Regulation of Enzymes of Lysine Biosynthesis in *Corynebacterium glutamicum*

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(Received 27 April 1988; revised 25 July 1988)

The regulation of the six enzymes responsible for the conversion of aspartate to lysine, together with homoserine dehydrogenase, was studied in *Corynebacterium glutamicum*. In addition to aspartate kinase activity, the synthesis of diaminopimelate decarboxylase was also found to be regulated. The specific activity of this enzyme was reduced to one-third in extracts of cells grown in the presence of lysine. Aspartate-semialdehyde dehydrogenase, dihydrodipicolinate synthase, dihydrodipicolinate reductase, and diaminopimelate dehydrogenase were neither influenced in their specific activity, nor inhibited, by any of the aspartate family of amino acids. Homoserine dehydrogenase was repressed by methionine (to 15% of its original activity) and inhibited by threonine (4% remaining activity). Inclusion of leucine in the growth medium resulted in a twofold increase of homoserine dehydrogenase specific activity. The flow of aspartate semialdehyde to either lysine or homoserine was influenced by the activity of homoserine dehydrogenase or dihydrodipicolinate synthase. Thus, the twofold increase in homoserine dehydrogenase activity resulted in a decrease in lysine formation accompanied by the formation of isoleucine. In contrast, repression of homoserine dehydrogenase resulted in increased lysine formation. A similar increase of the flow of aspartate semialdehyde to lysine was found in strains with increased dihydrodipicolinate synthase activity, constructed by introducing the *dapA* gene of *Escherichia coli* (coding for the synthase) into *C. glutamicum*.

INTRODUCTION

L-Lysine is economically one of the most important amino acids. Commercially, the largest amounts are produced by *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* (Kleemann *et al.*, 1985); therefore several studies have been done on the regulation of individual enzymes of lysine biosynthesis in these organisms with the aim of improving their productivity.

The entry of aspartate into lysine synthesis is controlled at the level of aspartate kinase activity (see Fig. 1). In contrast to *Escherichia coli*, only one aspartate kinase is present in *B. flavum* (Shiio *et al.*, 1970). This enzyme is feedback-inhibited when both lysine and threonine are present in excess (Shiio & Miyajima, 1969; Tosaka & Takinami, 1978; Nakayama *et al.*, 1966). If the enzyme is released from feedback inhibition, mutants excrete lysine in the range of 5–10 g l⁻¹ (Sano & Shiio, 1970; Tosaka *et al.*, 1978*a*). Other amino acids of the aspartate family are not excreted in these mutants, indicating the presence of further control elements at branching points. Thus, homoserine dehydrogenase in *B. flavum* (Miyajima & Shiio, 1971; Miyajima *et al.*, 1968) and *B. lactofermentum* (Tosaka *et al.*, 1979) is repressed by methionine and inhibited by threonine. Mutants with decreased homoserine dehydrogenase activity produce more lysine (Shiio & Sano, 1969).

Interestingly, a further control at this branching point has been reported to be the synthesis of dihydrodipicolinate synthase. Tosaka *et al.* (1978*b*) reported that in *B. lactofermentum* upon leucine limitation a tenfold derepression of the enzyme specific activity occurs. This latter

Fig. 1. Synthesis of lysine via the diaminopimelate dehydrogenase reaction.

observation coincides well with the fact that certain leucine-negative strains obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis show increased lysine productivity (Tosaka *et al.*, 1978*b*). In spite of the economic interest, there have been no reports on the regulation of diaminopimelate decarboxylase, located at a further branching point, or that of aspartate semialdehyde dehydrogenase, dihydrodipicolinate reductase or diaminopimelate dehydrogenase. We therefore studied the regulation of these enzymes and re-examined that of other enzymes responsible for the conversion of aspartate to lysine. We included homoserine dehydrogenase and did experiments to elucidate the 'leucine effect' on lysine formation.

METHODS

Organisms and growth. The organisms used were *Corynebacterium glutamicum* ATCC 13032, the Leu⁻ strain ATCC 14310, *Brevibacterium lactofermentum* ATCC 13869, and two independently isolated mutants of *Corynebacterium glutamicum* ATCC 13032 with deregulated aspartate kinase (strains DG52-5 and MH20). These were obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (250 µg ml⁻¹, 30 min) and selected on plates containing the lysine analogue *S*-(2-aminoethyl)-L-cysteine plus L-threonine (12 mM each). The medium for the plates was C.g. VIII, plus 2% (w/v) agar agar and 5% (w/v) urea, but with CaCO₃ omitted. C.g. VIII contained, per litre: 20 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 0.25 g MgSO₄·7H₂O, 10 mg MnSO₄·H₂O, 10 mg FeSO₄·7H₂O, 1 mg ZnSO₄·7H₂O, 0.2 mg CuSO₄, 0.2 mg biotin. After sterilization, 4% (w/v) glucose and 2% (w/v) CaCO₃ were added.

For preparation of cell extracts and for measurement of amino acid production, a preculture was grown overnight at 30 °C in complex medium (C.g. III) consisting of, per litre: 2.5 g NaCl, 10 g yeast extract, 10 g peptone and 20 g glucose. Cells from this culture were washed once with 0.9% NaCl and transferred to medium C.g. VIII to give an initial OD₆₀₀ of about 0.7 (corresponding to 2 × 10⁸ cells ml⁻¹). All cultivations were done in 100 ml medium at 30 °C on a rotary shaker at 100 r.p.m.

Preparation of extracts and enzyme assays. The cells were harvested by centrifugation and, after washing with 50 mM-potassium phosphate buffer pH 7.5, were suspended in the same buffer. Sonic extracts were prepared by disrupting the cells with a microtip-equipped Branson Sonifier at maximal settings (5 min, 0 °C). The resulting homogenate was centrifuged for 30 min at 10000 *g*, and the supernatant used as cell-free extract.

Fig. 2. Construction scheme of plasmid pJC1/*dapA*.

Aspartate kinase (EC 2.7.2.4) was assayed in extracts of cells suspended in 50 mM-(NH₄)₂SO₄, 100 mM-Tris/HCl pH 7.5 according to the method of Black & Wright (1955). The assay mixture consisted of 100 mM-Tris/HCl pH 7.5, 13.3 mM-ATP, 24 mM-MgCl₂, 468 mM-(NH₄)₂SO₄, 613 mM-(NH₃OH)Cl, 93 mM-potassium L-aspartate, and extract. The assay was incubated at 30 °C for 30 min and the reaction stopped by the addition of 1 ml 10% (w/v) FeCl₃·6H₂O, 3.3% (w/v) trichloroacetic acid in 0.7 M-HCl to 1.5 ml assay mixture. After centrifugation, the absorbance at 546 nm was measured and compared with standards obtained with aspartyl hydroxamate.

Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) was assayed in extracts of cells suspended in buffer containing 33% (v/v) glycerol and was assayed according to Hegemann *et al.* (1970) in the reverse of the biosynthetic reaction. The assay mixture consisted of 60 mM-diethanolamine. HCl pH 9, 0.8 mM-NADP, 40 mM-Na₂HAsO₄, 0.12 M-NaCl, 1.5 mM-L-aspartate semialdehyde, and extract. The reaction was followed at 25 °C and 340 nm.

Dihydrodipicolinate synthase (EC 4.2.1.52) was assayed by the method of Yamakura *et al.* (1974). The assay mixture consisted of 0.2 M-Tris/HCl pH 8, 2 mM-L-aspartate semialdehyde, 4 mM-sodium pyruvate, and extract. After incubation for 10 min at 30 °C, the 500 µl assay mixture was mixed with 450 µl 1 M-HCl and 50 µl *o*-aminobenzaldehyde (20 mg per ml ethanol). After 50 min incubation in the dark, the samples were centrifuged and their absorbance at 540 nm determined ($\epsilon = 1220 \text{ M}^{-1} \text{ cm}^{-1}$).

For determination of dihydrodipicolinate reductase (EC 1.3.1.26) the *dapA* gene of *Escherichia coli*, coding for dihydrodipicolinate synthase (Richaud *et al.*, 1986), was transferred via plasmid pJC1 (see below) into the *dapB*- (reductase-) negative *E. coli* strain AT999 (Bukhari & Taylor, 1971). Extracts of this strain with a dihydrodipicolinate synthase specific activity of 3.3 µmol min⁻¹ (mg protein)⁻¹ were used to synthesize the reductase substrate in 900 µl of the following reaction mixture: 111 mM-Tris/HCl pH 7.5, 5 mM-aspartate semialdehyde, 1.1 mM-NADPH, 6 mM-sodium pyruvate, and 50 µl extract of AT999 (pJC1/*dapA*) (= 1 U dihydrodipicolinate synthase). This assay was incubated for 10 min at 30 °C and the reductase reaction started by the addition of extract previously incubated for 3 min at 70 °C (the heat treatment of the extract was necessary to inactivate the homoserine dehydrogenase and NADPH oxidase activities). The NADPH-dependent reduction was followed at 340 nm and 30 °C.

meso-Diaminopimelate dehydrogenase (EC 1.4.1.16) was assayed in the reverse direction at 30 °C in the following assay mixture: 200 mM-glycine/NaOH pH 10.5, 2 mM-NADP, 4 mM-*meso*-diaminopimelate.

Diaminopimelate decarboxylase (EC 4.1.1.20) was assayed in a system containing 50 mM-potassium phosphate pH 7.5, 0.1 mM-pyridoxal phosphate, 0.5 mM-EDTA, 25 mM-diaminopimelate, and extract. After incubation at 30 °C for various times (at least four points), the lysine formed was determined by amino acid analysis (see below).

Homoserine dehydrogenase (EC 1.1.1.3) was assayed at 25 °C in 66 mM-potassium phosphate pH 7.0, 0.2 mM-NADPH, 0.1 mM-aspartate semialdehyde. The activity was followed at 340 nm or determined by direct homoserine analysis (see below), giving comparable results.

All assays were checked for linearity with respect to time and protein concentration. They were optimized for the pH of the incubation buffer and for the concentrations of substrates and coenzymes. Determinations were done with at least three independently generated extracts. Specific activities are given in $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

DNA manipulation. The shuttle vector pJC1 and the plasmid pDA3 containing the *dapA* gene of *E. coli* (Richaud *et al.*, 1986) were isolated from *E. coli* DH5 and RDA8, respectively, by the alkaline lysis method (Birnboim & Doly, 1979). The construction scheme for the recombinant plasmid pJC1/*dapA* is shown in Fig. 2. The source of the DNA-modifying enzymes was Boehringer, whose recommendations for use were followed throughout. The ligation mix was used to transform *E. coli* DH5 (Hanahan, 1985) using the RbCl method, and Kan^r transformants were selected. Four of the transformants assayed had a specific dihydrodipicolinate synthase activity of 0.8–1.1 (host 0.04) and gave the restriction pattern of pJC1/*dapA*. This plasmid was isolated on a large scale by caesium chloride density gradient centrifugation (Maniatis *et al.*, 1982) and used to transform spheroplasts of *C. glutamicum* (Thierbach *et al.*, 1988).

Amino acid analysis. Amino acids were analysed as their *o*-phthalaldehyde derivatives by reversed-phase chromatography. Derivatives were formed via automatic pre-column derivatization using the *o*-phthalaldehyde reagent (Pierce) with an LC 1090 HPLC (Hewlett-Packard). The separation was achieved on a Lichrospher RP-18 column (Merck) using a gradient consisting of 0.1 M-sodium acetate pH 7.2 and methanol. Detection was via UV absorbance at 340 nm or fluorescence at 450 nm with excitation at 230 nm.

RESULTS

Activities of the enzymes of lysine synthesis during growth in batch culture

Washed cells of *C. glutamicum* were inoculated in parallel and one flask harvested at each time point to prepare the extract for enzyme determinations. As shown in Fig. 3, the specific activities of aspartate-semialdehyde dehydrogenase and dihydrodipicolinate synthase exhibited a more than threefold increase during the early exponential growth phase. By contrast, the other four enzyme activities determined varied only slightly with cultivation time.

Repression and inhibition of the enzymes by amino acids

The amino acids used were those of the aspartate family, and leucine. All amino acids were taken up from cultures, and consequently might influence the internal amino acid pool. This was not the case with diaminopimelic acid since all attempts to force the organism to use this compound as a nitrogen source failed. This explains why no mutants of *C. glutamicum* requiring diaminopimelic acid have been found (Follettie *et al.*, 1986) and agrees with a similar situation in *Bacillus sphaericus* (White, 1983).

The only two enzymes found to be regulated in their synthesis were diaminopimelate decarboxylase and homoserine dehydrogenase (Table 1), both located at branching points. The decarboxylase was repressed by lysine to a degree similar to that found in *E. coli* (Stragier *et al.*, 1983). Our results are thus at variance with the recent report by Yeh *et al.* (1988*a*), who found that the levels of dihydrodipicolinate synthase, diaminopimelate dehydrogenase and diaminopimelate decarboxylase in *C. glutamicum* were all independent of the addition of lysine to the growth medium. The repression of homoserine dehydrogenase by L-methionine has already been reported for *Brevibacterium flavum* (Miyajima & Shiio, 1971). Surprisingly, the enzyme was derepressed about twofold by leucine; this is probably of relevance for the decrease in lysine formation obtained by added leucine in lysine-producing strains. No repression of dihydrodipicolinate synthase was found with any of the amino acids tested, though in *B. lactofermentum* an eightfold repression by leucine has been reported (Tosaka *et al.*, 1978*b*). To enhance any possible effect of L-leucine on the synthesis of the enzyme, the Leu⁻ mutant *C.*

Fig. 3. Growth-dependent activities of enzymes of lysine synthesis in *C. glutamicum*. ♦, Growth; △, aspartate kinase; ●, aspartate-semialdehyde dehydrogenase; ○, dihydrodipicolinate synthase (values divided by 10 before plotting); ▲, dihydrodipicolinate reductase; ■, diaminopimelate dehydrogenase (values divided by 10 before plotting); □, diaminopimelate decarboxylase.

glutamicum ATCC 14310 was used. This mutant was grown in the presence of 10 mM-L-leucine and the enzyme activity determined in one part of the cells. The other part was washed, transferred to leucine-free medium and incubated for 6 h. The resulting enzyme specific activity was 0.24 as compared to 0.16 in the extract prepared at the beginning of the transfer and comparable to that in the wild-type. In a similar experiment with a *Leu*⁻ mutant of *B. lactofermentum* a tenfold derepression of the enzyme activity has been reported (Tosaka *et al.*, 1978*b*). We determined a constant specific activity in *B. lactofermentum* ATCC 13869, whether or not 10 mM-L-leucine was included in the growth medium.

The same amino acids which were tested for their influence on the synthesis of the lysine-biosynthetic enzymes were also tested for a possible influence on enzyme activity (Table 2). No regulation of dihydrodipicolinate synthase, dihydrodipicolinate reductase, diaminopimelate dehydrogenase or diaminopimelate decarboxylase was found. Aspartate kinase was inhibited by L-threonine plus L-lysine, confirming earlier reports for *Micrococcus glutamicus* (Nakayama *et al.*, 1966). The activity of aspartate-semialdehyde dehydrogenase was inhibited by L-threonine and L-lysine, but since the enzyme was assayed in the reverse reaction at pH 10, any apparent regulatory effect must be considered with caution. Homoserine dehydrogenase was inhibited almost completely by inclusion of L-threonine in the enzyme assay, whereas L-isoleucine inhibited the activity to about 50%.

Control of the flow of aspartate semialdehyde to either dihydrodipicolinate or homoserine

Dihydrodipicolinate synthase and homoserine dehydrogenase, both competing for the aldehyde, have comparable specific activities. The apparent K_m of the dehydrogenase for the aldehyde is 0.7×10^{-3} M, whereas that of the synthase is 6.2×10^{-3} M. These values, determined from Lineweaver-Burk plots, are of the same order as those for the enzymes from *B. lactofermentum* (Tosaka *et al.*, 1979). This kinetic analysis, together with the regulation of homoserine dehydrogenase, suggests that the partitioning of aspartate semialdehyde is primarily controlled at the level of homoserine dehydrogenase.

Table 1. *Specific activities of enzymes of lysine synthesis and of homoserine dehydrogenase in extracts of C. glutamicum*
L-Amino acids were added at a concentration of 20 mM to the growth medium, and cells were harvested after 15–24 h cultivation. Specific activities are in $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. ND, Not done.

Growth conditions	Aspartate kinase	Aspartate semialdehyde dehydrogenase	Dihydrodipicolinate synthase	Dihydrodipicolinate reductase	Diaminopimelate dehydrogenase	Diaminopimelate decarboxylase	Homoserine dehydrogenase
Complex medium	0.012	0.020	0.23	0.03	0.15	0.020	0.02
Minimal medium (MM)	0.015	0.010	0.20	0.06	0.13	0.031	0.39
MM + Leu	0.012	0.015	0.22	0.05	0.13	0.030	0.87
MM + Ile	0.016	0.012	0.23	0.06	0.13	0.031	0.46
MM + Lys	0.013	0.013	0.21	0.07	0.13	0.011	0.32
MM + Met	0.012	0.015	0.20	0.05	0.13	0.033	0.07
MM + Thr	0.013	0.013	0.22	0.07	0.13	0.033	ND
MM + Lys/Met/Thr	0.012	0.010	0.23	0.04	0.13	0.014	0.08

Table 2. *Inhibition of enzymes in extracts of C. glutamicum by amino acids*

Extracts were prepared from cells grown in minimal medium. L-Amino acids were added to a final concentration of 10 mM in the assay system. Specific activities are in $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$.

Addition	Aspartate kinase	Aspartate-semialdehyde dehydrogenase	Homoserine dehydrogenase
None	0.012	0.019	0.47
Leu	0.014	0.015	0.50
Lys	0.012	0.012	0.42
Met	0.014	0.015	0.44
Ile	0.015	0.016	0.21
Thr	0.010	0.010	0.02
Lys/Met/Thr	0.003	0.011	0.02

To verify this view, two independently isolated lysine-producing mutants of *C. glutamicum* ATCC 13032 were grown under conditions repressing homoserine dehydrogenase (10 mM-methionine) and the amounts of lysine formed were compared. In strain DG52-5 the concentration of lysine in the growth medium was increased from 40 to 50 mM, and in strain MH20 from 37 to 43 mM, respectively. A similar effect of methionine was found with a strain of *B. flavum* (Miyajima & Shiiro, 1971). By contrast, increased homoserine dehydrogenase activity was attained by cultivation of the strains with 10 mM-leucine. In both cases the increased dehydrogenase activity was comparable to that of the wild-type and the lysine yield was reduced: from 37 to 27 mM with strain DG52-5, and from 36 to 30 mM with strain MH20. Additionally, the cultures with derepressed homoserine dehydrogenase formed traces of isoleucine (1–2 mM). This is further evidence for the drain of the aldehyde into the threonine branch.

To ascertain whether the flow of aspartate semialdehyde can also be altered via an increase in dihydrodipicolinate synthase activity, the *dapA* gene of *E. coli* (coding for the synthase) was used (Richaud *et al.*, 1986). The construction of the *C. glutamicum*/*E. coli* vector containing *dapA* is outlined in Methods. The plasmid pJC1/*dapA* was then introduced in the lysine-producer *C. glutamicum* MH20 and transformants were assayed for dihydrodipicolinate synthase specific activity. This was 1.6–2.2, as compared to 0.2 in the original strain. Since the synthase of *E. coli* is inhibited by lysine, the specific activity was determined with 50 mM-lysine in the assay system. Under these conditions the specific activity was 0.5; thus even under the unfavourable assumption of very high internal lysine concentrations the presence of the *E. coli* *dapA* gene could result in a 2.5-fold activity increase in the transformants. The fermentation of two transformants yielded 41 and 42 mM-lysine, respectively, whereas controls (strains containing the vector without the *E. coli* *dapA* gene) yielded 33 and 34 mM.

DISCUSSION

The synthesis of lysine from aspartate in *Corynebacterium glutamicum* proceeds via the diaminopimelate dehydrogenase reaction (Misono *et al.*, 1979). Possibly, an additional bypass via succinylated (or acetylated) intermediates might occur. The only evidence for this latter variant of lysine synthesis is one measurement of aminoketopimelate succinylase using a problematic assay (Tosaka & Takinami, 1978), and comparisons of the distribution of ^{13}C in lysine synthesized from labelled glucose based on the assumption that both pathways might occur (Yamaguchi *et al.*, 1986). A physiological need for the presence of both pathways occurring together is questionable. This is especially the case for coryneform bacteria, where there are usually simple types of regulation, which might be one reason for the successful use of these organisms for amino acid production (Kinoshita, 1985). It therefore appears that ignorance of the diaminopimelate dehydrogenase reaction in coryneform bacteria at the beginning of the research on lysine production resulted in the branched pathway currently presented.

The targets for the regulation of the diaminopimelate dehydrogenase pathway in *C. glutamicum* are aspartate kinase and diaminopimelate decarboxylase. The recognized effectors are L-threonine and L-lysine. Much attention has already been paid to the regulation of aspartate kinase activity. The enzyme of *C. glutamicum* is inhibited to 30% of its activity by threonine plus lysine, a pair of amino acids which represent both the lysine/diaminopimelate and the methionine/threonine/isoleucine branches of the biosynthesis of the aspartate family of amino acids. This type of control is sufficient to regulate the synthesis of all amino acids, provided that the remaining aspartate kinase activity supplies sufficient precursor for methionine synthesis.

Diaminopimelate decarboxylase has not previously been recognized as a regulatory enzyme in coryneform bacteria. In *Bacillus sphaericus* (Bartlett & White, 1986) no repression of the enzyme occurs, whereas in *E. coli* a complex type of regulation exists, involving the *lysR* gene which modulates the expression of *lysA*, coding for the decarboxylase (Stragier *et al.*, 1983). This results in repression of synthesis of the enzyme by lysine, which can be overcome by the addition of diaminopimelate. The cloning of the diaminopimelate decarboxylase gene of *C. glutamicum* has recently been reported (Yeh *et al.*, 1988*b*). Interestingly, a DNA sequence upstream of *lysA* was found which was necessary for full expression of the decarboxylase on a cloned fragment. We propose that the upstream region is the target for the lysine-dependent regulation of *lysA* integrated in the genome of *C. glutamicum*. Diaminopimelate is probably also involved in regulation, as is the case with *lysA* of *E. coli*. Experiments to elucidate this are possible with cloned DNA fragments in a host where diaminopimelate can enter the cell. In any case, the decarboxylase is, in addition to the long-recognized aspartate kinase, a further candidate for direct manipulation to achieve increased lysine formation.

At the branching of the flow of aspartate semialdehyde to either homoserine or diaminopimelate, a regulation of dihydrodipicolinate synthase activity has not been observed in *C. glutamicum*. This is in contrast to *E. coli* (Patte, 1983) or *Bacillus sphaericus* (Bartlett & White, 1986), where lysine inhibits the enzyme. In *C. glutamicum* the regulation of the flow of aspartate semialdehyde is only via homoserine dehydrogenase. The repression of this enzyme by methionine and its inhibition by threonine allow a balanced flow to the methionine/threonine/isoleucine branch, whereas no fine-tuning of lysine synthesis between aspartate kinase and diaminopimelate decarboxylase occurs. The flow of aspartate semialdehyde to lysine can be increased either by enhancement of dihydrodipicolinate synthase activity or by a decrease in homoserine dehydrogenase activity. This is in agreement with the fact that the profitable use of several lysine-producing *Brevibacterium flavum* strains could be subsequently attributed to a decreased homoserine dehydrogenase activity (Shiio & Sano, 1969). Such mutants are also advantageous for fermentations since no adjustment of the threonine and methionine supply is required as it is with mutants with no homoserine dehydrogenase activity.

The observed negative effect of leucine on lysine formation is most probably due to an increased drain-off of aspartate semialdehyde via increased homoserine dehydrogenase activity. If this is the only effect of leucine, this effect should not be found with homoserine-dehydrogenase-negative mutants. To confirm this, precisely defined mutants, perhaps obtained by transposon mutagenesis, will have to be analysed.

The authors thank Dr C. Richaud for pDA3. They thank Dr Bachmann and Dr Buchholz for aspartate semialdehyde and Dr Aragon for strain DG52-5. This work is part of a common project with DEGUSSA AG and Lehrstuhl für Genetik, Universität Bielefeld, supported by grants from the BMFT (038409).

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