Appl Microbiol Biotechnol (1989) 32:205–210

Applied and Microbiology Biotechnology

Requirement of chelating compounds for the growth of *Corynebacterium glutamicum* in synthetic media

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Summary. The effects of various iron-complexing substances on the growth of *Corynebacterium glutamicum* in synthetic medium were investigated. The data obtained indicate that *C. glutamicum* has an absolute requirement for the presence of an iron-complexing compound as a growth factor for rapid and abundant growth in synthetic media. This requirement can be met by adding low concentrations (10^{-5} *M*) of certain dihydroxyphenols (catechol, protocatechuate) or relatively high concentrations (0.1%) of citrate to the medium or by autoclaving a small amount of glucose together with other media components. The addition of catechol or protocatechuate to synthetic media has advantages over media preparation with citrate or autoclaved glucose. In the described synthetic broth supplemented with catechol or protocatechuate growth is largely independent of the iron content of the medium in a range between 0.037 and 0.37 m*M*.

Introduction

The production of amino acids by coryneform bacteria has become a process of great commercial interest during recent decades. Corynebacteria have also been found to be useful microorganisms for various other biotechnological applications, for example bioconversions (reviewed by Martin et al. 1987). Recent advances in the understanding of the molecular biology of these bacteria (Peoples et al. 1988) as well as the establishment of cloning systems (Santamaria et al. 1984; Katsumata et al. 1984; Yoshihama et al. 1985) have led to the development of new strategies for strain improvement via genetic engineering.

Knowledge of the nutritional requirements of a bacterial strain is of crucial importance if one wants to devise growth media for specific purposes. This is the case for industrial fermentations as well as for basic laboratory research concerned with many aspects of carbohydrate and amino acid metabolism. In the course of our work with *Corynebacterium glutamicum* it was observed that in order to reproducibly achieve abundant growth in glucose-containing synthetic medium it was helpful to autoclave a small portion of the total amount of glucose together with the other medium constituents (unpublished). Undefined glucose reaction products formed during this method of media preparation are thought to have effects similar to those of chelating agents (Sergeant et al. 1957; Lankford et al. 1957; Guirard 1958) and thus to supply iron in a soluble form suitable for uptake by the bacterial cells.

The effects of some chelating agents and autoclaved sugar on the growth of *C. glutamicum (Micrococcus glutamicus)* in synthetic medium have been described by Nakayama et al. (1964a, b). Ferrichrome was effective in promoting growth in synthetic medium and could be replaced by hydroxyaspergillic acid or large amounts of ascorbic acid or iron salts. These data reflect the significance of iron in supporting the growth of *C. glutamicum* in synthetic media.

Other chelating compounds (i.e. dihydroxyphenols) have been reported to stimulate the growth of *Bacillus subtilis* (Peters and Warren 1968) and *M. luteus* (Salton 1964; Walsh et al. 1971) in defined media. In this communication we report an investigation of the effects of various dihydroxyphenols and other chelators on the growth of *C. glutamicum* in synthetic media.

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Materials and methods

Organism. All experiments were carried out with *C. glutamicum* AS 019, a spontaneous rifampicin-resistant mutant of strain ATCC 13059 (Yoshihama et al. 1985).

Medial and chemicals. The basal medium used throughout this study, designated BMCG, was prepared in the following manner: 7 g $(NH_4)_2SO_4$ was dissolved in 850 ml deionized H_2O, mixed with 100 ml of 10× M9 stock (60 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl in 1 l H_2O, pH 7.3) and autoclaved, followed by the addition of 5 ml 200× salt stock (80 g $MgSO_4 \cdot 7H_2O$, 4 g $FeSO_4 \cdot 7H_2O$, 0.4 g $MnSO_4 \cdot H_2O$, 5 g NaCl in 1 l H_2O), 2 ml trace element solution (88 mg $Na_2B_4O_7 \cdot 10H_2O$, 40 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 270 mg $CuSO_4 \cdot 5H_2O$, 7.2 mg $MnCl_2 \cdot 4H_2O$, 870 mg $FeCl_3 \cdot 6H_2O$ in 1 l H_2O), 0.05 ml 1 *M* $CaCl_2$ (all autoclaved separately), 1 ml vitamin stock (1 mg biotin, 10 mg thiamine HCl per 1 ml H_2O) and 50 ml 20% glucose (both filter sterilized). Phenolic compounds or citrate (stocks adjusted to a neutral pH and sterilized by filtration) were added aseptically to the medium before inoculation. In experiments with increasing iron concentrations, appropriate amounts of a sterile 0.1 *M* stock of ferric chloride were added. Chemicals were purchased from Sigma (St. Louis, Mo, USA). Glucose was p.A. grade.

Culture conditions. Cultures were grown in 30 ml media in 100-ml erlenmeyer flasks. Overnight cultures used as inocula were generally grown in media of identical composition as the corresponding test media. The initial optical density (OD) at 578 nm was adjusted to a value of 0.2–0.3. Incubation was carried out on a rotary shaker set at 200 rpm at a temperature of 30° C. All growth experiments were carried out at least as duplicates. Growth was followed by measuring the OD of the cultures at 578 nm using a Beckman Model 24 (Beckman, Palo Alto, Calif, USA) spectrophotometer. The turbidometric readings were converted to dry cell weight (dw) by reference to a prepared standard curve. At an OD of 1.0 the cell concentration was found to be 0.28 mg dw/ml.

Results and discussion

Iron is an essential constituent of many enzymes, especially those involved in oxygen metabolism and electron transfer. Therefore, the acquisition of iron from the surrounding medium is of the utmost importance for almost all organisms. However, the extremely poor solubility of iron often causes this metal to be a limiting factor for microbial growth. This is particularly the case under conditions of aerobic growth and for pathogenic bacteria which multiply in habitats such as body fluids, where any free iron is bound to iron-binding proteins (Griffiths 1987).

A way to overcome the limited supply of iron widely distributed among microorganisms is the production of siderophores, that is low molecular weight compounds capable of binding iron with extremely high affinity. A number of coryneform and related bacteria produce siderophores, for example, *Mycobacterium* and *Rhodococcus* strains (Snow and White 1969; Macham and Ratledge 1975; Hall and Ratledge 1986) or *C. diphtheriae* (Russell et al. 1984). In amino-acid-producing corynebacteria, however, the production of iron-binding siderophores has not been reported to date.

Poor growth of *C. glutamicum* in certain synthetic broth formulations can be overcome by autoclaving glucose together with other medium constituents (Nakayama et al. 1964b; von der Osten et al. 1989; own unpublished results). However, this method of media preparation is often not desirable because it leads to the formation of certain glucose reaction products and therefore the medium is no longer chemically defined. Also, it seems that only certain carbohydrates develop stimulatory properties upon autoclaving (Lankford et al. 1957). If, however, the metabolism of other carbon sources than glucose is to be studied in *C. glutamicum*, it may be inappropriate to add even a small amount of glucose and autoclaving the alternative carbon source together with the medium may have no growth-stimulating effect. In these cases the availability of a synthetic medium which employs a chemically defined chelating agent potent in supporting iron uptake would be extremely useful.

A synergistic action between citric acid and iron salt for promoting growth of *C. glutamicum*

Table 1. Growth response of *Corynebacterium glutamicum* to supplementation of basal medium (BMCG) with various substances

Addition to BMCG (10^{-5} *M*)	OD_{578} after 24 h incubation at 30° C
Benzoate	0.23
Catechol	24.40
Chorismic acid	0.54
Citrate	0.16
2,3-Dihydroxybenzoate	5.80
2,4-Dihydroxybenzoate	0.28
2,6-Dihydroxybenzoate	0.29
3,4-Dihydroxybenzoate	22.30
p-Hydroxybenzoate	0.27
Salicylate	0.20
Shikimate	0.21
Tryptophane	0.18
Tyrosine	0.20
Phenylalanine	0.22
Nil	0.20

Photometric readings (578 nm) were made after 24 h incubation at 30° C. The initial optical density (OD) after inoculation was approximately 0.25

was noted very early by Nakayama et al. (1964a) and investigated in more detail recently by von der Osten et al. (1989). We have also found citrate to be effective in supporting growth of this species in synthetic broth (Fig. 1). However, the addition of relatively large amounts of citrate (0.1% w/v = 3.4 m*M*) is required in order to achieve abundant growth in BMCG broth. Citrate at a concentration of 10^{-5} *M* is not effective (Table 1). This is not surprising since a large excess of citrate (ratio of citrate to iron of 200:1) seems to be necessary for the generation of soluble ferric iron-dicitrate complexes (Spiro et al. 1967). Therefore, low concentrations of citrate are not sufficient to hold enough iron in solution to support abundant growth.

Von der Osten et al. (1989) found that citrate added to glucose-containing minimal medium was metabolized by *C. glutamicum* at a significant rate. However, it was not clear what the fate of the citrate was or which reactions were responsible for the almost total depletion of citrate from the culture medium within the first 10 h of fermentation. We have observed that upon inoculation of basal medium containing citrate (0.5%) as sole carbon source growth was weak but nevertheless significant (unpublished data). This circumstance may be a serious drawback for the use of citrate as a growth-promoting agent, particularly for research concerning certain aspects of carbon metabolism and metabolic regulation.

We have observed that *C. glutamicum* AS 019 grew well in BMCG broth if the inoculum was taken from a fresh LB (Luria-Bertani medium, Maniatis et al. 1982) agar plate or from LB broth. However, if the inoculum was from a previous BMCG culture or if washed inocula were used, growth was very poor, although in these cases growth occasionally occurred after extended lag periods (5 days or more). Similar observations were made by Salton (1964) and Walsh et al. (1971) for *Micrococcus lysodeikticus (M. luteus)*, where varying lag periods preceded the initiation of growth in defined media. The length of the lag period was directly correlated with the number of washing steps the cells experienced before inoculation. These effects were attributed to the requirement of *M. luteus* for dihydroxyphenolic compounds in the culture medium (Salton 1964; Walsh et al. 1971).

In order to achieve a better understanding of the nutritional requirements of *C. glutamicum* and ultimately obtain a recipe for an improved medium of defined composition, we have tested a variety of dihydroxyphenolic and other compounds for their ability to enhance growth of *C. glutamicum* in synthetic media.

In a preparatory experiment (no multiple cultures performed) the following substances were added to glucose (1%)-containing basal synthetic medium (BMCG) at a concentration of 10^{-5} *M* and their influence on the growth of *C. glutamicum* AS 019 was investigated: benzoic acid, catechol (1,2-benzenediol, pyrocatechol), chorismic acid, citric acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), *p*-hydroxybenzoic acid, salicylic acid and shikimic acid. The inoculum for these cultures was from an overnight BMCG culture which itself had been inoculated from a fresh LB plate with strain AS 019. After 24 h incubation at 30° C the OD of the cultures at 578 nm was determined. At the chosen concentration, benzoic acid, citric acid, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, *p*- hydroxybenzoic acid, salicylic acid and shikimic acid had no significant growth-stimulating effect, although some differences were noted (Table 1). Negative results were also obtained if the medium was supplemented (10^{-5} *M*) with one of the aromatic amino acids, L-tyrosine, L-tryptophane or L-phenylalanine (not shown). On the other hand, addition of catechol, protocatechuate (3,4-dihydroxybenzoic acid), 2,3-dihydroxybenzoic acid and, to a lesser extent, chorismate were clearly stimulatory for the growth of *C. glutamicum* AS 019 in BMGC broth (Table 1). Therefore, the growth-enhancing effect of these compounds was studied in more detail.

The effect of supplementation of BMCG with a wide range of concentrations (between 10^{-3} *M* and 10^{-8} *M*) of catechol or protocatechuate (3,4-dihydroxybenzoate) on the growth of *C. glutamicum* AS 019 in BMCG broth was studied. For these experiments the concentration of iron (ferric chloride) in the culture medium was always 20 mg/l. For catechol, the lowest concentration required for optimal growth was 10^{-5} *M* (Fig. 1). At 10^{-6} *M* growth was impaired, resulting in a decrease in the growth rate as well as the final culture density which only reached approximately 10% of the maximum value. For protocatechuate the optimal concentration in BMCG was 10^{-4} *M*, while supplementation at 10^{-5} *M* was suboptimal and at 10^{-6} *M* growth was significantly weaker. The growth rates and maximum culture densities reached with BMCG supplemented with the dihydroxyphenolic chelators catechol or protocatechuate at their respective optimal concentrations were similar to the values obtained with BMCG

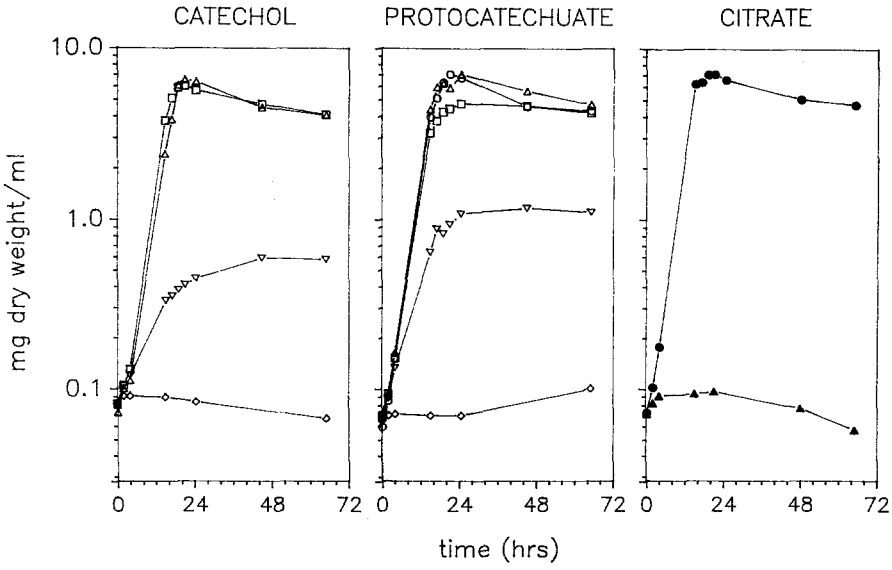

Fig. 1. Growth of *Corynebacterium glutamicum* in BMCG supplemented with various concentrations of catechol *(left)*, protocatechuate *(centre)* or 0.1% citrate *(right)*. Concentrations of phenolic compounds: 10^{-3} *M* (○), 10^{-4} *M* (△), 10^{-5} *M* (□), 10^{-6} *M* (▽), 10^{-7} *M* (◇). Growth in BMCG broth with 0.1% citrate (●) or without any chelating agent (▲) is shown in the graph at the *right*

containing 0.1% citrate (Fig. 1). Catechol or protocatechuate (10^{-5} *M*) added to complex LB broth exerted no effect on the growth of *C. glutamicum* (not shown).

The growth response of *C. glutamicum* to supplementation of BMCG with 2,3-dihydroxybenzoate at 10^{-3} *M* and 10^{-4} *M* was similar to that observed for protocatechuate. However, the negative effect on growth by decreasing the concentration of effector to 10^{-5} *M* was more severe in the case of 2,3-dihydroxybenzoate than found with protocatechuate (not shown). The level of chorismate supplementation required for good growth was more than one order of magnitude higher than observed with the other compounds (not shown). Therefore, the growth-enhancing properties of chorismate were not studied in further detail.

Iron-binding phenolic acids and phenolic acid-amino acid conjugates have been isolated from low-iron cultures of a variety of bacteria, although some of the identified phenolic substances may be hydrolysis products of more complex compounds (Lankford 1973). Ratledge and Chaudhry (1971) found that some organisms within the group Actinomycetales accumulated iron-chelating phenolic acids in the growth medium during extended incubation under conditions of iron deficiency. It is not known if iron limitation also results in the appearance of phenolic acids (which may aid in the assimilation of ferric iron) in the growth medium of the related species *C. glutamicum*, but a similar finding for this species could possibly provide an explanation for the occasional resumption of growth by *C. glutamicum* after an extended lag period in synthetic media without supplementation of iron chelators (unpublished observations).

In another set of experiments the effect of increasing concentrations of iron salt on the growth of *C. glutamicum* in BMCG containing 10^{-5} *M* catechol or protocatechuate, respectively, was investigated. For the given concentration of chelator the iron supplementation could be varied between 10 and 100 mg/l without changing the growth rate or maximum culture density more than approximately 10% (not shown). However, increasing iron concentrations had a slightly more pronounced effect on growth in protocatechuate-containing BMCG than in BMCG containing catechol.

The results presented above must be considered when designing a synthetic medium for *C. glutamicum*. Certain dihydroxyphenols (catechol, protocatechuate), citrate or autoclaved glucose are able to stimulate growth of *C. glutamicum* in synthetic broth (see above). However, the disadvantages of autoclaved glucose (undefined nature, not applicable when investigating the metabolism of carbohydrates other than glucose) and citrate (high concentrations necessary, unclear metabolism of citrate by *C. glutamicum*) indicate that the dihydroxyphenolic compounds mentioned above are superior for the preparation of synthetic media for *C. glutamicum*.

Our data fit together well with results obtained by others (Nakayama et al. 1964a, b; von der Osten et al. 1989) and lead us to believe that all *C. glutamicum* strains investigated to date are siderophore-auxotrophs. These findings indicate that

the production of strong iron-chelating siderophores may not be essential for the survival of this species in its natural habitat. On the other hand, (a) mechanism(s) with presumably low specificity exists for the acquisition of ferric iron when complexed with certain dihydroxyphenolic chelating agents. Additionally, a ferric dicitrate iron transport system, perhaps similar to those described for *E. coli* and *Mycobacterium,* may be present. However, the precise molecular mechanisms for the uptake of solubilized ferric iron by *C. glutamicum* remain to be elucidated.

In conclusion, the stimulating effects of various iron-complexing substances on the growth of *C. glutamicum* have been investigated. The results presented in this communication indicate that *C. glutamicum* requires an iron-complexing compound as a growth factor for rapid and abundant growth in synthetic media. Good growth occurs upon supplementation with low concentrations (10^{-5} *M*) of certain dihydroxyphenols (catechol, protocatechuate; Fig. 1). The addition of relatively high concentrations (0.1%) of citrate (Fig. 1) or autoclaving a small amount of glucose together with other media components is also effective, but in these cases disadvantages must be taken into account.

These results have consequences for the design of synthetic media for *C. glutamicum*. Catechol or protocatechuate are chemically defined substances and need only to be added at very low concentrations. Therefore, in comparison with the preparation of synthetic media with either citrate or autoclaved glucose, the supplementation with one of the dihydroxyphenolic substances mentioned above is clearly advantageous. In BMCG broth supplemented with catechol or protocatechuate the iron content of the medium can be varied over a wide range (0.037–0.37 m*M*) without seriously affecting growth (see results).

The dependence on supplementation with chelating agents for growth indicates that *C. glutamicum* is not able to produce strong iron-binding compounds like many other microorganisms. Instead, *C. glutamicum* seems to be siderophore-auxotrophic, a phenotypic trait which is also found with several other bacterial species (Lankford 1973).

Acknowledgement. This work was supported from the Deutsche Forschungsgemeinschaft (DFG) by grant B9 of the SFB145.

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Received 5 May 1989/Accepted 10 July 1989