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Pseudomonas oxalaticus: Requirement of a Cosubstrate for Growth on Formate

THOMAS HÖPNER and ANNEMARIE TRAUTWEIN

Fachgruppe Biochemie der Universität Heidelberg

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Summary. The strand *Pseudomonas oxalaticus* obtained from a culture collection was separated into three closely related variants, which have in common the following growth properties on formate:

1. Growth on formate is strongly dependent on the presence of bacterial contaminations or the addition of minimal amounts of a cosubstrate, for example pyruvate or glycine.

2. There is a linear dependence of the amount of bacteria obtainable in the stationary phase on the amount of pyruvate which is present in the medium. After total consumption of pyruvate growth continues for ca. one cell generation and then ceases.

3. Uptake of ^{14}C from formate by washed cells is increased in the presence of unlabelled pyruvate.

4. Even after growth in a mixture of labelled formate and unlabelled pyruvate, the specific radio-activity of the carbon in the bacterial protein reveals that two thirds of the carbon is derived from formate.

A possible explanation for these results may be seen in an anaplerotic pathway which in the presence of pyruvate generates a metabolite acting as a C_1 acceptor.

Pseudomonas oxalaticus was isolated by Khambata and Bhat (1953) as an organism able to grow on formate as the sole carbon source. C_1 fixation is reported by Quayle and Keech (1959) to proceed via the ribulose diphosphate cycle. The energy and the reduction equivalents needed for this fixation are obtained from the oxidation of formate catalyzed by a soluble formate : NAD oxidoreductase (Quayle *et al.*, 1961) or by an insoluble formate oxidase mentioned by Quayle and Keech (1959).

In attempts to gain large quantities of pure bacterial material for the purification and characterization of the Formate : NAD oxidoreductase, we tried to reproduce the growth results on formate obtained from Johnson *et al.* (1965) and Höpner and Knappe (1970). The experiments revealed the inability of the organism to use formate as a source of cell constituents in the absence of a cosubstrate. It could be demonstrated that two events lead to growth on formate alone: Contaminations by other bacteria or impurities in the formic acid used for the preparation of the medium.

Material and Methods

Maintenance and Growth of Bacteria. Cultures of *Pseudomonas oxalaticus* were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen GB (Culture No. 8642) and maintained on dishes of 1.7% Agar (Powder, Serva Heidelberg, Germany) containing 0.8% yeast extract (DIFCO) pH 7.5 to combine storing with control of purity. Stock cultures were grown at 30°C, stored at 3°C and subcultured weekly.

For growth experiments, 10 ml of 0.8% yeast extract were inoculated from the stock culture. After 6 h shaking at 30°C the bacteria were centrifuged under sterile conditions, washed three times with 5 ml of the medium to be inoculated and used as an inoculum suspended in the same medium. Growth took place in Fernbach flasks on a rotary shaker at 30°C. Cells were harvested in a Sorvall refrigerated centrifuge RC 2B. Cell densities were calculated from turbididy data (578 mμ, 1 cm cuvette, Photometer Eppendorf, Hamburg, Germany) by means of a standard curve obtained from a dilution series of a suspension of *Pseudomonas oxalaticus A* in formate salt medium and expressed as g/liter wet cells. For growth, washing and labelling of the bacteria, the formate salt medium of Johnson *et al.* (1965) was used throughout. The "formate-pyruvate salt medium" contains in addition 5 mM pyruvate unless otherwise stated. The denotation "salt medium" means a medium in which formate is replaced by chloride. The media were sterilized once after adjusting the pH value to 7.8.

Chemicals. Sodium pyruvate, sodium formate, amino acids, vitamins, and formic acid (containing utmost 0.2% acetic acid) were purchased from Merck, Darmstadt, Germany. NAD, NADH and L-lactate dehydrogenase (rabbit muscle) were from Boehringer, Mannheim, Germany. NAD (free acid) was lyophilized three times to remove formate. ^{14}C-Formate, 52 mCi/mMol (Buchler, Braunschweig, Germany) was purified by paper chromatography. Paper No. 2040b from Schleicher and Schüll, Dassel, Germany, was used with the solvent n-propanol/conc. ammonia 7:3 (v/v). Formate had an $R_f = 0.73$. Two impurities (ca. 5% of radioactivity, $R_f = 0.38$ and 0.24) were separated off.

Assays. Formate and formate : NAD oxidoreductase activity were measured according to Höpner and Knappe (1970). Pyruvate was determined enzymatically with lactate dehydrogenase, protein by the biuret method. Radioactive assays were performed in a Packard 3003 TriCarb scintillation spectrometer using Aquasol (NEN, Dreieichenhain, Germany) as the scintillation mixture.

Results

Separation of the Commercial Culture into Three Variants

The commercial lyophilisate was transferred to 0.8% yeast extract and streaked out for single colonies on yeast extract agar plates after 12 h. Three types of colonies (A, B, C = Fig. 1) in the ratio ca. 1:2:2 were observed. The three variants were subcultured and maintained separately for (hitherto) 7 months (ca. 25 transoculations) without any change in the properties which are summarized in the Table. Growth on oxalate seems to be a property unique to A, while only B can grow on lactose and citrate. None of the species can utilize formate exclusively, but all are able to grow on formate when supplemented with a cosubstrate, e.g. pyruvate. The variants are indistinguishable under the phase constrast microscope

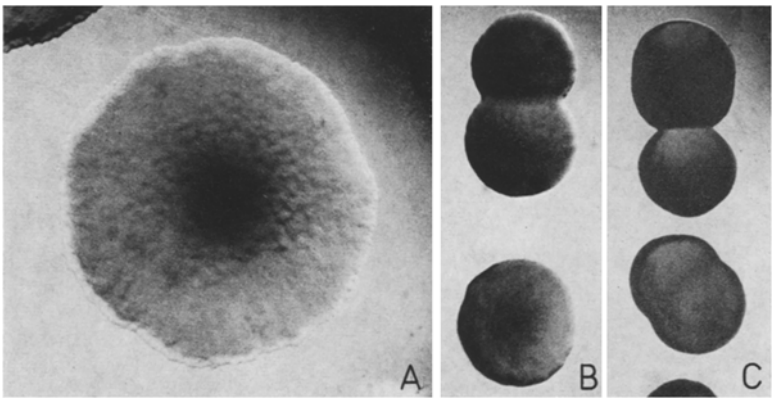

Fig. 1. Colony types isolated from *Pseudomonas oxalaticus* on yeast extract agar after 48 h at 30°C. ×8. We thank M. Maiwald for the photographs

after growing on yeast extract or formate medium. The three variants seem to be equally suitable as a starting material for enrichment of formate : NAD oxidoreductase. There are no significant differences in the amount and properties between the enzymes obtained from A, B and C. Clotting in suspension, as mentioned by Blackmore and Quayle (1968), is a property of C alone. A grows considerably faster than B and C. It was therefore chosen for further experiments.

Requirement of Auxiliary Substances for Growth on Formate

When *Pseudomonas oxalaticus* (A, B or C or the mixture) grown on yeast extract and washed three times with the formate salt medium is used as an inoculum of the formate salt medium, one observes essentially no growth (Fig. 2) or change of pH. Bacteria which are not washed or washed only once grow on for one or two cell generations. In order to determine whether growth in the presence of formate is dependent on auxiliary substances, the chessboard method of Holliday as modified by Clowes and Hayes (1968) was applied for checking the common vitamins, amino acids and other compounds. Growth (followed by turbididy and change of pH) was observed in the presence of serine, glycine, cysteine, phenylalanine and tyrosine. These substances could be replaced by pyruvate, lactate and (with lower activity) acetate, pyruvate being by far the most potent. The effect of pyruvate is shown in Fig. 2. The pH increased to a value of 8.5 only in the flasks with formate plus pyruvate (growth velocity is diminished only at pH-values above 8.9—9.0), indicating formate consumption. It remained constant (7.7) in the flasks with pyruvate or formate alone.

Table. *Properties of the* Pseudomonas oxalaticus *colony types*

Species	A	B	C
Properties of single bacteria[a]			
Shape (yeast extract) size (μm)	rod 0.6×3	rod 0.6×3	rod 0.6×3
Shape (formate medium) size (μm)	ovoid 1×1.5	ovoid 1×1.5	ovoid 1×1.5
Motility	motile	motile (more than A and C)	motile
Properties of colonies[b]			
Diameter after 48 h 30°C (mm)	5.5	2	2
Other properties	rough translucent granular slightly brownish flat with raised center	rough translucent granular slightly brownish convex	smooth opaque homogeneous almost white convex
Growth on C-sources[c]			
Lactate	+++	+++	+++
Pyruvate	+++	+++	+++
Acetate	+++	+++	+++
Oxalate	++	−	−
Formate	−	−	−
Formate/pyruvate 100:1	++	++	++
Glucose	++	+	+
Lactose	−	+	−
Citrate	−	+	−

[a] As visible in the phase contrast microscope. Enlargement 1600×.

[b] On agar dishes as described in "Material and Methods".

[c] 5 mg/ml in the salt medium. The inoculum was prepared as described in "Material and Methods". 30 ml tubes, covered with Kapsenberg-covers, were filled with 10 ml medium, sterilized twice, inoculated, and shaken in angled position on a rotary shaker for 72 h at 30°C.

Dependence of the Yield of Bacteria on the Amount of Pyruvate

In a series of 500-ml batches, the amount of bacteria in the stationary phase of growth was related to the amount of pyruvate (given as initial concentration) at the time of inoculation. It is clearly shown (Fig. 3) that there is an almost linear dependence. In any case, pyruvate was not detectable after reaching the stationary phase.

In the cultures containing initially 5 and 1 mM pyruvate respectively the kinetics of growth and pyruvate utilization were followed (Fig. 4a and b). It was observed, that growth proceeded for ca. one cell generation after total consumption of pyruvate and then ceased, while the formate consumption proceeded indicating a pyruvate independent formate respiration.

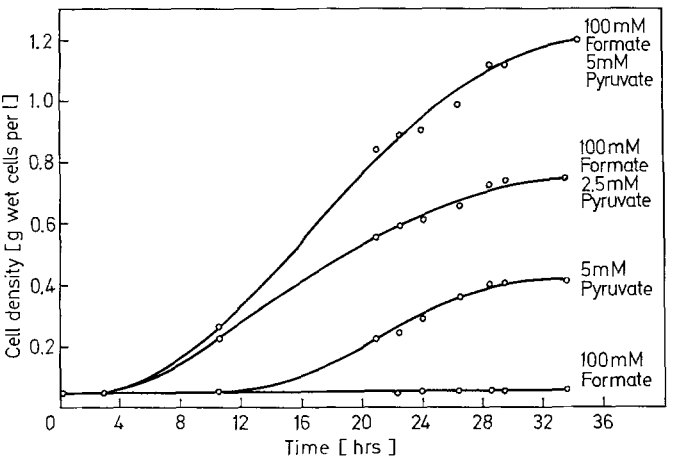

Fig. 2. Dependence of growth of *Pseudomonas oxalaticus A* on pyruvate in the presence of formate. The given concentrations of pyruvate and formate are initial concentrations at the time of inoculation

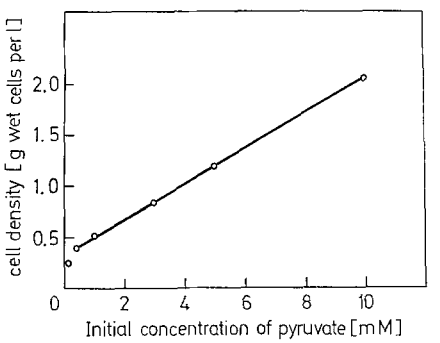

Fig. 3. Dependence of cell density in the stationary phase on initial concentration of pyruvate in the presence of 107 mM formate. Each point represents the cell density in the stationary phase of growth kinetics as shown in Figs. 2 and 4

Uptake of Radioactivity from ^{14}C-Formate by Whole Cells in the Presence of Unlabelled Pyruvate

If *Pseudomonas oxalaticus* is able to utilize formate for building up cell constituents only with the aid of pyruvate, the formate uptake would be expected to be dependent on pyruvate. To test this, bacteria grown in the formate-pyruvate salt medium were washed twice with the salt medium and shaken as a suspension in the salt medium for 1 h at 30° C. Cells were resuspended in the salt medium. After addition of

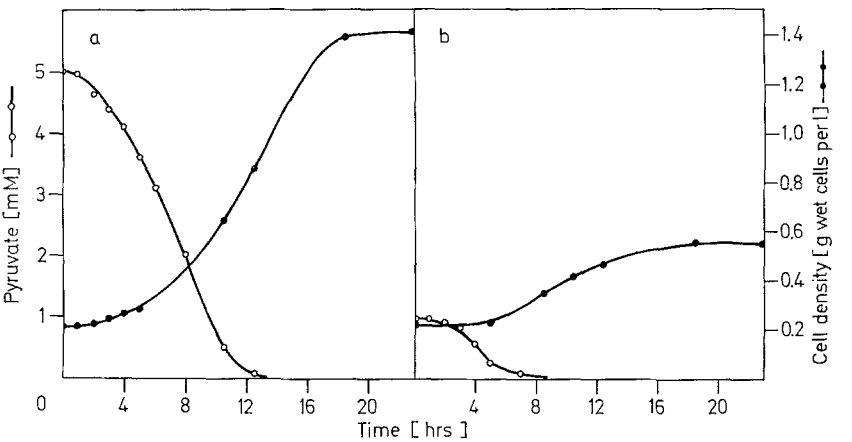

Fig. 4. Kinetics of growth and pyruvate consumption in the flasks of Fig. 3 having started with 5 (a) and 1 (b) mM pyruvate in the presence of 107 mM formate. In a formate concentration was 80 mM after 23 h further decreasing by ca. 1 mM per hour

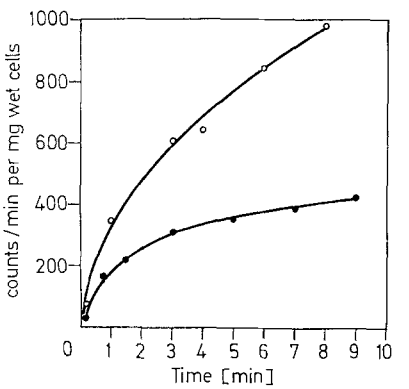

Fig. 5. Uptake of radioactivity by whole cells from ^{14}C-formate in the presence and absence of unlabelled pyruvate. Open circles: with pyruvate. Closed circles: without pyruvate. 0.05 ml sodium pyruvate (0.1 M) and immediately after it 0.05 ml sodium formate (0.1 M, 2×10^6 counts/min per μmole) were added to 5 ml cell suspension (100 mg wet cells per ml, for preparation see text) in a 15-ml-tube. To a second tube only the formate was added. During incubation at 20°C under vigorous shaking samples of 0.5 ml were withdrawn as indicated and washed as described in the text. Finally cells were suspended in 0.5 ml water and transferred into the scintillation vessels

^{14}C-formate and pyruvate as indicated in Fig. 5 samples of 0.5 ml were withdrawn and mixed into 0.5 ml cold salt medium containing 0.2 M sodium formate and 0.05 M sodium bicarbonate. Cells were centrifuged

off and washed five times with 1 ml formate salt medium containing 0.05 M carbonate. In the incubation mixture 20% of the added formate were still present after 10 min. Fig. 5 shows, that formate uptake is enhanced more then two fold by the presence of pyruvate.

Incorporation of Radioactivity from ^{14}C-Formate into Bacterial Protein in the Presence of Unlabelled Pyruvate

To further examine whether formate or pyruvate is the main source of carbon in the cell constituents, bacteria were grown in a mixture of labelled formate (109 mM, 3050 cpm/μmole) and unlabelled pyruvate (2.6 mM). 500 ml were inoculated with 0.32 g wet bacteria (grown in unlabelled medium as above) and aerated at 30° C. The pH-value was maintained at 7.5—8.0 by addition of phosphoric acid. Cells (0.70 g) were harvested after 24 h (stationary phase), washed twice with the unlabelled formate-salt-medium and fractionated for protein according to Clifton and Sobek (1961). After the final extraction of trichloroacetic acid by ethyl ether, the suspension was lyophilized. The remainder proved to be rather pure protein fitting exactly the biuret estimation (factor 17). The specific activity of the protein was 78350 cpm/mg after correction for the unlabelled inoculum. Carbon content determined by combustion analysis was found to be 49.06%. The data lead to a specific activity of 1918 cpm/μmole for the carbon in the protein of bacteria grown in the presence of the labelled formate. This shows clearly, that even in the presence of pyruvate almost two thirds of the carbon in the protein are derived from formate.

Discussion

The experimental results show that *Pseudomonas oxalaticus* is not able to grow on formate as the sole carbon source. Evidence for the dependence of growth and formate incorporation on pyruvate or a metabolite of it is presented.

The effect of pyruvate is observable only under strict exclusion of any contamination by other bacteria which enable *Pseudomonas oxalaticus* to grow on formate in the absence of a cosubstrate. Contaminations with this property can occur during the experiments especially after total consumption of endogenous auxiliary components when the increase of cell density ceased. This "pseudostationary phase" can be maintained for several days when the formic acid lost by respiration is replaced. The starvation period may end in the death of the culture (if it remained sterile) or in a surprisingly quick new start of growth. In the latter case contaminations are always detectable. These observations hold for the isolated species as well as for the mixture.

The parental culture itself was initially suspected to be the source of the contaminations. In spite of not being homogeneous, the culture con-

tained no impurities which supported growth of the main components on formate. It should be emphasized that the isolation of the three variants (no other species were detectable) was achieved without employing selective conditions. Their identical properties on formate therefore provide conclusive evidence that they are indeed components of the original culture and that no contamination occurred during the isolation experiments. The ratio 1:2:2 (A:B:C) between the three types of colonies on the first agar dish does not represent the true ratio in the lyophilisate obtained from the culture collection because the colony types have different growth rates in the yeast extract medium which had been inoculated with the lyophilisate. It is therefore interesting that variant A with the highest growth rate is present in the mixture in the smallest amount. If one assumes that A is not especially labile during lyophilisation, one can consider A as a mutant of the original strain which was enriched during maintenance of the culture. After inoculation of the formate pyruvate salt medium with the mixture, A supersedes B and C to an extent that after ca. 20 cell generations 95—99% of all bacteria are of the A type. On the other hand, the great biochemical similarity between A and C may lead to the conclusion that C is the smooth form of the rough form A or vice versa.

The inhomogeneity of the culture has little bearing on the main subject of this work, the ability of *Pseudomonas oxalaticus* to grow on formate alone. The three types which have been isolated behave identically with respect to their cosubstrate requirement for growth on formate. The experiments described here were performed with variant A, but the main results were also checked with B and C. No deviations have been found. The results show the unability of the organism to grow on a formate medium in the absence of a cosubstrate, e.g. pyruvate, the linear dependence of the amount of cells on the amount of pyruvate which is present in the medium, and the enhancement of ^{14}C uptake from ^{14}C-formate by pyruvate. Additionally it is demonstrated, that formate is the main source of carbon for building up cell protein even in the presence of pyruvate. These observations indicate that *P. oxalaticus* can utilize formate for the synthesis of the cell constituents only if a compound as pyruvate is present.

In the growth experiments the ratio pyruvate/formate is not constant during growth and reaches zero at the beginning of the stationary phase. Quantitative calculations, which might elucidate the fixation mechanism of C_1, therefore, are not possible. Even the fact, that in the presence of pyruvate two thirds of the carbon atoms of the bacterial protein were derived from formate, has only qualitative consequences regarding the kinetics of pyruvate consumption. In 10-liter-batches for preparative growth, excellent results are obtained by feeding the bacteria continually

with a mixture of formic acid/pyruvic acid 100:1 (moles per mole). This method of feeding maintains steady state conditions in the suspension as expressed by the constancy of the pH-value. Under these conditions the pyruvate concentration remains under the limit of detection. The consumption ratio 100:1 (or 33:1 for the carbon atoms) shows best, that there is no stoichiometric relationship between formate and pyruvate uptake. It is therefore concluded, that an anaplerotic pathway is maintained in the presence of pyruvate generating an acceptor molecule for the C_1-moiety.

Our observations could possibly be explained by a pathway proposed by Rabinowitz (1960) which was demonstrated to be involved in the C_1 metabolism of *Pseudomonas AM 1* (Large and Quayle, 1963). *AM 1* is reported to grow on formate as the sole carbon source (Peel and Quayle, 1961). By demonstrating the presence of the corresponding enzymes and their induction by C_1 substrates it was found that formate is fixed to tetrahydrofolate and transferred to glycine to yield serine (Heptinstall and Quayle, 1970). The crucial point is the regeneration of glycine because a mechanism for its synthesis from C_1 units via an aerobic main pathway is not known. The results given here show that *Pseudomonas oxalaticus* behaves like a mutant of *Pseudomonas AM 1*, the mutation being located in the pathway leading from C_1 to glycine. Glycine may be derived from the cosubstrate pyruvate. The superiority of pyruvate in this manner is perhaps only a question of permeability.

The results presented here differ from those of Quayle's laboratory in the question of the ability of the organism to grow on formate without any additives. It seems unlikely that Quayle's results can be explained by the assumption of slight impurities in his bacteria or impurities in the formate used. The latter is without doubt the reason for a large scale growth of *Pseudomonas oxalaticus* in 1967 using technical grade formic acid which was the basis for our preparation of formate : NAD oxidoreductase (Höpner and Knappe, 1970). The possibility that the incorrect bacteria are used in the work presented here is excluded by the method and the result of the isolation of the three types. In addition, the purity of the bacteria was checked by streaking out for single colonies after each experiment. It was not checked whether our method of maintenance of the subculturs on yeast extract agar (in contrast to subculturing on oxalate agar) is the reason for the non-autotrophy on formate. The disparity between the results presented here and those of Quayle's laboratory thus cannot be explained at this time.

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Dr. Thomas Höpner
Fachgruppe Biochemie
der Fakultät für Biologie
Organisch-Chemisches Institut
BRD-6900 Heidelberg
Im Neuenheimer Feld 7
Deutschland