A DICARBOXYLIC ACID TRANSPORT SYSTEM IN *BACILLUS SUBTILIS*

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1. Introduction

The transport of compounds related to the tricarboxylic acid cycle (TCA) in microorganisms has only recently been investigated in detail. In *Escherichia coli* and *Salmonella typhimurium* [1, 2] L-aspartate, fumarate, L-malate and succinate share a common transport system and mutants devoid of this system have been described. Citrate transport in *S. typhimurium* (W.W. Kay, unpublished results) is mediated by a separate system as in *Aerobacter aerogenes* [3] and *B. subtilis* [4] however, the specificity of this system in *B. subtilis* has not been reported although growth on C_4-dicarboxylic acids repressed citrate transport.

This report describes an investigation which determines the existence and properties of a C_4-dicarboxylic acid active transport system in *B. subtilis*.

The organism was selected for several obvious advantages: it is easily grown on simple media, it is genetically defined, and it is a gram-positive microorganism – a group of microorganisms in which transport systems have received little attention. The latter characteristic also permits the ready isolation of membrane preparations free of cell wall material [5, 6], an important advantage for detailed investigations into the biochemistry of transport systems.

2. Materials and methods

B. subtilis 168, an indole or tryptophan auxotroph, (Marburg strain), and a derivative 1Aa22 deficient in succinate dehydrogenase [5] were selected for this study. Stocks were kept either lyophilized or frozen at −20° in 10% glycerol/peptone media. Cultures were routinely grown on minimal salts medium [6] to which various sterile carbon sources (20 mM L-malate, 10 mM citrate or 10 mM glucose) were added. Cells were harvested, washed with minimal medium, and kept on ice prior to initiation of uptake experiments.

Measurements of the uptake of radioactive dicarboxylic acids were carried out at 37° in 10 ml reaction mixtures containing 10^{-5} 2,3-^{14}C-dicarboxilic acid and 1.1 mg/ml dry wt of cells. Cells were intermittently filtered through Millipore membrane filters (0.45 μ) and washed with 4 ml of media and assayed for cellular radioactivity using a scintillation spectrometer. An equal volume of cells was precipitated with cold 5% trichloracetic acid and the precipitate was similarly filtered, washed with trichloracetic acid and assayed for acid-insoluble radioactivity.

The labelled intracellular pool material was released from the cells by extraction of filtered cells with 1 ml of ice-cold distilled water containing a drop of toluene and lyophilized for chromatography.

The enzymes of the TCA cycle were assayed according to published methods [7]. Autoradiography of pool material was performed as previously described [2]. 2,3-^{14}C-Succinic acid, fumaric acid and U-^{14}C-malic acid were purchased from Amersham/Searle Corp.

3. Results

Preliminary experiments revealed that *B. subtilis* rapidly incorporates ^{14}C-dicarboxylic acids into pool material only when cells were previously grown on substrates closely related to intermediates of the TCA cycle. Fig.1 describes the incorporation of ^{14}C-malate into washed suspensions of *B. subtilis* which had been previously grown on L-malate. Uptake of the labelled

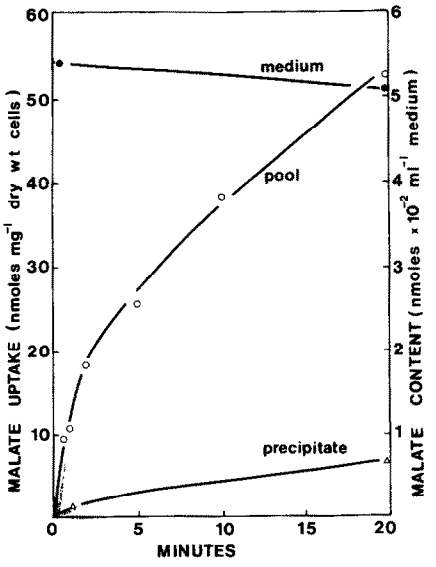

Fig. 1. U-^{14}C-L-malate uptake by *B. subtilis* 168 ind$^-$. Log phase cells harvested from L-malate minimal medium were incubated with 10^{-5} M laballed L-malate. The incorporation into filtered cells, trichloracetic acid precipitable material, and cell-free filtrate was measured.

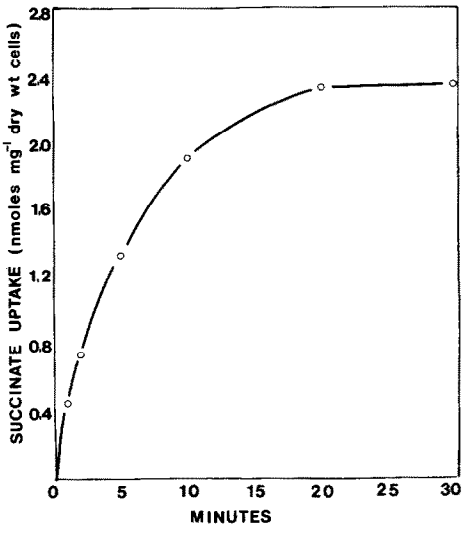

Fig. 2. 2,3-^{14}C-succinate uptake by *B. subtilis* 1Aa22. Log phase cells harvested from L-malate minimal medium were incubated with 10^{-5} M labelled succinate. Essentially all of the labelled succinate incorporated was found in the soluble pool.

^{14}C-dicarboxylic acid was rapid until the soluble "pool" reached a maximum level. Little incorporation into cellular material, or loss of $^{14}CO_2$ occurred under these conditions. A nearly identical pattern was found also for the uptake of ^{14}C-fumarate or ^{14}C-succinate. These results are in contrast to those obtained with the enteric bacteria which rapidly oxidize and assimilate the labelled substrate under similar conditions [2]. Radioautography revealed that the "pool" represents various TCA cycle intermediates and related compounds, the largest part (80–90%) of which was identified as glutamate, and malate did not accumulate.

When *B. subtilis* 1Aa22, a strain carrying a genetic lesion for the enzyme succinate dehydrogenase, was grown on malate minimal medium the mutant was found to be able to accumulate a large quahtity of labelled succinate (fig. 2). Under these conditions the intracellular/extracellular concentration gradient resulting was approx. 132-fold, which represents a massive concentration, especially when compared to a similar mutant of *E. coli* K12 which was unable to accumulate significant levels of dicarboxylic acids [2]. The level of ^{14}C-succinate accumulation was repeatedly higher than that resulting from citrate uptake in this microorganism [5]. Radioautography of the pool material extracted from *B. subtilis* 1Aa22 revealed no detectable compounds other than labelled succinate.

The stereospecificity and possibly the number of transport systems can be deduced from competitive inhibition studies on the uptake system and such studies can be carried out effectively with non-blocked mutants [2]. This was found to be particularly necessary in this study because of the difficulty in growing *B. subtilis* TCA cycle mutants on intermediates of the cycle.

The results of a series of competitive inhibition experiments with *B. subtilis* 168 ind$^-$ are shown in table 1. When cells were previously grown on L-malate, it was found that ^{14}C-malate was not only rapidly incorporated but that ^{12}C-malate was a strong competitive inhibitor of labelled fumarate or succinate uptake. These results indicated the existence of at least a single transport system with the relative affinity of L-malate > fumarate > succinate ⩾ aspartate. However, when cells were grown on citrate as a sole carbon source (table 1), this pattern was found to vary. In this case all the various C_4-acids (with the

Table 1
Competitive inhibition of ^{14}C-labelled dicarboxylic acid uptake by *B. subtilis* 168 ind$^-$ cells grown on L-malate or citrate.

Unlabelled acid added (1 mM)	Inhibition of rate of uptake of 0.0 mM ^{14}C-malate (%)	^{14}C-Fumarate (%)	^{14}C-Succinate (%)
Malate Grown			
None	0	0	0
L-Malate	78.1	85.7	90.9
Fumarate	56.1	85.7	92.7
Succinate	36.6	78.6	89.1
L-Aspartate	0	17.8	21.8
Citrate Grown			
None	0	0	0
L-Malate	77.8	90.9	88.2
Fumarate	77.8	95.5	91.2
Succinate	77.8	90.9	82.4
L-Aspartate	–	18.2	0

Log phase cells were harvested, washed twice in the cold and stored on ice until ready for use. Reaction mixtures consisted of 10 ml of cells at a concentration of 1.1 mg dry weight per ml in carbon-free minimal media. ^{14}C-dicarboxylic acids were added to a final concentration of 0.01 mM (0.1 μCi/ml). ^{12}C-dicarboxylic acid competitive inhibitors were added simultaneously with the labelled dicarboxylic acids to achieve a final concentration of 1 mM. Inhibition data were calculated from the initial slope of the curve obtained from the incorporation of label into the filtered cells.

exception of aspartate) were nearly equally competitive with one another for uptake into the cell. From the inhibition of the uptake of ^{14}C-fumarate and ^{14}C-succinate it is likely that the order of transport slightly favors fumarate and malate. This apparent shift in uptake pattern under conditions in which all the TCA cycle enzyme levels were relatively high and comparable points out that the differences observed were most likely due to an alteration in the transport properties of the cells toward the dicarboxylic acids. These results suggest the existence of perhaps two transport systems, one of which is inducible by and has a greater affinity for L-malate.

4. Discussion

The transport of C_4-dicarboxylic acids into *B. subtilis* differs significantly from that previously described for *E. coli* [2]. Although, like *E. coli*, the system is specific for C_4-dicarboxylic acids – oxaloacetate, glutamate, and citrate are not competitive inhibitors (unpublished results) – there is a difference in the relative order of affinity. Whereas in *E. coli* a single inducible system exists where the order of transport is apparently fumarate > succinate > malate ⩾ aspartate, in *B. subtilis* there appears to be one system predominant in malate grown cells with a relative transport order of malate > fumarate > succinate ⩾ ⩾ aspartate and likely a second system predominant in citrate grown cells of fumarate > malate > succinate ⩾ aspartate. Aspartate acts as a poor competitor for ^{14}C-dicarboxylic acid uptake in *B. subtilis*, however, the organism grows extremely well on aspartate as a sole carbon source. This is taken as presumptive evidence for the possible existence of a specific aspartate transport system in this microorganism. An aspartate specific transport system has been demonstrated in *E. coli* [8], however it does not appear to act as a system required for catabolism. The relative order of affinity of the C_4-acids for the transport system(s) in *B. subtilis* would seem to be

borne out also in growth experiments. The strain tested in our laboratory grows on the C_4-dicarboxylic acids in the order of malate > fumarate > succinate; in fact, our laboratory strains will hardly use succinate as a sole carbon source. It is likely that this is due to the low activity of the transport system with succinate as substrate.

The final resolution of the multiplicity of dicarboxylic acid transport systems in *B. subtilis* will depend on the isolation of specific transport negative mutants. Several attempts in this direction have been fruitless so far.

The high accumulation of dicarboxylic acids in suitably blocked mutants is noteworthy. *E. coli* transports these substrates by facilitated diffusion and is highly dependent on further metabolism [2]. Thus, *B. subtilis* must possess an energy generating mechanism to maintain these unusually high gradients. Such a system represents an ideal model for the investigation of the energetics of organic anion transporting systems.

Acknowledgements

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