potassium oxalate (111.4 mmol/l in 50 mmol/l phosphate buffer, pH 5.0) was incubated at 28°C and the reaction was terminated by adding 330 μl of phosphate buffer (100 mmol/l, pH 9.5) to increase the pH to 7.5. As a control, phosphate buffer (100 mmol/l, pH 9.5) was added to the enzyme solution before the enzyme was mixed with oxalate. Formate formation from oxalate was determined by coupling with formate dehydrogenase (FDH, Roche, Germany) and NAD (Roche, Germany) by following the increase in absorbance at 340 nm at 25°C. For this purpose, a Michaelis-Menten equation for FDH in the condition above was rearranged.

### Preparation of AtuOXDC-Specific Antiserum

To raise an antiserum against AtuOXDC, bacteria-expressed truncated AtuOXDC was used as an antigen, which was purified from inclusion bodies on SDS-PAGE gel by electroelution [25]. The prepared antigen was used in standard immunization protocols for New Zealand rabbits at the Experimental Animal Core Facility, Institute of Genetics and Developmental Biology, CAS. The resulting antiserum was incubated with an acetone-dried powder of *E. coli* BL21(DE3) cultures to eliminate cross-contaminants, as described by Sambrook *et al.* [25].

### Construction of Full-Length and Signal-Peptideless AtuOXDC Plasmids for Expression in *A. tumefaciens* C58

The full-length *AtuOXDC* with NdeI and SacI sites at the 5'- and 3'-terminus, respectively, was amplified using 5'-GCCCATGGAAA-CGTTGACCAG-3' and 5'-GCGAGCTCTCAGCCCGGCATGAT-3' primers, and then ligated into pBluescript II SK (+) at the SmaI site. After confirmation by sequencing, the DNA fragment was subcloned into the pTrc200 vector [27], generating pTrFOXDC (Fig. 3B). The procedures for constructing the plasmid designed to express the signal-peptideless AtuOXDC, designated as pTrTOXDC (Fig. 3B), were the same as those used to construct pTrFOXDC, except that the forward primer was replaced with 5'-GCCCATGGCAATCAAT-GCCAATGCCGCA-3', so that the resulting plasmid lacked the portion encoding the 39 amino-acid N-terminal sequence. The plasmid constructs were introduced into *A. tumefaciens* C58 and screened on LB media with the appropriate antibiotics.

### Site-directed Mutagenesis of AtuOXDC Signal Peptide

The full-length *AtuOXDC* sequence cloned in pBluescript II SK (+) for constructing the pTrFOXDC expression plasmid, mentioned above, was used as the template for site-directed mutagenesis. The conserved -RR- motif, which has been defined as a motif for the TAT signal, at amino acids 6 and 7, was substituted with -KK-, using the primers 5'-GAAACGTTGACCAAAAAGGCAGTCCTC-GCA-3' and 5'-TGCGAGGACTGCCTT TTTGGTCAACGTTTC-3', with a Quik Change Site-directed Mutagenesis Kit (Stratagene, U.S.A.). The mutant was subcloned into the pTrc200 vector at the NdeI and SacI sites to obtain the construct pTrMOXDC (Fig. 3B).

**Cellular Fractionation, Electrophoresis, and Immunoblotting**

To prepare cellular fractions, cultures of *A. tumefaciens* strain C58, containing different versions of *AtuOXDC* harbored in pTrc200, were grown in LB medium overnight at 28°C, and then 50 μl of the starter cultures were inoculated into 5 ml of AB minimal medium [28] and cultured at 20°C for 24 h before IPTG was added to a final concentration of 0.5 mmol/l. After continuous culture for 4 h with IPTG induction, the cells were harvested by centrifugation at 12,000 ×*g* for 10 min at 4°C. Periplasm and spheroplast fractions were prepared by the lysozyme-EDTA-cold osmotic shock method. Briefly, the collected cultures were resuspended in TES buffer (30 mmol/l Tris·HCl, 1 mmol/l EDTA, 20% sucrose, pH 8.0) containing 1 mg/ml lysozyme and incubated on ice for 10 min with gentle shaking to generate spheroplasts. The spheroplasts were collected by centrifugation at 12,000 ×*g* for 10 min at 4°C, and the supernatants were retained as the periplasmic components. The spheroplasts were disrupted by three cycles of freezing and thawing, and then were centrifuged at 12,000 ×*g* for 10 min at 4°C. The supernatants of the disrupted spheroplasts were collected and regarded as the cytoplasmic fraction. The periplasmic and cytoplasmic fractions were precipitated with 10% trichloroacetic acid (TCA), and washed three times with ice-cold acetone. The protein samples were dissolved in 1× SDS-PAGE sample buffer [9]. After fractionated on 10% SDS-PAGE gels, protein samples were electroblotted onto nitrocellulose membrane and analyzed by immunoblotting [9]. AtuOXDC was detected using antiserum raised against AtuOXDC prepared from rabbits as mentioned above, followed by horseradish peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz, U.S.A.). The bands were visualized by chemiluminescence (Roche, Germany) according to the manufacturer's manual and recorded on Kodak film.

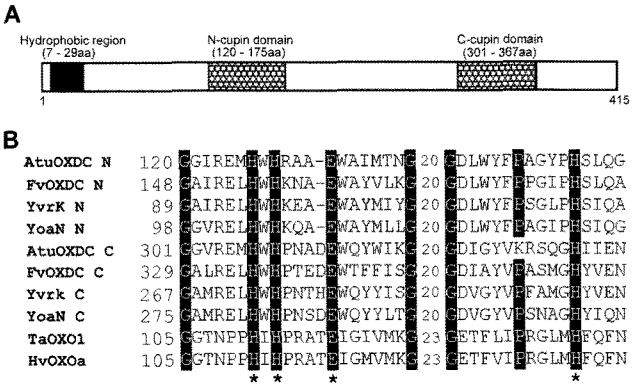


**Fig. 1.** Analysis of the AtuOXDC.

**A.** Structure of oxalate decarboxylase from *A. tumefaciens* C58. The putative signal peptide characterized by hydrophobia, and the two conserved cupin domains are indicated. **B.** Alignment of the cupin domains. Sequences of the N- and C-cupin domains of *Agrobacterium tumefaciens* oxalate decarboxylase (AtuOXDC; GenBank Accession No. 17938460), *Flammulina velutipes* (now *Collybia velutipes*) oxalate decarboxylase (FvOXDC; GenBank Accession No. 6468006), and those of the *Bacillus subtilis* bicupins YvrK (GenBank Accession No. 2635837) and YoaN (GenBank Accession No. 2619026) were aligned with those of wheat (*Triticum aestivum*) oxalate oxidase (TaOXO1; GenBank Accession No. 46408929) and barley (*Hordeum vulgaris*) oxalate oxidase (HvOXOa; GenBank Accession No. 539054). The intramotif spacing is indicated. Conserved residues are indicated with asterisks. The conserved residues, shown in shaded area, are known to bind a $Mn^{2+}$ ion in the active site of oxalate decarboxylase or oxalate oxidase.

## RESULTS

### Computational Analysis of AtuOXDC From *A. tumefaciens* C58

A locus predicted to encode a putative oxalate decarboxylase (*AtuOXDC*) is present in the chromosome of *A. tumefaciens* C58 (Accession No. 17938460). It contains a 1,248 bp open reading frame (ORF) for a 415 amino acid polypeptide with a predicated molecular mass of 44.7 kDa (Fig. 1A). AtuOXDC has two conserved cupin domains (Fig. 1A) shared by members of the cupin superfamily [12, 18]. Alignment of the two AtuOXDC cupin domains with those of oxalate decarboxylases from other species, and oxalate oxidases from *Triticum aestivum* and *Hordeum vulgaris* [20], revealed that three glycine residues, one glutamic acid residue, and three histidine residues are highly conserved, amongst which the glutamic acid residue and histidine residues are presumed to be involved in ligation to the active-site manganese ion (Fig. 1B). In addition, one proline residue in the N-cupin motif is conserved; however, another proline residue in the C-cupin motif is replaced with a lysine residue in AtuOXDC in contrast to other original OXDCs or OXOs [10–12, 18] (Fig. 1B). Interestingly, analysis of the amino acids revealed a prominent hydrophobic segment of 23 amino acid residues, near the N-terminus of the AtuOXDC amino acid sequence, with a length characteristic of a secretion signal sequence (Fig. 1A).

### AtuOXDC Overexpressed in *E. coli* has Oxalate Decarboxylase Activity

To confirm that the *AtuOXDC* does indeed encode an oxalate decarboxylase, the truncated *AtuOXDC* lacking the putative signal portion was expressed in *E. coli*, and its enzymatic activity was determined. After induction with IPTG, an additional band with 43 kDa molecular mass, corresponding to the estimated molecular mass, appeared in SDS-PAGE-separated crude extracts of *E. coli* BL21 (DE3) containing pET-AtuOXDC. Analysis of the sonicated cell extracts showed that the expression products were present either in the supernatants as soluble protein or in the pellets as inclusion bodies (Fig. 2A). Using Ni-NTA affinity chromatography, the homologous AtuOXDC was purified, taking advantage of the fused 6×Histidine tag at the N-terminus of AtuOXDC, which can bind to nickel ions in the Ni-NTA resin. The enzymatic activity assays detected little activity in the supernatants of crude extracts from BL21 (DE3) cells containing pET-30 as a control, but substantially activity (2.487 μmol formate/min/mg) was present in crude extracts of induced bacteria containing pET-AtuOXDC. The purified truncated AtuOXDC showed enzymatic activity at 6.567 μmol formate/min/mg (Fig. 2B).

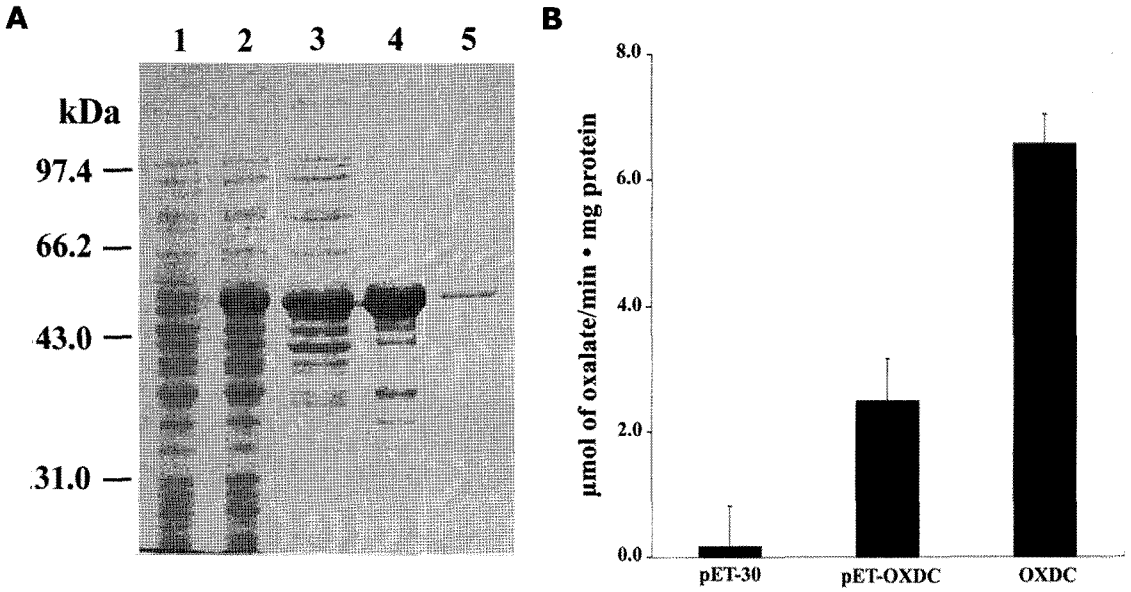


**Fig. 2.** Prokaryotic expression and enzymatic activity assays of AtuOXDC.
**A.** Expression and purification of recombinant truncated AtuOXDC in *E. coli*. Lane 1, crude cell extracts before induction; lane 2, crude cell extracts after induction; lane 3, supernatants from induced *E. coli* lysate; lane 4, pellets from induced *E. coli*; lane 5, affinity-purified AtuOXDC. **B.** Enzymatic activity assays of crude extracts from BL21 (DE3) cells containing pET-30 vector as a blank control (left column), soluble proteins of induced cells containing pET-AtuOXDC (middle column), and purified AtuOXDC (right column).

### AtuOXDC is Localized in Periplasm

Computational analysis detected a putative secretion signal peptide in AtuOXDC. To confirm that AtuOXDC is secretory, a full-length version (AtuFOXDC) and a signal-peptideless version (AtuTOXDC) were cloned into the pTrc200 plasmid, which can function as an expression vector in both *E. coli* and *A. tumefaciens* (Fig. 2B). The vectors were introduced into *A. tumefaciens* C58 and their expression was induced by adding IPTG. The endogeneous expression of AtuOXDC in wild-type *A. tumefaciens* C58 was not detected under our experimental conditions. After induction, the periplasmic and cytoplasmic components were separated, and the localization of the two versions of AtuOXDC was detected by immunoblotting using antiserum raised against AtuOXDC. Two forms of AtuOXDC, with molecular masses of 40 kDa and 43 kDa, were found in whole-cell crude extracts of induced cultures carrying the full-length construct, whereas cultures carrying the signal-peptideless gene yielded only the 40 kDa protein. In cultures expressing the full-length version, most of the 40 kDa protein was found in the periplasmic fractions, but a considerable proportion was also found in the cytoplasmic fractions, possibly due to cross-contamination of the fractions. In extracts of cultures expressing the signal-peptideless version, only the 40 kDa product was present in the cytoplasmic fractions, and no components reacting with the antiserum were detected in periplasmic fractions (Fig. 3C). Therefore, the results support the prediction that a secretion signal is present in the AtuOXDC protein.

### The -RR- Motif in Secretion Signal is Essential for AtuOXDC Secretion

The results described above show that the N-terminal located signal peptide is involved in the translocation of AtuOXDC to the periplasm. Further alignment analysis of the signal peptide sequence revealed that there is a conserved -RR- motif (Fig. 3A), which is a signature motif in TAT signal peptides. It has been established that -RR- motifs are essential for the translocation of proteins *via* the twin-arginine pathway [4, 8]. When the -RR- motif of the AtuOXDC signal peptide was replaced with -KK- using site-directed mutagenesis and the resulting mutant version (AtuMOXDC) of the full-length AtuOXDC protein was expressed in *A. tumefaciens* strain C58, subsequent immune-blotting analysis showed that OXDC translocation of the mutant version was completely blocked (Fig. 3C), implying that the AtuOXDC protein is translocated by the TAT pathway.

## Discussion

The experiments reported here showed that the putative oxalate decarboxylase locus in *A. tumefaciens* strain C58 definitely encodes an oxalate decarboxylase, and analysis of the amino acid sequence deduced from its ORF revealed that it contains a putative signal peptide. Deletion of the DNA sequence encoding for the putative signal peptide resulted in disappearance of AtuOXDC from the periplasm of transformed *A. tumefaciens* C58 expressing the protein,

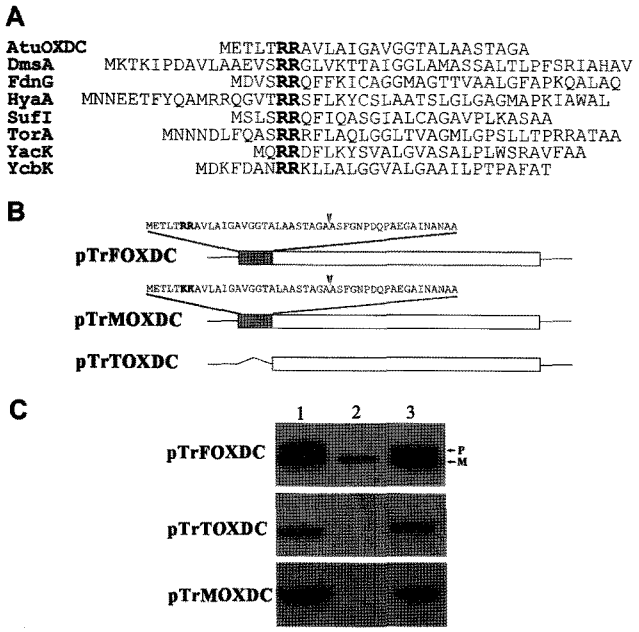


**Fig. 3.** Confirmation of periplasmic export of AtuOXDC *via* the TAT pathway.

**A.** Sequence alignment of signal peptides of AtuOXDC and several proteins exported *via* the TAT pathway. *Agrobacterium tumefaciens* OXDC (AtuOXDC; GenBank Accession No. 17938460), *Escherichia coli* K12 anaerobic dimethyl sulfoxide reductase chain A precursor (DMSO reductase α-subunit) (DmsA, GenBank Accession No. 9911072), *Escherichia coli* K12 nitrate-inducible formate dehydrogenase α-subunit (FdnG, GenBank Accession No. 16129433), *Escherichia coli* K12 hydrogenase-1 small subunit (HyaA, GenBank Accession No. 16128938), *Escherichia coli* K12 multicopy suppressor of an ftsI mutation (SufI, GenBank Accession No. 16130913), *Escherichia coli* K12 trimethylamine N-oxide reductase (TorA, GenBank Accession Nos. 16128963), *Escherichia coli* K12 multicopper oxidase (laccase) (YacK, GenBank Accession No. 1786314), *Escherichia coli* K12 unknown Protein (YcbK, GenBank Accession No. 1787157). **B.** Schematic diagrams of pTrc200-derived vectors expressing in *A. tumefaciens* C58. pTrFOXDC contains full-length *AtuOXDC*; pTrTOXDC contains signal-peptideless *AtuOXDC*; pTrMOXDC contains the -RR- site-directed *AtuOXDC* mutant in which the two consecutive conserved arginines residues were substituted with two lysine residues. **C.** Localization of AtuOXDC in different cellular fractions detected by Western-blotting. Lanes: 1, total cell lysate; 2, periplasmic fractions; 3, soluble cytoplasmic fractions. Here, "P" indicates the precursor of AtuOXDC and "M" indicates the mature form of AtuOXDC.

whereas cells expressing the full-length version showed AtuOXDC in the periplasm. The majority of proteins in the bacterial periplasm are transported by the general common Sec (secretory) protein exporting pathway in an unfolded conformation, driven by a combination of ATP hydrolysis and the transmembrane proton electrochemical gradient [5, 6]. However, a novel transport pathway, called the twin arginine translocation pathway, has recently been found in bacteria. This pathway, which was originally found in plant chloroplasts, is involved in the transport of certain proteins from the stroma into thylakoids, using energy supplied by the transmembrane pH gradient [29]. The substrates exported by this pathway are fully folded proteins, or even enzyme complexes, often containing bound redox cofactors, and play important roles in energy conservation [4, 8]. Additionally, the TAT mechanism is also capable of exporting the green fluorescent protein, which folds in the cytoplasm after translation [14, 32]. These proteins are specifically recognized by the TAT mechanism by cleavable N-terminal signal peptides with a (S/T) RRXFLK motif, in which the consecutive arginine residues are almost invariant [14, 32]. It needs to be emphasized that the charge of Sec transport is conserved, and Sec reads the positive charge and not the protein sequence, but TAT transport normally requires the exact -RR- sequence. Here, further analysis of the AtuOXDC signal peptide detected two consecutive arginine residues that were shared by TAT substrates, implying that secretion of AtuOXDC is mediated by the TAT pathway. For this reason, we carried out site-directed mutagenesis involving the -RR- motif. After the -RR- motif in the wild-type version was replaced with -KK-, translocation of the mutated version was blocked, corroborating the hypothesis.

Some fungi produce oxalic acid, which is involved in lignin degradation, nutrition, and competition. The fungi-derived OXDCs are induced by oxalate, secreted, and appear to control excessive concentrations of oxalate and to regulate the pH balance in extra-environment [3, 17, 23, 24]. The first prokaryotic oxalate decarboxylase identified was OxdC from *B. subtilis*. This protein is acid-inducible [30, 31]. Recently, OxdC from *B. subtilis* was identified as the most abundant cell wall protein, which was induced at the transcriptional level owing to the acidic conditions caused by phytate, and was not detected in the cytoplasmic fraction [2]. Most of the cell wall proteins of *B. subtilis* are synthesized with N-terminal signal peptides, secreted *via* the Sec pathway, and subsequently linked to the cell wall *via* wall-binding domains. However, the sorting mechanism whereby OxdC is directed to the cell wall is unknown, since OxdC lacks a signal peptide and conserved wall-binding domains [2]. Moreover, another oxalate decarboxylase from *B. subtilis*, OxdD, has been found to be specifically associated with the spore coat, demonstrating that it must be secreted. OxdD is induced $\sigma^K$-dependently under sporulation conditions and associated with the inner spore-coat layers. However, no oxalate decarboxylase activity was detected in spores [7]. In addition, no significant TAT signal peptide tag was found in it. Here, we found that AtuOXDC has oxalate decarboxylase activity and is exported by the TAT pathway. Blast search against the GenBank database revealed that a TAT signal is present in putative oxalate decarboxylases/glucose-6-phosphate isomerases from the genomes of *A. tumefaciens*, *Bradyrhizobium* spp., and *Burkholderia* spp., which are members of the cupin superfamily (Fig. 4). Because *A. tumefaciens*, like *Bradyrhizobium* spp. and *Burkholderia* spp., is a soil-dwelling bacterium, it is reasonable that AtuOXDC's secretion was possibly associated

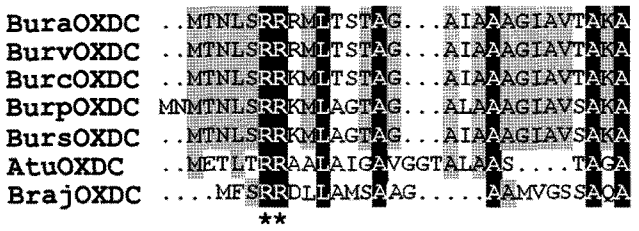


**Fig. 4.** Alignment of the TAT signal motifs in AtuOXDC and putative oxalate decarboxylases (OXDC)/glucose-6-phosphate isomerases (G6PI) from several soil-dwelling bacterial strains. *Burkholderia ambifaria* MC40-6 putative OXDC (BuraOXDC; GenBank Accession No. 118694971), *Burkholderia vietnamiensis* G4 OXDC (BurvOXDC; GenBank Accession No. 134293789), *Burkholderia cenocepacia* HI2424 OXDC (BurcOXDC; GenBank Accession No. 116692682), *Burkholderia pseudomallei* K96243 OXDC (BurpOXDC; GenBank Accession No. 53721988), *Burkholderia* sp. 383 G6PI and related metalloenzyme (putative OXDC) (BursOXDC; GenBank Accession No. 78062106), *Agrobacterium tumefaciens* C58 OXDC (AtuOXDC; GenBank Accession No. 17938460), *Bradyrhizobium japonicum* USDA110 OXDC (BrajOXDC; GenBank Accession No. 27375392). The conserved -RR- residues are indicated with asterisks.

with these bacterial existent environments. Now, it could be affirmed that AtuOXDC located in the periplasm could not be involved in cytosolic decarboxylative phosphorylation as in *O. formigenes* [22]. Thus, it is a highlight question of what physiological function AtuOXDC plays in bacterial life. Regarding this, we just have acquired initial results. AtuOXDC was localized in the periplasm but was not found in the medium, and endogeneous expression of AtuOXDC in the wild-type bacterium was not found when oxalate, acid (*i.e.*, HCl), alkali (*i.e.*, NaOH), IPTG, and even acetosyringone were added (unpublished data). In addition, an *OXDC* deletion mutant of *A. tumefaciens* strain C58 was acquired in our laboratory, but no obvious impact on the organism's pathogenicity was observed when wild-type *Nicotiana tabacum* was inoculated with wild-type *A. tumefaciens* C58 and the *AtuOXDC*-deletion mutant (unpublished data). Hence the physiological aspects would be interesting, and their analysis would be facilitated when the *AtuOXDC*-deletion mutant strain has been analyzed in more detail.

In summary, our studies show that OXDC from *A. tumefaciens* C58 clearly has oxalate decarboxylase activity, that the protein is localized in the periplasm of cells, and that the TAT system is most likely responsible for the translocation of the protein. To our knowledge, this is the first experimental demonstration that a member of the cupin superfamily is transported by the TAT apparatus.

## Acknowledgments

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