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A TRAP Transporter for Pyruvate and Other Monocarboxylate 2-Oxoacids in the Cyanobacterium *Anabaena* sp. Strain PCC 7120 ▽

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In the cyanobacterium *Anabaena* sp. strain PCC 7120, open reading frames (ORFs) *alr3026*, *alr3027*, and *all3028* encode a tripartite ATP-independent periplasmic transporter (TRAP-T). Wild-type filaments showed significant uptake of [^{14}C]pyruvate, which was impaired in the *alr3027* and *all3028* mutants and was inhibited by several monocarboxylate 2-oxoacids, identifying this TRAP-T system as a pyruvate/monocarboxylate 2-oxoacid transporter.

The tripartite ATP-independent periplasmic transporter (TRAP-T) family of proteins (family 2.A.56 in the transporter classification database [19]) comprises transporters that consist of three components: a small membrane protein usually bearing 4 transmembrane segments (TMSs), a large membrane protein usually bearing 12 TMSs that is the membrane translocator, and a periplasmic substrate binding protein (10). The TRAP transporters use the energy of an electrochemical ion gradient to drive uphill substrate transport (7, 14). TRAP-T family members are widely present in bacteria and archaea, but only a few substrates, including different types of carboxylates, have been identified for them (20). *In vitro* binding analyses with the periplasmic solute binding proteins RRC01191 from *Rhodobacter capsulatus* (20) and TakP from *Rhodobacter sphaeroides* (8) have shown that they bind monocarboxylate 2-oxoacids, including pyruvate. Additionally, pyruvate induces the TRAP-T periplasmic solute binding protein SMb21353 in *Sinorhizobium meliloti* strain 1021 (13). We are not aware, however, of any study showing a direct role of any of these proteins in pyruvate transport *in vivo*.

Cyanobacteria are a morphologically diverse group of photoautotrophic bacteria that includes unicellular and multicellular (filamentous) organisms (18). Most cyanobacteria can use ammonium or nitrate ions as nitrogen sources, and some can also assimilate urea or fix atmospheric N_2 (5). Some filamentous cyanobacteria fix N_2 in differentiated cells called heterocysts that are formed under combined nitrogen deprivation (6). A TRAP transporter is involved in sodium-dependent glutamate uptake in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (17). It is composed of proteins GtrA and GtrB (small and large membrane subunits, respectively) and GtrC (periplasmic substrate binding protein). A cluster of open reading frames (ORFs), *alr3026*, *alr3027*, and *all3028*, encoding proteins similar to TRAP-T proteins, is found in the genome of the filamentous, heterocyst-forming *Anabaena* sp. strain PCC 7120 (9). The proteins are Alr3026, with 4 predicted TMSs; Alr3027, with 13 predicted TMSs (however, the N-terminal TMS is a predicted signal peptide that could be removed, producing a mature protein of 12 TMSs); and All3028, a predicted periplasmic solute binding protein. Whereas the two membrane proteins are most similar to proteins of the *Synechocystis* Gtr glutamate transporter (Alr3026 shares 63% identity with GtrA, and Alr3027, 77% identity with GtrB), the periplasmic solute binding protein, All3028, is more similar to *Rhodobacter capsulatus* RRC01191 (47% identity) and *Rhodobacter sphaeroides* TakP (49% identity) than to *Synechocystis* GtrC (about 18% identity in a 300-amino-acid overlap). It was of interest, therefore, to determine the substrate(s) for this *Anabaena* transporter, which we approached by mutation and transport analysis.

Methods. *Anabaena* sp. strain PCC 7120 was grown in medium BG11 (nitrate-containing medium [18]) or $BG11_0$ (BG11 medium lacking $NaNO_3$) at 30°C in 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light in a shaker. For the mutants, the medium was supplemented with 10 to 40 μg of $Nm \cdot ml^{-1}$ or 2 to 5 μg of $Sp \cdot ml^{-1}$ and 2 to 5 μg of $Sm \cdot ml^{-1}$. For plating, the medium was solidified with 1% separately sterilized agar. Cyanobacterial cell masses were estimated by measuring the concentrations of chlorophyll *a* (Chl) in the cultures (12). *Escherichia coli* strain DH5α was used for plasmid constructions and was grown in LB medium supplemented when appropriate with antibiotics at standard concentrations (1), as were strains HB101 and ED8654, which were used for conjugations with *Anabaena* sp.

To inactivate *alr3027*, an internal 565-bp fragment was amplified by PCR using primers alr3027-7120-1 and alr3027-7120-2, which contain BamHI restriction sites in their 5′ ends (Table 1), and as a template, strain PCC 7120 genomic DNA was used (2). The amplified fragment was cloned into vector pGEM-T and transferred to BamHI-digested pCSV3 (16), producing pCSR11 (Sm^r Sp^r), or to BamHI-digested pRL424 (4), producing pCSR18 (Nm^r). To inactivate *all3028*, an internal 553-bp fragment was amplified by PCR using primers all3028-7120-1 and all3028-7120-2, which contain BamHI restriction sites in their 5′ ends; strain PCC 7120 genomic DNA was used as a template. The amplified fragment was cloned into vector pGEM-T Easy and transferred to BamHI-digested

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TABLE 1. Oligodeoxynucleotide primers used in this work[a]

Primer	Sequence (5′→ 3′)
all3028-7120-1	**GGA TCC** CTG TGG AAT GTG GTC ATA C
all3028-7120-2	**GGA TCC** GTT AGC TTC TAC AGT CGC CG
all3028-7120-3	GGT GCA GAA ACC GTT GCC
all3028-7120-4	CAT TAA CCC GAT TCC AGG CG
alr3027-7120-1	**GGA TCC** GTG GTG GAC TTG CTT TAG C
alr3027-7120-2	**GGA TCC** GGT AGT ATC GCA GAC TTG GC
alr3027-7120-3	GAC ACT GGC TTA TGA ATG G
alr3027-7120-4	GCA AAG CCA AAG GGT GG

[a]Introduced restriction enzyme cutting sites are in boldface type.

pCSV3, producing pCSR56 (Sm^r Sp^r). Conjugation of *Anabaena* sp. strain PCC 7120 with *E. coli* HB101 carrying pCSR11, pCSR18, or pCSR56 with helper and methylation plasmid pRL623 was effected by the conjugative plasmid pRL443, carried in *E. coli* ED8654, and was performed as described previously (3), with selection for resistance to Sm and Sp or Nm. The genetic structures of selected clones were studied by PCR with DNA from the clones and primer pairs alr3027-7120-3/alr3027-7120-4 or all3028-7120-3/all3028-7120-4 for the *alr3027*::pCSV3 and *all3028*::pCSV3 mutants, respectively, or by Southern analysis performed by standard procedures (1), with EcoRV-digested DNA from the wild type and the *alr3027*::pRL424 mutant and a probe corresponding to the DNA fragment amplified for inactivation of the gene (see above). *Anabaena* strains homozygous for the mutant chromosomes were obtained and named CSR7a (*alr3027*::pCSV3), CSR7b (*alr3027*::pRL424), and CSR13a (*all3028*::pCSV3), respectively.

For uptake assays, filaments of the wild-type and mutant strains were grown in BG11 or BG11$_0$ medium (supplemented with antibiotics for the mutants), harvested at room temperature, washed, and suspended in 25 mM N-tris(hydroxymethyl)-methylglycine (tricine)-NaOH buffer (pH 8.1) to give a cell density corresponding to 3 to 10 μg of Chl · ml^{-1}. After a preincubation at 30°C in 200 μE · m^{-2} · s^{-1} of light for 5 to 15 min, the assays were started by mixing the suspensions of cells with a solution of [1-^{14}C]pyruvate (23 to 28 μCi · μmol^{-1}; GE Healthcare, United Kingdom), D-[1-^{14}C]mannitol (59 μCi · μmol^{-1}; Amersham Biosciences, United Kingdom), or ^{14}C-labeled amino acids as previously described (16). After being incubated for the indicated time periods, 0.3- to 1-ml samples were filtered (0.45-μm-pore-size Millipore HA filters were used), and the filters carrying the cells were washed with tricine buffer and immediately immersed in a scintillation cocktail to measure their radioactivity. Retention of radioactivity by boiled cells was used as a blank. Each study was done two or three times with different substrate concentrations, and the results of representative experiments are presented in Fig. 2 and 3. Transport kinetics data were analyzed with SigmaPlot (version 11.0, build 11.0.1.80).

Impaired transport of pyruvate. Two strains with mutations in *alr3027*, strains CSR7a and CSR7b, and one with a mutation in *all3028*, strain CSR13a, were generated as described above (Fig. 1) and used in transport assays that were performed in the presence of 10 mM Na$^+$ ions. Because of the homology of the membrane proteins Alr3026 and Alr3027 to proteins of the *Synechocystis* Gtr system, the uptake of a set of amino acids, including glutamate (16), was tested in BG11-grown filaments of CSR7b and compared to that of the wild type, but no consistent impairment was observed (data not shown). Because the periplasmic solute binding protein, All3028, is annotated in some databases as an element of a mannitol transporter, we also tested the uptake of [^{14}C]mannitol (88 μM), but we found a very low level of uptake activity in wild-type filaments either grown in BG11 medium or incubated for 24 h in BG11$_0$ medium and no indication of impairment in mutant strain CSR7b.

To investigate whether the *Anabaena* TRAP system could be involved in the transport of monocarboxylate 2-oxoacids, we studied pyruvate uptake. Linear uptake was observed in the wild type for at least 10 min with different concentrations of [1-^{14}C]pyruvate (0.44 to 85 μM), and the uptake rates were similar for BG11- or BG11$_0$-grown filaments. The uptake of [1-^{14}C]pyruvate was scarcely affected in the mutants compared to that of the wild type for substrate concentrations below 15 μM, but it was affected significantly and to a similar extent for the three mutants at substrate concentrations from 30 to 90 μM (Fig. 2). These results suggest that there is a high-affinity mechanism for the uptake of pyruvate that is not inactivated in the mutants. However, the fact that a similar impairment in

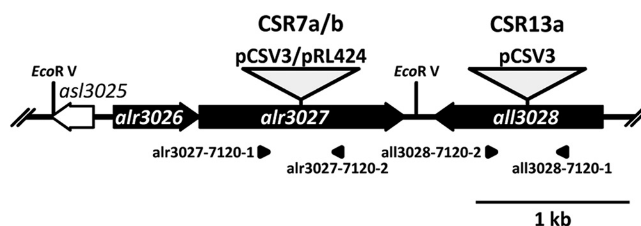

FIG. 1. Schematic of the *Anabaena* sp. strain PCC 7120 *alr3026-alr3027-all3028* genomic region. The approximate locations of plasmid insertions and PCR primers used to clone internal fragments of the genes are shown.

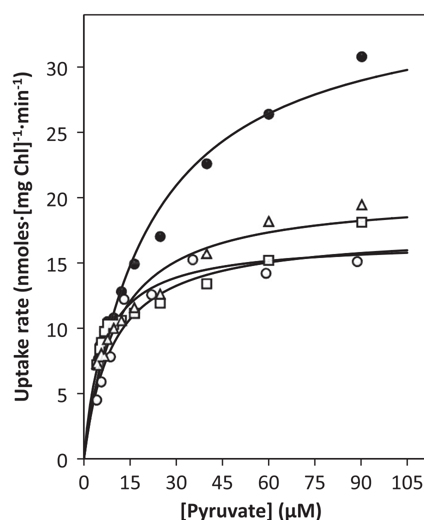

FIG. 2. Effect of inactivation of ORFs *alr3027* and *all3028* on pyruvate uptake. Filaments of strains PCC 7120 (closed circles), CSR13a (*all3028*::pCSV3; triangles), CSR7a (*alr3027*::pCSV3; squares), and CSR7b (*alr3027*::pRL424; open circles) grown in BG11 medium were used in 10-min assays of uptake of [1-^{14}C]pyruvate supplied at the indicated concentrations.

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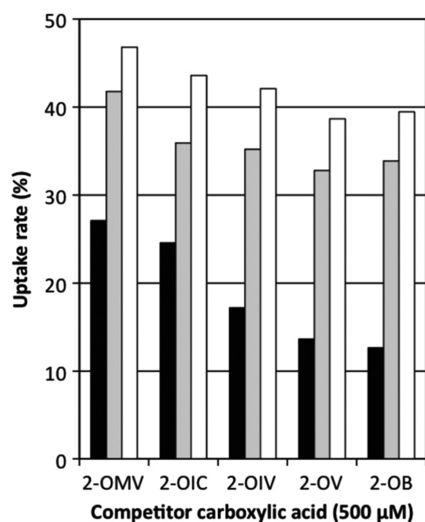

FIG. 3. Effects of some monocarboxylate 2-oxoacids on pyruvate uptake in strains PCC 7120, CSR7b, and CSR13a. The uptake of 50 μM [1-^{14}C]pyruvate was tested in 10-min assays in the absence or presence of 500 μM 2-oxobutyrate (2-OB), 2-oxovalerate (2-OV), 2-oxoisovalerate (2-OIV), 2-oxoisocaproate (2-OIC), or 2-oxo-3-methylvalerate (2-OMV). The bars represent the percentages of activity in the presence versus the absence of competitor. Black bars, strain PCC 7120; gray bars, strain CSR7b (*alr3027*::pRL424); white bars, strain CSR13a (*all3028*::pCSV3).

pyruvate uptake was observed for the three mutants suggests that Alr3027 and All3028 are proteins involved in the same process and that this system can transport pyruvate, albeit with a relatively low affinity. To deduce the kinetic parameters of pyruvate uptake by the TRAP transporter, the activities obtained with the mutants were subtracted from the activities obtained with the wild type with different substrate concentrations. From the resulting values, we deduced a K_m of 66.9 ± 20.5 μM and a V_{max} of 21.3 ± 3.71 nmol (mg Chl)$^{-1}$ min^{-1}.

Inhibition by monocarboxylate 2-oxoacids. To investigate whether the TRAP transporter could recognize other monocarboxylate 2-oxoacids, the effects of several monocarboxylate 2-oxoacids on the uptake of [1-^{14}C]pyruvate were tested. Figure 3 shows that different monocarboxylate 2-oxoacids, tested at 500 μM, inhibited the uptake of pyruvate (50 μM) more in the wild type than in the mutant strains CSR7b and CSR13a. The different monocarboxylate 2-oxoacids tested showed different degrees of inhibition, in the following order (from more to less inhibitory): 2-oxobutyrate>2-oxovalerate>2-oxoisovalerate>2-oxoisocaproate>2-oxo-3-methylvalerate. These results indicate that the system missing in the mutants is sensitive to the tested monocarboxylate 2-oxoacids and, therefore, that the *Anabaena* TRAP transporter recognizes monocarboxylate 2-oxoacids other than pyruvate.

Concluding remarks. The *Anabaena* sp. strain PCC 7120 *alr3026-alr3027-all3028* gene cluster encodes a TRAP transporter for pyruvate and possibly more generally for monocarboxylate 2-oxoacids. Although this transporter is annotated in some databases as a mannitol transporter, neither our results nor those of other authors studying homologues from *Rhodobacter* spp. support such an assignment (8, 20). Whereas the *Anabaena* TRAP transporter shows a relatively low affinity for pyruvate, the dissociation constants of pyruvate binding to the corresponding *Rhodobacter* binding proteins are very low, suggesting high affinity (8, 20). It is possible that other steps of the transport process, such as translocation through the membrane, affect the apparent affinity of uptake. Our results also show the important role that the solute binding protein has in determining the substrate selectivity of the transporter (20). The *Synechocystis* and *Anabaena* TRAP-T membrane proteins are highly similar. However, whereas the *Synechocystis* system includes a binding protein that, among characterized proteins, is most similar to glutamate binding proteins such as the *Campylobacter jejuni* PEB1a protein (11) and mediates the uptake of glutamate (17), the *Anabaena* system includes a binding protein that is most similar to monocarboxylate 2-oxoacid binding proteins such as *Rhodobacter sphaeroides* TakP (8) and mediates the uptake of pyruvate. In filaments of *Anabaena* sp. strain PCC 7120 incubated with [1-^{14}C]pyruvate, a wide distribution of labeling among metabolic products was observed (15), which indicates that pyruvate is a usable substrate, thus rationalizing the presence of pyruvate transporter(s) in this organism.

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