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# MINICELLS OF *BACILLUS SUBTILIS*
# A UNIQUE SYSTEM FOR TRANSPORT STUDIES

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## SUMMARY

Ultrasound-purified minicells produced by *Bacillus subtilis* mutant div IV-B1 have been studied for their ability to transport and incorporate into macromolecules a variety of amino acids, uracil and thymine. Minicells transport all 12 amino acids examined, but are unable to incorporate them into macromolecules. No significant differences were found in the initial uptake rates of glutamic acid, aspartic acid, and alanine by minicells and parental cells. The uptake of methionine and proline by minicells was shown to be inhibited by metabolic poisons, indicating an energy-metabolism requirement for transport in this system. The proline pool in minicells was found to be readily exchangeable with exogenous proline. In contrast metabolically poisoned minicells only slowly lose their pool proline, indicating an energy requirement for pool maintenance. Packed-cell experiments reveal that minicells accumulate proline against a concentration gradient.

In addition to amino acids, minicells are able to transport uracil but cannot incorporate uracil into acid-precipitable material (RNA). Neither thymine transport nor its incorporation into macromolecules can be demonstrated in minicells.

Minicells appear to be a new system, therefore, in which transport may be studied in the absence of macromolecular biosynthesis.

## INTRODUCTION

In recent years much effort has been devoted to the study of transport in bacterial cells [1, 2]. The approach followed in general has been to study the uptake of a particular substrate into either intact cells [e.g. 3–6] or into specially prepared membrane vesicles [7, 8], and where appropriate to then trace its metabolic fate. In this report we will demonstrate a new bacterial system in which transport may be investigated which is not as artificial as isolated vesicles nor as complex as growing cells.

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We have recently reported the isolation of several *Bacillus subtilis* mutants which because of a cell-division defect produce small spherical minicells from the cell pole regions. We have isolated and purified the minicells and shown them to be free of DNA and incapable of growth [9]. Nevertheless minicells remain structurally intact for long periods after separation from the parent cell and ultrastructure studies have revealed that the minicell-surface structure is identical to that of intact genome-containing cells. Ultrasound-purified minicells can respire and generate ATP [10]. We have examined the ability of such minicells to transport and utilize a variety of amino acids, uracil and thymine. The data indicate transport in the absence of macromolecular synthesis.

## MATERIALS AND METHODS

### *Bacteria*

*B. subtilis* CU 403 div IV-Bl, thy$^-$, met B$^-$ was used for all experiments. Minicells produced by this strain were purified using ultrasound as described previously [10].

### *Media*

Cultures were grown at 30 °C with aeration in the minimal medium as described previously [9].

### *Determination of transport and incorporation into macromolecules*

10 ml of cells or minicells were suspended at $A_{660\ nm}$ of 0.2 in minimal growth medium and aerated for 1 h at 30 °C before the addition of the radioactive compound being measured. Total uptake and incorporation into material precipitated by cold 5% trichloroacetic acid was measured. 0.5-ml samples were either rapidly filtered through membrane filters (Millipore Corp., Bedford, Mass.) followed by five washes, at room temperature (26 °C) each with 2 ml of minimal medium containing 200 μg/ml of the appropriate unlabelled compound (the entire procedure was completed within 30 s) or pipetted directly into 4 ml of ice-cold 5% trichloroacetic acid. The trichloroacetic acid-treated samples were held at 0 °C for a minimum of 30 min, filtered through membrane filters and washed 3 times each with 5 ml of ice-cold 5% trichloroacetic acid, followed by three washes, each with 5 ml of distilled water. The membranes were dried and the radioactivity carried on them counted by standard liquid scintillation techniques in a Packard Tri-Carb system (Packard Instrument Co., Inc., LaGrange, Ill.).

Membrane filters of 0.45-μm porosity were used for cell suspensions, whereas 0.22-μm porosity membranes were used to retain minicell suspensions.

The following radioactive compounds were utilized (concentrations and specific activities for amino acids are given in Table I): L-[3,4-$^3$H]proline; L-[3-$^3$H]-arginine; L-[2,3-$^3$H]aspartic acid; L-[3-$^3$H]glutamic acid; L-[$^3$H]valine; L-[4,5-$^3$H]-isoleucine; L-[3-$^3$H]alanine; L-[3-$^3$H]phenylalanine; L-[$^3$H]serine; L-[$^3$H]histidine; [2-$^3$H]glycine. In addition, [*Me*-$^3$H]methionine, [2-$^{14}$C]uracil and [*Me*-$^3$H]thymine uptake and incorporation into macromolecules was measured. Concentrations and specific activities of these compounds are given in the appropriate results section. All radioactive materials were obtained from New England Nuclear Corp., Boston, Mass.

Metabolic inhibitors, where employed, consisted of $5 \cdot 10^{-3}$ M $N_3^-$ plus $10^{-2}$ M iodoacetamide.

*Removal of isotope*

Minicells were removed from radioactive medium by filtration on 0.22-$\mu$m membranes, washed rapidly on the membrane with complete medium minus the radioactive compound and suspended in the non-radioactive medium to the same $A_{660\ nm}$ as in the original suspension prior to filtration and washing.

*Packed-cell experiments*

A modification of the technique described by Rhinehart and Copeland [11] was used. A standard curve was constructed by counting the radioactivity in known volumes of supernatant in which minicells had been incubated.

## RESULTS

In Fig. 1 the uptake and incorporation of methionine into macromolecules is shown for both cells and minicells. It appears that genome-containing cells rapidly utilize the transported amino acid to build macromolecules, leaving only a small pool of labeled methionine. In contrast, minicells transport methionine but do not incorporate any of the labeled amino acid into macromolecules. As a result, a pool of labeled methionine accumulates in minicells. The transport of methionine by cells and minicells is completely abolished by $5 \cdot 10^{-3}$ M $NaN_3$ plus $1 \cdot 10^{-2}$ M sodium iodoacetate, metabolic inhibitors which block energy metabolism. These results are found also for many other amino acids. Table I summarizes a series of experiments, each of which involves a different amino acid. Representatives of all of the major classes of amino acids were examined. The format of each experiment was identical to that described for the methionine experiments reported in Fig. 1. These data demonstrate that minicells can transport all of the amino acids tested, but cannot incorporate any of them into macromolecules. The data for normal genome-containing cells, shown also in table I, indicates that the amino acids studied can all normally be utilized by *B. subtilis*, under the conditions reported, as substrates for macromolecular synthesis. Table I also includes examples of apparent differences in transport rates between cells and minicells for certain amino acids.

The fact that minicells represent a unique region of the cell surface prompted us to explore further differences in the transport properties of minicells and normal genome-containing cells. We compared the initial uptake kinetics of four different labeled amino acids in cells and minicells using a variety of non-labeled, carrier, amino acid concentrations. The data obtained using cells are shown in the left panels and minicells in the right panels of Fig. 2. The kinetics of glutamic acid, aspartic acid and alanine transport by cells and minicells are very similar. Minicells apparently transport much less arginine than do normal cells. Since all four amino acid experiments were performed using aliquots of the same minicell preparation, the arginine data cannot simply reflect a particular minicell preparation in poor physiological condition. Table I includes other examples of apparent differences in amino acid-transport rates between cells and minicells.

The transport of proline by minicells has been examined in more detail.

TABLE I

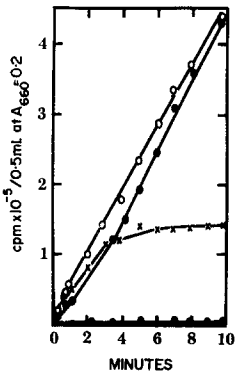
TOTAL UPTAKE AND INCORPORATION INTO TRICHLOROACETIC ACID-PRECIPITABLE MATERIAL OF $^3$H-LABELED-AMINO ACIDS BY CU403 div IV-B1 CELLS AND PURIFIED MINICELLS

| $^3$H-labeled amino acid[a] | Concentration (mM) | Specific activity (Ci/mmole) | Cells[b] | | | | Minicells[c] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total (cpm/ml $\times 10^{-3}$) after | | Trichloroacetic acid ppt. (cpm/ml $\times 10^{-3}$) after | | Total (cpm/ml $\times 10^{-3}$) after | | Trichloroacetic acid ppt. (cpm/ml $\times 10^{-3}$) after | |
| | | | 2 min | 10 min | 2 min | 10 min | 2 min | 10 min | 2 min | 10 min |
| Glutamic acid | $4.9 \cdot 10^{-5}$ | 20.4 | 125 | 360 | 65 | 315 | 70 | 140 | 1 | 1.5 |
| Aspartic acid | $3.8 \cdot 10^{-5}$ | 26 | 8 | 26 | 2 | 17 | 3.5 | 12.5 | 0 | 0.5 |
| Alanine | $3.3 \cdot 10^{-5}$ | 30.3 | 38 | 81 | 11 | 80 | 14 | 33 | 1 | 5 |
| Valine | $9 \cdot 10^{-4}$ | 1.11 | 16 | 50 | 9 | 44 | 9 | 13 | 0 | 0 |
| Isoleucine | $1 \cdot 10^{-5}$ | 10.5 | 14 | 55 | 1 | 7 | 4 | 10 | 0 | 1 |
| Arginine | $4.9 \cdot 10^{-5}$ | 20.6 | 480 | 650 | 325 | 550 | 35 | 75 | 0 | 1 |
| Histidine | $3.4 \cdot 10^{-4}$ | 2.92 | 35 | 165 | 21 | 145 | 16 | 20 | 0 | 0 |
| Phenylalanine | $7.9 \cdot 10^{-5}$ | 12.8 | 50 | 43 | 120 | 115 | 6 | 6.5 | 0 | 0 |
| Glycine | $1 \cdot 10^{-4}$ | 10.2 | 10 | 8 | 52 | 50 | 2.5 | 4.5 | 0 | 0 |
| Serine | $4 \cdot 10^{-4}$ | 1.23 | 21 | 11 | 77 | 71 | 6.5 | 11 | 0 | 1 |
| Proline | $2.6 \cdot 10^{-5}$ | 38 | 115 | 310 | 50 | 260 | 90 | 165 | 0 | 0 |

[a] See Materials and Methods for isotope-labeling details.

[b] Cell concentration adjusted to $A_{660\ nm} = 0.2$, equivalent to $3.7 \cdot 10^7$ colony-forming units/ml.

[c] Minicell concentration adjusted to $A_{660\ nm} = 0.2$, maximum colony-forming units/ml, $3 \cdot 10^4$, minicell concentration, $1 \cdot 10^9$/ml.



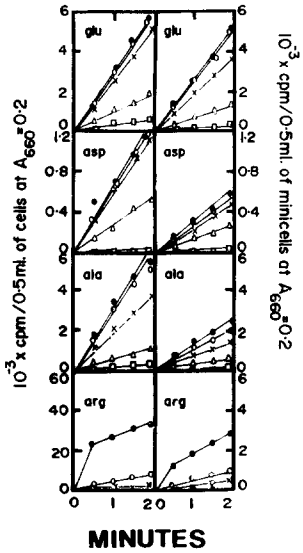




Fig. 1. Total uptake and incorporation into trichloroacetic acid-precipitable material of [*Me*-$^3$H]-methionine by CU403 div IV-B1 cells and minicells. [*Me*-$^3$H]-Methionine (final concentration $5 \cdot 10^{-4}$ mM, specific activity 4 Ci/mmole) was added to cells and minicells at 0 time. Samples were removed at intervals and total incorporation into cells (○) and minicells (×) measured. Incorporation into trichloroacetic acid-precipitable material by cells (●) and minicells (⊗) was also measured as described in Materials and Methods.

Fig. 2. Initial uptake rates of amino acids by CU403 div IV-B1 cells and minicells. At 0 time [3-$^3$H]-glutamic acid ($4.9 \cdot 10^{-4}$ mM, 20.4 Ci/mmole); [3-$^3$H]aspartic acid ($3.8 \cdot 10^{-4}$ mM, 26 Ci/mmole); [3-$^3$H]alanine ($3.3 \cdot 10^{-4}$ mM, 30.3 Ci/mmole) or [3-$^3$H]arginine ($4.9 \cdot 10^{-4}$ mM, 20.6 Ci/mmole) was added to a suspension of CU403 div IV-B1 cells or purified minicells (●). Unlabeled homologous amino acid was added at final concentrations of $1 \cdot 10^{-3}$ mM (○); $1 \cdot 10^{-2}$ mM (×); $1 \cdot 10^{-1}$ mM (△); 1 mM (□). Total uptake was measured into cells (left-hand column) and into minicells (right-hand column) as described in Materials and Methods.

The series of curves shown in Fig. 3 indicate proline transport without incorporation into macromolecules as previously shown for other amino acids. If a sample of minicells, loaded with labeled proline, is removed and transferred to an environment containing a high concentration of non-radioactive proline, an exchange reaction takes place which results in the rapid loss of labeled proline from the minicells. Virtually all of the labeled proline in the minicell pool is subject to exchange (Fig. 3). The effects of metabolic poisoning on proline-labeled minicells is shown in Fig. 4. Poisoned minicells slowly lose their labeled proline indicating an energy-metabolism requirement for pool maintenance. The rate of loss following poisoning is independent of the exogenous proline concentration. This is shown by the fact that poisoned minicells which begin to lose their proline pool, continue to lose proline at the same rate following transfer to an environment with a high exogenous proline concentration. The rapid exchange reaction in non-poisoned minicells is also included in Fig. 4 for comparison.

The equilibration of proline in minicells preloaded with proline and then transferred to a proline-free environment is shown in Fig. 5. A new equilibrium is

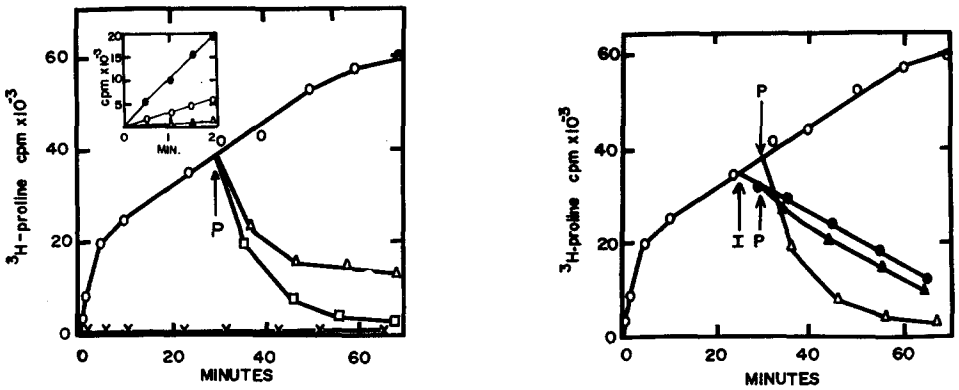



Fig. 3. Uptake of proline by CU403 div IV-B1 minicells and competition by high concentrations of unlabeled proline. Proline (final concentration $1 \cdot 10^{-2}$ mM) plus [3,4-$^3$H]proline (final concentration $2.6 \cdot 10^{-4}$ mM, specific activity 38 Ci/mmole) was added to a suspension of minicells at 0 time. Total uptake (○) and incorporation into trichloroacetic acid-precipitable material (×) measured as in Materials and Methods. At 30 min the suspension was subdivided and proline added at final concentrations of $1 \cdot 10^{-1}$ mM (△) and 1 mM (□) to portions of the suspension. Total incorporation of proline into these suspensions was measured. The insert shows the initial uptake rates of [3,4-$^3$H]-proline (final concentration $2.6 \cdot 10^{-4}$ mM, specific activity 38 Ci/mmole) by the minicell suspension when labeled proline is also added at final concentrations of $1 \cdot 10^{-3}$ mM (●); $1 \cdot 10^{-2}$ mM (○) and $1 \cdot 10^{-1}$ mM (△).

Fig. 4. Uptake of proline by CU403 div IV-B1 minicells and maintenance of accumulated proline in the presence of energy-metabolism inhibitors. Proline (final concentration $1 \cdot 10^{-2}$ mM) and [3,4-$^3$H] proline (final concentration $2.6 \cdot 10^{-4}$ mM, specific activity 38 Ci/mmole) were added to a suspension of minicells at 0 time and total incorporation measured (○). At 25 min a portion of the suspension was added to energy-metabolism inhibitors (I) ($NaN_3$, final concentration $5 \cdot 10^{-3}$ M plus sodium iodoacetate, final concentration $1 \cdot 10^{-2}$ M) (●). At 30 min proline (P) (final concentration 1 mM) was added to portions of both the inhibited (▲) and the uninhibited (△) minicell suspensions. Total uptake was followed for each suspension.

achieved shortly after transfer to the proline-free environment. If metabolic inhibitors or a high concentration of non-labeled proline are then added, the remaining labeled proline is free to exit. As in the previous experiments, none of the labeled proline becomes trichloroacetic acid-precipitable during the period of observation. In the same experimental system we have found that proline-loaded minicells in the presence of excess exogenous glutamic acid exchange their pool proline in a manner similar to that observed with exogenous proline. Other amino acids such as arginine do not stimulate proline exchange.

We have used a modification of the dextran-exclusion method described by Rhinehart and Copeland [11] to determine whether minicells accumulate proline against a concentration gradient. The data shown in Table II are based on a comparison of counts using $^{14}$C-labeled dextran to measure the void volume in packed-cell pellets. The intracellular concentration of radioactive proline is approx. 150 times that remaining in the supernatant from which the minicells have been removed. These data clearly demonstrate transport of proline against a concentration gradient by minicells.

Also included in Table II are data concerning thymine transport by minicells. Minicells do not accumulate a significant thymine pool, nor do they incorporate

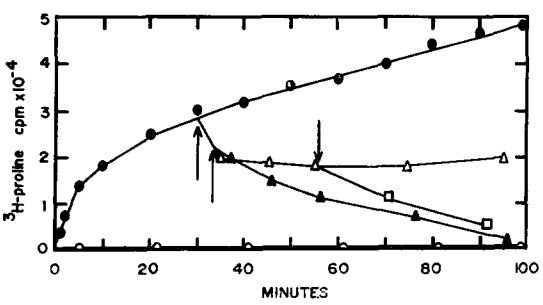
TABLE II

ABILITY OF CU403 div IV-B1 MINICELLS TO CONCENTRATE PROLINE AGAINST A CONCENTRATION GRADIENT AND INABILITY TO CONCENTRATE THYMINE

| Substrate for uptake | Concentration of isotope inside minicells (cpm/$\mu$l minicell) | Concentration of isotope outside minicells (cpm/$\mu$l supernatant) |
|---|---|---|
| [3,4-$^3$H]Proline | 27 300 | 180 |
| [*Me*-$^3$H]Thymine | 4 500 | 6 650 |

labeled thymine into trichloroacetic acid-precipitable material (results not shown).

In view of the fact that minicells cannot grow or synthesize proteins, and contain no DNA, we searched for RNA synthesis by examining labeled-uracil transport and incorporation into trichloroacetic acid-precipitable material. Fig. 6 illustrates our findings. Uracil is transported and incorporated into macromolecules by normal cells, but only transported by minicells. Virtually no RNA synthesis can be detected in minicells. A pool of labeled uracil is formed in minicells in a manner similar to that reported for amino acids above.



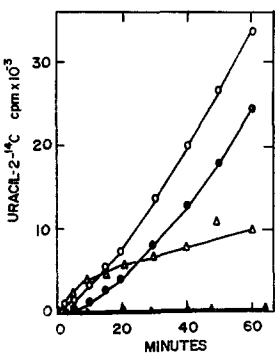




Fig. 5. Uptake of proline by CU403 div IV-B1 minicells and loss of proline following removal of proline from suspending medium. Proline (final concentration $1 \cdot 10^{-2}$ mM) and [3,4-$^3$H]proline (final concentration $1.3 \cdot 10^{-4}$ mM, specific activity 38 Ci/mmole) were added to a suspension of minicells at 0 time. Total uptake (●) and incorporation into trichloroacetic acid-precipitable material (○) were measured as in Materials and Methods. At 30 min a portion of the suspension was resuspended at the same $A_{660\ nm}$ in the same medium minus proline (△). At 35 min energy-metabolism inhibitors ($NaN_3$, final concentration $5 \cdot 10^{-3}$ M, plus sodium iodoacetate, $1 \cdot 10^{-2}$ M) were added to a portion of the suspension lacking proline (▲). At 55 min proline (final concentration $1 \cdot 10^{-1}$ mM) was added to a second portion of the suspension previously lacking proline (□). Total uptake of radioactive proline was measured in suspension (△), (▲) and (□).

Fig. 6. Total uptake and incorporation into trichloroacetic acid-precipitable material of [2-$^{14}$C]-uracil by CU403 div IV-B1 cells and minicells. [2-$^{14}$C]Uracil (final concentration $3.5 \cdot 10^{-2}$ mM, specific activity 0.1 mCi/0.374 mg) was added to cells and minicells at 0 time. Samples were removed at intervals and total incorporation into cells (○) and minicells (△) measured. Incorporation into trichloroacetic acid-precipitable material by cells (●) and minicells (△) was also measured as described in Materials and Methods.

## DISCUSSION

Minicells are unique biological systems. They are cells with no genetic material. They cannot grow or synthesize DNA, RNA or proteins. They are formed from the cell-pole regions and thus consist of a particular compartment of cell structures. We have previously observed that *B. subtilis* minicells are stable over long periods of time, respire, generate ATP, are motile and contain a normal cell-surface ultrastructure [9]. These observations led us to consider the possibility that minicells might be an ideal system in which to study transport phenomena. In the preliminary study reported here, we investigated the transport of 12 different amino acids, uracil and thymine by minicells.

The properties of amino acid transport by minicells demonstrated in our experiments include: (1) amino acids of all major categories are transported by minicells; (2) the transport of amino acids is energy dependent; (3) once a pool of labeled amino acids is formed, minicells must continue energy metabolism in order to maintain the pool; (4) labeled-amino acid pools in minicells are accessible to rapid exchange with exogenous homologous, and in some cases heterologous amino acids; (5) the rapid exchange of amino acids from minicell pool to the environment requires energy metabolism in the minicells; (6) labeled amino acids transported into minicells are not incorporated into macromolecules; and (7) the concentration of labeled proline in minicells has been examined and shown to greatly exceed the extracellular labeled-proline concentration. It appears therefore that minicells can concentrate amino acids against a gradient. Taken together, these data indicate that minicells are capable of transporting amino acids via a classical "active-transport system" [1–3, 12].

In addition to amino acid transport we have shown that minicells can transport uracil but do not synthesize RNA hence the uracil accumulates in a pool. The inability to synthesize RNA is presumably a reflection of the lack of DNA in minicells, which also accounts for the lack of thymine incorporation into trichloroacetic acid-precipitable material. However, the inability of minicells to transport thymine is probably not a ramification of the anucleate nature of minicells, but rather a property of *B. subtilis* in general [11].

The metabolic fate of substrates transported but not utilized for macromolecular synthesis in minicells remains to be elucidated.

## ACKNOWLEDGEMENTS

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