SHORT COMMUNICATIONS

Characterization of a cym Mutant of *Bacillus subtilis*

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(Received 23 December 1974)

INTRODUCTION

Bacillus subtilis can grow with sulphate as the only source of sulphur. Activation and reduction of sulphate occur by reactions that are similar to the reactions in *Escherichia coli* (Pasternak, 1962; Pasternak *et al.* 1965). Less is known about the later steps of S-amino acid synthesis although a number of *cys* mutants and *met* mutants have been reported (see Dubnau, 1970). One strain, BD112, has an absolute requirement for cysteine, and this was originally considered to be caused by a single mutation designated *cysA14* which was mapped near the origin of replication of the chromosome close to *rif* (resistance to rifampicin). There are now several reports that, in genetic crosses by transformation, *cysA14* behaves as two linked auxotrophic mutations (Harford & Sueoka, 1970; Haworth & Brown, 1973; Piggot, 1973). One of these is a cym mutation (Piggot, 1973), that is a mutation causing a requirement for either cysteine or methionine for growth; such mutants are known in *Salmonella typhimurium* (see Smith, 1971).

The relationship between cysteine and methionine biosynthesis in *B. subtilis* is similar to that in *S. typhimurium* and *E. coli*. However, the cym mutant differs from those of *S. typhimurium* (Qureshi, Smith & Kingsman, 1975) in that the toxicity of selenate is not reversed by cysteine.

METHODS

Strains. *Bacillus subtilis* 168 was used as the standard strain, and is referred to as the wild type. It is a prototrophic derivative of the original *trpC2* strain. All auxotrophic strains are derivatives of *B. subtilis* 168. Strain BD112, *cys-I* cym-1 (formerly *cysA14*) was obtained from Dr D. Dubnau. Strains MB14, *rif-I* *trpC2*, and MB18, *rif-I* *cys-I* cym-1 have been described previously (Piggot, 1973).

Isolation of strains MB22 and MB23. Strain MB18 was transformed by DNA from the wild type. Transformants were selected that grew on minimal agar + methionine. Two colony types were observed amongst the transformants: the type giving the larger colony grew equally well on minimal agar with no supplement, while that giving the smaller colony type grew very poorly on minimal agar and needed either cysteine or methionine for good growth. Colonies of this latter type of transformant, having the cym phenotype, were isolated. One, MB22 (cym-1), having the *rif*⁺ genotype of the donor, and one, MB23 (cym-1 *rif-I*), retaining the *rif-I* of the recipient, were used for further study.

Transformation. Recipient cells were grown to competence and transformed by the method of Ayad & Barker (1969). DNA was purified from the donor by the method of Marmur (1961).

Media. Spizizen salts medium (SSM) consisted of (for 900 ml): (NH₄)₂SO₄, 2 g; K₂HPO₄,

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14 g; KH_2PO_4 , 6 g; trisodium citrate. $2\text{H}_2\text{O}$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g (Anagnostopoulos & Spizizen, 1961). Basal medium consisted of: SSM, 900 ml; monosodium glutamate (1.5 %, w/v) adjusted to pH 7.0 with NaOH, 90 ml; glucose (40 %, w/v), 5 ml; L-tryptophan (0.2 %, w/v), 5 ml. These components were sterilized separately by autoclaving. Low sulphate basal medium was similar to basal medium except that NH_4Cl (1.6 g) and MgCl_2 (0.16 g) were used in place of $(\text{NH}_4)_2\text{SO}_4$ and MgSO_4 in SSM. Sporulation media and the minimal medium used for plates have been described previously (Piggot, 1973). Unless otherwise stated, supplements, when required, were added as follows (per litre): L-tryptophan, 20 mg; L-methionine, 2 mg; L-cysteine-HCl, 2 mg; DL-allo-cystathionine, 40 mg; homocysteine thiolactone-HCl, 100 mg; rifampicin, 2.5 mg. Sodium salts of possible cysteine precursors were used at the following concentrations: thiosulphate, 0.3 and 30 mM; thiosulphite, 0.3 and 30 mM; sulphite, 0.03 and 0.3 mM; sulphide, 0.03 and 0.3 mM. Higher concentrations of sulphite and sulphide inhibited growth of the wild type. All supplements were sterilized by passage through a bacterial filter (Millipore, HA 0.45 μm); solutions of cysteine and its precursors were prepared and sterile-filtered immediately before use.

Growth. Inocula were grown overnight in low sulphate basal medium + 2 μg cysteine-HCl/ml. Bacteria were collected by centrifugation and resuspended in low sulphate basal medium with no supplement. This was used to inoculate fresh medium to a bacterial density of up to 5 μg dry wt/ml. After an initial lag, growth was generally exponential until a bacterial density of about 75 to 100 μg dry wt/ml, depending on strain and medium, was reached. Growth of inocula of *cys*⁺ strains with methionine in place of cysteine did not affect results. All media were pre-warmed to 37 °C. Cultures were grown in conical flasks at 37 °C. The culture volume was not greater than 12 % of the flask volume. Flasks were aerated by shaking on a reciprocal shaker (Warburg Apparatus type A, Mickle Laboratory Engineering Co., Gomshall, Surrey) at 80 to 100 cycles/min. Growth was followed by measuring the E_{600} with a Unicam SP600 spectrophotometer. The E_{600} was converted to mg dry wt/ml by means of a standard calibration curve.

RESULTS AND DISCUSSION

Selenate inhibition of growth of the wild type

Selenate inhibits growth by preventing sulphate activation (Postgate, 1952). In *E. coli* and *S. typhimurium* this inhibition is overcome non-competitively by cyst(e)ine or sulphide but not by methionine (Fells & Cheldelin, 1949; Pasternak, 1962). In *Saccharomyces cerevisiae*, however, methionine, but not cyst(e)ine, reverses the toxic effect of selenate. This is indicative of the different pathways of incorporation of S into amino acids. In the bacteria, cysteine is an intermediate in the biosynthesis of methionine from sulphate but cysteine cannot be formed from methionine (Smith, 1971), whereas in yeasts, cysteine can be formed from methionine and methionine cannot be formed from cysteine (De Robichon-Szulmajster & Surdin-Kerjan, 1971).

In low sulphate basal medium containing cysteine, addition of selenate (10 or 50 μM) did not appreciably alter the growth rate (mass doubling times of 62 and 66 min respectively, as against 59 min with no selenate). However, with low sulphate basal medium containing methionine, 10 μM -selenate reduced the growth rate (mass doubling time 105 min against 57 min with no selenate), and 50 μM -selenate stopped growth completely. Sulphide had a comparable effect to cysteine. Thus *B. subtilis* showed a similar response to that of *E. coli*. This suggested that it had a similar pathway for sulphate utilization, namely:

Phenotype of the cym mutant

Strain MB22 grew in low sulphate basal medium with either L-cysteine-HCl (0.5 µg/ml) or L-methionine (0.5 µg/ml) as supplement, with mean generation times of 65 min in each case. With no added S-amino acid, the mean generation time was greater than 400 min but there was perceptible growth, suggesting either a leaky mutation or low activity of some alternative pathway.

The possible methionine precursors, DL-allo-cystathionine and homocysteine thiolactone, supported growth of MB22 as effectively as methionine. Sulphide (0.3 mM) had a similar effect, but no other cysteine precursor tested (thiosulphite, thiosulphate, sulphite) supported growth. This suggested that MB22 had a defect in the reactions converting sulphite to sulphide. Sulphide did not support the growth of the parental strain BD112, confirming that this strain had a second block in the cysteine biosynthetic path.

In low sulphate basal medium, 10 µM-selenate greatly reduced the growth rate with either methionine or cysteine as supplement (mass doubling times 300 and 270 min respectively). At 50 µM, selenate completely inhibited growth. The inhibition was not reversed by a mixture of methionine, cysteine and sulphide. This contrasts with the behaviour of the wild type. The result is difficult to explain in terms of the known action of selenate; it may indicate that selenate has some additional site of action in MB22. In this respect, the *cym* mutant differs from *cym* mutants of *S. typhimurium* which showed the same response as the wild type to selenate inhibition (Qureshi *et al.* 1975).

Genetic mapping of cym-1 and cys-1

The mutation *cym-1* was linked to *rif-1* in transformation crosses. Selecting for *cym*⁺, the markers were 24 % cotransformed; selecting for resistance to rifampicin, they were 12 % cotransformed.

Strain BD112 harboured, in addition to *cym-1*, a second mutation that gave it the absolute requirement for cysteine. This second mutation is in a *cys* locus (Haworth & Brown, 1973) and is designated *cys-1*. Transformation of *cys-1* to *cys*⁺ was selected for by selecting transformants of BD112 (or MB18) which could grow on minimal agar + methionine. Mutation *cys-1* was 29 % cotransformed with *cym-1* and 48 % cotransformed with *rif-1*. An unambiguous order could not be deduced from these crosses.

In a three-factor transformation cross with wild type as donor, MB18 as recipient, and selection for *cys*⁺, significant numbers of all four possible recombinant classes were obtained (Table 1). It is assumed that transformants result mainly from double crossovers between donor and recipient, and that quadruple crossovers are rare. Of the three possible map orders, the one that is most compatible with the appearance of all recombinant classes is *cym-cys-rif*, as no quadruple crossovers need be postulated; for the order *cys-cym-rif*, quadruple crossovers are required to give the recombinant class *cys*⁺ *cym-1* *rif*⁺ (which accounted for 38 out of 126 colonies tested); the order *cys-rif-cym* would require quadruple crossovers to give *cys*⁺ *cym*⁺ *rif-1* transformants (11 out of 126). In the second three-factor cross, MB14 was donor, BD112 was recipient, and selection was for resistance to rifampicin (Table 1). This cross was of more limited value than the first three-factor cross, as two of the four possible recombinant classes could not be distinguished. However, the order *cys-cym-rif* is most unlikely as this would require quadruple crossovers to give *rif-1* *cym-1* *cys*⁺ transformants (28 out of 69 colonies tested). Both the other possible map orders are compatible with the results of this cross.

In a cross with selection for double transformation of BD112 to *cys*⁺ and *cym*⁺ using

Table 1. *Three-factor transformation crosses*

Donor	Recipient	Selected marker	Class of transformant	No. in class	Suggested order
Wild type (<i>cym</i> ⁺ <i>cys</i> ⁺ <i>rif</i> ⁺)	MB18 (<i>cym</i> -1 <i>cys</i> -1 <i>rif</i> -1)	<i>cys</i> ⁺	<i>cys</i> ⁺ <i>cym</i> -1 <i>rif</i> -1 <i>cys</i> ⁺ <i>cym</i> ⁺ <i>rif</i> -1 <i>cys</i> ⁺ <i>cym</i> -1 <i>rif</i> ⁺ <i>cys</i> ⁺ <i>cym</i> ⁺ <i>rif</i> ⁺	52 11 38 19	<i>cym</i> - <i>cys</i> - <i>rif</i>
MB14 (<i>rif</i> -1 <i>cym</i> ⁺ <i>cys</i> ⁺)	BD112 (<i>rif</i> ⁺ <i>cym</i> -1 <i>cys</i> -1)	<i>rif</i> -1	<i>rif</i> -1 <i>cym</i> -1 <i>cys</i> ⁺ <i>rif</i> -1 <i>cym</i> ⁺ <i>cys</i> ⁺ <i>rif</i> -1 <i>cym</i> -1 <i>cys</i> -1 <i>rif</i> -1 <i>cym</i> ⁺ <i>cys</i> -1 } *	28 1 40	<i>cym</i> - (<i>cys</i>) <i>rif</i>

* These two classes of transformant could not be distinguished.

DNA from MB14 (i.e. selection on minimal agar), only 26 % of the transformants received the donor *rif*-1 marker. This confirms that *rif*-1 does not lie between the other markers.

When the data from the last cross and the three-factor crosses are considered together, then the only consistent map order for the mutations is *cym*-1-*cys*-1-*rif*-1.

I thank Dr M. A. Foster, Dr M. Jones-Mortimer, Professor J. Mandelstam and Dr D. A. Smith for helpful discussions. I am indebted to Mrs F. C. Grant and Mrs D. Torgerson for their skilled technical assistance. This work was supported by the Science Research Council.

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