FEMS Microbiology Letters 77 (1991) 67–72
Published by Elsevier



FEMSLE 04238

# Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*

M.P. Stephan, M. Oliveira, K.R.S. Teixeira, G. Martinez-Drets and J. Döbereiner

*CNPBS/EMBRAPA, Seropédica, Rio de Janeiro, Brazil*

Received 17 May 1990
Revision received 17 July 1990
Accepted 26 July 1990

Key words: $N_2$-dependent growth; $N_2$-fixation, in sugar cane; *Acetobacter diazotrophicus*



## 1. SUMMARY

The recently described species *Acetobacter diazotrophicus* isolated from sugar cane roots and stems was found capable of growth at pH 3.0 and showed high nitrogenase activity even at pH 2.5. No growth occurred at pH 7.5. Extracellular oxidation of glucose followed by gluconic acid formation was necessary for initiation of logarithmic growth, which proceeded with $N_2$ as the sole nitrogen source. $N_2$-dependent growth did not occur in N-free liquid medium under air, but starter doses of 0.6 to 1 mM $(NH_4)_2SO_4$ led to active $N_2$-fixing cultures after 34 h. Nitrogenase activity was only partially inhibited by 20 mM $(NH_4^+)_2SO_4$ and several amino acids showed similar effects. However, $NO_3^-$ did not inhibit or repress nitrogenase activity.

## 2. INTRODUCTION

Nitrogen balance studies, confirmed by $^{15}N$ isotope dilution, showed that more than 50% of the 200–400 kg N per hectare assimilated by a sugar cane crop can be obtained through biological $N_2$ fixation (BNF) in certain sugar cane genotypes [1,2]. Earlier observations indicated that $N_2$-fixing organisms of the genus *Beijerinckia* selectively multiply in the rhizosphere of sugar cane [3].

A new diazotroph, *Acetobacter diazotrophicus*, was found to occur in high numbers in roots and stems of sugar cane all over Brazil [4] and in Australia [5]. The bacterium which seems to be specific for this crop [6], was identified through DNA-rRNA hybridization and DNA-DNA homology values as a new species of *Acetobacter* [5,7]. Within the *Acetobacter* genus, seven species are described; however up to now, *Acetobacter diazotrophicus* is the only one able to fix $N_2$ [5].

In this paper we report several physiological characteristics of this new diazotroph.

## 3. MATERIALS AND METHODS

### 3.1. Strains

The bacterium used for this study was strain PAL 3 of *Acetobacter diazotrophicus* isolated from roots of sugar cane from a field in Alagoas, one of the main cane-growing regions in Brazil. All observed characteristics of this strain were identical

*Correspondence to:* J. Döbereiner, EMBRAPA, CNPBiologia do Solo, 23851, Seropédica, Rio de Janeiro, Brazil.

to those of the strain PAL 5 (ATCC 49037) also isolated from sugar cane roots in Alagoas.

*3.2. Culture media*

Media used in this study were modified forms of potato medium [8]. (i) Modified potato medium utilized for refreshing cultures contained (per liter): 500 ml potato extract; 100 g sucrose; 2 mg $Na_2MoO_4 \cdot 2H_2O$; 2.4 mg $MnSO_4 \cdot H_2O$; 2.8 mg $H_3BO_3$; 0.08 mg $CuSO_4 \cdot 5H_2O$; 0.24 mg $ZnSO_4 \cdot H_2O$; 0.01 mg biotin; and 0.2 mg pyridoxal · HCl. The medium was adjusted with 0.1 N KOH or 0.1 N HCl to pH 6.0 (medium potato P). (ii) Modified liquid potato medium utilized for inoculum preparation contained (per liter): 500 ml of potato extract and 2.5 g of sucrose adjusted to pH 6.0 (medium potato S). Potato extract (500 ml) was prepared from sliced potatoes (200 g) gently boiled in 500 ml of distilled water for 30 min and filtered through cotton-wool. (iii) Defined minimal media were a modified form of LGI medium [4] with the following composition (per liter): 5 g glucose; 0.2 g $K_2HPO_4$; 0.6 g $KH_2PO_4$; 1.32 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.02 g $CaCl_2 \cdot 2H_2O$; 0.002 g $Na_2MoO_4 \cdot 2H_2O$; 0.01 g $FeCl_3 \cdot 6H_2O$ (LGIM medium). To buffer LGIM medium at pH 5.5 the phosphate buffer was substituted by a buffer of 5 g of citric acid (26 mM) plus 4 g mono- and 5 g dipotassium phosphates (58 mM) (LGITA medium). The study of response to pH changes used different buffer mixtures of citric acid and potassium phosphates which were added to obtain media with pH ranging from 2.5 to 7.5. In order to obtain $N_2$-fixing cells some modifications were done in LGITA and LGIM media by reducing the concentration of $(NH_4)_2SO_4$ to 0.132 g $l^{-1}$ (1 mM) (LGITAF and LGIMF media, respectively).

*3.3. Nitrogenase activity in vivo*

Nitrogenase activity was monitored by the whole cell acetylene reduction method (ARA) [9]. Into 100-ml vials containing 20 ml liquid cultures acetylene was injected to give 10 kPa. Cultures were incubated at 30 °C for the time mentioned in each experiment and ethylene produced was analyzed by gas chromatography.

*3.4. Analytical methods*

Protein was determined after Lowry et al. [10] as modified by Herbert et al. [11], which was standardized with bovine serum albumin. Glucose, measured as reducing sugar, was determined by the method of Nelson [12], and $NH_4^+$ by the method of Giusti [13]. Gluconic acids formed were determined by HPLC. For this procedure the culture was centrifuged and the acidic components of the supernatant were extracted 3 times with ethyl acetate after acidification with 5 N $H_2SO_4$ to pH 2.5. After evaporation of the ethyl acetate phase at 30 °C and desiccation with $Na_2SO_4$, the material was resuspended in 40 mM phosphate buffer (pH 6.0), filtered through a Millipore filter and injected onto a reverse-phase RP-18 column using the same buffer as solvent. The method did not permit separation of gluconic acid, 2-ketogluconic acid and 5-ketogluconic acid, all of which had the same retention time. Using gluconic acid as standard, 60% of the sample was recovered during separation procedures.

## 4. RESULTS

*A. diazotrophicus* has a pronounced acid tolerance and was able to grow on $NH_4^+$ as nitrogen source at an initial pH as low as 3.0. At the optimal pH 5.5, the initial concentration of $3.3 \times 10^7$ cells $ml^{-1}$ increased to $320 \times 10^7$ cells $ml^{-1}$ after 41 h of incubation (Fig. 1). The optical density curve agreed well with the cell number curve, indicating no effect of pH on substances affecting the OD at 560 nm. Visually, the cultures showed pronounced increased viscosity at pH 5.5–6.5. Growth at an initial pH of 3.5 was half that observed at optimal pH 5.5 and there was no growth at pH 7.5. Growth of *A. diazotrophicus* on various carbon substrates is shown in Table 1. Glucose and Na salts of gluconate, lactate, pyruvate and acetate supported growth of *A. diazotrophicus*. However, no growth was observed with Na salts of dicarboxylic acids (succinate, malate, fumarate), ketoglutarate, tartrate, formate or with Na citrate (data not shown), as sole carbon source, at concentrations of 10, 20 and 30 mM.

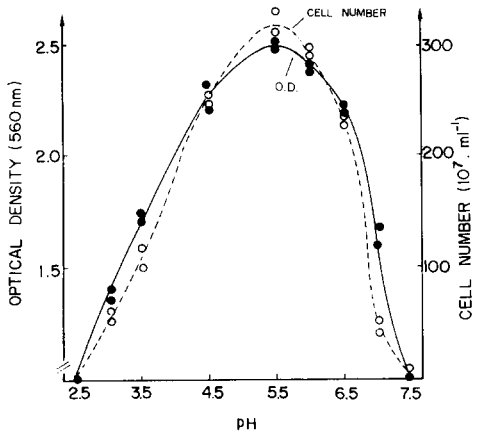


Fig. 1. Effect of pH on growth of *A. diazotrophicus*. Cultures were grown in 100 ml-flasks containing 20 ml of LGITA medium (range of pH 2.5–7.5). A 24 h-inoculum (0.2 ml) was added and the cultures were incubated at 30 °C in a rotatory shaker (190 rpm) for 41 h. Data shown are mean values of two replicate parallel cultures. ○——○, cell numbers; ●——●, optical density.

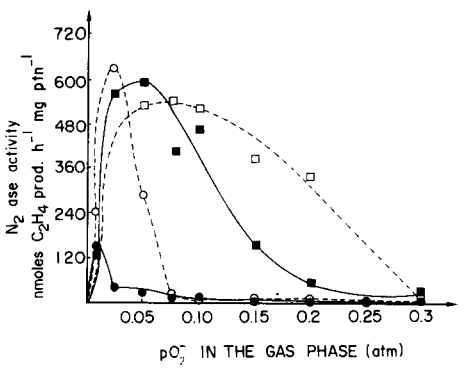


Fig. 2. Effect of $pO_2$ on nitrogenase activity of *A. diazotrophicus*. A 24-h inoculum was added to 6-l batch cultures containing LGIM medium with sucrose (100 g l$^{-1}$) and 1 mM $(NH_4)_2SO_4$. Cells were centrifuged, washed and resuspended in the same medium without carbon and nitrogen source to a concentration of 0.152 mg protein ml$^{-1}$. Incubations were performed in 100-ml flasks containing cell suspension (20 ml) and the mentioned carbon source (30 mM). Nitrogenase was measured after agitation at 190 rpm for 15 min under the given $pO_2$. ●——●, without carbon source; ○——○, cane sugar; ■——■, gluconic acid; □——□, glucose.

Nitrogenase activity of *A. diazotrophicus* was more tolerant to $O_2$ than that of most microaerobic diazotrophs, and cells grown on glucose showed the broadest oxygen tolerance (Fig. 2).

There was no $N_2$ dependent growth in N-free liquid media under air unless a nitrogen starter dose was used. Fig. 3 shows that cultures with

Table 1

Growth of *A. diazotrophicus* PAL 3 on several carbon sources

Cells were grown for 48 h in 100-ml flasks containing LGITA medium without glucose, supplemented with the indicated carbon source (5 g l$^{-1}$) at a shaking rate of 180 rpm. The initial OD was 0.03 and the number of cells was $3.7\times10^7$ cells ml$^{-1}$.

| Carbon source | Optical density (560 nm) * | No. of cells ($10^7$) ml$^{-1}$ |
|---|---|---|
| Malate | 0.05 $^{e}$ | 4.6 $^{e}$ |
| Succinate | 0.065 $^{e}$ | 6.7 $^{e}$ |
| Fumarate | 0.035 $^{e}$ | 4.1 $^{e}$ |
| α-ketoglutarate | 0.05 $^{e}$ | 6.0 $^{e}$ |
| Tartrate | 0.05 $^{e}$ | 5.5 $^{e}$ |
| Formate | 0.03 $^{e}$ | 3.8 $^{e}$ |
| Acetate | 0.23 $^{d}$ | 29.5 $^{d}$ |
| Lactate | 0.17 $^{d}$ | 22.4 $^{de}$ |
| Pyruvate | 0.61 $^{c}$ | 79.0 $^{c}$ |
| Gluconate | 2.07 $^{b}$ | 265 $^{b}$ |
| Glucose | 2.25 $^{a}$ | 288 $^{a}$ |

* Data were subjected to analysis of variance. Values in each column, followed by the same letter are not significantly different at $P = 0.05$.

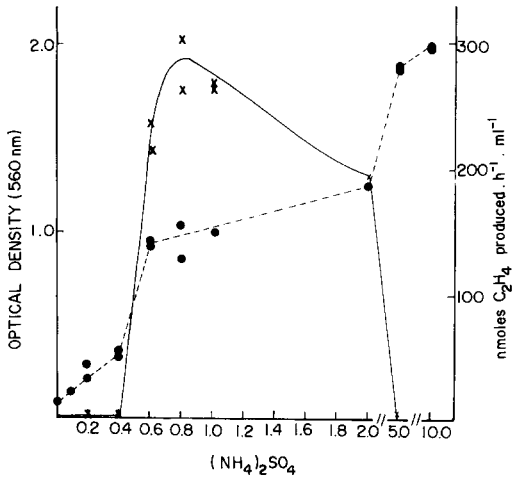


Fig. 3. Effect of $NH_4^+$ starter doses on expression of nitrogenase in *A. diazotrophicus*. Cells ($1.9\times10^7$) were grown at 30 °C and 190 rpm in 100 ml-flasks containing LGITA medium (20 ml), with different concentrations of $(NH_4)_2SO_4$ as starter doses. After 34 h of incubation nitrogenase activity (ARA) was measured. ●——●, optical density; ×——×, nitrogenase activity (nmol $C_2H_4$ h$^{-1}$ ml$^{-1}$).

Table 2

Inhibition of nitrogenase activity by various N sources

$N_2$ fixing cells were grown in 2-l batch culture for 34 h in LGIMF medium. Aliquots of 20 ml were distributed in 100-ml flasks. ARA was estimated 2 h after addition of the various N sources. A control, without addition of N source, was used to calculate the % inhibition.

| Nitrogen source | Percent of inhibition | |
|---|---|---|
| | Concentration of nitrogen source: | |
| | 1 mM | 10 mM |
| L-Methionine | 15.4 | 22.3 |
| L-Threonine | 17.2 | 25.4 |
| L-Glutamine | 20.5 | 27.3 |
| L-Glutamate | 10.4 | 12.5 |
| L-Leucine | 14.2 | 18.9 |
| $(NH_4)_2SO_4$ | 27.3 | 40.5 |
| $KNO_3$ | 0 | 0 |

$(NH_4)_2SO_4$ in the range of 0.6 to 1 mM increased the OD from 0.05 to 1.0 with derepression of nitrogenase and activity levels of about 300 nmol $C_2H_4$ $h^{-1}$ $ml^{-1}$ were reached after 34 h of incubation. Higher initial levels of $(NH_4)_2SO_4$ (5 mM) proportioned faster growth, but did not permit expression of nitrogenase activity during the 34-h incubation period.

Results on the effect of $NH_4^+$ on nitrogenase activity of whole cells are shown in Table 2. A partial inhibition of nitrogenase activity has also been demonstrated with various amino acids. Nitrate had no effect on growth or $N_2$ fixation by *A. diazotrophicus* even at higher doses and after prolonged incubation (data not shown). Based on initial studies in shaken cultures a minimal medium (LGIMF) was used for the growth of *A. diazotrophicus* on $N_2$. In order to control aeration, temperature and pH for a longer period of time a fermentor was used. The growth curve of *A. diazotrophicus* with dissolved $O_2$ control and with starter N (1 mM $(NH_4)_2SO_4$) is shown in Fig. 4A,B. Maximal OD and protein concentration was detected after 24 h incubation. A 10 h slow-growing phase during which glucose reached the concentration of 6 mM, was followed by the formation of gluconic acids and the initiation of the logarithmic growth phase (Fig. 4A). HPLC analyses did not allow the separation of the three possible gluconic acids formed during glucose oxidation. However, 2-keto- and 5-keto-gluconic acids gave a positive reaction with the reducing sugar test, which would explain the second peak observed in Fig. 4A. The continued protein accumulation after $NH_4^+$ exhaustion showed that under these conditions the cells were growing on $N_2$ as sole nitrogen source (Fig. 4B). These cultures were grown without adjusting the pH, reaching a final pH of 2.5 (Fig. 4A,B). Growth curves had a similar pattern for glucose, $NH_4^+$ utilization and gluconic acid formation as those grown at pH 6.0 [14]. The maximal rate of nitrogenase activity observed was 400 nmol $h^{-1}$] (mg protein)$^{-1}$ for

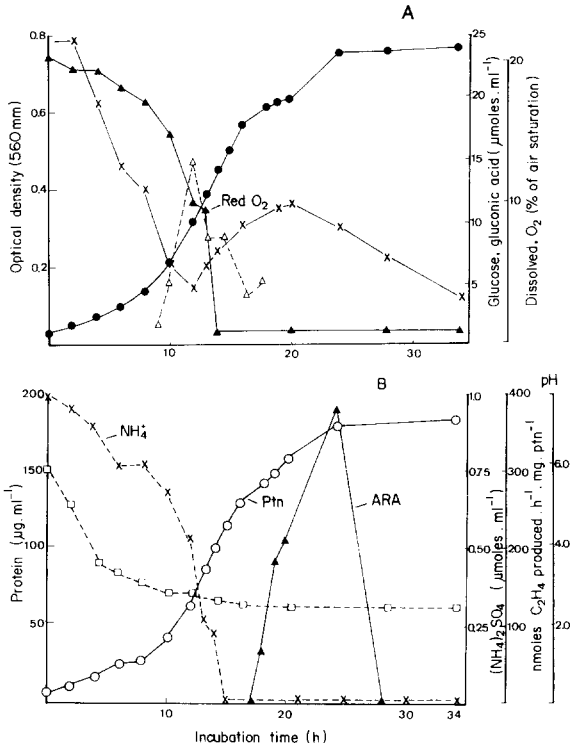


Fig. 4. Growth of *A. diazotrophicus* in a fermentor. Cells were grown at 30 °C in LGIMF medium at airflow of 2.5 l $min^{-1}$. Cells were centrifuged, washed, suspended in the same medium and added to the fermentor. At 13 h incubation, $pO_2$ was reduced to 0.01 atm. Samples were taken every 2 h and data shown are mean values of duplicate assays. (**A**) ●———●, optical density; ×———×, glucose utilized; ▲———▲, dissolved $O_2$; △- - - - -△, gluconic acids. (**B**) ○———○, protein; ▲———▲, nitrogenase activity; ×———×, $NH_4^+$ utilized; □- - - - -□, pH.

growth at pH 2.5 (Fig. 4B) and 130 nmol $h^{-1}$ (mg protein)$^{-1}$ at pH 6.0 [14].

## 5. DISCUSSION

The capacity of growth and $N_2$ fixation at the extreme acid conditions (Figs. 1 and 4A,B) of *Acetobacter diazotrophicus* is unique and suggests the existence of a very effective protection mechanism of the cell. The nitrogenase activity rates observed at such low pH are in the range reported for *Azospirillum* [15] or *Azotobacter* [16]. Very little change of ARA in the pH range between 2.5 and 16.5 was observed previously by us [17]. The need for extracellular oxidation of glucose, which seems to occur in the periplasmic space as described for *Pseudomonas aeruginosa* [18], explains the difficulties in initiating growth at low pH (Fig. 1) with glucose, which has to be oxidized to gluconic acids before the logarithmic growth phase begins.

An extracellular mechanism of glucose oxidation with the formation of gluconic acid has been described for *Pseudomonas aeruginosa* [18], and *Gluconobacter oxidans* (*Acetomonas oxidans*) [19]. HPLC analyses showed that the accumulation of gluconic acids was detected only after the glucose concentration had dropped to 6.0 mM (Fig. 4A). This suggests the formation of an intermediate product of glucose oxidation, perhaps gluconolactone as described for *Pseudomonas ovalis* [20]. Extracellular metabolism of glucose did not occur anaerobically confirming the oxidative nature of this process.

The extracellular oxidation of glucose and other reducing sugars [4] might represent a new oxygen protection mechanism of nitrogenase. The higher oxygen protection observed with glucose than with gluconic acid could be explained by the higher oxidation state of gluconic acid (Fig. 2).

The relative tolerance of the nitrogenase of *A. diazotrophicus* to $NH_4^+$ and to several amino acids (Table 2) might have considerable ecological importance, especially since this organism lacks also a nitrate reductase [4]. There are only two other diazotrophs known to lack nitrate reductase and which fix $N_2$ in the presence of nitrate completely independent of its concentration: *Azotobacter paspali* [21,22] and *Bacillus azotofixans* [23]. Nitrogenase of *Azotobacter vinelandii* is completely inhibited within 15 min after addition of even much lower concentrations of $NH_4^+$ [24]. Similar observations were reported for three *Azospirillum* spp. which showed immediate switch-off of nitrogenase in the presence of 0.25 mM $NH_4^+$ [25].

The optimal ammonium sulphate concentration for obtaining $N_2$-fixing cells of *A. diazotrophicus*, in liquid medium, was 0.8 mM (Fig. 3). Concentrations lower than 0.6 mM were probably not sufficient to increase the cell number to levels necessary to reduce the $pO_2$ to a concentration that permits the derepression of the nitrogenase system.

The above mentioned characteristics suggest that *A. diazotrophicus* is a quite different organism when compared to other root-associated diazotrophs and extensive studies of the mechanism of plant-bacteria interaction involving this bacterium are necessary for its exploitation in agriculture.

## ACKNOWLEDGEMENTS

Financial support from the NAS/BOSTID project and fellowships for all authors from CNPq are acknowledged. We thank Teresa Cristina Souto Silva for typing the manuscript, Dorimar dos Santos Felix and Zilda Martim Lourenço for organizing the bibliography and Wanderlei Pires for laboratory assistance.

## REFERENCES

[1] Lima, E., Boddey, R.M. and Döbereiner, J. (1987) Soil Biol. Biochem. 19, 165–170.
[2] Urquiaga, S., Botteon, P.B.L. and Boddey, R.M. (1989) in Nitrogen Fixation with Non-Legumes (Skinner, F.A., Boddey, R.M. and Fendrik, I., eds.), pp. 311–319, Kluwer Academic Publ., Dordrecht.
[3] Döbereiner, J. (1961) Pl. Soil 15, 211–216.
[4] Cavalcante, V.A. and Döbereiner, J. (1988) Pl. Soil 108, 23–31.
[5] Gillis, M., Kersters, K., Hoste, B., Janssens, D., Krop-

penstedt, R.M., Stephan, M.P., Teixeira, K.R.S., Döbereiner, J. and De Ley, J. (1989) Int. J. Syst. Bacteriol. 39, 361–364.
[6] Döbereiner, J., Reis, V.M. and Lazarini, A.C. (1988) in Nitrogen Fixation: Hundred Years After (Bothe, H., De Bruijn, F.J. and Newton, W.E., eds.), pp. 717–722, Fischer, Stuttgart.
[7] Gillis, M., Janssens, D., Kersters, K., Hoste, B., Döbereiner, J. and De Ley, J. (1988) in Nitrogen Fixation: Hundred Years After (Bothe, H., De Bruijn, F.J. and Newton, W.E., eds.), p. 793, Fischer, Stuttgart.
[8] Döbereiner, J. (1980) in Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F.J., ed.), 535–555, John Wiley, Chichester.
[9] Burris, R.H. (1972) Methods Enzymol. 24B, 415–431.
[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Herbert, D., Phipps, P.J. and Stranger, R.E. (1971) in Methods in Microbiology, Vol. 5B (Norris, J.R. and Ribbons, D.W., eds.), pp. 209–344, Academic Press, New York.
[12] Nelson, N. (1944) J. Biol. Chem. 153, 375–380.
[13] Giusti, G. (1970) in Methoden der Enzymatischen Analyses (Bergemeyer, H.V., ed.), pp. 1049–1056, Verlag Chemie, Weinheim.
[14] Oliveira, M., Stephan, M.P. Teixeira, K.R.S. and Döbereiner, J. (1990) Revista Brasileira de Microbiologia (in press).
[15] Pedrosa, F.O., Stephan, M.P. and Döbereiner, J. (1981) in Associative $N_2$-Fixation (Vose, P.B. and Ruschel, A.P., eds.), pp. 15–25, CRC Press, Inc., Boca Raton, FL.
[16] Yates, M.G., Walker, C.C. Partridge, C.D.P., Pedrosa, F.O., Stephan, M.P. and Döbereiner, J. (1981) in Current Perspectives in Nitrogen Fixation (Gibson, H. and Newton, W.E., eds.), pp. 97–100, Australian Academy of Science, Canberra.
[17] Stephan, M.P., Teixeira, K.R.S. and Döbereiner, J. (1988) in Nitrogen Fixation: Hundred Years After (Bothe, H., De Bruijn, F.J. and Newton, W.E., eds.), p. 287, Fischer, Stuttgart.
[18] Whiting, P.H., Midgley, M. and Dawes, E.A. (1976) J. Gen Microbiol. 92, 304–310.
[19] Glattli, H. and Ettlinger, L. (1970) Arch. Mikrobiol. 74, 273–282.
[20] Humphrey, A.E. and Reilly, P.J. (1965) Biotechnol. Bioeng. Z, 229.
[21] Peña, J.J. and Döbereiner, J. (1974) R. Latinoam. Microbiol. 16, 33–44.
[22] Döbereiner, J., Day, J.M. and Dart, P.J. (1972) J. Gen. Microbiol. 71, 103–116.
[23] Seldin, L., Van Elsas, J.D. and Penido, E.G.C. (1984) Int. J. Syst. Bacteriol. 34, 451–456.
[24] Kurz, W.G.W., La Rue, T.A. and Chatson, K.B. (1975) Can J. Microbiol. 21, 984–988.
[25] Hartmann, A., Fu, H.A. and Burris, R.H. (1986) J. Bacteriol. 165, 864–870.