The Sodium Effect of *Bacillus subtilis* Growth on Aspartate

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(Received 7 December 1979)

aspH mutants of *Bacillus subtilis* have a constitutive aspartase activity and grow well on aspartate as sole carbon source. *aspH aspT* mutants, which are deficient in high affinity aspartate transport as a result of the *aspT* mutation, grow as well as *aspH* mutants in medium containing high concentrations of aspartate and Na⁺. This Na⁺ effect is not due to an enhancement of aspartate transport but is the result of increased cellular metabolism. The ability to grow rapidly in sodium aspartate is induced by prior growth in the presence of Na⁺. In potassium aspartate, the addition of arginine, citrulline, ornithine, Δ^1 -pyrroline-5-carboxylate or proline instead of Na⁺ also allows rapid growth; but in a mutant deficient in ornithine-oxo-acid aminotransferase, only pyrroline-carboxylate or proline can replace Na⁺. The amino acid pool of cells growing slowly in potassium aspartate contains proline at a low concentration which increases upon addition of proline (but not Na⁺) to the medium. Thus, Na⁺ addition does not increase the synthesis of proline, but proline or pyrroline-carboxylate acts similarly to Na⁺ either in preventing some inhibitory effect (by aspartate or the accumulating NH₄⁺) or in overcoming some deficiency (e.g. in further proline metabolism).

INTRODUCTION

Whereas wild-type strains of *Bacillus subtilis* grow only slowly on L-aspartate as sole carbon source until they have adapted to this compound, mutants (*aspH*) can be isolated which always grow rapidly on aspartate; the *aspH* mutation renders the strain constitutive in aspartase (Iijima *et al.*, 1977). At high concentrations of sodium aspartate, growth occurs at the same rate irrespective of whether the cells possess a high affinity active aspartate transport system or whether an *aspT* mutation has eliminated it (Whiteman *et al.*, 1978). The growth on high aspartate concentrations utilizes low affinity but high capacity aspartate transport, the conversion of aspartate to fumarate and ammonia via aspartase, and the subsequent metabolism of fumarate via the citric acid cycle and by gluconeogenesis. It was noticed in the earlier studies in this laboratory that the cultures grew at a much lower rate if sodium aspartate was replaced by potassium aspartate and that addition of 10 mM or more Na⁺ was optimal for rapid growth on aspartate. In other micro-organisms, growth on L-glutamate requires Na⁺ ions (Frank & Hopkins, 1969) which apparently are needed to activate glutamate transport, as was reported for *Escherichia coli* (Halpern *et al.*, 1973; Miner & Frank, 1974; Tsuchiya *et al.*, 1977) and *Mycobacterium phlei* (Hirata *et al.*, 1974). In the presence of Na⁺, *B. subtilis aspH* mutants, which contain constitutive aspartase, reach the maximal rate of growth only at concentrations ≥ 30 mM-aspartate (the maximal rate of active aspartate transport, into isolated membrane vesicles, is obtained at about 6 mM-aspartate). Apparently, up to 30 mM-aspartate the rate of growth is limited by the rate (low affinity) of aspartate transport rather than by that of aspartate catabolism (Whiteman *et al.*, 1978). Since, in the presence of Na⁺ the growth rates increased more

Table 1. *Bacillus subtilis* strains

Strain	Genotype*	Origin†
61501	<i>aspH1 trpC2</i>	Spontaneous mutant of 60002 isolated for rapid growth on aspartate
61507	<i>aspH1 aspT13 trpC2</i>	Spontaneous mutant of 61501 resistant to DL-threo- β -hydroxy-aspartate
60337	<i>Amy Hom-2 trpC2</i>	Ethyl methanesulphonate mutagenesis of 60021 (<i>Amy trpC2</i>)
61702	<i>Amy aspH1 aspT13 Hom-2 trpC2</i>	Tf of 60337 by 61507
60961	<i>metC7 Pro-2 trpC2</i>	Nitrosoguanidine treatment of 60015
60019	<i>argO trpC2</i>	E. W. Nester
60198	<i>OrdA1</i>	Ultraviolet mutagenesis of 60009
61859	<i>aspH1 aspT13 OrdA1</i>	Tf of 61507 by 60198

* The first letter of a genotype symbol is capitalized if the marker has not yet been mapped.

† Tf implies transformation of the first strain by DNA of the second strain.

rapidly with the aspartate concentration and reached a higher plateau than in the absence of Na^+ , Na^+ might increase the maximal velocity of aspartate transport. However, it also appeared possible that Na^+ increased the rate of aspartate metabolism, for example, by preventing the inhibitory effect of high concentrations of aspartate or a compound such as NH_4^+ derived from it. This paper demonstrates that the latter is the case.

METHODS

Bacteria. The strains used are listed in Table 1. All were derived from the transformable strain 168 of *Bacillus subtilis* Marburg.

Media and growth conditions. The bacteria were grown in the synthetic salts mixture (S6) described previously (Whiteman *et al.*, 1978). Aspartic acid was used as carbon source, usually at a final concentration of 25 or 50 mM and adjusted to pH 7.0 with NaOH or KOH before use. Growth was followed by the increase in absorbance at 600 nm (A_{600}). To express values per cell mass (e.g. in uptake measurements) we have used the 'absorbance mass' $AM_{600} = A_{600}$ ml, which is proportional to the cell mass.

Transformation. DNA of strains 61507 and 60198 was obtained by the method of Saito & Miura (1963). Portions of the donor DNA were added to recipient bacteria grown in the minimal glucose/citrate medium of Anagnostopoulos & Spizizen (1961).

Isolation of an *aspH aspT Hom* strain. DNA of strain 61507 (*aspH1 aspT13 trpC2*) was added to strain 60337 (*Hom-2 trpC2*) to obtain an *aspT Hom trpC* strain which was isolated on S6+glucose (25 mM) plates containing 25 μg L-homoserine ml^{-1} , 25 μg L-tryptophan ml^{-1} , 0.01 % (w/v) casein hydrolysate and 100 μM DL-threo- β -hydroxyaspartate. This strain was again transformed with DNA of strain 61507; *aspH aspT Hom trpC* mutants were isolated by selection in S6 medium containing 25 mM-sodium aspartate, 25 μg L-tryptophan ml^{-1} and 25 μg L-homoserine ml^{-1} . Single colonies which grew rapidly on the sodium aspartate were isolated and retested for their resistance to threo-hydroxyaspartate (on S6+glucose) and for their homoserine requirement.

Isolation of an *aspH aspT Ord* strain. DNA of strain 60198 (*OrdA1*) was added to strain 61507 (*aspH1 aspT13 trpC2*). Single colonies which grew rapidly on sodium aspartate (50 mM) as sole carbon source in the absence of added tryptophan were isolated and tested for the *Ord* property by plating on S6 in which K_2SO_4 (10 mM) replaced $(\text{NH}_4)_2\text{SO}_4$, and 2 mM-potassium glutamate or 5 mM-ornithine was added as a nitrogen source; 25 mM-glucose was used as carbon source. One such mutant, 61859 (*aspH1 aspT13 OrdA1*), was used in this work. This mutant was unable to use ornithine as sole nitrogen source but it could use glutamate or proline instead of ammonia.

L-Ornithine:2-oxo-acid aminotransferase assay (EC 2.6.1.13). Bacteria were grown to an A_{600} of 1.0 in S6 containing 50 mM-potassium aspartate with or without 10 mM-NaCl and with or without 100 μg L-ornithine ml^{-1} . Chloramphenicol (100 μg ml^{-1}) was then added, and the cells were harvested by centrifuging and washed once with 100 mM-Tris/HCl pH 8.0 at room temperature. The pellet containing 60 AM_{600} units was resuspended in 1 ml of similar buffer containing lysozyme (100 μg ml^{-1}). After incubation at 37 °C for 30 min the extract was centrifuged for 20 min at 27000 g and the supernatant was used for assay.

Ornithine-oxo-acid aminotransferase was assayed by a method based on that of Vogel & Kopac (1960); the L-glutamic semialdehyde produced in the reaction is spontaneously converted to L- Δ^1 -pyrroline-5-

carboxylate which reacts with *o*-aminobenzaldehyde giving a yellow colour (Vogel & Davis, 1952). The reaction mixture (1 ml) contained 100 mM-Tris/HCl pH 8.0, 5 mM-L-ornithine, 25 μ M-pyridoxal 5-phosphate, 1 mM-*o*-aminobenzaldehyde and cell extract containing 0.5 to 1.0 mg protein. After equilibration at room temperature 2-oxoglutarate (5 mM) was added and the increase in absorbance at 400 nm was followed.

DL- Δ^1 -Pyrroline-5-carboxylic acid was prepared from DL- Δ^1 -pyrroline-5-carboxylate-2,4-dinitrophenyl-hydrazone. HCl according to the method given by the manufacturer (Calbiochem) and used to determine its molar absorption coefficient as 2160 l mol⁻¹ cm⁻¹.

L-Aspartate:2-oxoglutarate aminotransferase assay (EC 2.6.1.1). Bacterial extracts were prepared in 50 mM-Tris/HCl buffer pH 8.0 as described for ornithine-oxo-acid aminotransferase. The assay mixture (1 ml) contained 50 mM-Tris/HCl pH 8.0, 25 μ M-pyridoxal phosphate, 10 mM-potassium 2-oxoglutarate (pH 8), 1.5 international units malate dehydrogenase (Boehringer) and cell extract containing 0.1 to 0.5 mg protein; after equilibration at room temperature for 5 min, 0.5 mM-NADH (dipotassium salt, prepared just prior to use in 50 mM-Tris/HCl pH 8.0) was added followed by 20 mM-potassium L-aspartate (pH 8). The difference in the rate of decrease of A_{340} in the presence and absence of aspartate was used to determine enzyme activity. The effect of NaCl (10 mM) on the activity was determined.

Glutamate- γ -semialdehyde dehydrogenase assay. Attempts were made to demonstrate this enzyme activity in extracts prepared as described above in 100 mM-Tris/HCl pH 8.0. Two methods were tried: (i) The assay mixture (1 ml) contained 100 mM-Tris/HCl pH 8.0, 10 mM-ATP (dipotassium salt), 5 mM-MgCl₂, 0.3 mM-NADPH or NADH (dipotassium salt) and cell extract containing 0.5 to 1.0 mg protein. The decrease in A_{340} was followed and when the rate was constant, 10 mM-potassium L-glutamate (pH 8) was added. No effect was seen.

(ii) *o*-Aminobenzaldehyde was used to measure the formation of the product of the reaction, L- Δ^1 -pyrroline-5-carboxylate. The assay mixture (1 ml) contained 100 mM Tris/HCl pH 8.0 or 100 mM-sodium phosphate buffer pH 6.6, 10 mM-ATP (dipotassium salt), 5 mM-MgCl₂, 10 mM-NaCl, cell extract containing 0.5 to 1 mg protein and 2 mM-*o*-aminobenzaldehyde. The absorbance at 440 nm was followed after the addition of potassium L-glutamate (10 mM). No reaction was detected, and the same was true when acetyl-CoA (1 mM) replaced ATP. Use of the crude lysozyme extract (without centrifugation) also produced no effect.

Amino acid analysis. Bacteria from an exponentially growing culture were harvested in less than 10 s on to membrane filters (pore size 0.45 μ m; Millipore) and washed once with an equal volume of warmed S6 medium (without a carbon source). The filters were immediately placed in ice-cold 0.3 M-HClO₄ plus 0.1 mM-EDTA (1 ml for every 10 A_{600} units collected) in a 30 ml Corex centrifuge tube (Corning Glass Works, Corning, N.Y., U.S.A.). (We found this rapid extraction to be essential for reproducible measurements of amino acid pools.) The filters were kept on ice for 5 min with frequent mixing (on a Vortex mixer). They were then centrifuged at 17000 *g* for 10 min at 4 °C. The supernatant was removed to another cold Corex tube, adjusted to about pH 6 with cold 1 M-K₂CO₃, and then centrifuged at 4 °C for 10 min at 12000 *g*. The resulting supernatant was carefully removed to another tube and the pH was adjusted to < 2 with ice-cold 1 M-HCl. The extract was rapidly frozen in dry ice/acetone and stored at -70 °C until analysed.

Amino acid analysis was performed on a Beckman model 120C amino acid analyser. The acidic and neutral amino acids were determined on a 56 $\times$ 0.9 cm column of HP AN-90 resin (Hamilton Co., Reno, Nev., U.S.A.) in the Li⁺ form. The basic amino acids were determined on a 26 $\times$ 0.9 cm column of Aminex A-5 resin (Bio-Rad) in the Na⁺ form.

Uptake studies. During exponential growth in S6 or S6 + Na⁺, containing aspartate (25 or 50 mM) as carbon source, the bacteria were harvested (usually at A_{600} = 0.5 unless otherwise noted) by centrifugation (12000 *g* at room temperature for 5 min), washed once with an equal volume of S6 and resuspended in the same medium. Portions were incubated at 37 °C in a water bath shaker.

Uptake was initiated by the addition of L-[U-¹⁴C]aspartate (0.1 μ Ci μ mol⁻¹, 3.7 kBq μ mol⁻¹) to give the desired final concentration (usually 25 mM and 2.5 μ Ci ml⁻¹). The radioactivity in samples (0.2 ml) was measured as described previously (Whiteman *et al.*, 1978). The initial rates of uptake (nmol AM_{600}^{-1} min⁻¹) were determined from the linear portion of the uptake curves obtained within 2 min (four to six points).

Purification of [¹⁴C]aspartate. L-[U-¹⁴C]Aspartic acid [125 μ l; 203 mCi mmol⁻¹, 7.5 GBq mmol⁻¹) was applied to an MN300 cellulose plate and run in a solvent system composed of ether/formic acid/water (7:2:1, by vol.) for 1.5 h (Myers & Huang, 1966). The radioactive aspartate was located by autoradiography, eluted with water and freeze-dried. The purified material was added to sodium or potassium aspartate to give 50 μ Ci ml⁻¹ and 500 mM. The uptake of this aspartate was measured as above and compared with that of unpurified aspartate.

Fig. 1. Aspartate concentration dependence of growth of (a) *aspH* strain 61501 and (b) *aspH aspT* strain 61507. Cultures were grown in S6 plus different concentrations of potassium L-aspartate in the presence (●) or absence (○) of 10 mM-NaCl.

RESULTS

Effect of sodium on growth

All strains used contained the *aspH* mutation which confers on the organism the ability to produce aspartase constitutively (Whiteman *et al.*, 1978). The dependence of the growth of an *aspH* mutant (61501) on the concentration of aspartate is shown in Fig. 1(a). As has been previously shown (Iijima *et al.*, 1977), the growth rate greatly increased in the presence of Na^+ (with an optimum of 10 mM or more). The *aspH aspT* double mutant lacking the high affinity transport of aspartate grew more slowly than the *aspH* mutant at low aspartate concentrations but both strains grew at the same rate at high aspartate concentrations exceeding 20 mM; both strains showed the same Na^+ effect (Fig. 1b). Thus, growth at high aspartate concentrations requires only the low affinity aspartate transport system.

Effect of sodium on aspartate uptake

To determine whether Na^+ increased the transport of aspartate or rather exerted its growth stimulatory effect through increased cellular metabolism, the initial rate of aspartate uptake was measured in the presence and absence of 10 mM-NaCl in both the single and double mutants (Fig. 2). The measurements were made while the cells continued to grow on and to metabolize aspartate. Only a small stimulation by Na^+ on the initial rate of aspartate uptake was observed which was probably due to an influence of aspartate metabolism on the uptake points obtained within the first 2 min after aspartate addition. But this effect of Na^+ was clearly much less pronounced than that on growth (Fig. 1). For example, with 25 mM-aspartate, the initial rate of aspartate uptake in the presence of Na^+ was less than twice that in its absence. In contrast, the rate of growth of the *aspH aspT* strain was 15 times higher in the presence of Na^+ than in its absence. Apparently the Na^+ effect on growth was mainly due to stimulation of cellular metabolism rather than to increased aspartate transport.

Following the initial rapid uptake of aspartate, a slower uptake rate was maintained, as is shown in Fig. 3 for the case of 25 mM-aspartate; it reflects the rate of aspartate metabolism. In the presence of Na^+ (in cells previously grown on sodium aspartate), these ultimate uptake rates were about $32 \text{ nmol min}^{-1} \text{ AM}_{600}^{-1}$ for the *aspH* strain and $23 \text{ nmol min}^{-1} \text{ AM}_{600}^{-1}$ for the *aspH aspT* strain; they were four and eight times higher than in

Fig. 2. Aspartate concentration dependence of the initial rate of uptake by (a) *aspH* strain 61501 and (b) *aspH aspT* strain 61507. Each strain was grown in S6+50 mM-sodium L-aspartate to $A_{600} = 0.5$, centrifuged, washed and resuspended in S6. Potassium L-[U- ^{14}C]aspartate ($0.1 \mu\text{Ci} \mu\text{mol}^{-1}$) was added to give the final concentration indicated, and the uptake was measured as described in Methods in the presence of 10 mM-NaCl (●) or 10 mM-KCl (○). The initial rate was determined from the points obtained within the first 2 min.

Fig. 3. Uptake of 25 mM-L-aspartate by (a, b) *aspH* strain 61501 and (c, d) *aspH aspT* strain 61507. The cells were grown in S6+25 mM sodium or potassium aspartate to $A_{600} = 0.1$ (61507) or 0.25 (61501), centrifuged, washed and resuspended in warm (37°C) S6. The uptake of sodium (●) and potassium (○) L-aspartate ($0.1 \mu\text{Ci} \mu\text{mol}^{-1}$) was measured as described in Methods.

the absence of Na^+ (7.7 and $2.8 \text{ nmol min}^{-1} AM_{600}^{-1}$, respectively). Interestingly, at this concentration of aspartate (25 mM) the *aspH aspT* strain could still concentrate aspartate in the absence of Na^+ to an intracellular level of $100 \text{ nmol } AM_{600}^{-1}$, i.e. 100 mM if it is assumed that $1 AM_{600}$ represents $1 \mu\text{l}$ intracellular volume. In spite of the high level of intracellular aspartate the strain was unable to grow. This amount of aspartate was not due to non-specific binding because the aspartate uptake was prevented by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide ($100 \mu\text{M}$), an inhibitor of the electron transport system and thus of active amino acid transport. In the presence of this inhibitor, the uptake was reduced to about $25 \text{ nmol } AM_{600}^{-1}$ which would be expected by the equilibration of intracellular and extracellular aspartate. Furthermore, starvation of the cells (absence of carbohydrates in the medium) for about 1 h before assaying the aspartate uptake also abolished the concentrative uptake of aspartate.

The possibility existed that the radioactivity observed in the cell in the absence of Na^+ was due to a radioactive contaminant of the aspartic acid. When a sample of the aspartic acid solution was chromatographed on a thin layer, traces of radioactive impurities were seen. To purify the $[^{14}\text{C}]$ aspartate, portions were subjected to preparative thin-layer chromatography using MN300 cellulose plates. The purified potassium aspartate showed an initial rate of uptake of $60 \text{ nmol min}^{-1} AM_{600}^{-1}$ in the *aspH aspT* strain and the intracellular concentration reached $170 \text{ nmol } AM_{600}^{-1}$. Thus, the purified material was taken up to an even greater extent than the unpurified commercial aspartate, which indicates that any impurity did not contribute to the observed uptake.

Figure 3 also shows that aspartate was taken up at the same rate, regardless of the presence of Na^+ or K^+ , if cells had been pre-grown in the absence of Na^+ . After the initial rapid uptake was complete, only a very slow continued rate of aspartate uptake was observed even in the presence of Na^+ . This result shows that the Na^+ stimulation of growth and metabolism required the adaptation of the cells to Na^+ .

Attempts to explain the effect of sodium on metabolism

The requirement for Na^+ was limited to growth on aspartate as sole carbon source. If the medium contained, as carbon sources, glucose, other carbohydrates or L-malate alone or in addition to aspartate, no Na^+ requirement was found. The enzymes immediately required for aspartate catabolism, i.e. aspartase, fumarase, malate dehydrogenase and aspartate:2-oxoglutarate aminotransferase, did not exhibit any Na^+ dependence when assayed in extracts prepared from the *aspH aspT* strain grown in 25 mM sodium or potassium aspartate.

In the aspartate-dependent branch of amino acid synthesis, homoserine dehydrogenase is an enzyme known to require K^+ and to be inhibited by Na^+ . Conceivably, cells grown with aspartate as sole carbon source could accumulate homoserine or threonine at inhibitory concentrations, and the addition of Na^+ might reduce this accumulation. To check this possibility, a homoserine-requiring mutant (*Hom*) was transformed twice by DNA of the *aspH aspT* strain to produce an *aspH aspT Hom* triple mutant (strain 61702). This triple mutant grew in S6+ sodium aspartate with a doubling time of 1.1 h only if the medium was supplemented with homoserine ($25 \mu\text{g ml}^{-1}$) or with threonine ($25 \mu\text{g ml}^{-1}$) + methionine ($10 \mu\text{g ml}^{-1}$). In S6+ potassium aspartate, the doubling time was 3 h with homoserine and 4.8 h with threonine + methionine. Since Na^+ still significantly increased the growth rate of this triple mutant, the accumulation of homoserine or any compound derived from it on the pathways to threonine or methionine cannot be responsible for the inhibition of growth.

Thus, it would seem that either the high concentrations of aspartate or the large amount of ammonia, necessarily produced by cells growing on aspartate as sole carbon source, was responsible for the inhibitory effect that could be counteracted by Na^+ . Ammonia (or rather NH_4^+ generated from it in the neutral pH range) could be excreted as such, by co- or countertransport with other ions, or in the form of urea produced in the arginine cycle.

Fig. 4. Growth of *aspH aspT* strain 61507 in S6+50 mM-potassium L-aspartate and different additions. Cells were pre-grown in S6+50 mM-sodium aspartate, washed and resuspended at $A_{600} = 0.05$ in S6+50 mM-potassium aspartate. They were then distributed into flasks containing additions giving the following final concentrations: (a) 10 mM-NaCl (●) or 10 mM-KCl (○), (b) L-arginine (200 $\mu\text{g ml}^{-1}$), (c) L-ornithine (200 $\mu\text{g ml}^{-1}$), (d) L-proline (200 $\mu\text{g ml}^{-1}$), (e) L-glutamine (500 $\mu\text{g ml}^{-1}$), (f) potassium L-glutamate (1.5 mg ml^{-1}).

In the latter case, addition of arginine or a precursor of it in the arginine cycle might replace Na^+ , because the urea-producing enzyme arginase is induced by arginine in *B. subtilis* (Harwood & Baumberg, 1977). In order to check this possibility and the possible relief from inhibition by aspartate, the ability of the normal amino acids to restore growth in a potassium aspartate medium was determined.

Restoration of growth in potassium aspartate medium by certain amino acids

The *aspH aspT* mutant (61507) was grown in S6+50 mM-sodium aspartate, the cells were sedimented by centrifuging, resuspended in S6+50 mM-potassium aspartate and distributed into flasks each containing a different amino acid (final concentration 200 $\mu\text{g ml}^{-1}$); all amino acids normally present in proteins were tested, with the exception of L-cysteine. As shown in Fig. 4, arginine, citrulline and ornithine restored growth in the absence of Na^+ but so did proline; glutamine was less effective and glutamate was ineffective. All other amino acids tested had little or no effect. *N*-Acetyl-L-glutamate or *N*-acetyl-L-ornithine (1 mg ml^{-1}) and the potassium salts of acetate, succinate, 2-oxo-glutarate or citrate (all 10 mM) were also ineffective. L-Cysteine (50 $\mu\text{g ml}^{-1}$), DL-homocysteine (100 $\mu\text{g ml}^{-1}$) and DL-homoserine (200 $\mu\text{g ml}^{-1}$) inhibited growth. The direct proline precursor L- Δ^1 -pyrroline-5-carboxylate (100 $\mu\text{g ml}^{-1}$) also restored growth. The compounds capable of restoring growth are metabolically related as shown in Fig. 5. The

Fig. 5. Metabolic relationship of compounds that allow rapid growth of *aspH aspT* strain 61507 in S6 + 50 mM-potassium L-aspartate.

question now raised was whether the compound actually required for rapid growth on potassium aspartate was a member of the arginine cycle or whether it was proline. Since a proline auxotroph (60961) could also grow on media supplemented with ornithine whereas an ornithine auxotroph (60019) could grow on ornithine but not on proline, ornithine apparently can be converted to proline but not the reverse. This suggested that compounds capable of supporting rapid growth in potassium aspartate did so by being converted to proline. This was confirmed by use of a mutant (*Ord*) which had been isolated in our laboratory as being unable to use ornithine as sole nitrogen source. The *Ord* mutation creates a block in the ornithine-oxo-acid aminotransferase which converts ornithine to glutamate semialdehyde (Table 2). A triple mutant *aspH aspT Ord* (strain 61859) was isolated by its ability to grow on plates containing glutamate, as sole nitrogen source, but not on those containing ornithine. Table 2 shows that ornithine-oxo-acid aminotransferase was induced by growth of the *aspH aspT* strain (61507) in the presence of ornithine. But the triple mutant (61859) showed no or only little activity of this enzyme, regardless of whether it was grown in the presence or absence of ornithine. The *aspH aspT Ord* strain could not grow rapidly in potassium aspartate supplemented (1 mg ml⁻¹) with arginine, citrulline or ornithine, but it grew well in the presence of proline or pyrroline-carboxylate; in sodium aspartate this triple mutant grew as well (doubling time 1.0 h) as the *aspH aspT* strain (Fig. 6).

Amino acid composition of cells grown under different conditions

The major enzyme leading from glutamate to glutamate semialdehyde (L-glutamate- γ -semialdehyde dehydrogenase) has never been demonstrated (Adams, 1970), and we were also unable to do so. Therefore, we could not determine whether this enzyme might possibly require activation by Na⁺. The pyrroline-5-carboxylate reductase, the other enzyme needed for proline synthesis, was not Na⁺-dependent.

To see whether the production of proline might be deficient in cells growing on potassium aspartate but not in those growing on sodium aspartate as sole carbon source or whether some other differences in amino acids pools were apparent, extracts of cells grown in these media and also of cells grown in the presence of proline were prepared by rapid filtration

Table 2. Activity of ornithine-oxo-acid aminotransferase

Cells were grown in S6 containing 50 mM-potassium aspartate (Asp) with or without 10 mM-NaCl and with or without 100 μ g L-ornithine (Orn) ml⁻¹. Extracts were prepared and assayed as described in Methods. Specific activity is expressed as pmol min⁻¹ (mg protein)⁻¹.

Strain	Specific activity		
	Cells grown in S6 (Na) + Asp		Cells grown in S6 (K) + Asp
	- Orn	+ Orn	+ Orn
<i>aspH aspT</i>	2.83	85.1	192.1
<i>aspH aspT Ord</i>	2.47	1.47	No growth

Fig. 6. Growth of *aspH asp1 Ord* strain 61859 in S6 + 50 mM-potassium aspartate in the presence of (a) 10 mM-NaCl (●) or 10 mM-KCl (○), (b) proline (○, ●), ornithine (□, ■) or arginine (△, ▲) at 200 μ g ml⁻¹ (open symbols) or 1 mg ml⁻¹ (closed symbols).

and formic acid extraction. The amounts of amino acids found are shown in Table 3. First, it is apparent that all cells contained large amounts of aspartate. Although the concentrations of the other amino acids differed to some extent in cells grown in different media, only for citrulline and proline were differences of more than a factor of two observed. As would be expected, the cells grown in the presence of proline contained more than ten times the concentration of proline than did those grown without extracellular proline. But no significant difference was observed in the concentration of proline in cells grown with sodium or potassium aspartate. Thus, the ability of proline to support growth must be related either to some aspect of its transport into the cells or to its intracellular concentration. The accumulation of citrulline in cells growing with potassium aspartate indicated that argininosuccinate synthetase is (always or only in the absence of Na⁺) rate-limiting. Thus, aspartate or NH₄⁺ metabolism via the arginine cycle and the removal of nitrogen by urea production appears to be rather inefficient in *B. subtilis*. Apparently, the accumulation of citrulline is avoided only in rapidly growing cells where arginine is rapidly utilized for protein synthesis. In any case, these processes are not relevant to this paper.

Table 3. *Amino acid composition of aspH aspT strain 61507*

Amino acid	Amino acid content (nmol A_{600}^{-1})			
	K^+	Cells grown with:		
		K^+ + proline	Na^+	Na^+ + proline
Alanine	8.0	7.0	6.5	5.7
α -Aminoadipate	ND	<0.1	2.2	3.3
α -Aminobutyrate	0.5	<0.1	0.2	0.3
Aspartate	206.3	274.1	201.3	163.1
Arginine	<0.1	<0.1	<0.1	<0.1
Citrulline	23.3	8.9	11.2	8.4
Cystine	8.8	8.7	8.8	7.5
Glutamate	490.4	503.0	313.0	276.9
Glutamine and homoserine	42.7	24.7	23.2	20.1
Glycine	2.1	1.7	2.9	2.3
Histidine	0.8	ND	<0.1	<0.1
Isoleucine and leucine	3.7	5.8	6.0	5.5
Lysine	1.4	0.8	1.3	1.6
Methionine	2.3	2.2	2.2	2.2
Ornithine	1.4	2.5	2.1	1.9
Phenylalanine	0.9	<0.1	ND	ND
Proline	6.2	77.4	5.4	43.4
Serine	7.6	5.6	4.6	4.1
Threonine	2.2	2.8	3.4	3.1
Tyrosine	0.4	<0.1	ND	ND
Tryptophan	<0.1	<0.1	<0.1	<0.1
Valine	3.9	7.0	3.9	3.7
Unknown 1	3.2	3.6	3.0	2.6
Unknown 2	ND	1.1	2.3	2.0

ND, Not detected.

DISCUSSION

aspH strains of *B. subtilis*, producing aspartate constitutively, grow on aspartate as sole carbon source much faster if the medium contains Na^+ than if it contains only K^+ ; this effect is specific to Na^+ (Iijima *et al.*, 1977). At high concentrations of aspartate the growth rate is not affected by the presence of the high affinity aspartate transport system since that is saturated at about 200 μM -aspartate. The low affinity aspartate transport system also seems to involve active transport because the inside concentration of aspartate is several-fold higher than the outside concentration even in the absence of Na^+ ; in an *aspH aspT* mutant, lacking the high affinity aspartate transport system, this accumulation is prevented by starvation or by inhibition of the electron transport system. This observation and the fact that the initial rates of aspartate uptake show much less Na^+ dependence than the rates of growth (compare Figs 1*b* and 2*b*) demonstrate that the much higher growth rates on aspartate observed in the presence of Na^+ do not result from a Na^+ -stimulated increase of aspartate transport but from an effect on cell metabolism. (There was some increase in the initial rate of aspartate uptake if the medium contained Na^+ . But it is well known that transport and metabolism cannot be strictly separated in whole cells. Although we have tried to optimize this separation by rapid sampling after aspartate addition, it is not clear whether the observed increase in aspartate uptake is due to a Na^+ effect on aspartate transport or whether it also results from the Na^+ effect on metabolism. However this may be, it does not affect our major conclusion.)

Since Na^+ does not affect growth on other carbon sources, and since all initial enzymes metabolizing aspartate as carbon source are not stimulated by Na^+ , the slow growth without Na^+ must be caused by the presence of high intracellular concentrations of aspartate, or of NH_4^+ which is immediately formed from the NH_3 liberated from aspartate via

aspartase. The possibility that Na^+ stimulates growth by inhibiting the K^+ -dependent homoserine dehydrogenase has been excluded. Conceivably, the inhibition of some other enzyme by aspartate or NH_4^+ could be counteracted by Na^+ . But it is also possible that Na^+ allows the rapid export of (inhibitory) NH_4^+ by countertransport; in this case Na^+ itself would have to be exported by a Na^+ export system, whose existence has been indirectly demonstrated in several bacteria (Silver, 1978). The Na^+ stimulation effect of *B. subtilis* growth on aspartate (i.e. possibly the Na^+ export mechanism) is inducible by prior growth in the presence of Na^+ .

Growth in potassium aspartate can also be greatly enhanced by arginine, citrulline, ornithine, pyrroline-5-carboxylate or proline. Proline (or pyrroline-carboxylate) is the compound actually needed; all other compounds can be converted into it. An *Ord* mutant, deficient in ornithine-oxo-acid aminotransferase, can still grow rapidly in potassium aspartate plus proline (or pyrroline-carboxylate) but not with arginine, citrulline or ornithine. Since this mutant can grow on glucose or other carbon sources without addition of ornithine or arginine it is apparent that ornithine-oxo-acid aminotransferase differs from acetylornithine aminotransferase (which is needed for the synthesis of *N*-acetylornithine and thus ornithine from glutamate). This is also supported by the fact that ornithine-oxo-acid aminotransferase is inducible by ornithine (or arginine) as was previously shown by Harwood & Baumberg (1977) whereas the acetylornithine aminotransferase is repressed by arginine (Vogel & Vogel, 1963).

It is not clear why proline allows good growth in the presence of potassium aspartate whereas other amino acids do not. Conceivably, aspartate might inhibit one of the steps in the formation of proline from glutamate; the first enzyme of this pathway (glutamate- γ -semialdehyde dehydrogenase) has still not been detected in any organism (Adams, 1970) so that its possible inhibition by aspartate (as an analogue of glutamate) or the possible counteraction of such inhibition by Na^+ could not be measured. However, *B. subtilis* is able to produce some proline in the absence of Na^+ because we have found the same intracellular concentration of proline in cells grown in either sodium or potassium aspartate. Thus, it is rather the more than 10-fold higher concentration of proline found in cells grown in the presence of proline, which allows growth on potassium aspartate. Possibly, proline counteracts the inhibition of some metabolic reaction by aspartate or NH_4^+ , such as the proline-activating enzyme. Alternatively, proline might activate some enzyme needed for growth on potassium aspartate. But it is also possible that proline specifically allows the more rapid export of inhibitory NH_4^+ either by countertransport or, following its own active import, by symport.

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